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ABE Fermentation From Low Cost Substrates

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Graduate Program in Chemical and Biochemical Engineering

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Abstract

The high cost of substrate and product inhibition in the fermentation broth remain two major problems associated with bio-butanol production. This thesis aims to solve these problems by examining abundant lignocellulosic biomass as potential feedstocks and exploring novel substrates such as carbohydrates derived from microalgae for ABE (Acetone-butanol-ethanol) fermentation. The commonly observed toxic effect after pretreatment of lignocellulosic biomass was removed by resin adsorption, where the resin could also serve as an in-situ butanol recovery device.

Corn cobs (an agricultural waste), switchgrass (an energy crop) and phragmites (an invasive plant in North America) were investigated as substrates for ABE fermentation by *Clostridium saccharobutylicum* DSM 13864. NaOH pretreatment followed by a washing step was used to reduce the biomass recalcitrance and facilitate the subsequent enzymatic hydrolysis. Total sugar yields for corn cobs, switchgrass and phragmites were 475, 365, and 385 g/kg of raw biomass, respectively. After the subsequent fermentation, an ABE yield of 166, 146, and 150 g/kg raw biomass was obtained.

Although biofuel production from lignocellulosic biomass is considered more sustainable than biofuel from food crops, it still faces many challenges. In order to demonstrate a possible biofuel production strategy using microalgal biomass, lipid extracted microalgae (LEA) was also used as substrates for ABE fermentation. To convert the carbohydrate fraction into solvents (ABE), LEA was either acid hydrolysed into glucose or directly fermented. The highest butanol titers (8.05 g/L) was obtained with the fermentation of acid hydrolysates. However fermenting the hydrolysate required detoxification via a resin, while direct fermentation did not, significantly simplifying the LEA to butanol process.

The resin that was used to detoxify acid hydrolysates of LEA was further investigated for detoxification of lignocellulosic hydrolysates and in-situ butanol recovery. Detoxification of acid hydrolyzed phragmites by resin L-493, improved the fermentability significantly. Resin L-493 was efficient in removing phenolic compounds present in the phragmites hydrolysates, as well as butanol produced during fermentation.

Keywords

lignocellulosic biomass, NaOH pretreatment, ABE fermentation, resin, pervaporation, microalgae

Co-Authorship Statement

Contents of Section 2 were published to refereed journals and were co-authored by Dr. Lars Rehmman, who provided editorial and technical advice.

Simone Boiano provided experimental assistance to portions of the work performed in section 2.4 and is listed as an co-author; Dr. Antonio Marzocchela provided advice on the draft of manuscript and is listed as a co-author.

In section 2.5, Valerie Orr contributed to cultivation and lipid extractions of microalgae, and revision of the first draft of manuscript, and is listed as a co-author; Kai Gao designed and conducted all fermentation experiments, composition analysis and collected data; Kai Gao drafted the manuscript.

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Section 1 - Introduction and Literature Review

1.1. Introduction

Today's transportation infrastructure (freight and passenger) is almost entirely dependent on petroleum-based fuels such as gasoline, diesel fuel, LPG (liquefied petroleum gas), and (CNG) compressed natural gas (Fulton et al., 2015). Electric vehicles are increasingly available and might become mainstream with the coming decades, while solar and wind energy installations are increasingly providing renewable electricity. However, in the intermediate future internal combustion engines will be dominant means to propel personal vehicles, and the entire aviation sector will require liquid transportation fuels with high energy density in the foreseeable future. In order to decrease the dependence on fossil fuels, the need for alternative technologies to produce liquid fuels from renewable resources increased for the past two decades, which were characterized by political instability in many oil producing regions and highly volatile prices.

For both developing and industrialized countries, development of biofuels can help reduce reliance on imported fossil fuels, improve the socioeconomics in rural areas, and bring many environmental benefits such as green house gas (GHG) reductions and less air pollution (Demirbas, 2009). Ethanol from corn, wheat, sugar beet, and sugar cane is the most commonly produced biofuel, of which the two major producing countries are Brazil and the USA (Jonker et al., 2015). In Brazil, sugarcane is used as the major feedstock, and about 20.7 million m³ ethanol (corresponding to 30% of domestic consumption of gasoline energy-wise) was produced in 2011 by fermentation of sugars extracted from sugarcane; whereas in the US, 54.2 million m³ corn ethanol were produced, corresponding to 7% of the domestic consumption of gasoline energy-wise; the two countries combined contribute to 87% of the world's total ethanol production, which however only accounts for 5% by energy of the world's total gasoline consumption (Chum et al., 2012). In the US, ethanol is mostly blended with gasoline at a ratio of 10% by volume, whereas in Brazil it is blended at a higher ratio from 18-25%, and this blending ratio can only be achieved with flexible-fuel vehicles in both countries (Chum et al., 2012).

A large demand on dense liquid fuel is still expected in this century (80% of transportation in 2050 and 50% in 2075), and this assumption is made based on significant fuel efficiency improvement and deep market penetration of vehicles running on electricity and hydrogen; the demand will mainly come from aviation, ocean shipping, and long-haul trucking (Fulton et al., 2015). To limit the global temperature increase within 2 degrees, a full transition to near-zero emissions carbon fuels is difficult to achieve without the widespread use of biofuels (Fulton et al., 2015).

While biofuels have attracted a lot of attention from the public, renewable chemicals produced through biological pathways is considered as another opportunity for commercialization of industrial biotechnology in today's markets, at lower costs and with potentially higher returns (Erickson et al., 2012). Butanol is one of the platform chemicals that can be produced through microbial processes by conversion of sugars; it is a building block that has many applications in solvents, rubber and transportation fuels (Erickson et al., 2012). As a superior biofuel, it can be blended with gasoline at any ratio, or even completely substitute gasoline without the need for engine modification; it contains more energy compared to ethanol, which provides a similar performance to gasoline; it can be transported through existing pipelines, significantly decreasing the potential transportation cost of fuels, etc (Dürre, 2007). Furthermore, butene (easily obtained from butanol) can be catalytically converted into jet fuels at high efficiency (Wright et al., 2008). Used as a solvent, butanol often appears in paints and cosmetics such as nail polish. The solvent, rubber, and fuel industries each worth several billion dollars.

However, large areas of high-quality arable land has been used to produce food crops for biofuel production, thus causing concerns over increasing food prices, reducing food production as well as competition of resources such as land and water with food-oriented crops (Rajagopal et al., 2009). To avoid this so-called food versus fuel dilemma, cellulosic biomass which include perennial plants grown on degraded lands, wastes abandoned from agricultural use, crop residues, and sustainably harvested wood and forest residues are suggested to be used as non-food feedstocks for biofuel production (Tilman et al., 2009). However, extraction of sugars from cellulosic biomass is much more difficult compared to the traditional crop feedstocks such as corn and sugarcane,

which is mainly due to the recalcitrant nature of lignocellulosic biomass (Naik et al., 2010). To breakdown the structural barrier and facilitate the hydrolysis of sugar polymers into fermentable simple sugars, different types of pretreatment methods have been developed in order to improve the sugar yields (Alvira et al., 2010). A number of studies have reported bio-butanol production from lignocellulosic biomass including wheat straw, barley straw, corn stover, corn cobs, switchgrass and dried distilled grains and solubles (DDGS). However, the biomass was mainly pretreated by similar pretreatment method (dilute sulfuric acid) and fermentation results varied significantly depending on the biomass species (Ezeji and Blaschek, 2008; Qureshi et al., 2010a, 2010b, 2007). The variation in fermentation results is likely due to the formation of fermentation inhibitors during pretreatment of certain biomass species at harsh conditions. Different strategies have been employed to alleviate the toxic effect of lignocellulosic hydrolysates, which include the use of different pretreatment method with milder operating conditions and less strong chemicals, or an extra detoxification step is integrated after pretreatment to remove potential fermentation inhibitors (Martinez et al., 2001; Millati et al., 2002; Parekh et al., 1988). Having said that, to make an impact at industrial level, a biorefinery plant can not rely on single feedstocks and should possess the ability to process a wide range of biomass species. Therefore, it is necessary to look for a pretreatment method that is universally applicable to different types of lignocellulosic biomass, which facilitate high sugar yields in the subsequent enzymatic hydrolysis while it does not require complicated processes to remove fermentation inhibitors.

Biofuel production from cellulosic biomass also has its own limitations in terms of competition of resources such as land and water with food crops, long harvesting period, and requirement of both energy and chemical intensive pretreatment. As an alternative to terrestrial biomass, algae may become a promising feedstock for biofuels. High biomass yields can be obtained without requiring any arable land; algae cells grow well in saline, brackish and waste water, whereas terrestrial plants require fresh water to grow (Daroch et al., 2013). So far, biofuels produced from algal biomass are mainly separated into two categories, fermentative biofuel production from algal carbohydrates and biodiesel produced from algal oil (Daroch et al., 2013). Biomass recalcitrance is one of the major problems associated with biofuel conversion from lignocellulosic feedstocks, however,

saccharification of algal biomass is much easier due to absence of lignin, and some algae species are able to accumulate significant amount of starch (40-60% dry weight), which can be hydrolysed either by dilute acid or enzymes into fermentable sugars at high efficiency (Brányiková et al., 2011). In recent years, bio-ethanol production has been reported in a number of researches (Choi et al., 2010; Lee et al., 2011; Nguyen et al., 2009); however, limited research has been reported on butanol production from algal feedstocks, and butanol titer are relatively low compared to fermentation with glucose as substrate (Ellis et al., 2012a; Potts et al., 2012).

In addition to the problem of substrate cost, another major limitation of bio-butanol production is the low solvent titer in the product stream, which is mainly due to butanol toxicity on cell growth and nutrient uptake (Ezeji et al., 2010). In a normal batch fermentation, butanol titer rarely exceed 12-13 g/L (Papoutsakis, 2008); resulting in high distillation costs. In recent years, a number of butanol removal technologies have been developed including adsorption, gas stripping, liquid-liquid extraction, and pervaporation to relieve butanol toxicity and reduce the energy cost in downstream processing (Lin et al., 2012; Qureshi et al., 2001; Xue et al., 2012a; Yen and Wang, 2013). Though each of these technologies has its own limitations, in-situ butanol removal through adsorption seems to have extra advantages in the context of cellulosic bio-butanol production compared to other technologies, mainly due to the fact that adsorption has also been studied as an effective method for removal of fermentation inhibitors generated during pretreatment (Ranjan et al., 2009; Weil et al., 2002). Therefore, it is theoretically possible to employ adsorption to solve two problems at one time, namely alleviate butanol toxicity and removal of fermentation inhibitors, and as a result of that, the process of cellulosic bio-butanol production could be significantly simplified.

1.2. Literature review

1.2.1. Butanol: Platform chemical and superior biofuel

Butanol (n-butanol) is a four carbon alcohol with a formula of C_4H_9OH . It is considered as a superior fuel compared to ethanol and a valuable chemical feedstock with major applications as a direct solvent. Compared to ethanol, butanol has an energy content

closer to gasoline, which means a smaller effect on the vehical's milage and no need to adjust the engines at high blend ratios (e.g. fuel volume to be pumped to reach similar torque, etc); it is safer to handle as it is less volatile; last but not least, it can be transported through existing pipelines (the more hygroscopic ethanol has to be transported by trucks and blended at gas stations (Dürre, 2007)).

When sold into the bulk chemical market, n-butanol's value is three times higher than the value as a fuel (Lee, 2015). Butanol has an immediate market value at more than USD 6 billion and is projected to reach USD 9.9 billion by 2020 (Marketsandmarkets.com, 2015). The most dominant application of butanol is manufacturing of butyl acrylate, which is mainly used as a component of water-based latex paints and pressure sensitive adhesives (Lee, 2015). The second most important application is the production of butyl acetate, which is an active solvent for film-formers such as nitrocellulose and polyesters and is widely used in automotive coatings (Eastman, 2016).

1.2.2. History of butanol production

Currently, n-butanol is industrially produced from petrochemical feedstock propylene (also known as propene). A process called the oxo process or more formally known as hydroformylation was used to produce butanol from propylene. Briefly, this process involves the reaction of propylene with syngas (CO and H₂) in the presence of a catalyst (rhodium); the product of this reaction is aldehyde, which can be further hydrogenated to butanol (Georgios, 2012). In the industry of oil refining, propylene is obtained by cracking larger hydrocarbon molecules and separated by distillation.

However, prior to 1950s, butanol was mainly produced through fermentative pathways, or also known as ABE fermentation. As early as 1910, Chaim Weizmann and coworkers successfully separated butanol producing microorganisms for synthetic rubber production. The strain, later renamed as *Clostridium acetobutylicum*, showed strong ability to utilize starch substrates for production of acetone and butanol (Jones and Woods, 1986). During the first World War, the demand of cordite (smokeless gunpowder), mainly produced from acetone, drastically increased, which accelerated the development of ABE fermentations in England with acetone as the target product,

whereas butanol became a useless byproduct stored in tanks. It should be noted that maize was the most popular substrate for the Weizmann process compared to other substrates tested including potatoes or chestnuts (Jones and Woods, 1986). As the WWI came to the end (1918), the demand for acetone dropped significantly, however, butanol became the focus of the booming auto industry as butanol and its derivative such as butyl acetate turned out to be excellent solvents for nitrocellulose lacquers (Jones and Woods, 1986). With the outbreak of the Second World War, interests in acetone started to rise again, and many alcohol distilleries have been repurposed to produce more acetone. New clostridial strains were isolated to ferment different substrates such as molasses with higher solvent titers.

The ending of the Second World War marked the beginning of the decay of ABE fermentation. In fact, solvent production through ABE process almost stopped in Western Countries as the fast-developing butanol production through petrochemical pathways were shown to be more economic (substrates for ABE fermentation such as maize and molasses became unaffordable) and less labour intensive. However, in the 21st century, ABE fermentation has attracted tremendous interests again due to the price fluctuation and limited supply of fossil fuels, environmental concerns over petrochemical industry, global governments' determination to lower the carbon footprint and green house gas (GHG) emission, and the renewable nature of ABE process (Ueda et al., 2011).

1.2.3. Fermentative Solvent Production

Solvents (acetone, butanol, ethanol, isopropanol, and 1,3-propanediol) can be produced by a number of clostridia, including *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*. Among these species, *C. acetobutylicum* is one of the earliest strains isolated for the production of acetone and butanol from starchy substrates. Other similar strains that are isolated later focused on utilization of substrates such as molasses. In general, clostridia are spore-forming Gram positive anaerobic bacteria. It has the ability to use a wide range of substrates from monosaccharides including many pentose (five-carbon sugars) and hexose (six-carbon sugars) to polysaccharides such as starch (Jones and Woods, 1986).

A typical ABE fermentation can be separated into two phases: the exponential growth phase also known as "acidogenesis" and the stationary phase also known as "solventogenesis". In acidogenesis, the fast-growing clostridium cells started to convert carbohydrates into organic acids including acetic and butyric acids, thus lowering the external pH (<5). In response to this, the clostridium cells enter into the second phase, "solventogenesis" or stationary phase, where the cells started to reassimilate the acids previously produced (mainly acetate and butyrate) and convert them into neutral solvents such as acetone, butanol and ethanol.

Accompanying with the production of solvents, the clostridial cells also exhibit significant morphology change as the rod-shaped cells at acidogenesis stage became swollen and look like a cigar, as carbohydrates are accumulated inside the cells in the form of granulose, which is mainly composed of α -1,4-linked polyglucan (Shaheen et al., 2000). Being able to convert acids into solvents helps clostridial cells to maintain a proton gradient along the cell membrane. In the ending stage of acidogenesis (low pH), significant amount of acetic and butyric acids present in undissociated form, which are lipophilic and can easily diffuse across the cell membrane (Skrivanová and Marounek, 2007). Once they are inside the cytoplasm, they can dissociate again and thus causing the proton gradient along cell membrane to collapse. Such H^+ gradient are essential to many important cellular activities such as synthesis of ATP and transport of various solutes (Mitchell, 2011). Meanwhile, when solvents (especially butanol) are accumulated to a certain amount, it tends to increase the fluidity of membrane and disrupt the function of membrane-related proteins (Bowles and Ellefson, 1985). In the meantime, the cells start synthesizing endospores by consuming the previously stored granulose to guarantee long-term survival. The spores can tolerate various stress conditions such as UV light, heat, drought, or frost. Hence, when provided with a better condition, the spores will germinate and start the cell cycle again (Wang et al., 2014).

1.2.4. Metabolism of monosaccharides

Glucose metabolism

As mentioned above, solventogenic clostridia can utilize a wide variety of monosaccharides including many hexose and pentose. Glucose represents the most

common hexose and was preferred by the bacteria compared to other monosaccharides such as xylose. At the beginning stage of fermentation, the cells employ EMP (Embden-Meyerhof-Parnas Pathway) or glycolysis to convert 1 molecule of glucose into 2 molecules of pyruvate, with net generation of 2 molecules of ATP and 2 molecules of NADP (as shown in Fig 1.1). Glycolysis occurs nearly in all organisms, both aerobic and anaerobic; its ubiquitous existence suggest that it is one of the most ancient metabolic pathways (Romano and Conway, 1996).

The glycolysis pathway can be separated into two phases, the first phase (reactions 1 to 5) is referred to as energy investment phase, where 2 ATPs are consumed to phosphorylate 1 molecule of glucose; the second phase is energy generation phase (reactions 6-10), where 2 NADHs and 4 ATPs are generated for generations of 2 pyruvate. It is interesting to note that some reactions are not favourable at standard conditions due to a positive Gibbs energy, such as reaction 4, however, the concentrations of product (in the case of reaction 4, Dihydroxyacetone phosphate and D-Glyceraldehyde-3-Phosphate) are kept low enough in the cells to favour the forward reaction.

The product of glycolysis (pyruvate) was further used as substrates for production of acids and solvents (as shown in Fig 1.2). Although many clostridia contain lactate dehydrogenase which catalyzes the conversion of pyruvate to lactic acid, however, lactate is not a major fermentation product, except under stress conditions (such as iron or sulphur limitation) where the pyruvate to acetyl-CoA pathway is partially blocked (Bahl and Gottschalk, 1984; Bahl et al., 1986).

In reaction 1, pyruvate-ferredoxin oxidoreductase (PFOR) cleaved the pyruvate that resulted from glycolysis in the presence of CoA to produce carbon dioxide and acetyl-CoA, meanwhile, the ferredoxin in oxidized-form was reduced and two electrons was transferred (Uyeda and Rabinowitz, 1971). The PFOR of clostridia is very unstable in the presence of oxygen, upon exposure to pure oxygen, the enzyme lost 50% of its activity within one hour, however, the inactivation was not observed under nitrogen within 24 h (Gheshlaghi et al., 2009).

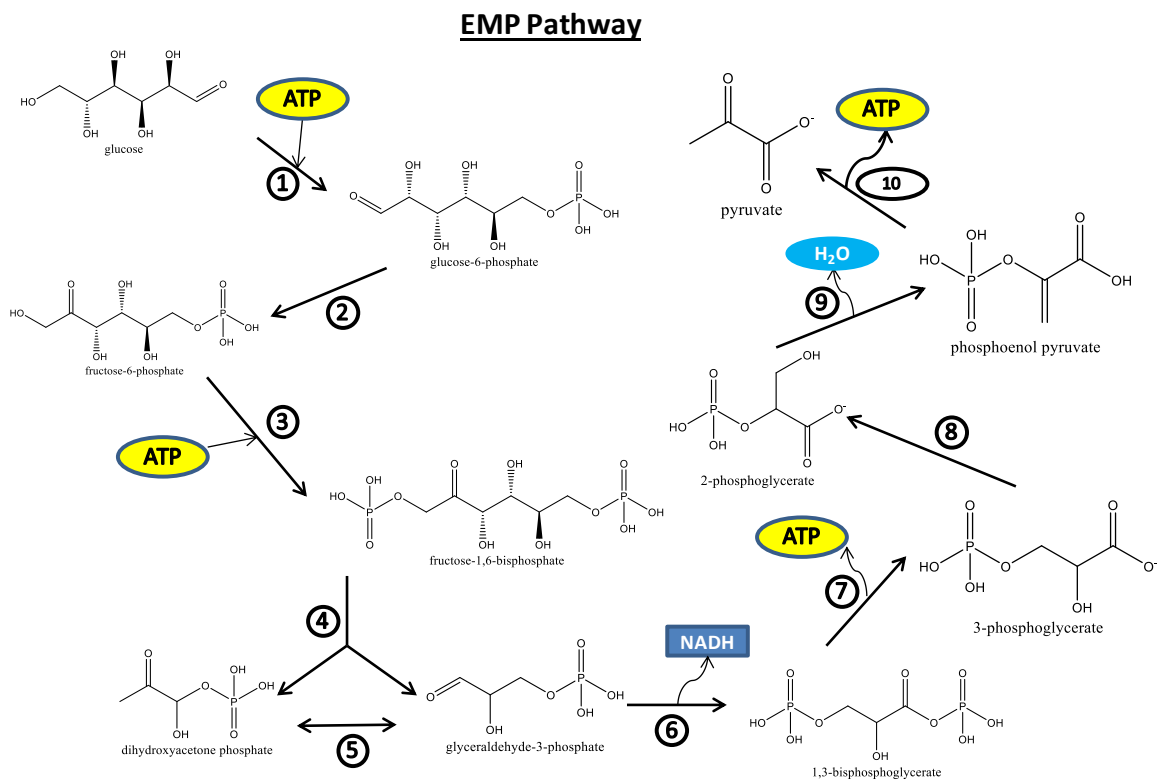


Figure 1.1 An overview of glycolysis of solventogenic clostridia (Romano and Conway, 1996). Solid arrow represent intracellular reactions which are numbered. The enzymes for each reaction are provided as follow: 1. Hexokinase; 2. Phosphoglucosomerase; 3. Phosphofructokinase; 4. Fructose-1,6-Bisphosphate Aldolase; 5. Triose Phosphate Isomerase; 6. Glyceraldehyde-3-Phosphate Dehydrogenase; 7. Phosphoglycerate Kinase; 8. Phosphoglycerate Mutase; 9. Enolase; 10. Pyruvate Kinase.

In the next reaction (reaction #2), under the catalyst of thiolase, two molecules of acetyl-CoA were condensed into 1 molecule of acetoacetyl-CoA, which is the precursor of butyric acid and butanol (Bennett and Rudolph, 1995). Thiolase compete with phosphate acetyltransferase in reaction #10 during acidogenesis and acetylaldehyde dehydrogenase during solventogenesis in reaction #12 for the available acetyl-CoA from last step.

Production of acetic acid through reaction #10 can generate twice the amount of ATP compared to the formation of butyric acid following reaction #2 from one molecule of acetyl-CoA. Thus, the regulation of ATP is highly dependent on thiolase. On the other hand, the ratio of butanol to ethanol and acetone could also be influenced by thiolase.

From this point, acetoacetyl-CoA was sequentially catalysed to become butyryl-CoA by β -hydroxybutyryl-CoA dehydrogenase (reaction #3), enoyl-CoA hydratase (crotonase in reaction #4) and butyryl-CoA dehydrogenase (in reaction #5) before branched into butyric acid and butanol.

Meanwhile, acetoacetyl-CoA is also the precursor for acetone. In reaction #14 and #15 (Figure 1.2), CoA transferase transfers CoA from acetoacetyl-CoA to either acetate or butyrate, respectively (Andersch et al., 1983). The product, acetoacetate, is subsequently decarboxylated by acetoacetate decarboxylase (reaction #16) to produce acetone and carbon dioxide. These two steps were found to be solely responsible for the uptake of acetate and butyrate and the latter step was implied to provide the driving force due to its large equilibrium constant (Hartmanis et al., 1984).

The formation of acetyl-P (butyryl-P) from acetyl-CoA (butyryl-CoA) was catalyzed by phosphate acetyltransferase (phosphate butyryltransferase) as shown in reaction #10 or #17, and was subsequently catalyzed by acetate kinase or butyrate kinase to form ATP and acetate or butyrate in reaction #11 or #18. The activity of acetate kinase was found to decrease as the acetate concentration increased, however, the activity of butyrate kinase remain constant throughout the fermentation and was not inhibited by elevated butyrate concentration (Ballongue et al., 1986).

During the solvent production phase, acetaldehyde/butyraldehyde dehydrogenase catalyzes the conversion of acetyl-CoA/butyryl-CoA to acetaldehyde/butyraldehyde with the formation of CoA. In fact, a single enzyme protein was considered to carry both the activities of acetaldehyde dehydrogenase and butyraldehyde dehydrogenase (Palosaari and Rogers, 1988). In the end, butanol is produced from reduction of butyraldehyde by butanol dehydrogenase at the expense of a reduced NAD(P). In a typical ABE fermentation, multiple products are produced at different phases, thus making it difficult to have a simple equation to summarize the complicated biochemical reactions associated with ABE fermentations; however, the reactions during acidogenesis and solventogenesis can be best approximated by the following equations (Lütke-Eversloh and Bahl, 2011).

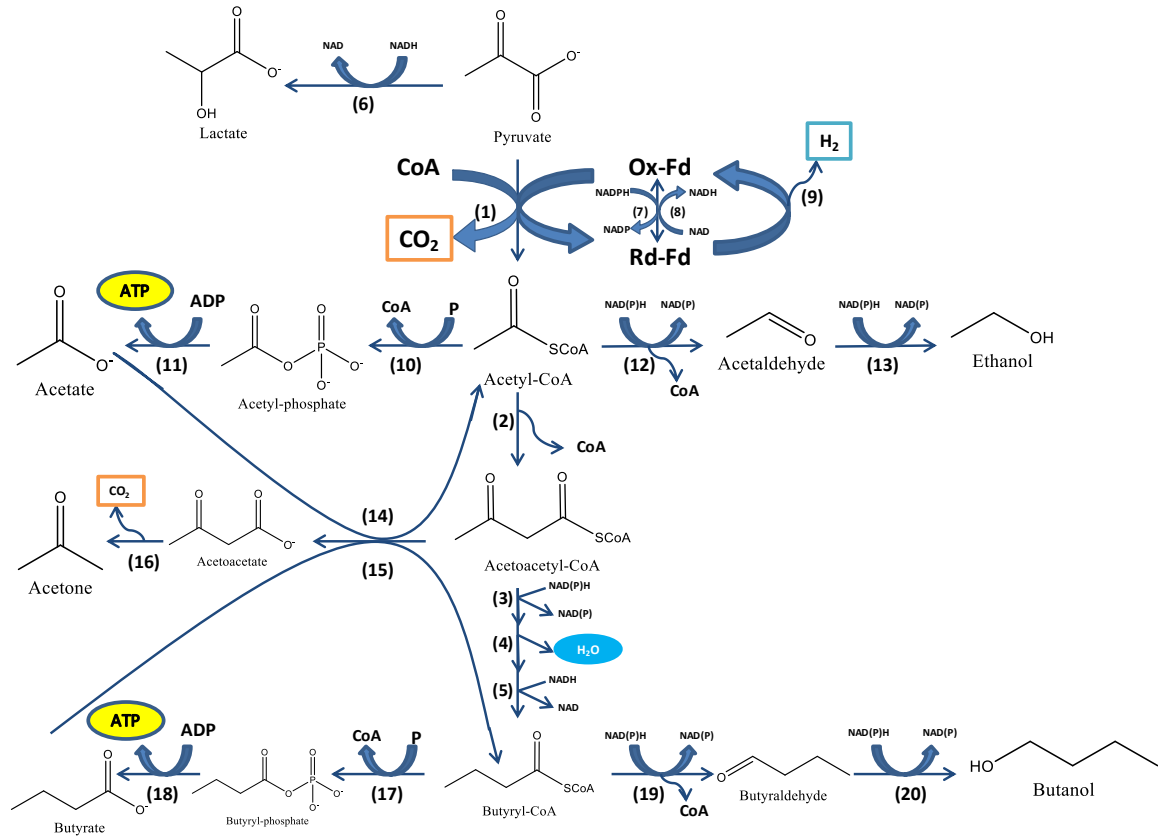
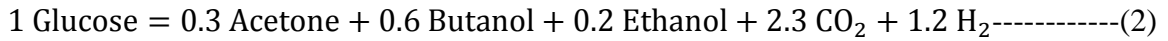
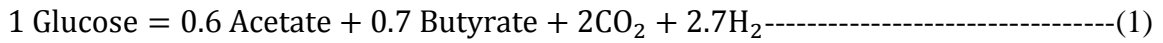


Figure 1.2 Solventogenic pathways of clostridia (modified from Gheshlaghi et al., 2009). The names of enzymes for each numbered reaction are provided as follow: 1. pyruvate-ferredoxin oxidoreductase; 2. thiolase; 3. β -hydroxybutyryl-CoA dehydrogenase (BHBD); 4. enoyl-CoA hydratase (crotonase); 5. butyryl-CoA dehydrogenase; 6. lactate dehydrogenase; 7. Ferredoxin-NADP reductase/NADPH-ferredoxin oxidoreductase; 8. Ferredoxin-NAD reductase/NADH-ferredoxin oxidoreductase; 9. Ferredoxin hydrogenase; 10. Phosphotransacetylase (phosphate acetyltransferase); 11. Acetate kinase; 12. Acetaldehyde dehydrogenase; 13. Ethanol dehydrogenase (NADH or NADPH linked); 14. Acetoacetyl-CoA: acetate: transferase; 15. Acetoacetyl-CoA: butyrate: CoA-transferase; 16. Acetoacetate decarboxylase; 17. Phosphotransbutyrylase; 18. Butyrate kinase; 19. Butyraldehyde dehydrogenase; 20. Butanol dehydrogenase.

Xylose metabolism

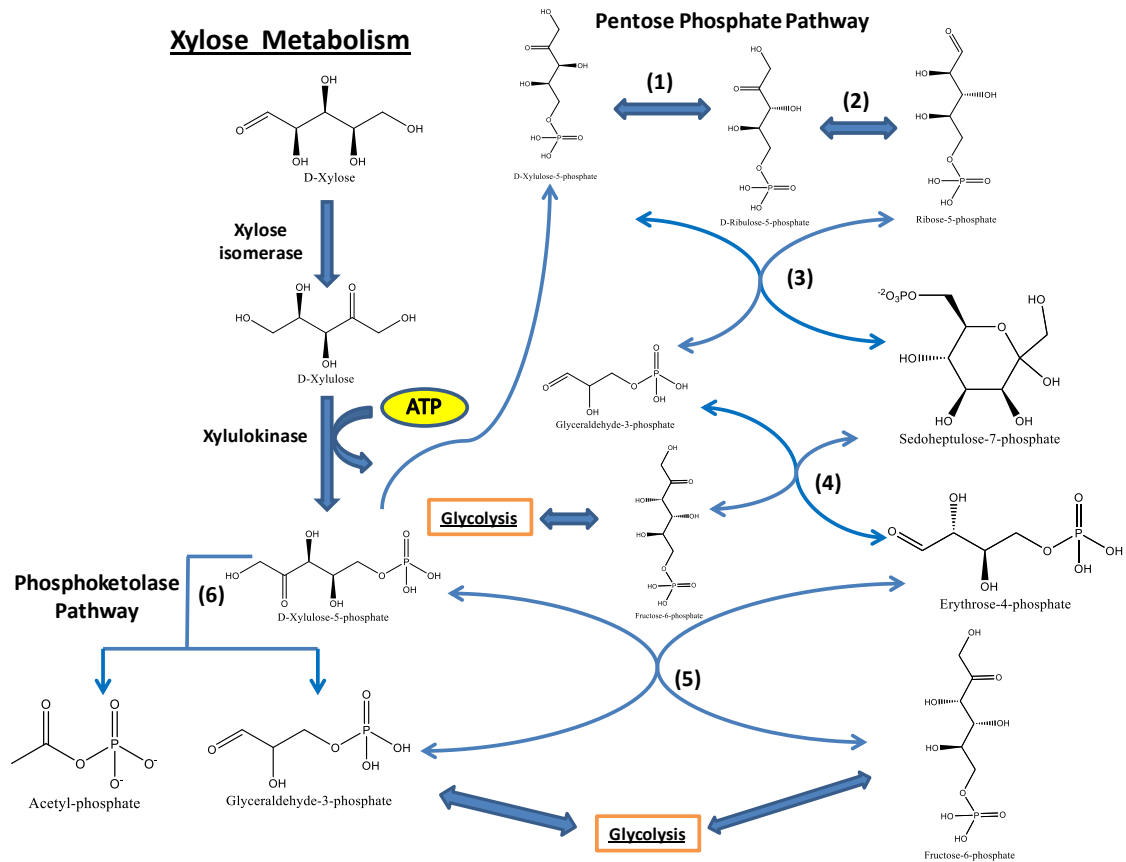


Figure 1.3 Xylose metabolism in solventogenic clostridia (modified from Liu et al., 2012). Double-headed arrows represent reactions assumed to be reversible. The enzyme-catalyzed reactions are numbered and the names of enzymes are listed below: (1), Ribulose 5-Phosphate 3-Epimerase; (2), Ribulose 5-Phosphate Isomerase; (3), Transketolase; (4), Transaldolase; (5), Transketolase; (6), Phosphoketolase.

Xylose, as the major product of hemicellulose degradation, can be fermented by clostridia for solvent production. Solvent yield of 28% (g/g) from xylose was obtained with *C. acetobutylicum* close to the solvent yield of 32% from glucose (Ounine et al., 1983). However, when a mixture of both glucose and xylose was fermented with *C. acetobutylicum*, xylose uptake was repressed until glucose was almost exhausted (Ounine et al., 1985).

In general, there are two major xylose metabolic pathways: xylose isomerase pathway used by bacteria and oxidoreductase pathway used by fungi. The xylose metabolic pathways for clostridia has been summarized in Fig 1.3. In this pathway, xylose is first converted into xylulose catalyzed by xylose isomerase, however, the reverse reaction is actually more thermodynamically favourable; to promote the equilibrium moving toward the formation of xylulose, xylulose was phosphorylated by xylulokinase to form xylulose-5-phosphate in the next reaction with the consumption of ATP. When xylose is used as the only carbon source, xylulose-5-phosphate is generated as mentioned above and enter into the non-oxidative metabolic steps of the Pentose Phosphate Pathway (PPP), where xylulose-5-phosphate are converted back to fructose-6-phosphates and glyceraldehyde -3-phosphate, both of which are intermediates of the glycolysis pathway. The reactions are highly reversible and at equilibrium, which result in a pool of 3- to 7-carbon sugar phosphates (Jeffries, 1983).

Another possible xylose pathway is the phosphoketolase pathway, where xylulose-5-phosphate is converted into glyceraldehyde-3-phosphate and acetyl phosphate catalyzed by phosphoketolase (Fig 1.3). The acetyl-phosphate generated can be further linked to the production of acetate and ATP by acetate kinase (Fig 1.2) (Jeffries, 1983). In a recent study, the activity of phosphoketolase was revealed: at low xylose concentration (10 g/L), 85% xylose was metabolized through the pentose phosphate pathway and 15% entered the phosphoketolase pathway; however, at high xylose concentration (20 g/L), the phosphoketolase pathway contributed up to 40% of the xylose catabolic flux in *C. acetobutylicum* with an elevated xylose uptake rate (Liu et al., 2012).

1.2.5. Starch degradation by solventogenic clostridium

Starch is a cheap and widely available sugar polymer, and it has been used as a substrate for industrial productions of bio-ethanol and ABE. As shown in Fig 1.4, starch is composed by two components: amylose, which is linear chains of glucose connected by α -1,4-glycosidic bonds; and amylopectin, where glucose units are linked linearly by α -1,4-glycosidic bonds, however, branching also take place with α -1,6-glycosidic bonds. The highly branched amylopectin provide more reducing ends compared to amylose for

enzymes to attach, therefore it can be degraded faster; the amylose, in contrast, is more compact, less amorphous and slower to hydrolyze (Byrnes et al., 1995).

For bio-ethanol production from starch substrates, a separate saccharification stage that converts starch to simple sugars has to be included before the subsequent fermentation. However, solvent-producing clostridia have been shown to synthesize different amylolytic enzymes that contribute to the breakdown of starch. The enzymes that are responsible of hydrolysing α -1,4-glycosidic bonds include the endo-acting α -amylase (act in the middle of the polysaccharide chain), β -amylase and glucoamylase (which remove maltose and glucose units from the non-reducing end of the polymer chain, respectively). On the other hand, the α -1,6-glycosidic bonds in amylopectin is hydrolysed by pullulanase, which is widespread among saccharolytic clostridia (Mitchell, 1998).

Starch is insoluble in cold water or alcohol. The molecules of starch arrange themselves in semi-crystalline structure that are resistant to enzymatic hydrolysis; however, with the presence of heat, starch can become soluble in water. This process is called starch gelatinization (Eliasson, 1986). When heated, water are absorbed and causes the granules to swell. The crystalline structure of starch is lost and opened up for enzyme actions. Meanwhile, the viscosity of the mixture is increased significantly (Uthumporn et al., 2010). When gelatinized starch is cooled or left at lower temperature for long period of time, the viscosity of the mixture further increases to form a gel, this process is called starch retrogradation. This is mainly because amylose and the linear parts of amylopectin will rearrange themselves to form a crystalline structure again through the formation of hydrogen bonds between parallel linear chains. During retrogradation, a small amount of water can be observed on top of the gel, and the starch become less digestible.

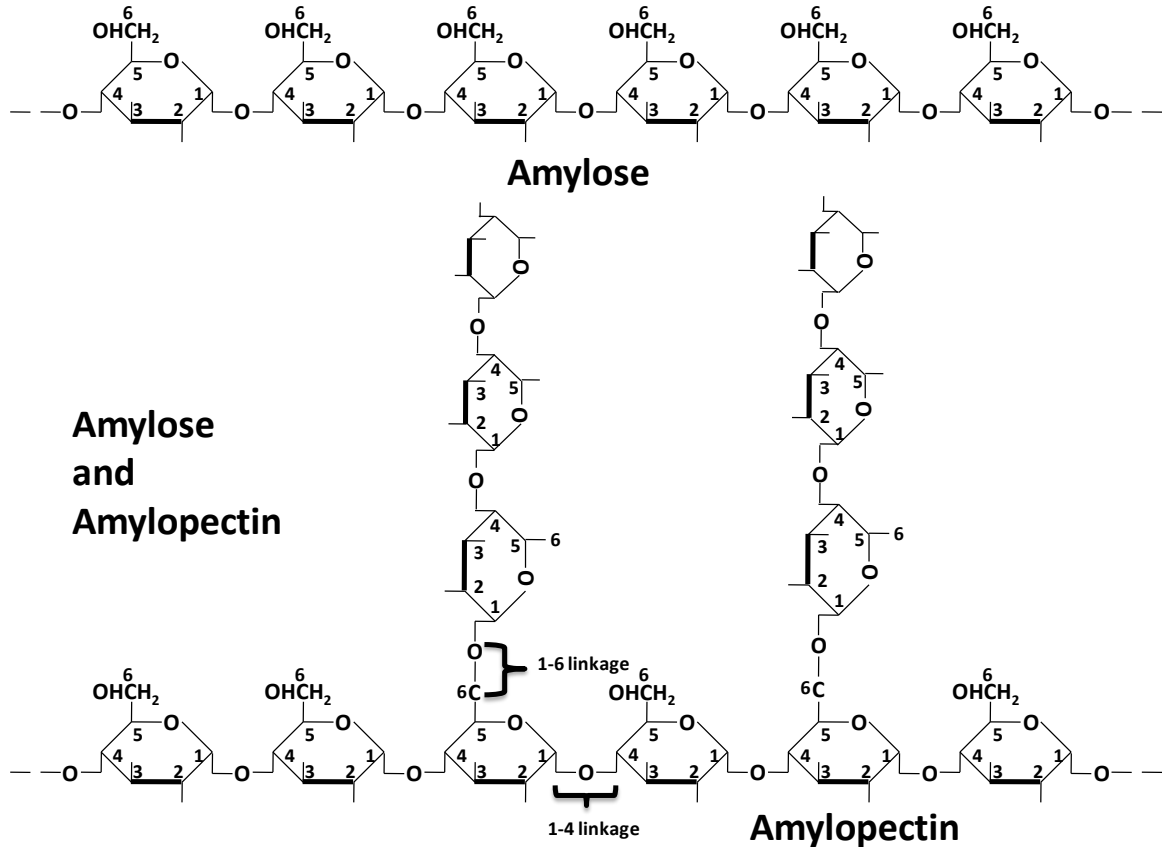


Figure 1.4 Compositions of starch: Amylose and amylopectin (adapted from Generalic, 2015).

In recent years, novel starchy substrates have been utilized for the production of bio-butanol in aims of lowering the substrate cost. As summarized in Table 1.1, butanol fermentation from cassava starch, corn starch, sago starch and waste packing peanuts were compared with glucose fermentation. Similar butanol productions (15-16 g/L) was obtained from both starch-based and glucose-based substrates. However, the utilization rate of glucose-based substrate is much higher (>95% of the initial substrate concentration) compared to those with starch-based substrate (69.1 to 84.1%). From an economic point of view, the un-utilized starch substrate at the end of fermentation will result in a waste of carbohydrate resource. On the other hand, solvent productivity is also much slower with starch substrate compared to glucose, this is probably due to an extra phase of starch hydrolysis by the action of amylolytic enzymes from the strain.

Table 1.1 Butanol fermentation from starch-based substrates

Substrates	Strain	Initial Carbohydrate Conc. (g/L)	Substrate utilization (%)	Butanol/ABE Conc. (g/L)	Butanol/ABE Yield (g/g)*	Solvent Productivity (g/L/h)	Ref.
Glucose	1	65.9 ^a	96.4	16.2 (24.2)	0.26/0.38	0.67	
Cassava chips hydrolysate	1	65.1 ^a	96.2	16.4 (23.1)	0.25/0.37	0.64	
Cassava chips	1	60 ^b	78.3	15.5 (19.4)	0.30/0.38	0.40	(Thang et al., 2010)
Cassava starch	1	60 ^b	78.2	16.9 (21.0)	0.33/0.41	0.44	
Corn starch	1	50 ^b	78.6	16.2 (20.7)	0.37/0.48	0.31	
Sago starch	1	60 ^b	69.1	15.5 (19.6)	0.34/0.43	0.27	
Waste Packing Peanuts	2	69.6 ^b	84.1	15.7 (21.7)	0.24/0.34	0.20	(Jesse et al., 2002)
Sago	3	30 ^b	-	8.38 (11.03)	0.25/0.33	0.26	
Corn	3	30 ^b	-	8.61 (11.87)	0.26/0.36	0.18	(Madiah et al., 2001)
Potato	3	30 ^b	-	3.34 (4.62)	0.10/0.14	0.06	
Tapioca	3	30 ^b	-	4.89 (6.74)	0.15/0.20	0.16	

1. *C. saccharoperbutylaceonicum*; 2. *C. beijerinckii* BA101; 3. *C. acetobutylicum* P262;
^a Glucose; ^b Starch;
*. Yield was calculated as the concentrations of butanol (ABE) divided by glucose concentrations consumed by the strain, starch substrates was converted into potential glucose by multiplying a factor of 1.1.

1.2.6. Bio-butanol Production from Lignocellulosic Biomass

One of the factors that severely affect the economics of ABE fermentations is high cost of substrate (Qureshi and Blaschek, 2001). As mentioned earlier, the once commercialized ABE fermentation was ceased due to the rising prices of conventional substrates such as corn and molasses and the competition with the more economically favourable chemical synthesis of butanol from cheaper fossil fuel resources. According to an economic analysis, the cost of feedstock accounts for 65% of the total production cost based on

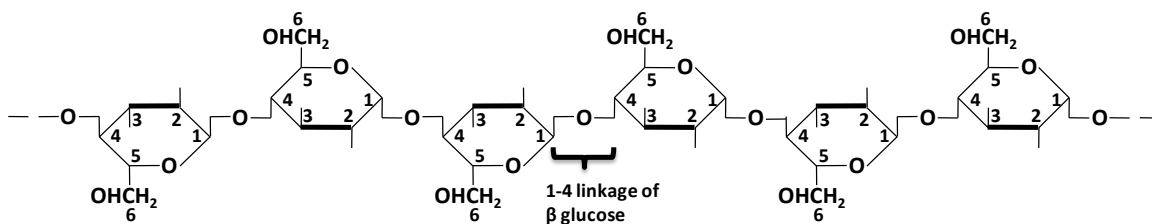
10,000 tons butanol production/year (M. Kumar et al., 2012). Therefore, the revival of bio-butanol production is mostly dependent on search of cheap and widely available carbohydrate source.

In recent years, successful utilization of low-cost lignocellulosic raw materials has motivated the development of bio-butanol production. Lignocellulosic biomass represent the most abundant renewable energy source on the planet (Qureshi et al., 2013). The annual production of lignocellulosic biomass is predicted at 1.3 billion dry tons in US, enough to generate biofuels to meet the current domestic demand for one-third of transportation fuels. Agricultural residues and wastes are common examples of lignocellulosic biomass. These materials are normally the non-edible parts of food-crops, such as corn stover, corn cobs, wheat straw, barley straw, sugarcane bagasse, which are readily available in agricultural-based countries. The other example is dedicated energy crops including woody crops such as willow and poplar and perennial grasses such as switchgrass and miscanthus. Recently, the cost of raw lignocellulosic biomass is in the range of \$33 to 61/ton compared to \$1110/ton for glucose, \$190/ton for corn, and \$740/ton for sago (M. Kumar et al., 2012). It is obvious that non-cellulosic biomass are much more expensive compared to cellulosic biomass, however, with the exception of sugarcane (market price at \$47/ton). Although the growth of dedicated energy crops may still result in competition of land and water with food crops, the maintenance and harvest cost for these materials are much lower compared to food crops.

What is lignocellulose?

In general, lignocellulose biomass are mainly composed by three different types of polymers, namely cellulose, hemicellulose and lignin.

Cellulose, as depicted in Fig 1.5, is a long linear chain of glucose units. Due to the β -1,4-linkages, every other glucose are flipped over, thus promoting the formation of hydrogen bonds between parallel chains and contributing to the resistance of crystalline to degradation (Rubin, 2008).



Cellulose

Figure 1.5 Molecular structure of cellulose (adapted from O'sullivan, 1997)

Hemicelluloses, in comparison, are polysaccharides that contains many different types of sugar monomers, such as xylose, mannose, galactose, rhamnose, and arabinose. In most cases, xylose is present in the biggest amount (Karimi et al., 2006). Structures and compositions of these polysaccharides including xyloglucan, glucuronoxylan, glucuronoarabinoxylan, (gluco) mannan, galactoglucomannan, and β -(1-3, 1-4)-glucan have been well discussed in a recent review paper (Scheller and Ulvskov, 2010). The suffix of these polysaccharides such as -glucan, -xylan and -mannan indicates the composition of the backbone of each polysaccharide, respectively. For example, xyloglucan has a backbone of glucose units connected by β -1,4 linkages, with xylose as the major component in the side chains (Scheller and Ulvskov, 2010). Compared to the cellulose, the polysaccharides of hemicelluloses have shorter chain length with branched structure, and are more vulnerable to reactions and easier to hydrolyze (Chen, 2014).

Lignin is an aromatic polymer composed by three basic phenolic compounds, which are p-coumaryl alcohol (H), coniferyl-alcohol (G), and sinapyl alcohol (S) as shown in Fig 1.6 (Rubin, 2008). The ratio between the building blocks within the polymer may vary between plants species, for example, coniferyl-alcohol (G) accounts for 90-95% of the lignin components in softwoods (Chen, 2014). The basic units of lignin are linked to each other through ether (C-O) or carbon-carbon (C-C) bonds, with β -O-4 as the dominant ether bonds, and β -5 linkage as the major C-C bonds (Chen, 2014). Generally, lignin has two major biological functions. First, it increases the strength and rigidity of the lignocellulosic matrix by cross-linking with carbohydrate polymers, thus providing resistances to pathogens and insects (K. Ritter, 2008). Second, lignin plays a crucial part

in conducting water through plant stems. Lignin is hydrophobic whereas cellulose and hemicellulose are hydrophilic, by cross-linking with the above carbohydrate polymers, it stops water from permeating through cell walls, thus making the cell wall more efficient in conducting water (Iiyama et al., 1994).

Table 1.2 summarized the percentage of the major components in common lignocellulosic biomass. The ratio of each components varies from one plant species to another, however, for most lignocellulosic biomass, cellulose is normally the dominant polysaccharides in plant cell walls, followed by hemicellulose and lignin (Mussatto and Teixeira, 2010).

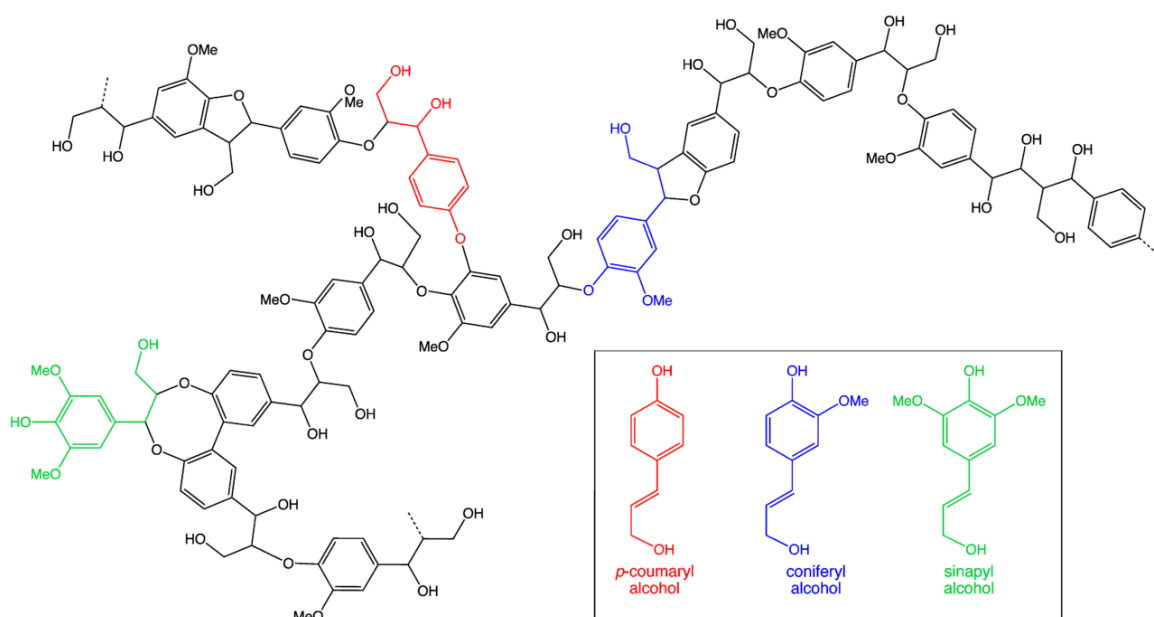


Figure 1.6 Lignin structure and three building blocks of lignin(Retrieved April 20, 2016, from <https://commons.wikimedia.org/wiki/File:Lignin.png>)

Table 1.2 Percentage of major components of common lignocellulosic biomass (Adopted from Iqbal et al., 2013)

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Sugar cane bagasse	42	25	20	(Kim and Day, 2011)
Sweet sorghum	45	27	21	(Kim and Day, 2011)
Hardwood	40-55	24-40	18-25	(Malherbe and Cloete, 2002)
Softwood	45-50	25-35	25-35	(Malherbe and Cloete, 2002)
Corn cobs	30	33	13	(Syawala et al., 2013)
Corn stover	39	28	18	(Zhu et al., 2009)
Rice Straw	32.1	24	18	(Prasad et al., 2007)
Nut shells	25-30	25-30	30-40	(Abbasi and Abbasi, 2010)
Newspaper	40-55	25-40	18-30	(Howard et al., 2003)
Grasses	25-40	25-50	10-30	(Malherbe and Cloete, 2002)
Wheat straw	29-35	26-32	16-21	(McKendry, 2002)

Factors limit the accessibility of lignocellulosic sugars

The utilization of the carbohydrates in lignocellulosic biomass is not an easy task. Solventogenic clostridia cannot directly utilize cellulose or hemicellulose as substrate, therefore, these polysaccharides need to be hydrolysed into simple sugars either chemically or enzymatically prior to fermentation (Dong et al., 2014). The enzymatic approach is preferred by biofuel industry due to many reasons including milder conditions, lower energy costs, and potential for high yields and selectivity (Yang et al., 2011). However, both chemical and enzymatic hydrolysis is limited by the recalcitrant structure of lignocellulosic biomass.

Lignin is considered as a physical barrier that prevents the enzymes from accessing the polysaccharides of lignocellulose (Hu and Ragauskas, 2012). On the other hand, enzymes tend to irreversibly bind to lignin through hydrophobic interactions, which resulted in significant loss in enzyme activity and led to high enzyme dosage (L. Kumar et al.,

2012). In fact, positive relations have been found between sugar yield and lignin removal in a number of different types of lignocellulose materials (Han et al., 2012; Nlewem and Thrash, 2010; Yan et al., 2014; Zhang et al., 2016).

Hemicelluloses, as a family of polysaccharides, also serves as another protective coat to cellulose. On one hand, hydrogen bonds can be formed between the unbranched part of hemicellulose and the surface of cellulose fibrils; on the other hand, hemicellulose can be covalently bonded to lignin to form cross-links which makes the enzymes difficult to penetrate (Chundawat et al., 2011). Since enzymes need to bind to the surface of cellulose before the hydrolysis takes place, lignin and hemicellulose need to be removed or hydrolysed without losing hemicellulose sugars (Öhgren et al., 2007).

Other factors that may be influential to the deconstruction of lignocellulose include cellulose crystallinity, DP (degree of polymerization), particle size, pore volume and specific surface area of cellulose (Hu and Ragauskas, 2012). The crystallinity of cellulose is the result of the intra- and inter-molecular hydrogen bonds. Although generally cellulose with higher amorphous content tend to be digested by enzymes more easily, it is not recommended to use CrI (crystallinity index) as the only indicator for cellulose digestibility (Park et al., 2010). Degree of polymerization is also considered to play a role in the extent of enzymatic hydrolysis. A decrease in the degree of polymerization (DP) will produce more reactive ends available to the exo-cellulases (Kleman-Leyer et al., 1994). It is a common practice to grind lignocellulose into smaller particle size for higher conversion efficiency. However, the concept of "the smaller, the better" has not necessarily been the case. Many studies showed that further reduction in particle size (already at the level of millimetres) does not result in any significant change in the sugar yield (B. Rivers and H. Emert, 1987; Ballesteros et al., 2002).

The surface area of cellulose has been considered as a critical factor for enzymatic hydrolysis yield and rate since the contact between substrate and enzymes is necessary for hydrolysis to occur. Surface area of substrate can be divided into interior and exterior surface area (Wang et al., 2012). Exterior surface area is mainly determined by particles size, which has been discussed briefly earlier; however, interior surface area, which is essentially reflected by biomass porosity, has been show to contribute more than 90% of

the enzymatic digestibility (Wang et al., 2012). It has been found that the lower enzymatic digestibility of corn stover at bigger range of particles size (425–710 μm) was compensated to the similar level of corn stover at smaller particle size (53–75 μm) after both types of particles were pretreated with liquid hot water. The authors attribute this phenomena to the disruption of inner structure of corn stover particles due to pretreatment, thus potentially increasing the pore volume and the accessible surface area of the substrates (Zeng et al., 2007); however, the effect of lignin removal was not evaluated in the research, which could have contributed to the digestibility increase.

Pretreatment of lignocellulose for ABE Fermentation

Due to the recalcitrant nature of lignocellulosic biomass, pretreatment is required to break the restriction imposed by lignin and hemicellulose structure. In the past decade, a number of pretreatment methods was developed and studied, including dilute sulfuric acid (DAP), liquid hot water (LHW), steam explosion, alkali pretreatment, and ammonia fiber explosion (AFEX), etc (Hendriks and Zeeman, 2009; Hu and Ragauskas, 2012).

Dilute sulfuric acid pretreatment

Dilute sulfuric acid pretreatment (DAP) has been applied to a wide range of biomass including hardwood, softwood and agricultural residues (Jensen et al., 2010; Nlewem and Thrash, 2010). It is normally performed at a temperature range of 120 to 210 $^{\circ}\text{C}$, with acid concentration less than 4% (w/v), and residence time from seconds to hours, in different types of reactors (Hu and Ragauskas, 2012). During DAP, most xylan is hydrolyzed to xylose and accumulated in the liquor, and the resulted xylose may be further converted into furfural at elevated pretreatment severity (Hendriks and Zeeman, 2009). However, the cellulose component will not be hydrolyzed unless the conditions are too severe, but an increase in crystallinity may be observed due to solubilisation of the amorphous portion of cellulose (Foston and Ragauskas, 2010). Interestingly, DAP does not lead to significant delignification. Although the employment of DAP can improve enzymatic digestibility of lignocellulosic biomass, undesired by-products such as furfural, 5-hydroxymethylfurfural (HMF), levulinic acid and formic acid may be generated. These compounds have been shown to be toxic in the subsequent enzymatic hydrolysis and fermentation stage (Larsson et al., 1999; Palmqvist and Hahn-Hägerdal, 2000).

Liquid hot water pretreatment

Liquid hot water (LHW) and un-catalyzed steam explosion are the two major hydrothermal pretreatment technologies, where water in liquid or vapour state is used to pretreat lignocellulosic biomass. Compared to DAP and other pretreatment methods, no chemical catalyst is required, which resulted in significant reduction in both chemicals and the materials for construction cost (Hu and Ragauskas, 2012). In LHW, water is pressurized to maintain liquid state at elevated temperatures (160-240 °C) (Alvira et al., 2010). At this temperature, water acts as a weak acid and hydrolyze hemicellulose by selectively breaking down the glycosidic linkages, with the formation of acetic acid and uronic acid (Allen et al., 2001). Since pH is maintained at a much milder range (4-7), much less sugar degradation product (furfural and HMF) is produced during LHW compared to DAP. Similar to DAP, most cellulose and lignin is preserved in solid form during LHW, and due to solubilisation of hemicellulose, significant increases in lignin content are observed among many substrates after pretreatment (Hu and Ragauskas, 2011; Kristensen et al., 2008; Kumar et al., 2009; Lee et al., 2010; Xiao et al., 2011).

Steam explosion

In steam-explosion, lignocellulosic materials is treated with high-pressure saturated steam from several seconds to a couple of minutes at a temperature range of 160-260 °C, and then the pressure is suddenly released, which causes the materials to explode and fibers to separate (Sun and Cheng, 2002). Addition of chemical catalyst such as SO₂ and H₂SO₄ can significantly increase the hemicellulose yield (Wyman et al., 2005). Similar to LHW, the hemicelluloses are dissolved as oligosaccharides due to water at high temperature, and partially hydrolyzed into monosaccharides. An increase in lignin content (acid-insoluble) has been observed after steam-explosion pretreatment, which is mainly due to the solubilisation of the hemicellulose and the very little solubilised lignin; when more severe conditions are employed, cellulose begin to degrade and pseudo-lignin are formed, which results in further increase in lignin content (Sergey M. Shevchenko and Saddler, 1999). Another risk with steam explosion pretreatment is the production of fermentation inhibitors such as furfural, HMF and phenolic compounds (Hendriks and Zeeman, 2009). Due to less water input, steam explosion consumes less energy compared to LHW ,

however, more toxic compounds could be generated (Hu and Ragauskas, 2012).

Alkali pretreatment

Alkali pretreatment is one of the major chemical pretreatment technologies that received extensive studies. It was found to be more effective on hardwoods, herbaceous crops and agricultural wastes with relatively low lignin contents compared to softwood. Alkali pretreatment is basically a delignification process, it utilizes various bases including NaOH, Ca(OH)₂, and ammonia (Zheng et al., 2009). Alkali pretreatment efficiencies are mainly affected by three parameters, reaction temperature, pretreatment time and alkali loading (Hu and Ragauskas, 2012). The mechanism of alkali pretreatment is mainly involved with cleavage of the ester bonds (saponification) that crosslink lignin and hemicelluloses under base catalyst (Zheng et al., 2009). On the other hand, hemicellulose solubilisation, although normally at a degree lower than that of LHW, in combination with substantial lignin removal, contribute to the increase in enzymatic digestibility of cellulose (Silverstein et al., 2007). Acetyl and the various uronic acid substitutions on hemicellulose that lowers the accessibility of enzymes to the surface of cellulose and hemicelluloses are also removed during alkali pretreatment (Mosier et al., 2005). Other effects include the decrease of DP of cellulose, swelling of cellulose, and thus leading to an increase in the internal surface area (Rojo et al., 2013).

Ammonia fiber explosion pretreatment

Ammonia is a desirable chemical agent for pretreatment of lignocellulosic biomass due to the following reasons: a) it effectively causes the lignocellulosic materials to swell; b) it selectively reacts with lignin over carbohydrates and cleaves the linkages in the lignocellulose complex (LCCs); c) it is a non-corrosive and easy to recover and reuse (Kim et al., 2003). During ammonia pretreatment, lignocellulosic materials is treated with liquid ammonia at temperatures between 60 to 100 °C at high pressure for a period of time (such as 30 min), and then the pressure is swiftly released (Hu and Ragauskas, 2012). The effect of ammonia treatment or ammonia fiber explosion pretreatment (AFEX) can be considered as a combination of steam explosion and alkali pretreatment, which leads to disruption of complex cell wall structure and partial solubilisation of hemicellulose (Carvalho et al., 2008). Similar to alkali pretreatment, ammonia is able

to cleave the linkages between lignin and hemicellulose, thus facilitating the solubilisation and removal of lignin and hemicellulose, which makes the embedded cellulose microfibrils more accessible to enzymes (Bals et al., 2010). Furthermore, the sudden pressure release may lead to the formation of large pores at the middle lamella, which makes the cellulose much more accessible to enzymes (Chundawat et al., 2010).

Ionic liquid pretreatment

During the past two decades, Ionic liquids (ILs) have received increasing popularity in many fields of research (Hallett and Welton, 2011). Ionic liquids are families of salts that are liquid at room temperature or melt at slightly elevated temperatures, with negligible vapour pressure and high thermal stability (Blokhin et al., 2006; Brandt et al., 2011; Domańska and Bogel-Lukasik, 2005). One of its applications is as a pretreatment solvent to solubilise the lignocellulosic biomass, of which the solubilised cellulose can be precipitated by adding an anti-solvent such as water or alcohols with reduced crystallinity, thus making the cellulose more accessible compared to those without pretreatment by ionic liquids (Brandt et al., 2011). Another factor that could contribute to the enhanced glucose release from the precipitated cellulose has a correlation with lignin and hemicellulose removal (Doherty et al., 2010; Lee et al., 2009). The major disadvantage of ILs as pretreatment solvents are their relatively high cost, compared to conventional molecular solvents, the prices of ILs are normally 5-20 times higher (Lee et al., 2009; Plechkova and Seddon, 2008), and most of ILs are developed only at lab scale (Li et al., 2013). Although many processes have been developed to lower the production cost, typical ILs remain expensive.

Effects of different pretreatment methods

As summarized in the Table 1.3, the major effects of most pretreatment methods include increase in accessible surface area, cellulose decrystallization, removal of lignin and/or hemicellulose, and alteration in lignin structure, which contribute to the increased enzymatic digestibility of lignocellulosic biomass (Mosier et al., 2005). As mentioned above, the ability to utilize a wide range of substrates (including both hexose and pentose)

provides solventogenic clostridia the potential to fully convert lignocellulosic carbohydrates into desired products compared to glucose-restricted ethanol-producing yeast. Therefore, pretreatment methods that resulted in significant removal of hemicellulose are not optimal for ABE fermentation due to possible loss of five-carbon sugar stream.

Table 1.3 Effect of various pretreatment methods (Modified from Mosier et al., 2005)

	Increases accessible surface area	Decrystalizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Uncatalyzed steam explosion	■		■		■
Liquid hot water	■	ND	■		■
pH controlled hot water	■	ND	■		ND
Flow-through liquid hot water	■	ND	■	■	■
Dilute acid	■		■		■
Flow-through acid	■		■	■	■
AFEX	■	■	■	■	■
ARP	■	■	■	■	■
Lime	■	ND	■	■	■
Ionic liquid	■	■	■	■	■

■: major effect;
 ■: minor effect;
 ND: not determined

Inhibitors generated during pretreatment

Table 1.4 Comparison of major inhibitor concentrations obtained after different pretreatment methods (adapted from Du et al., 2010)

Compounds	Concentration (mg/L)							
	0.7 % H ₂ SO ₄	0.07 % H ₂ SO ₄	LHW	Wet oxidation	Buffer	NH ₃	Lime	Oxidative lime
Levulinic acid	41	1.5	0.48	1.9	1.5	1.7	1.1	1.6
Formic acid	120	76	55	79	110	250	43	92
Syringic acid	2	1.5	1.8	2.17	2.3	1.54	1.7	1.4
Benzoic acid	1.5	1.3	0.16	0.31	0.36	1.3	1.7	1.3
4- Hydroxycoumaric acid	5.6	14	11	11	14	11	17	8.1
Ferulic acid	6.6	2.6	2.2	1	5.1	4.2	6.6	0.76
5-HMF	44	11	2.3	2.8	4.3	0.89	2.3	3.8
Furfural	220	26	8	6.5	3.8	0.4	1.5	3.2
Syringaldehyde	1.8	0.6	1	2	1.7	0.31	1	0.084

The other factor that cannot be ignored during evaluation of pretreatment methods is the generation of fermentation inhibitors as shown in Table 1.4. Treatment of lignocellulose at high temperature and acidic conditions often results in formation and release of a range of inhibitors, which can be separated into two categories based on their origins, namely furan aldehydes and aliphatic acids from the degradation of carbohydrates, and phenolic compounds from lignin (Jönsson et al., 2013). As shown in Fig 1.7, xylose, one of the major components of hemicellulose, can be further degraded into furfural; similarly, HMF is formed from hexose (such as mannose, galactose and glucose) degradation. Further degradation of furfural or HMF will result in the formation of formic acid. Another degradation product of HMF is levulinic acid (Palmqvist and Hahn-Hägerdal, 2000).

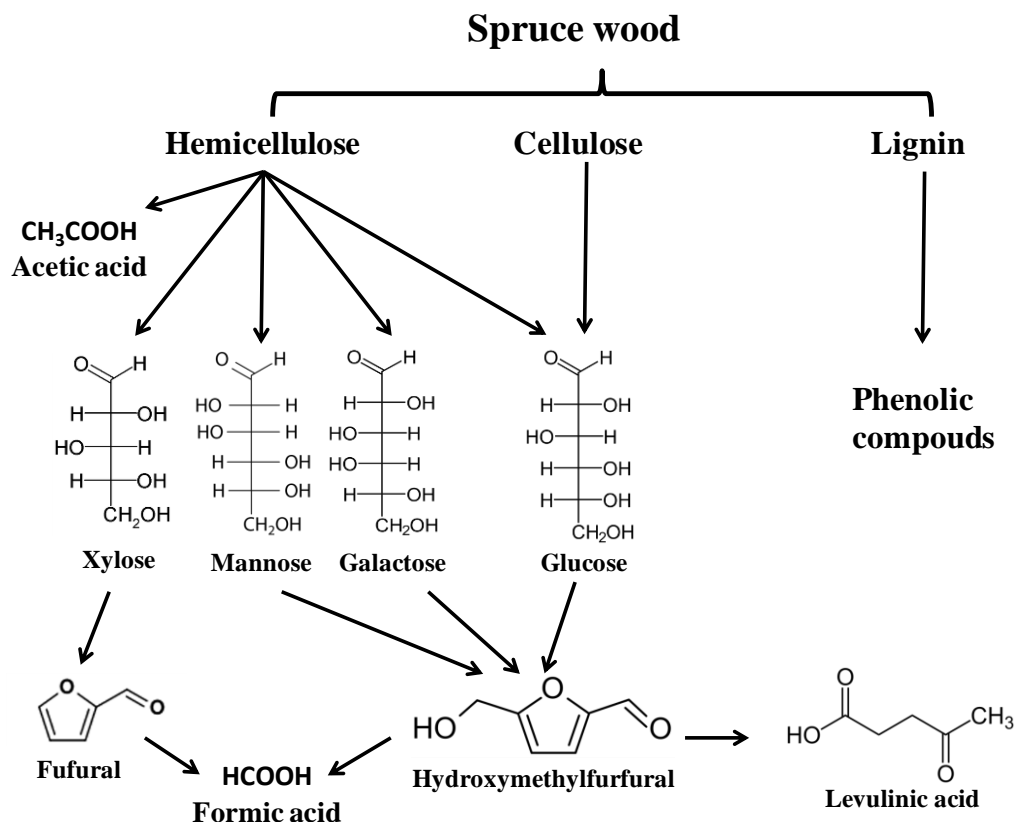


Figure 1.7 Inhibitors generated and their origins during pretreatment (Adapted from Palmqvist and Hahn-Hägerdal, 2000).

Mechanisms of inhibitors

The mechanism of inhibition by different degradation products has been previously reviewed in detail (Palmqvist and Hahn-Hägerdal, 2000). Briefly, weak acids have been shown to inhibit the growth of microorganisms and are used widely as food preservatives (Lambert and Stratford, 1999). Two theories have been proposed to explain the mechanisms of inhibition induced by weak acids. In undissociated form, acids are liposoluble and can cross the plasma membrane into cytosol; dissociation of the weak acids takes place under neutral intracellular pH, thus decreasing the cytosolic pH (Pampulha and Loureiro-Dias, 1989). According to the uncoupling theory, the decrease in intracellular pH is compensated by the action of plasma membrane ATPase, which pumps protons outside of the cell at the expense of ATP (Verduyn et al., 1992). To maintain the intracellular pH, additional ATP must be generated, and in the case of yeast, this is achieved by increased ethanol production under anaerobic condition at the expense of

biomass formation (Carmelo and Bogaerts, 1996). However, when exposed to high acid concentration, the proton pumping capacity of cells is exhausted, which resulted in depletion of intracellular ATP, dissipation of proton driving force, and acidified cytoplasm (Imai and Ohno, 1995). In comparison, the anion accumulation theory attributes the toxicity of weak acids to intracellular anion accumulation since only one proton is imported into the cell with each molecule of undissociated acid, which will diffuse into the cell until an equilibrium is reached (Palmqvist and Hahn-Hägerdal, 2000).

Both furfural and HMF have been reported to inhibit the growth of yeast at high concentrations (Larsson et al., 1999; Navarro, 1994). However, both compounds can be metabolized to less toxic forms and adapted by yeast (Banerjee et al., 1981; Taherzadeh et al., 2000), and their toxic effects can be mitigated by increased inoculum size (Navarro, 1994). Interestingly, furfural and HMF concentrations up to 3 g/L do not introduce significant negative effect on the growth and solvent production by clostridium, hence the concentrations of these two compounds in most lignocellulosic hydrolysates are within or around this range, thus making furfural and HMF less concerning as fermentation inhibitors (Cavka and Jönsson, 2013; Chandel et al., 2011; Ezeji et al., 2007).

The inhibitory effect of phenolic compounds on fermentation of lignocellulosic hydrolysates are much stronger compared to furfural and HMF at the similar levels (Ezeji et al., 2007; Lin et al., 2015). Phenolic compounds can partition into the cell membrane, increase the membrane fluidity, and cause leakage of cellular content, thereby destroying membrane as selective barriers and its structural integrity (Heipieper et al., 1994). However, the mechanism of inhibition have not been elucidated. During a fermentation test by *Clostridium beijerinckii* BA101 with glucose as substrate, ferulic acid was found to be the most toxic compound, with solvent production completely shut down and weak cell growth at a concentration as low as 0.3 g/L; followed by p-coumaric acid that resulted in 30% reduction in ABE production at 0.5 g/L; furthermore, syringaldehyde (as low as 0.3 g/L) has been found to selectively inhibit ABE production pathways rather than the growth of clostridial cells (Ezeji et al., 2007). A synergistic effect have been found when multiple inhibitors are present in the same lignocellulosic hydrolysates (Chandel et al., 2011).

Methods for inhibitor removal (Detoxification)

To overcome the issue of fermentation inhibitors resulted from lignocellulosic hydrolysis, a number of detoxification methods have been developed to remove inhibitors from the hydrolysates, which include physical (evaporation, membrane mediated detoxification), chemical (neutralization, calcium hydroxide overliming, activated charcoal treatment, ion-exchange resins, and extraction with ethyl acetate) and biological detoxifications (enzymatic mediated using laccase, lignin peroxidase) (Chandel et al., 2011).

Treatment of lignocellulosic hydrolysates with calcium hydroxide (overliming) has become one of the most effective detoxification methods and has been studied extensively with different types of hydrolysates (Jönsson et al., 2013). The mechanism of detoxification by overliming is still unclear, one possible explanation could be due to the precipitation of inhibitors (Zyl et al., 1988), however, chemical conversion of toxic compounds under alkali conditions was suggested to be the major reason (Persson et al., 2002). Although employment of overliming can result in significantly improved fermentability in lignocellulosic hydrolysate, it results in excessive sugar loss at harsher conditions (from 30% to 60%), which further deteriorate the economics of biofuel production (Martinez et al., 2001; Millati et al., 2002). On the other hand, overliming seems to be not effective when it comes to removal of phenolic compounds, which as mentioned above has been considered as the most toxic fermentation inhibitors. As was summarized recently, phenol decrease is commonly around 10-20% after the treatment (Jönsson et al., 2013), which is not satisfactory when trace amount of these toxic compounds can still exert considerable inhibitions on fermentation.

Solid-liquid detoxification methods have also been studied extensively for detoxification of lignocellulose hydrolysates, such as the application of activated carbon and ion exchange resin (Jönsson et al., 2013), which can effectively remove inhibitors and lead to hydrolysates that show a fermentation performance similar to that of an inhibitor-free model substrate (Larsson et al., 1999; Ranjan et al., 2009; Weil et al., 2002). Unlike overliming, no significant decrease in fermentable sugars was observed after the treatment with most ion-exchange resins (Nilvebrant et al., 2001). Furthermore, both anion exchanger resin and neutral resin have been proven to be efficient in removal of

phenolic compounds, which resulted in more than 90% reduction in the content of vanillin (the most abundant phenols) compared to the untreated hydrolysates (Nilvebrant et al., 2001). Another attractive feature of using resins for detoxification is that they can be regenerated after treatment, and the desorbed inhibitors can be used as valuable compounds (Ranjan et al., 2009; Weil et al., 2002). Although activated carbon was also shown to be effective in removal of inhibitors from hydrolysates without interfering with fermentable sugars, it is considered expensive since powdered activated charcoal cannot be regenerated and granular activated charcoal always incurs a 10% loss during each thermal reactivation cycle (Ranjan et al., 2009).

Other detoxification methods also include ethyl acetate extraction and biotechnological detoxification. Although ethyl acetate extraction is an efficient method for removal of a wide range of inhibitors including phenolic compounds, the residual ethyl acetate could impose certain degree of toxicity to the fermenting organism; on the other hand, regeneration of ethyl acetate is necessary to make this process more economically competitive, which, unavoidably, results in more energy input due to distillation. Some microorganisms have proved to be able to use fermentation inhibitors as substrates for growth, thereby significantly decreasing the inhibitor levels when the acid hydrolysates was pre-cultivated with these microorganisms prior to subsequent fermentation (López et al., 2004; Okuda et al., 2008). However, biological detoxification normally requires a much longer reaction time (> 1 day) and sometimes consumes the fermentable sugars present in the hydrolysates (Bin and Charles E, 2008).

One of the major objection against the detoxification in general is that it introduces an extra step in the process, which further increases the complexity of biofuel production (Jönsson et al., 2013). One strategy to avoid this is to use pretreatment that generates less inhibitors or at milder conditions. As was shown in Table 1.4, alkali pretreatment produces the least amount of formic acid among other pretreatment methods, the levels of other inhibitors are also maintained relatively low. Meanwhile, as was shown in Table 1.5, a sugar yield of 33 g sugars/100 g switchgrass was obtained with 0.5% NaOH, which was considerably higher than the results with dilute sulfuric acid or hot water under similar conditions. Although similar sugar yields were obtained from pretreatment

methods such as ionic liquid and AFEX, the relatively low solid loading and the expensive cost of chemicals still remain the major obstacle for the industrial application of these methods.

Table 1.5 Sugar yields from switchgrass pretreated by various pretreatment methods

Pretreatment method	Biomass Loading (w/w %)	Initial quantity of biomass (g)	Remaining mass of switchgrass after pretreatment (g)	Sugar yield (grams of sugars/100 grams of initial quantity of biomass)	References
0.5% NaOH	15	10	6.5	33	Nlewem and Thrash, 2010
2% NaOH	15	10	5.3	21	
5% NaOH	15	10	4.2	14	
10% NaOH	15	10	3.9	12	
0.5% H ₂ SO ₄	15	10	9.5	17	
2% H ₂ SO ₄	15	10	9.5	10	
4% H ₂ SO ₄	15	10	9.6	8.6	
6% H ₂ SO ₄	15	10	9.6	8.6	
100 °C Water	15	10	9.8	8.8	
Ionic liquid	3	0.3	0.148	58	Li et al., 2010
1.2% H ₂ SO ₄	3	0.3	0.178	46	
Ionic liquid	15	900	497.5	~400	Li et al., 2013
AFEX	3	3	NA	28.0-38.5	Bals et al., 2010

NA, not available

Butanol fermentation from lignocellulosic substrates

Table 1.6 summarized the recently reported fermentation results from a variety of lignocellulosic materials pretreated by different methods. Among these results, DAP was the most studied pretreatment methods for the purpose of lignocellulosic butanol production; however, direct fermentations of hydrolysates resulted from DAP have been shown to have limited success, and a strong inhibition on the following fermentation is observed with a number of agricultural residues (with the exception of wheat straw), which could be alleviated at different extent by complicated detoxification methods such as overliming and resin adsorption or combined (Ezeji and Blaschek, 2008; Qureshi et al., 2010a, 2010b, 2008). Similarly, lignocellulosic hydrolysates resulted from steam-explosion pretreatment also contains significant amount of potent inhibitors to the fermenting organisms, which need to be removed by detoxification methods such as activated charcoal or alkali peroxide treatment in combination with washing (Wang and Chen, 2011).

The other approach to circumvent the issue of inhibition introduced by pretreatment is to reduce the severity of pretreatment, such as the use of milder chemicals in liquid hot water and AFEX, both of which produced hydrolysates that resulted in decent solvent yields from corn fiber and DDGS; however, the sugar yields were significantly lower than their counterparts with higher pretreatment severity, thus preventing the full utilization of lignocellulosic substrates (Ezeji and Blaschek, 2008; Qureshi et al., 2008). Alkali pretreatment, on the other hand, has been shown to be effective on different types of lignocellulosic materials, and only requires a simple washing step to remove degradation products and generate a highly fermentable sugar stream for fermentation (Cho et al., 2013; Ibrahim et al., 2015; Marchal et al., 1984).

Table 1.6 Comparisons of ABE fermentation from lignocellulosic materials(Adapted from Jurgens et al., 2012)

Substrate	Pretreatment	Saccharification	Detoxification	Total initial sugars (g/L)	Butanol yield (g/g)	Butanol/ABE (g/L)	Solvent productivity (g/L/h)	References
Wheat straw	1% H ₂ SO ₄	Enzymes	-	~60	0.24	12.0/25.0	0.6	(Qureshi et al., 2007)
Corn fiber	0.5% H ₂ SO ₄	-	-	29.8	0.05	1.4/1.7	0.03	
Corn fiber	0.5% H ₂ SO ₄	Enzymes	-	54.3	NA	NA/1.6	NA	(Qureshi et al., 2008)
Corn fiber	0.5% H ₂ SO ₄	Enzymes	Overliming + XAD4 (resin)	46.3	0.14	6.4/9.3	0.1	
DDGS	Dilute H ₂ SO ₄	Enzymes	-	52.6	NA	NA	NA	(Ezeji and Blaschek, 2008)
DDGS	Dilute H ₂ SO ₄	Enzymes	Overliming	~52.6	0.14	7.3/12.1	0.1	
DDGS	Liquid Hot Water	Enzymes	-	48.8	0.18	8.9/12.9	0.18	
DDGS	AFEX	Enzymes	-	41.4	0.17	7.0/11.6	NA	
Barley straw	1% H ₂ SO ₄	Enzymes	-	58.8	0.06	4/7.09	0.1	(Qureshi et al., 2010a)
Barley straw	1% H ₂ SO ₄	Enzymes	Overliming	63.4	0.28	18.01/26.64	0.39	
Corn stover	1% H ₂ SO ₄ , 160 °C	Enzymes	-	~60	NA	NA	NA	(Qureshi et al., 2010b)
Corn stover	1% H ₂ SO ₄ , 160 °C	Enzymes	Overliming	60.3	0.24	14.50/26.27	0.31	
Switchgrass	1% H ₂ SO ₄	Enzymes	-	60.0	0.02	0.97/1.48	0.02	
Switchgrass	1% H ₂ SO ₄	Enzymes	Overliming	~60	NA	NA	NA	
Yellow poplar	0.145 g NaOH/g biomass, 170°C	Enzymes	Washing	49	0.22	10.9/18.1	0.38	(Cho et al., 2013)
Corn stover	Steam explosion	Enzymes	Washing	53.52	0.01	0.36/3.71	0.05	(Wang and Chen, 2011)
Corn stover	Steam explosion	Enzymes	Washing + Activated charcoal	~49	0.17	8.5/12.38	0.17	
Corn stover	Steam explosion	Enzymes	Washing + Alkali peroxide treatment	~49	0.17	8.5/12.38	0.17	
Oil palm empty fruit bunch	0.2 g NaOH /g biomass	Enzymes	Washing	25	0.08	1.94/2.61	0.04	(Ibrahim et al., 2015)

-, step omitted in this process;

NA, no significant results is reported due to poor fermentation performance.

1.2.7. Product inhibition/butanol toxicity

Butanol toxicity is another major issue associated with the ABE fermentation. In a conventional batch fermentation, butanol concentration rarely exceeds 13 g/L, thus resulting in high energy cost for product separation through distillation and limited utilization of substrates. It was proposed that the production cost would be cut in half if the final butanol titer is raised to 19 g/L (Papoutsakis, 2008). However, when exposed to 7-13 g/L butanol, clostridia cells showed a 50% reduction in growth and sugar uptake; interestingly, unlike butanol, it would take up to 40 g/L acetone and ethanol to achieve similar inhibition effect, which makes butanol the most toxic solvent product in the ABE fermentation (Jones and Woods, 1986).

Mechanism of butanol toxicity

Butanol is more toxic compared to other solvent products mainly due to its hydrophobic nature. By disruption of the phospho-lipid component of cell membrane, long-chain alcohols such as butanol (in a concentration of 10 g/L) increases the membrane fluidity by 20-30% (Vollherbstschneck et al., 1984). The increase in membrane fluidity may result in destabilization of the membrane and disruption of membrane-linked functions (Jones and Woods, 1986). For example, after addition of butanol, membrane-bounded ATPase activity was found to be inhibited instantly (Moreira et al., 1981), the ability of cells to maintain its internal pH was weakened, and the membrane pH gradient was abolished (Bowles and Ellefson, 1985). Furthermore, butanol is also inhibitory to the uptake of sugars and amino acids, a 50% reduction in glucose uptake was observed in the presence of 7.4 g/L butanol (Moreira et al., 1981).

One approach to solve the problem of butanol toxicity is to enhance the cell's tolerance against butanol, which could be achieved by manipulation of fermentation conditions such as addition of saturated fatty acids and decreased fermentation temperature (Brosseau et al., 1985; Jones and Woods, 1986). Efforts have also been made to obtain strains that can tolerate and produce higher concentrations of butanol, however, limited success have been achieved to date. This is mainly due to the multiple toxic effects

induced by butanol toxicity and the development of butanol-tolerant strains may not necessarily lead to higher butanol titers (Papoutsakis, 2008).

In-situ butanol recovery

In-situ butanol removal techniques are considered as efficient methods to solve the problem of butanol toxicity and the low solvent titer that leads to high separation cost at the same time (Lee et al., 2008). So far, different types of butanol recovery processes including pervaporation, adsorption, liquid-liquid extraction, gas stripping, and reverse osmosis have been integrated with the ABE fermentation to obtain more concentrated solvent stream and better fermentation performance (Dürre, 1998).

Gas stripping

Gas stripping is a simple but efficient way to recover butanol from fermentation broth (Lee et al., 2008). Basically, the fermentation gas is collected and sparged through the fermentation; the vapour that contains volatile solvents is then passed through a condenser, and recycled back to the fermentor to strip more solvents (N. Qureshi and Blaschek, 2000). The application of gas stripping results in less butanol inhibition and thus enabling the clostridia to utilize higher concentration of sugars. More importantly, a solvent stream with higher butanol concentrations compared to the fermentation broth is obtained, which will significantly reduce the cost of downstream processing such as distillation. It has been observed that the butanol concentration in the condensate is largely dependent on the butanol concentration in the fermentation broth, for example, butanol concentration in the condensate was increased from ~100 g/L to ~150 g/L when its concentration in fermentation broth was increased from 8 to 10 g/L; however, gas stripping is shown to be ineffective when butanol concentrations is below 5 g/L, and the cells in the fermentation broth could have a negative effect on the performance of gas stripping. Therefore, cell concentration should be controlled as low as possible in the fermentation broth, which can be achieved by cell immobilization such as the use of a fibrous bed bioreactor (FBB), and the butanol concentrations in the fermentation broth should be controlled at a reasonable range depending on the procedure (such as 8 g/L to 10 g/L) (Xue et al., 2012).

Pervaporation

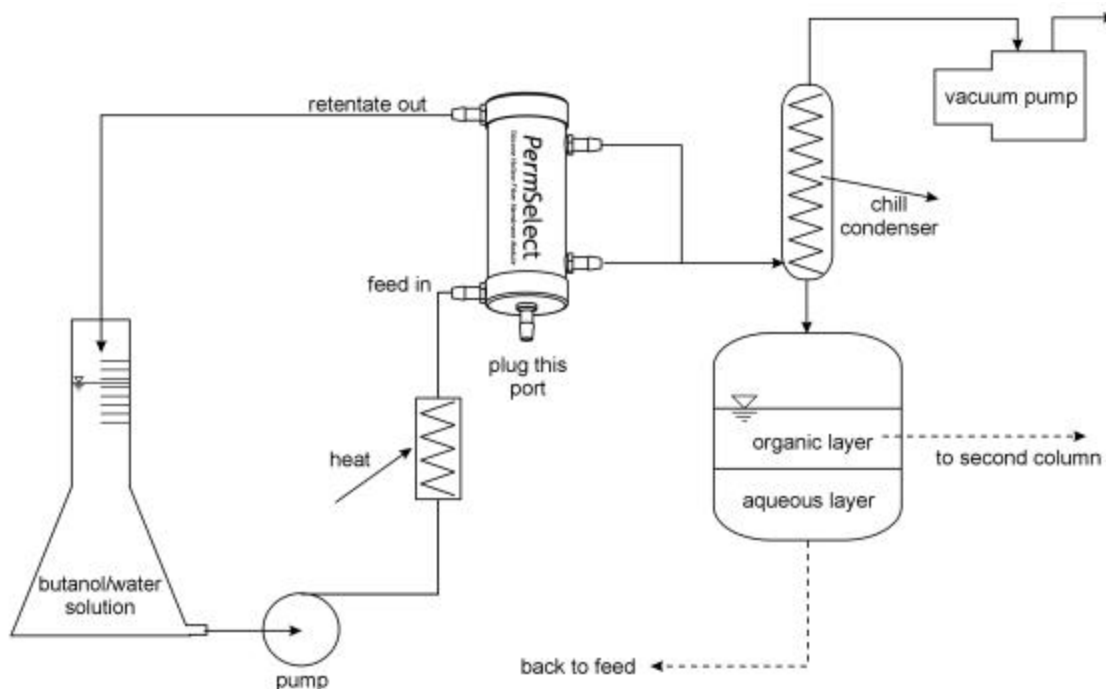


Figure 1.8 Pervaporation of butanol using a membrane module (Retrieved April 15, 2016, from https://www.permselect.com/Platform_Technology/Butanol_Pervaporation)

Similarly, the pervaporation process employs a membrane module to separate volatile solvents (mainly butanol) from the fermentation broth as shown in Fig 1.8. The fermentation broth is pumped through one side of the membrane and vacuum or a sweeping gas is applied to the other side. Since the permeability of butanol through the membrane is much higher compared to that of water, the vapour resulted from the vacuum side can be condensed into a highly concentrated butanol solution, which could automatically separate into two layers ($> 8\%$ v/v butanol). A pervaporation experiment was conducted with cell-free ABE fermentation broth from 60 g/L glucose, the initial acetone and butanol concentration were 6.10 g/L and 12.00 g/L; after starting the pervaporation process for 6 hours, the acetone and butanol concentration on the retentate side was reduced to 2.53 g/L and 2.00 g/L, respectively, and their concentrations in the condensate side were 105.86 g/L and 233.00 g/L, respectively (Qureshi et al., 2001). A continuous operation of pervaporation process to completely remove solvents from the fermentation broth is not considered necessary due to longer running time, lower butanol

flux through membrane due to more diluted feed stream, and lower butanol concentration in the condensate. In fact, butanol concentration below 5 g/L showed little to no inhibition on cell growth (Jones and Woods, 1986). One of the disadvantages associated with pervaporation process is that membrane fouling could occur if pervaporation is directly coupled with ABE fermentation, which is mainly due to the adsorption of cells on the membrane surface; however, the fouling is reversible and the membrane performance can be easily recovered by simple water rinse (Liu et al., 2011).

Liquid-liquid extraction

Liquid-liquid extraction has also been used to remove butanol from the fermentation broth. In this process, the aqueous fermentation broth is placed to contact with an organic phase (immiscible to the aqueous fermentation broth), and since butanol has a higher solubility in the organic phase (extractant), it is selectively concentrated in the organic phase, thus alleviating the butanol toxicity to the fermenting microorganism (Yen and Wang, 2013). In a fed-batch butanol fermentation from glucose, oleyl alcohol was used as extractant to remove solvents from fermentation broth. The butanol productivity was increased from 0.58 g/L/h in a control batch fermentation to 1.5 g/L/h in the fed-batch fermentation with in-situ butanol removal by liquid-liquid extraction; meanwhile, the organic phase (oleyl alcohol) accumulated near 35 g/L butanol in the end of fermentation (Roffler et al., 1987). However, when it comes to the choice of extractant solvent, it has to be non-toxic to the fermentative clostridia, which limits the use of solvents with higher butanol selectivity; on the other hand, compared to other in-situ butanol recovery techniques, butanol concentrations in the extractant are significantly lower, which indicates decent amount of energy input is still required for further purification of the final product.

Adsorption

Adsorption is a process where particles from a liquid or gas mixture are preferentially attached on a solid face (Kujawska et al., 2015). It is considered to be the most energy efficient among other butanol recovery techniques (Hughes and Cotta, 2005). So far, a number of different types of butanol adsorbents have been developed and characterized for their potential use as in-situ butanol recovery devices in ABE fermentations, such as

activated carbon, silicalite, and polymeric resins (Hughes and Cotta, 2005; Nielsen and Prather, 2009). In terms of screening the most promising butanol adsorbents for butanol recovery, the following characteristics should be considered, which include adsorption capacity, adsorption rate, selectivity for the desired product, and ease of desorption (Abdehagh et al., 2015).

Nielsen et al. (2009) evaluated the butanol affinities and selectivities of a number of commercially available polymeric resins for their potential use as in-situ butanol recovery devices for ABE fermentations. Among the resins they evaluated, Dowex Optipore L-493 and SD-2 were found to have the highest specific butanol loading of 175 and 152 g/kg-resin among the resin tested in the experiment. The uptake of butanol by polymer resins is mainly driven by hydrophobic interactions between the ligands in the polymer matrix and the alkyl chain of butanol (Anemasa et al., 2003). Butanol fermentation carried out with the addition of 50 g/L resin SD-2 resulted in a cumulative butanol production of 22.2 g/L from 160 g/L glucose; on the other hand, resin regeneration experiments were conducted by thermal treatment of butanol-saturated resin, a butanol recovery of 83% was obtained for multiple cycles, and phase separation was observed in the liquid mixtures obtained in the cold trap (Nielsen and Prather, 2009). Despite this, the prices of polymeric resins remain very expensive which could limit their applications in industrial scale.

Section 2 - Experimental data and interpretation

2.1 Structure of the thesis

Various biomass feedstocks and different pretreatment techniques were investigated in this thesis. The experimental section presented here are structured in four subsections representing the evolution of the investigated biofuel production process. Initially (section 2.3) corncobs are evaluated as a feedstock for ABE fermentation after caustic pretreatment and enzymatic hydrolysis. A similar process is subsequently used on an extended range of lignocellulosic feedstocks (section 2.4). Lipid-extracted algae, as a non-lignocellulosic carbohydrate resource is then evaluated as feedstock for butanol production, which allows simpler saccharification and direct fermentation (section 2.5). While the concluding subsection (2.6) returns to lignocellulosic biomass and includes an innovative technique to combine feedstock detoxification and in-situ product removal. Each of the subsection has been published in peer reviewed journals.

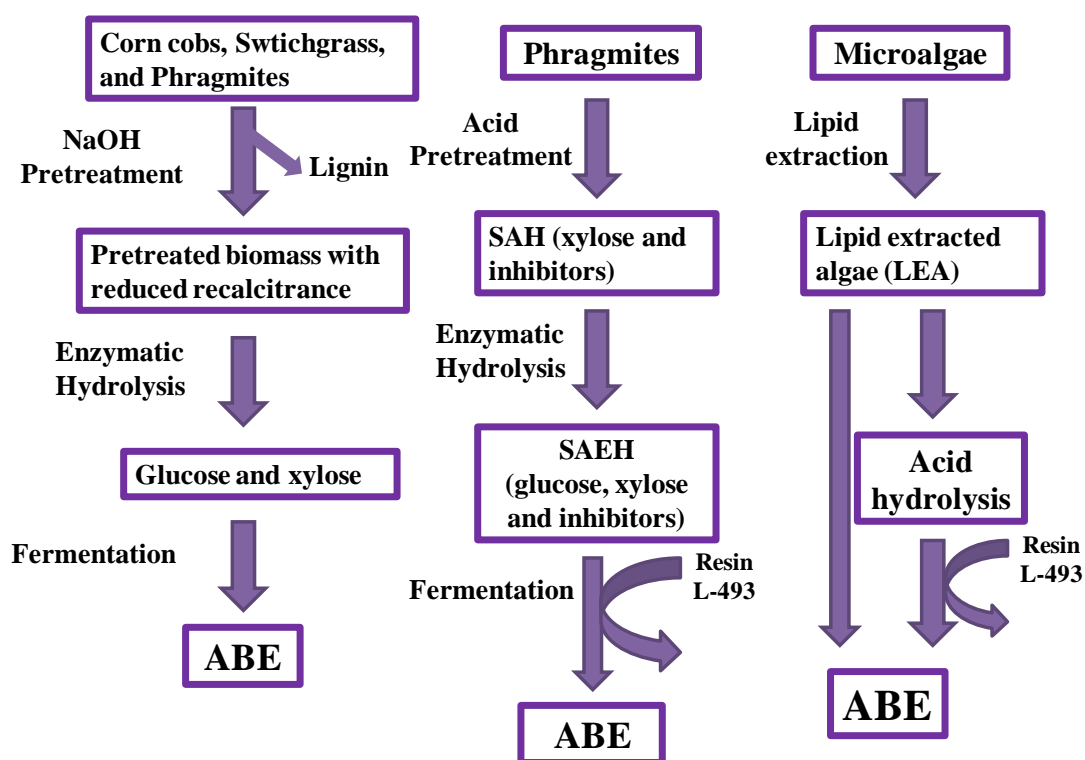


Figure 2.1 Overview of the research and major tasks

2.2 General Objective

The overall objective of this thesis is to explore potential low-cost substrates for fermentative butanol production, which include lignocellulosic biomass and microalgae, and strives to solve fermentation-related problems including butanol toxicity and inhibitors generated during pretreatment. Based on the types of biomass, different processes have been developed for efficient utilization of the carbohydrate components. To remove fermentation inhibitors, a hydrophobic resin has been employed to detoxify biomass hydrolysate; meanwhile the same resin has also been used to reduce butanol toxicity by simultaneously adsorbing butanol during fermentation.

2.3 Specific Objectives

Objective 1 ABE fermentation from enzymatic hydrolysate of NaOH-pretreated corncobs

A typical agricultural waste, corn cob, was evaluated as feedstock for ABE fermentation. Alkali pretreatment was used to remove lignin and improve the sugar yield in the subsequent enzymatic hydrolysis. The effect of post-pretreatment washing on enzymatic hydrolysis and fermentation was evaluated. Fermentation of corn cob hydrolysates was carried out with and without solids removal by centrifugation.

Objective 2 Cellulosic butanol production from alkali-pretreated switchgrass (*Panicum virgatum*) and phragmites (*Phragmites australis*)

Switchgrass and phragmites were evaluated as feedstocks for ABE fermentation. The effect of alkali pretreatment on the biomass compositions of both feedstocks were compared. Enzymatic hydrolysis were carried out with both un-treated and pretreated biomass and the sugar yields were compared. Finally, hydrolysates from pretreated switchgrass and phragmites were fermented by *Clostridium saccharobutylicum* DSM 13864 for butanol production.

Objective 3 Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction

Algal biomass was evaluated as an alternative non-food feedstock for ABE fermentation. Lipid extracted algae (LEAs) were obtained with either Hexane/Isopropanol or ionic liquid for lipid removal, which resulted in hexane-extracted algae (HEA) and ionic liquid-extracted algae (ILEA). Composition analysis was carried out to both qualify and quantify the carbohydrates of potential substrates. Subsequently, starch from HEA and ILEA were directly fermented for ABE fermentation. Alternatively, acid hydrolysates of both LEA were either detoxified by hydrophobic resin (Optipore L-493) first prior to fermentation or fermented as it is for butanol production. The effect of organic carbon source and algal biomass loading were also investigated.

Objective 4 Combined Detoxification and In-situ Product Removal by a Single Resin During Lignocellulosic Butanol Production

Poor solvent yield and partial sugar utilization revealed fermentation inhibitors present in the acid hydrolysates of phragmites. To overcome this issue, resin (L-493) was used to remove toxic compounds present in the hydrolysate. The profiles of toxic compounds including furfural, hydroxyl furfural (HMF), and phenolic compounds before and after resin treatment were compared; batch fermentations were carried out with resin-detoxified hydrolysates to demonstrate the improved fermentability. To reduce the butanol toxicity during fermentation and facilitate the use of more concentrated sugar stream, resin was kept inside detoxified phragmites hydrolysate and fermentation was carried out with externally added glucose to evaluate the resin's function as in-situ butanol recovery devices.

2.4 ABE fermentation from enzymatic hydrolysate of NaOH-pretreated corncobs

Kai Gao, Lars Rehm

Preface

The information in section 2.4 has been slightly changed to fulfill formatting requirements. It is substantially as it appears in *Biomass & Bioenergy*, July 2014, Vol 66, pages 110-115.

Every year, large amounts of corn are produced in countries like the US and China, however, corn cobs, as a by-product of corn industry, are left un-utilized in the field. Section 2.4 describes a process where carbohydrates from corn cobs are efficiently converted into solvents by ABE fermentation. NaOH pretreatment was used to reduce the recalcitrance of the biomass, thus facilitating the sugar yields during enzymatic hydrolysis of corn cobs. The effect of post-pretreatment washing on enzymatic hydrolysis and fermentation were evaluated. It has been found that both sugar yield and fermentability of the corn cobs hydrolysates were significantly improved after washing. The improved sugar yield was probably due to the removal of residual lignin after NaOH pretreatment. It has been reported that lignin preferentially binds to certain sites of cellulose which are also the target of cellulolytic enzymes for hydrolysis, thus reducing the reactivity of substrates; on the other hand, lignin also binds to the cellulose-binding module of enzymes, which may result in low enzymatic activity (Vermaas et al., 2015). To further simplify the process, ABE fermentation was carried out without removing the solids after enzymatic hydrolysis, and similar solvent yield and productivity was obtained compared to those with solids removal.

Although ABE fermentation from corn cobs or corn stover has been reported previously, however, this is the first time NaOH-pretreated corn cobs were used for butanol production. In the present study, solvent yield and productivity from NaOH-pretreated corn cobs were comparable to those with glucose as substrates, suggesting that corn cobs is a promising alternative feedstocks for ABE fermentation. The study presented in

section 2.4 fulfilled the first objective of this thesis. It developed a viable process for conversion of corn cobs into solvents. Besides, it provided valuable information and guidance on the studies described in the following subsection, where similar processes were used to convert different types of lignocellulosic materials (switchgrass and phragmites) to ABE.

2.4.1. Introduction

Looking beyond corn-based ethanol, there has been renewed interest in fermentative production of butanol. Butanol is a platform chemical and alternative biofuel which has a higher energy density compared to its ethanol counterpart and can also be mixed with petroleum at any ratio (Dürre, 2007). High substrate costs remain significant bottlenecks that preclude large-scale implementations of the ABE fermentation.

Lignocellulosic biomass is an abundant, renewable, and underutilized global carbon source (Yinbo et al., 2006), however, pretreatment is required to enzymatic release of fermentable sugars. High crystallinity of cellulose and the presence of lignin minimize enzyme access to the carbohydrates and result in poor yields of fermentable sugars (Kumar and Wyman, 2009). Alkaline pretreatment is one of several chemical pretreatment technologies that have been intensively investigated. The major effect of alkaline pretreatment is the delignification of lignocellulosic biomass, thus enhancing the reactivity of the remaining carbohydrates (Kim and Holtzapfle, 2005). After enzymatic hydrolysis, a relatively clean sugar stream (mainly glucose and xylose) can be obtained at reasonably high yield with economically relevant enzyme dosages (Chen et al., 2013). The resulting mixed sugar stream can be a suitable feedstock for biofuel production, especially when targeting butanol via solvent-producing bacteria which, unlike most yeast strains used in ethanol production, can utilize both hexose and pentose (T. C. Ezeji et al., 2007a).

Corn cobs (central core of maize ear) are an important lignocellulosic by-product of the sweet corn processing industry and are available in sufficient quantity (Sahare et al., 2012). Recently, it was reported that the hydrolysis of corn cobs could be increased from 16% to near 100% after pretreatment with NaOH (Sahare et al., 2012), while it was reported elsewhere that NaOH-pretreated corn stover could successfully be fermented by

a butanol-tolerant strain, with an ABE yield of 0.41 g g⁻¹ and a production rate of 0.21 g L⁻¹ h⁻¹ (Gao et al., 2012). However, no studies have reported butanol production from NaOH-pretreated corncobs.

In this study, alkali-pretreated corncobs were enzymatically hydrolyzed at solid loadings of 10% to evaluate the pretreatment efficiency. The resulting hydrolysates were further fermented by *Clostridium saccharobutylicum* DSM 13864 for ABE production. The effect of post pretreatment washing on both hydrolysis and fermentation was also investigated.

2.4.2. Materials and methods

Raw material

Corncoobs (central core of maize ear) from local farms near Chatham, Ontario (42°24'40.9"N 82°15'40.2"W) were kindly provided by Greenfield Ethanol (Chatham, Ontario, Canada). Kernel-free cobs were air dried and stored at 4 °C prior usage (less than two weeks). Before pretreatment, the corncoobs were grounded by a hammer mill to pass a 2 mm sieve and stored in sealed plastic bags at room temperature until used (less than two days).

Pretreatment

Pretreatment of corncoobs was performed in 500 mL media bottles with screwed caps. Biomass (20 g) was soaked in 160 mL of NaOH aqueous solution (0.5 mol L⁻¹), corresponding to an NaOH loading of 0.16 g g⁻¹, and kept in an autoclave (2041, AMSCO) for 30 min at 121 °C. If post pretreatment washing was applied, solid residues were thoroughly washed with tap water until neutral pH was achieved (at a washing intensity of 100 mL g⁻¹), and then filtered by nylon cloth (200 mesh). The washed and unwashed corncoobs from three replicates were recovered, oven-dried (6901, Fisher Scientific) at 80 °C for 48 h and weighed for evaluation of solid recovery. It was stored in sealed plastic bags at 4 °C until enzymatic hydrolysis was performed (less than 24 h).

Enzymatic hydrolysis

Batch enzymatic hydrolysis was conducted in 125 mL Erlenmeyer flasks. In each flask, 5 grams of treated sample (dried weight) was mixed with 50 mL distilled water. The pH was adjusted to 5.0 using 1 mol L⁻¹ H₂SO₄ or NaOH before hydrolysis started and maintained at pH 5.0 until the end of hydrolysis. Cellic CTec2 (kindly donated by Novozyme) was dosed to give a filter paper activity of 15 FPU g⁻¹ dry matter. The enzyme properties were reported recently (Cannella et al., 2012). Hydrolysis was performed in an incubator (InforsMulitron, Infors Switzerland) at 50 °C, 2.5 Hz (150 rpm) for 72 h. Samples were periodically taken for sugar analysis. The hydrolysate was either directly stored at 4 °C or centrifuged at 2739 × g (3500 rpm) for 10 min to remove sediments and stored in pre-sterilized flasks for fermentation studies.

Strain and culture conditions

Clostridium saccharobutylicum DSM 13864 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures). Laboratory stocks of cells were routinely maintained as spore suspensions at 4 °C. For vegetative growth, the cells were inoculated in an anaerobic chamber (Model 855-ACB, PLAS LABS) to seed medium (autoclaved at 121 °C for 20 min) containing 3 g L⁻¹ yeast extract, 5 g L⁻¹ peptone, 5 g L⁻¹ soluble starch, 5 g L⁻¹ glucose, 2 g L⁻¹ ammonium acetate, 2 g L⁻¹ NaCl, 3 g L⁻¹ MgSO₄, 1 g L⁻¹ KH₂PO₄, 1 g L⁻¹ K₂HPO₄, 1 g L⁻¹ FeSO₄, and cultured at a temperature of 37 °C for 10-12 h without any agitation as the inoculum for fermentation studies.

Fermentation

All fermentation studies were performed using 125 mL Erlenmeyer flask containing 50 mL fresh fermentation media in the anaerobic chamber with a mini incubating shaker (VWR) at the speed of 3.3 Hz (200 rpm), the temperature was controlled at 37 °C. Mixed sugar fermentation medium was prepared to mimic the sugar compositions in the hydrolysate, containing a glucose and xylose concentration of approximately 35 and 25 g L⁻¹, respectively. Other nutrients were added at the same concentration of the seed medium except that the carbon source (both glucose and soluble starch) was not used in the fermentation medium. The medium was autoclaved at 121 °C for 20 min and cooled down in the anaerobic chamber for anaerobiosis before inoculation. The enzymatic hydrolysates of the NaOH-pretreated corncobs was further diluted with water to have a

sugar concentration of 60 g L^{-1} before fermentation. Nutrients were added the same way as in mixed sugar fermentation medium. Subsequently the medium was transferred into an anaerobic chamber for anaerobiosis before fermentation started. No further sterilization was conducted due to possible loss of sugars during autoclaving. The anaerobic nature of clostridia further reduces the risk of contamination thus potentially simplifying industrial scale processes. Five mL (10% of total volume) of actively growing cells were transferred into the fermentation medium to initiate the fermentation. Samples were taken every 12 h for sugar and product analysis. The growth profiles of the clostridia were not monitored due to interference of the hydrolysate with optical density measurements.

Analytical methods

Samples of hydrolysates and fermentation broth were centrifuged at $13,300 \times g$ (12,000 rpm) for 10 min to remove insoluble particles. Acetone, butanol, ethanol, acetic acid, glucose and xylose in the fermentation broth were analyzed by high performance liquid chromatography (HPLC) using an Agilent 1260 liquid chromatography system (Agilent Technologies, Inc., CA, USA) equipped with a Hi-plex H column ($7.7 \times 300 \text{ mm}$) at $15 \text{ }^\circ\text{C}$ and a refractive Index detector. $0.005 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$ was used as mobile phase with a flow rate of 0.5 mL min^{-1} .

The overall production rate ($\text{g L}^{-1} \text{ h}^{-1}$) was calculated as the maximum ABE concentration achieved (g L^{-1}) divided by the fermentation time (h). Yields were calculated as the total amount of solvents (ABE) produced divided by the amount of fermentable sugar utilized and are expressed as g g^{-1} .

Solid recoveries were calculated as the dried weight of pretreated corncobs after washing divided by the dried weight of raw material before pretreatment times 100%.

The results presented here are averages of triplicate experiments with a standard error.

2.4.3. Results and discussion

Pretreatment and enzymatic hydrolysis

A solid recovery of 65.3% was obtained after the pretreatment with washing. It has been reported that a lower weight loss (21%) can be obtained at milder alkali pretreatment conditions (0.25 mol L⁻¹ NaOH at 50 °C) (Sahare et al., 2012). Although numerous studies have suggested that high severity pretreatment conditions lead to increased delignification, which resulted in improved substrate digestibility, it may also lead to substantial loss of hemicellulose (Chen et al., 2013; Xu et al., 2010a). Therefore, it is suggested that a balance between the extent of delignification and sugar preservation should be established in future research in order to achieve maximum sugar yield from corncobs. HPLC analysis showed 0.76 ± 0.01 g L⁻¹ glucose and 1.17 ± 0.24 g L⁻¹ xylose present in the waste treatment water (black liquor), indicating that the majority of the weight loss was due to lignin removal. The lignin content of corncobs is typically between 13.1 and 21.4 % (Liu et al., 2010; Zhang et al., 2010). The dried samples (both washed and unwashed) were subsequently used for enzymatic hydrolysis.

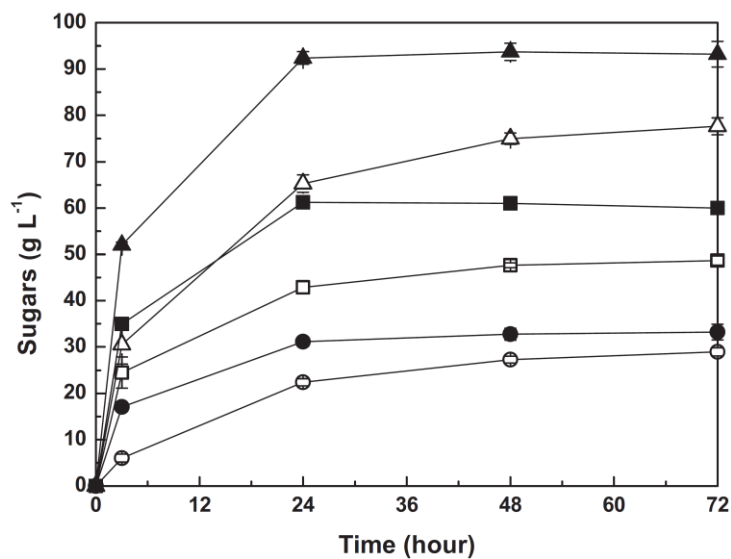


Figure 2.2 Enzymatic hydrolysis of washed and unwashed NaOH-pretreated corncobs (50°C, 150 rpm, and pH controlled at 5.0 with an enzyme loading of 15 FPU; -■- and -□-, glucose with and without washing; -●- and -○-, xylose with and without washing; -▲- and -△-, glucose and xylose with and without washing)

As shown in Fig 2.2, $34.95 \pm 1.06 \text{ g L}^{-1}$ glucose and $17.08 \pm 0.96 \text{ g L}^{-1}$ xylose was produced after 3 h of hydrolysis with washed samples, and 99.1% of the total sugar was produced within 24 h, indicating that an operational time of 24 h was sufficient for complete enzymatic hydrolysis. At the end of the hydrolysis (72 h), a total of $93.22 \pm 2.76 \text{ g L}^{-1}$ sugars with $59.98 \pm 1.07 \text{ g L}^{-1}$ glucose and $33.23 \pm 1.70 \text{ g L}^{-1}$ xylose was generated, corresponding to a sugar yield of 932 mg g^{-1} pretreated and washed corncobs (dried weight). HPLC results also showed trace amounts of arabinose, mannose and galactose presented in the resulting hydrolysates, which were not further quantified.

Enzymatic hydrolysis of unwashed pretreated corncobs was inhibited compared to washed samples. At the end of hydrolysis at 72 h, $48.68 \pm 0.97 \text{ g L}^{-1}$ glucose and $28.96 \pm 0.90 \text{ g L}^{-1}$ xylose was obtained, resulting in a total sugar yield of 776 mg g^{-1} pretreated corncobs. The higher sugar yield with washed samples indicates that there are enzyme inhibitors associated with NaOH pretreatment that can be removed by washing. Table 2.1 compares the total reducing sugar concentration resulting from enzymatic hydrolysis of corn stover and corncobs with different pretreatment method. All experiments were conducted using the same substrate loading and adequate enzyme loading, which provide good indication of the individual pretreatment efficiency. As was shown in Table 2.1, the total reducing sugar (93.22 g L^{-1}) obtained with NaOH-pretreated corncobs was much higher than that obtained with Ca(OH)_2 (49.3 g L^{-1}) and wet disk milling (39.7 g L^{-1}), indicating that NaOH pretreatment is a much more effective pretreatment method for corncobs. A comparison between the current study and a previous study using corn stover with the same pretreatment method shows that corncobs can release much more sugars than corn stover. This could be due to the higher carbohydrate contents and the lower crystallinity of corncobs that makes the substrate more accessible to enzymes (Kumar et al., 2009; Sahare et al., 2012).

Table 2.1 Comparison of enzymatic hydrolysis of corncobs and corn stover pretreated with alkali and wet disk milling

Feed Stock	Pretreatment Method	Substrate loading (g kg ⁻¹)	Total reducing sugar (g L ⁻¹)	Reference
Corncoobs	Ca(OH) ₂	100	49.3	(W. L. Zhang et al., 2012)
Corncoobs	Wet disk milling	100	39.7	(J. Zhang et al., 2012)
Corn stover	NaOH with washing	100	57.08	(Gao et al., 2012)
Corncoobs	NaOH with washing	100	93.22	The present study
Corncoobs	NaOH without washing	100	77.64	The present study

Mixed Sugar Fermentation

A control experiment using mixed sugars mimicking the sugar profiles of the hydrolysate was performed to compare the results obtained in this study. The fermentation was initiated with 35.28 ± 0.34 g L⁻¹ glucose and 21.68 ± 0.23 g L⁻¹ xylose. 99.8% of the glucose and 89.0% xylose was utilized after 36h. In a similar study, 35 g L⁻¹ glucose and 25 g L⁻¹ xylose was fermented by *C. acetobutylicum* SE-1, only 44% of the xylose was consumed after 72 h of fermentation, though glucose was depleted after 60 h (J. Zhang et al., 2012). In the present study, an ABE production of 16.81 g L⁻¹ with 6.09 g L⁻¹ acetone, 0.46 g L⁻¹ ethanol and 10.26 g L⁻¹ butanol was obtained at 36 h, resulting in an ABE yield of 0.30 g g⁻¹ and a production rate of 0.47 g L⁻¹ h⁻¹. The above results showed that the strain has the ability to utilize both glucose and xylose, indicating that it has the potential to be used for efficient fermentation of mixed sugars present in lignocellulosic enzymatic hydrolysates.

Additionally $1.70 \pm 0.16 \text{ g L}^{-1}$ of acetic acid was present at the beginning of fermentation, which was reduced to $0.62 \pm 0.09 \text{ g L}^{-1}$ after 36 h of fermentation.

Fermentation of enzymatic hydrolysates

Fig 2.4 and 2.5 show the fermentation results from enzymatic hydrolysates of washed NaOH-pretreated corncobs with and without sediments removal by centrifugation, respectively. In Fig 2.4, the initial sugar concentration was $55.30 \pm 0.28 \text{ g L}^{-1}$ with $35.32 \pm 0.07 \text{ g L}^{-1}$ glucose and $19.98 \pm 0.21 \text{ g L}^{-1}$ xylose. No significant sugar utilization and solvent production was observed within the first 12 h, however, the concentration of acetic acid was increased from $1.44 \pm 0.08 \text{ g L}^{-1}$ to $2.25 \pm 0.24 \text{ g L}^{-1}$, indicating a typical acidogenesis phase that the cultures were going through. Both glucose and xylose were utilized rapidly after 12 h and depleted after 36 h of fermentation (leaving 0.16 g L^{-1} sugars unutilized). A solvent production of $19.15 \pm 0.36 \text{ g L}^{-1}$ was obtained, including $12.36 \pm 0.01 \text{ g L}^{-1}$ butanol, $6.49 \pm 0.37 \text{ g L}^{-1}$ acetone and $0.31 \pm 0.02 \text{ g L}^{-1}$ ethanol, resulting in an ABE yield of 0.35 g g^{-1} and a production rate of $0.53 \text{ g L}^{-1} \text{ h}^{-1}$. Meanwhile, acetic acid was re-assimilated by the culture and its concentration was reduced to $0.61 \pm 0.11 \text{ g L}^{-1}$ after 36 h of fermentation. The significant solvent production and acetic acid re-assimilation indicated a typical solventogenesis phase.

Fermentation of the enzymatic hydrolysates of NaOH-pretreated corncobs resulted in ~20% more butanol productions, higher ABE yield and production rate compared to that of the mixed sugar fermentation. A lack of typical acidogenesis during mixed sugar fermentation could be the major reason to explain the inferior results. As can be seen from Fig 2.3, solvent productions started right after the inoculation, and no significant acetic acid generation was observed during the fermentation. It is well known that acidogenesis is closely related to cells exponential growth phase (Janssen et al., 2010). The lack of acidogenesis may indicate less cell growth in the mixed sugar fermentation, resulting in less solvent production.

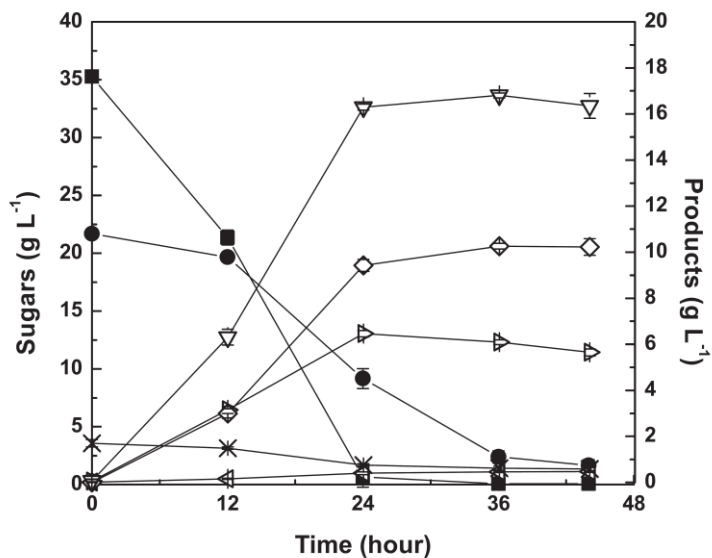


Figure 2.3 Fermentation of mixed sugar medium by *C. saccharobutylicum* DSM 13864 (37°C and 150 rpm; ■-, Glucose; ●-, Xylose; ▶-, Acetone; ◀-, Ethanol; ◆-, Butanol; ▼-, ABE; ×-, Acetic acid)

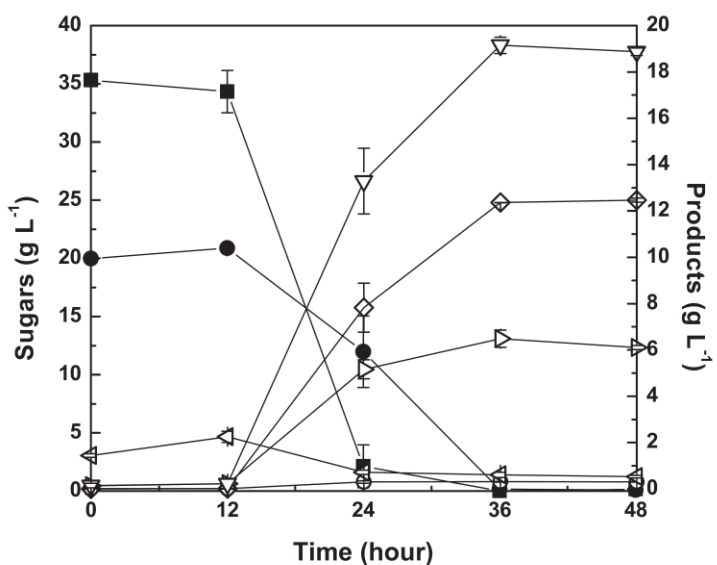


Figure 2.4 Fermentation of enzymatic hydrolysates of washed pretreated corn cobs with sediments removal (37°C and 150 rpm; ■-, Glucose; ●-, Xylose; ▶-, Acetone; ◀-, Ethanol; ◆-, Butanol; ▼-, ABE; ×-, Acetic acid)

To potentially further reduce the production cost and to simplify the process of cellulosic butanol production, the enzymatic hydrolysates of washed NaOH-pretreated corncobs was used directly for fermentation without sediments removal by centrifugation. As shown in Figure 2.5, the initial glucose and xylose concentration were $35.12 \pm 0.61 \text{ g L}^{-1}$ and $20.10 \pm 0.32 \text{ g L}^{-1}$, respectively. Rapid sugar utilization (both glucose and xylose) was observed after 12 h and sugars were depleted after 36 h of fermentation. The acetic acid concentration was $1.37 \pm 0.08 \text{ g L}^{-1}$ at the beginning of fermentation and subsequently increased to $2.30 \pm 0.05 \text{ g L}^{-1}$ at 24 h, indicating that the culture was going through acidogenesis. At 24 h, the culture started to reassimilate the acetic acid and to produce solvents rapidly. After 36 h of fermentation, $0.65 \pm 0.02 \text{ g L}^{-1}$ acetic acid was present in the fermentation broth. An ABE production of $19.44 \pm 0.33 \text{ g L}^{-1}$ (including $12.27 \pm 0.04 \text{ g L}^{-1}$ butanol, $6.86 \pm 0.38 \text{ g L}^{-1}$ acetone, and $0.32 \pm 0.01 \text{ g L}^{-1}$ ethanol) was obtained, resulting in an ABE yield of 0.35 g g^{-1} and a production rate of $0.54 \text{ g L}^{-1} \text{ h}^{-1}$. These results were similar to those obtained with sediments removal, indicating that it is applicable to use the enzymatic hydrolysates of NaOH-pretreated corncobs without sediment removal directly as substrate for ABE fermentation.

It has been reported that many species of *C. acetobutylicum* have a great preference in utilizing glucose over other sugars (e.g. xylose); xylose utilization will not start until a relatively low glucose concentration is reached (Gao et al., 2012; Raganati et al., 2013, 2012), and xylose is often left unutilized at the end of fermentation in glucose-xylose mixtures (He and Chen, 2013). However, in the present study, although glucose has been utilized more rapidly by strain *C. saccharobutylicum* DSM 13864, complete xylose utilization has also been observed in all fermentation studies, indicating that the strain has a great potential for fermenting mixed-sugar streams.

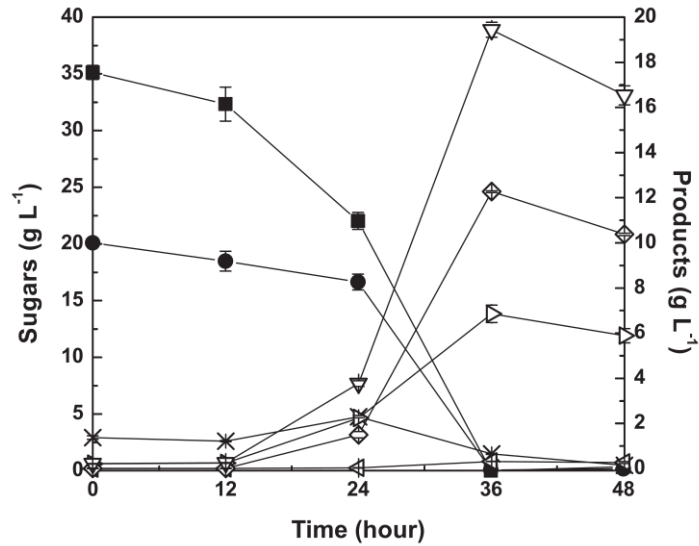


Figure 2.5 Fermentation of enzymatic hydrolysate of washed pretreated corn cobs without sediments removal (37°C and 150 rpm; -■-, Glucose; -●-, Xylose; -►-, Acetone; -◄-, Ethanol; -◆-, Butanol; -▼-, ABE; -x-, Acetic acid)

Another advantageous property of *C. saccharobutylicum* DSM 13864 was its higher ABE production rate. As summarized in Table 2.2, an ABE yield of 0.35 g g⁻¹ and a production rate of 0.54 g L⁻¹ h⁻¹ was obtained in this study, which was consistent with previous study results using the same strain (Ni et al., 2013, 2012). According to previous results (data not shown), shaking has an important role on sugar utilization by the strain. When the bacteria was used to ferment 60 g L⁻¹ glucose without agitation, about 20 g L⁻¹ glucose was left unutilized, thus resulting in low ABE productions. However, no agitation was conducted during the fermentation studies employing *C. acetobutylicum* listed in Table 2.2.

Table 2.2 Comparison of ABE production from alkali-pretreated corncobs and reported ABE production from lignocellulosic materials pretreated with other method

Strain	Substrate	Pretreatment & Detoxification	ABE Production (g L ⁻¹)	ABE Yield (g kg ⁻¹)	Production Rate (g L ⁻¹ h ⁻¹)	References
Mutant of <i>C. acetobutylicum</i> ATCC 824	Corn Stover	NaOH&Washing	15.42	410	0.21	(Gao et al., 2012)
<i>C. beijerinckii</i> P260	Corn Stover	Dilute sulfuric acid & Dilution with water	16.0	430	0.17	(Qureshi et al., 2010b)
<i>C. saccharobutylicum</i> DSM 13864	Cane Molasses	Washing and Sediments Removal	17.88	330	0.5	(Ni et al., 2012)
<i>C. beijerinckii</i> NCIMB 8052	Corncoobs	Ca(OH) ₂	16.0	330	0.33	(W. L. Zhang et al., 2012)
<i>C. acetobutylicum</i> SE-1	Corncoobs	Wet disk milling	14.12	360	0.20	(J. Zhang et al., 2012)
<i>C. saccharobutylicum</i> DSM 13864	Corncoobs	NaOH&Washing	19.44	350	0.54	The present study

Only trace amounts of ABE were produced during the fermentation of hydrolysate of unwashed NaOH-pretreated corncobs (data not shown). At the beginning of the fermentation, 32.88 ± 1.94 g L⁻¹ glucose and 18.61 ± 1.29 g L⁻¹ xylose were present in the hydrolysate. After 48 h, 27.49 ± 0.54 g L⁻¹ glucose and 18.18 ± 0.57 g L⁻¹ xylose were left unutilized, and 3.94 ± 0.09 g L⁻¹ acetic acid was produced. The inhibiting effect of unwashed substrate could be due to higher soluble lignin compounds (SLC) present in the hydrolysate. It has been reported that the ABE fermentation was inhibited when SLC exceeded 1.77 g L⁻¹ in steam exploded corn stover hydrolysate (Wang and Chen, 2011). Also, higher acetic acid concentration in this experiment could also result in premature cessation of ABE production. It has been reported that "acid crash" occurs when the

concentration of undissociated acids in the broth exceeds 57-60 mmol L⁻¹ (Maddox et al., 2000).

Results above showed that washing is an effective method to remove the toxic compounds generated during NaOH pretreatment of corncobs. To make this process more economical, the volume of water used for washing could be further optimized to reduce water consumption.

2.4.4. Conclusions

Alkaline-pretreated corncobs can be efficiently enzymatically hydrolysed and used for ABE fermentation without sediment removal. Post pretreatment washing is necessary to ensure a successful ABE fermentation. The higher solvent yield and production rate obtained during the fermentation of enzymatic hydrolysates compared to a synthetic mixed sugar fermentation indicates the presence of stimulating compounds in the corncobs hydrolysates. *C.saccharobutylicum* DSM 13864 was shown to be efficient in co-utilizing glucose and xylose. This study shows great potential for industrial application as efficient fermentation results were obtained without sediments removal and sterilization.

2.5 Cellulosic butanol production from alkali-pretreated switchgrass (*Panicum virgatum*) and phragmites (*Phragmites australis*)

Kai Gao, Simone Boiano, Antonio Marzocchella, Lars Rehm

Preface

The information in section 2.5 has been slightly changed to fulfill formatting requirements. It is substantially as it appears in *Bioresource Technology*, December 2014, Vol 174, pages 176-181.

This section focused on conversion of two different types of lignocellulosic biomass, namely switchgrass and phragmites into acetone, butanol and ethanol. Switchgrass (*Panicum virgatum*) is a C4 perennial grass that is native to the southern and eastern part of Canada. It is considered as a suitable feedstock for biofuel production due to many attractive characteristics, which include high biomass yield with low requirement on fertilisers, resistance to pest and disease, potential to be cultivated on marginal land, and low production cost, etc (McLaughlin and Kszos, 2005). Phragmites (*Phragmites australis*), also known as common reed, have caused increasing concern due to its rapid expansion in north America, and subsequently degrade waterfowl habitat and reduce biodiversity (Wilcox et al., 2003). On the other hand, phragmites have been considered as an ideal feedstock for biofuel production due to similar physical and chemical properties to switchgrass, as well as the ability to thrive without cultivation and produce large amounts of harvestable biomass annually in single species stands (Zhao et al., 2011).

Few studies have reported bio-butanol production from switchgrass. In a recent study, poor solvent yield and low cell concentrations were obtained from fermentation of dilute sulfuric acid pretreated switchgrass (Qureshi et al., 2010b), and as far as the authors' concern, butanol production from alkali-pretreated switchgrass has never been reported before. On the other hand, although phragmites has been studied experimentally as feedstocks for bio-energy production in different forms (biogas, pyrolytic oils, biochar,

ethanol, etc), but no butanol production from this feedstock has ever been reported (Vaičekonytė et al., 2014).

In this study, same alkali pretreatment conditions were employed as described in Section 2.4 to reduce the recalcitrance of biomass and facilitate the subsequent sugar yield during enzymatic hydrolysis. Compositional analysis of both types of biomass before and after pretreatment were carried out to study the effect of pretreatment. Sugar yields obtained from both pretreated switchgrass and phragmites were compared with those without any pretreatment. Finally, hydrolysates from both feedstocks were subjected to fermentation for butanol production.

This part of research fulfills objective 2 described in section 2.3. Results obtained from this study can be summarized as two major contributions. First, robust solvent yields comparable to control fermentation with glucose as substrates were obtained from both alkali pretreated switchgrass and phragmites, indicating that these feedstocks are suitable substrates for ABE fermentation; secondly, unexpected similar sugar and solvent yield were obtained from both biomass, given the fact that the chemical compositions of each biomass varies significantly in both glucan and lignin. The lignin content seems to play an important role on achieving this result. For example, phragmites with higher lignin content seems to be more resistant against alkali pretreatment, thus resulting in a higher solid recovery; although more glucan was preserved after the pretreatment, their enzymatic accessibility was more constrained by the higher content of residual lignin of phragmites compared to the switchgrass.

2.5.1. Introduction

Butanol has many industrial applications as an important chemical feedstock (Qureshi et al., 2013) and also exhibits the potential to become a superior biofuel compared to its ethanol counterpart mostly due to its higher energy density (Ni and Sun, 2009). One of the major problems that preclude the development of butanol production through biological pathway is high substrate cost (T. Ezeji et al., 2007; Qureshi et al., 2013, 2010b; Sarchami and Rehmann, 2014). However, the utilization of abundant lignocellulosic biomass as a feedstock may provide a possibility to lower the substrate

cost, depending on the source and type of biomass. Possible sources of biomass can be agricultural by-products such as corn-stover or cobs, dedicated energy crops, by-products from the food-processing industry, forestry products, municipal solids waste, etc., as reviewed in detail elsewhere (Kim and Dale, 2004; Sánchez and Cardona, 2008; Somerville et al., 2010). Switchgrass (*Panicumvirgatum L.*) has been considered as a promising lignocellulosic feedstock for biofuel production (Keshwani and Cheng, 2009). Switchgrass is native to North America and can be cultivated on land otherwise producing low agricultural crop yield. So far, numerous studies have been conducted on the pretreatment of switchgrass including dilute sulfuric acid, sodium hydroxide and hot water, etc (Nlewem and Thrash, 2010; Xu et al., 2010a), however, only one paper reported butanol production from dilute sulfuric acid pretreated switchgrass (Qureshi et al., 2010b), and no studies has been conducted on butanol production from alkali pretreated switchgrass. *Phragmites australis*, also known as common reed, is an invasive weed in North America which rapidly spread in the past 150 years (Saltonstall, 2002). Due to its high biomass yield, it is increasingly recognized as a potential feedstock for biofuel production (Szijártó et al., 2009). Like other lignocellulosic biomass, pretreatment is required to increase the enzymatic digestibility. Pretreatment methods that have been applied to phragmites include alkali pretreatment (Nath Barman et al., 2014), solvent based lignocellulose fractionation (Sathitsuksanoh et al., 2009), and wet oxidation (Szijártó et al., 2009). To date, no study has investigated bio-butanol production from phragmites. Unlike the feedstock for first generation biofuel production (including corn and molasses), lignocellulosic materials are composed of three major components: cellulose, hemicellulose and lignin. These components are in tight association with each other thus making the breakdown of polysaccharides through enzymatic digestion ineffective (Lacayo et al., 2013). Therefore, a pretreatment step is necessary to increase the digestibility of lignocellulosic materials.

Alkali pretreatment was shown to be an effective pretreatment method for lignocellulosic materials such as corn-stover (Chen et al., 2013), switchgrass (Xu et al., 2010a), Bermuda grass (Wang et al., 2010), etc. The major effect of alkali pretreatment is the removal of lignin from lignocellulosic biomass (Kim and Holtzapple, 2005). In a recent study, enzymatic hydrolysates of alkali-pretreated corncobs was successfully fermented by

Clostridium saccharobutylicum DSM 13864, resulting in an ABE production of 19.44 g L⁻¹ in a batch fermentation (Gao and Rehmann, 2014) and this current study intends to expand the substrate spectrum.

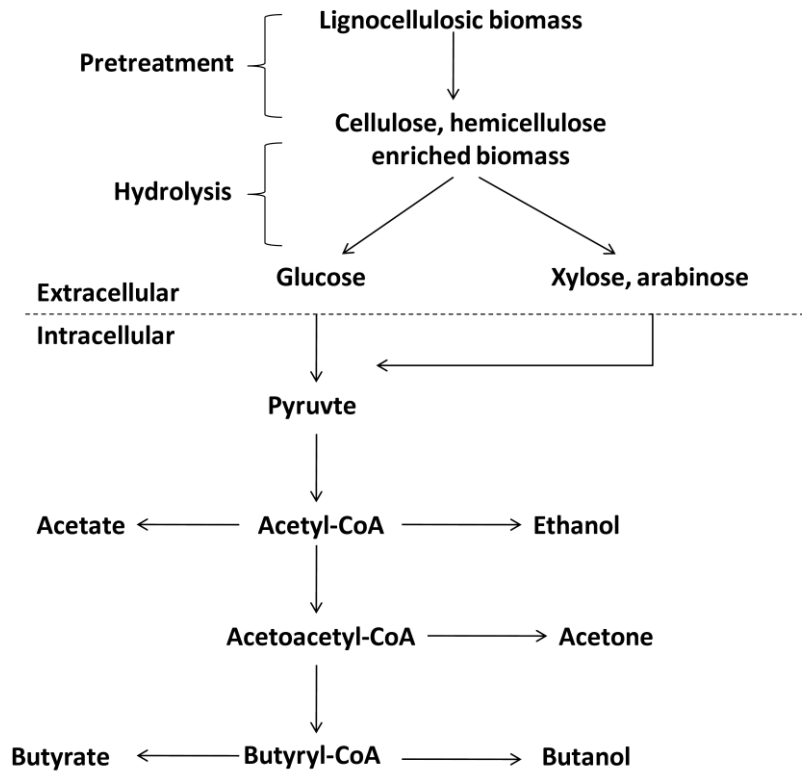


Figure 2.6 Simplified metabolic pathways from biomass to ABE by solventogenic clostridia(modified from T. C. Ezeji et al., 2007). 1, Glucose uptake by the phosphotransferase system (PTS) and conversion to pyruvate by the EMP pathway; 2, xylose/arabinose uptake and subsequent breakdown via the transketolase-transaldolase sequence producing fructose 6-phosphate and glyceraldehydes 3-phosphate with subsequent metabolism by the Embden-Meyerhof-Parnas (EMP) pathway; 3, pyruvate-ferrodoxin oxidoreductase; 4, thiolase; 5, 3-hydroxybutyryl-CoA dehydrogenase, crontonase and butyryl-CoA dehydrogenase; 6, phosphate acetyltransferase and acetate kinase; 7, acetaldehyde dehydrogenase and ethanol dehydrogenase; 8, acetoacetyl-CoA: acetate/butyrate: CoA transferase and acetoacetate decarboxylase; 9, phosphate butyltransferase and butyrate kinase; 10, butyraldehyde dehydrogenase and butanol dehydrogenase.

The objective of this study was therefore to use the aforementioned two lignocellulosic materials as feedstock for bio-butanol production. Both materials were pretreated with aqueous NaOH and compositional analysis before and after alkali pretreatment were provided. Enzymatic hydrolysis was carried out using commercial enzymes to evaluate the pretreatment efficiency. Finally, the lignocellulosic hydrolysates were subjected to ABE fermentation by *Clostridium saccharobutylicum* DSM 13864 without further treatment; a simplified flowchart is shown in Fig 2.6 (T. C. Ezeji et al., 2007b).

2.5.2. Materials and Methods

Strain and Culture Conditions

C. saccharobutylicum DSM 13864 purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) was used as the fermenting microorganism in this study. Details of culture propagation and inoculum development can be found elsewhere (Gao and Rehm, 2014).

Raw Biomass

Raw phragmites were collected near London, Ontario, Canada, while switchgrass was grown on experimental plots at Bioindustrial Innovation Canada (Sarnia, Ontario).

Air-dried raw switchgrass and phragmites (moisture content ~5%) were first cut into small pieces (~5 cm × 2 cm) and then grounded (IKA[®] MF10, Sigma Aldrich) to pass a 2 mm sieve and stored in sealed plastic bags at room temperature until used for characterization and pretreatment.

Pretreatment

Same pretreatment conditions were applied to both biomass samples. Biomass (10 g per replicate) was mixed well with 100 mL of 1% (w/v) sodium hydroxide solutions (solid to liquid ratio of 1:10) in 250 mL screw-capped media bottles in an autoclave (2041, AMSCO) for 30 min at 121 °C. The pretreated biomass was recovered by filtration through nylon cloth (200 mesh) and thoroughly washed with 1000 mL tap water (a washing intensity of 100 mL tap water per 1 gram raw biomass). The wet solids were

collected and transferred to pre-weighed plastic bags, weighed and stored sealed at 4°C for enzymatic hydrolysis. The moisture content of the wet solids was around 80%.

Enzymatic Hydrolysis

The enzymatic hydrolysis of the untreated and pretreated biomass samples was carried out in 125 mL erlenmeyer flasks. In each flask, 5 grams of sample (oven dried weight) was mixed with 50 mL of pre-sterilised distilled water. Cellic CTec 2 cellulase (kindly donated by Novozyme) was dosed to give a filter paper activity of 15 FPU/g dry matter. The hydrolysis was performed in an air-bath incubator (Infors Multitron, Infors Switzerland) at 50 °C and 150 rpm for 72 h. Flasks were sealed properly to prevent evaporation. The pH was controlled at 5.0 by adding 1 M NaOH/H₂SO₄ during the process. Samples were taken at the end for sugar analysis. The hydrolysates were stored at 4 °C for fermentation studies (no longer than 48 h).

ABE Fermentation

ABE fermentation from switchgrass and phragmites were conducted as described for corncobs hydrolysate in previous paper (Gao and Rehmann, 2014). In brief, enzymatic hydrolysates were transferred to pre-sterilised 50 mL falcon tubes for sediments removal (centrifugation at 3500×rpm for 20 min). The supernatant was decanted to pre-sterilised 125 mL ermenleyer flasks. Nutrients were added as followed: 3 g L⁻¹ yeast extract, 5 g L⁻¹ peptone, 2 g L⁻¹ ammonium acetate, 2 g L⁻¹ NaCl, 3 g L⁻¹ MgSO₄, 1 g L⁻¹ KH₂SO₄, 1 g L⁻¹ K₂HPO₄, and 1 g L⁻¹ FeSO₄. The only carbon source was sugars present in the hydrolysate. Prior to fermentation, the pH of the fermentation media was adjusted to 6.5 by adding 1 M NaOH or H₂SO₄. Following the pH adjustment, the bottles containing the fermentation media were transferred to a water bath at 85 °C for 15 min to remove dissolved oxygen and kept in an anaerobic chamber at 37 °C for 12-24 h for anaerobiosis (Plas-Labs, Inc., Lansing, MI). After this period, the bottles were inoculated with 10-12 h old seed culture (5 mL cell culture in 50 mL medium). Samples were taken at a time interval of 12 h until 48 h and were analysed for cell density, glucose, xylose, ABE, acetic acid and butyric acid. At least duplicate experiments were carried out for all fermentation experiments, average numbers and standard errors were shown in the text and figures.

Analytical Methods

The composition of switchgrass and phragmites before and after alkali pretreatment was determined by the procedures outlined by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012, 2008). Glucan, xylan and arabinan in the biomass were determined by measuring the concentration of corresponding monomeric sugars in the hydrolysate. The acid soluble lignin content was measured by UV-Vis spectroscopy (Evolution 60S, Thermo Scientific). Sugars (glucose and xylose) and fermentation products (acetic acid, butyric acid, acetone, ethanol and butanol) in the fermentation broth were determined by high performance liquid chromatography (HPLC) reported previously (Gao and Rehm, 2014). An Agilent 1260 Infinity HPLC with a refractive index detector (RID) was used, column temperature (HiPlex-H, Agilent) was 80 °C with a flow rate of 0.6 mLmin⁻¹ (water as mobile phase).

ABE production (g L⁻¹) was defined as the maximum amount of ABE produced during the fermentation. ABE productivity (g L⁻¹h⁻¹) was calculated as the ABE production (g L⁻¹) divided by the fermentation time required (h). ABE yield (g g⁻¹) was calculated as the ABE production (g L⁻¹) divided by the amount of sugars utilized by the bacteria (g L⁻¹).

2.5.3. Results and discussion

Compositional analysis before and after alkali pretreatment

The composition of the raw materials (phragmites and switchgrass) before and after alkali pretreatment is summarized in Table 2.3. For both untreated biomass, glucan was the major carbohydrate, followed by xylan, which is the major hemicellulose constituent. These two components comprised 44.4% of the total biomass for switchgrass and 46.0% of the total biomass for phragmites. Small amounts of arabinan were detected in all samples. It is important to note that the initial lignin content of phragmites is higher than that of switchgrass, which may bring extra recalcitrance to phragmites since lignin is predominantly believed to hinder the enzymatic hydrolysis (Lacayo et al., 2013). The composition of switchgrass and phragmites used in this study agreed with those of different varieties reported before (Szijártó et al., 2009; Xu et al., 2010a).

Table 2.3 Chemical compositions of raw and pretreated biomass

Component (g/100 g raw biomass)	Switchgrass		Phragmites	
	Untreated	Pretreated	Untreated	Pretreated
Solid recovery	100 (0.00)	52.04 (0.76)	100 (0.00)	68.27 (3.21)
Glucan	30.05 (0.12)	48.07 (1.51)	34.40 (0.22)	49.28 (0.52)
Xylan	14.38 (0.28)	19.71 (0.42)	14.63 (0.75)	16.38 (0.39)
Arabinan	1.21 (0.01)	1.97 (0.21)	1.53 (0.37)	0.75 (0.20)
lignin ^a	24.49 (0.12)	11.84 (1.70)	28.83 (0.63)	16.15 (0.52)

^aIncluding acid soluble lignin and acid insoluble lignin;

Data are means of three replicates with standard error reported in the brackets.

Significant lignin removal was observed for both switchgrass and phragmites after the pretreatment with NaOH (Table 2.3). The resulting pretreated biomass showed prominent increase in glucan content for both switchgrass and phragmites. However, for pretreated switchgrass the recoverable amount of glucan and xylan (g/100 g raw biomass) decreased by 16.7% and 28.7% after pretreatment due to significant solid loss, indicating partial solubilisation of the carbohydrate component. In a related study (Xu et al., 2010a), partial solubilisation also occurred to all biomass component of switchgrass pretreated 1% NaOH at 121 °C, which is in accordance to the results of the present study. In contrast, the total amount of glucan in the phragmite samples was only mildly reduced after pretreatment (~2%) compared to a 23% reduction in xylan (Table 2.3). Therefore, the weight loss during pretreatment of phragmites is mainly due to the solubilisation of lignin and xylan, however, solubilisation of glucan also contributed to weight loss during the pretreatment of switchgrass. It was suggested that milder pretreatment conditions should be considered for pretreatment of switchgrass to reduce the loss of glucan.

Enzymatic Hydrolysis

After pretreatment and enzymatic hydrolysis, glucose, xylose, cellobiose and arabinose in the hydrolysate were measured. Total reducing sugar was reported as the summation of the sugars measured. The total reducing sugar yields were employed to indicate the overall pretreatment efficiency. For pretreated switchgrass (Fig 2.7), after 72 h of enzymatic hydrolysis, $44.81 \pm 1.75 \text{ g L}^{-1}$ glucose and $19.89 \pm 0.58 \text{ g L}^{-1}$ xylose were present

in the hydrolysate, resulting in a sugar production of $70.86 \pm 2.99 \text{ g L}^{-1}$, which was 3.84 times higher than that of untreated biomass. Glucose and xylose yields were 2.06 and 1.41 times higher than that of untreated biomass, respectively. The total reducing sugar yield reached $364.7 \pm 15.4 \text{ g kg}^{-1}$ raw biomass, which is almost twice as high as that of untreated biomass. For pretreated phragmites (Fig 2.8), $34.71 \pm 1.05 \text{ g L}^{-1}$ glucose and $17.41 \pm 0.29 \text{ g L}^{-1}$ xylose were present in the hydrolysate, resulting in a total sugar production of $55.26 \pm 1.57 \text{ g L}^{-1}$, which was 7.35 times higher than that of untreated biomass. Glucose and xylose yield were 5.28 and 4.11 times higher than that of untreated biomass. The total reducing sugar yield reached $384.5 \pm 10.9 \text{ g kg}^{-1}$ raw biomass, which was 5.11 times higher than that of untreated biomass.

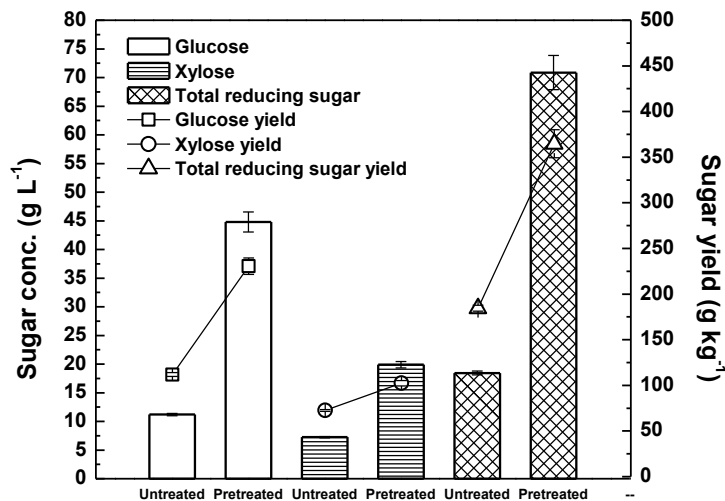


Figure 2.7 Comparison of suar production and yield from pretreated and untreated switchgrass. Sugar concentration are shown on the left axis. White columns represent glucose concentration in the hydrolysate; columns with horizontal and crossed stripes corresponds to xylose concentration and total reducing sugar concentration, respectively. Glucose yield (open squares), xylose yield (open circles) and total reducing sugar yield (open triangles) are shown on the right axis.

A higher sugar concentration was obtained during the enzymatic hydrolysis of switchgrass (both untreated and pretreated) compared to phragmites, this might be due to

a lower lignin content of switchgrass before and after pretreatment since lignin not only create a physical barrier that limits the access of substrates to cellulase (Ding et al., 2012), but also unproductively binds to cellulases, thus further reducing their activity (L. Kumar et al., 2012). However, the total reducing sugar yield of pretreated switchgrass was less than that of phragmites due to higher weight loss during pretreatment (Table 2.3). Optimization of the pretreatment conditions such as NaOH loadings, pretreatment temperatures and residence times for both types of biomass are recommended in order to maximize the sugar yield. For example, a total reducing sugar yield of 433.4 g kg⁻¹ raw biomass was achieved when switchgrass was pretreated at 50°C for 24 h with 0.10 g Ca(OH)₂/g raw biomass (Xu et al., 2010b), however the pretreatment conditions for both substrates were kept constant in this study for comparison purposes.

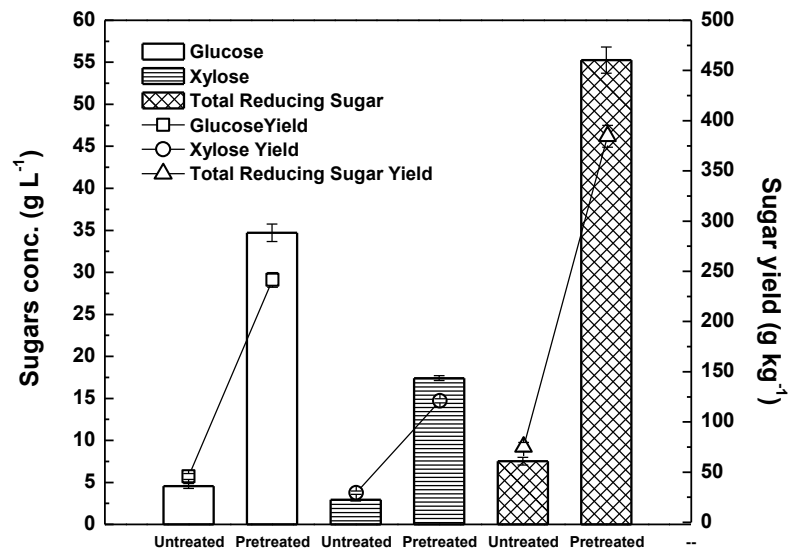


Figure 2.8 Comparison of sugar production and yield from pretreated and untreated phragmites. Sugar concentration are shown on the left axis. White columns represent glucose concentration in the hydrolysate; columns with horizontal and crossed stripes corresponds to xylose concentration and total reducing sugar concentration, respectively. Glucose yield (open squares), xylose yield (open circles) and total reducing sugar yield (open triangles) are shown on the right axis.

ABE Fermentation

A control fermentation with media containing pure glucose ($\sim 60 \text{ g L}^{-1}$) as the sole carbon source was performed to compare the results from fermentation of switchgrass and phragmites hydrolysates. After 24 h of fermentation, glucose was depleted by the culture, which resulted in the production of 23.02 g L^{-1} total ABE (acetone 9.58 g L^{-1} , ethanol 1.26 g L^{-1} , and butanol 12.18 g L^{-1}). During the fermentation, an ABE yield and productivity of 0.38 g g^{-1} and $0.96 \text{ g L}^{-1} \text{ h}^{-1}$ was obtained, respectively. A maximum cell concentration of 5 g L^{-1} was reached at 12 h (data not shown).

Switchgrass hydrolysate was prepared and subjected to ABE fermentation as described above. No extra detoxification or sugar supplementation was performed. Initially 40.37 g L^{-1} glucose and 16.52 g L^{-1} xylose were present in the fermentation media (Figure 2.9). Rapid glucose utilization was observed in the first 12 h of fermentation until all glucose was consumed (24 h), while the culture showed a delay of 12 h before xylose was utilized. After 36 h of fermentation (when the fermentation stopped), 22.70 g L^{-1} ABE including 9.13 g L^{-1} acetone, 0.56 g L^{-1} ethanol, and 13.00 g L^{-1} butanol were produced, which represents an ABE yield and productivity of 0.40 g g^{-1} and $0.63 \text{ g L}^{-1} \text{ h}^{-1}$. Insignificant amounts of residual sugars (both glucose and xylose) were detected at the end of fermentation. During the course of the fermentation, 3.12 g L^{-1} acetic acid was present initially in the fermentation media which was reduced to 1.17 g L^{-1} after 36 h of fermentation; butyric acid increased from 0.17 g L^{-1} at time 0 to 1.10 g L^{-1} at 36 h. A maximum cell density of 2.25 g L^{-1} was reached at 12 h.

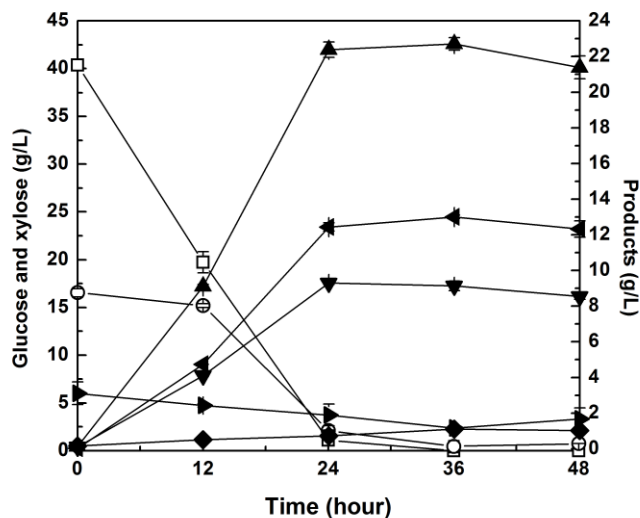


Figure 2.9 ABE fermentation from enzymatic hydrolysate of alkali pretreated switchgrass(-□-, glucose; -○-, xylose; -▶-, acetic acid; -◆-, butyric acid; -▼-, acetone; -◀-, butanol; -▲-, ABE).

To date, no paper has reported butanol production from alkali pretreated switchgrass. A recent study showed that ABE fermentation from enzymatic hydrolysate of dilute sulphuric acid pretreated switchgrass was weak, and the poor ABE production (1.48 g L^{-1}) was improved to 14.61 g L^{-1} only by dilution with water and sugar supplementation (Qureshi et al., 2010b). In the present study, switchgrass hydrolysate was used directly as substrate for ABE fermentation, and a total solvent production of 22.70 g L^{-1} including 13.00 g L^{-1} butanol was obtained, which is comparable to the control fermentation with glucose as substrate. These results indicate that enzymatic hydrolysates of alkali pretreated switchgrass, compared to those pretreated with dilute sulphuric acid, may contain less toxic compounds that could potentially inhibit biobutanol production by clostridium species.

For ABE fermentation of phragmites, there was initially 35.69 g L^{-1} glucose and 15.21 g L^{-1} xylose present in the media (Figure 2.10). The sugar utilization pattern was similar to that of the fermentation with switchgrass hydrolysate. The culture was able to utilize glucose rapidly right after inoculation, and a delay of 12 h was observed before the

culture started to utilize xylose, indicating glucose is preferred by the strain over xylose. In a relevant study, similar sugar utilization pattern was observed when mixtures of glucose and xylose was fermented by *Clostridium acetobutylicum* DSM 792 (Raganati et al., 2014). At 36 h, the fermentation stopped, with 1.5 g L⁻¹ glucose and 1.63 g L⁻¹ xylose left unutilized. An ABE production of 19.75 g L⁻¹ including 9.15 g L⁻¹ acetone, 0.47 g L⁻¹ ethanol and 10.14 g L⁻¹ butanol was obtained, which resulted in an ABE yield and productivity of 0.39 g g⁻¹ and 0.55 g L⁻¹h⁻¹, respectively. These results are similar to what was obtained from switchgrass fermentation in this study. The slightly lower butanol production from phragmites hydrolysate is mainly due to the lower initial glucose concentration compared to that of switchgrass fermentation. It is also worthy to note that more than two-fold ethanol production was obtained from control fermentation than those from hydrolysate, thus making ABE fermentation from lignocellulosic hydrolysates more desirable since less byproducts were produced. However, the reason that causes this difference is not clear. A maximum cell concentration of 1.05 gL⁻¹ was reached at 24 h.

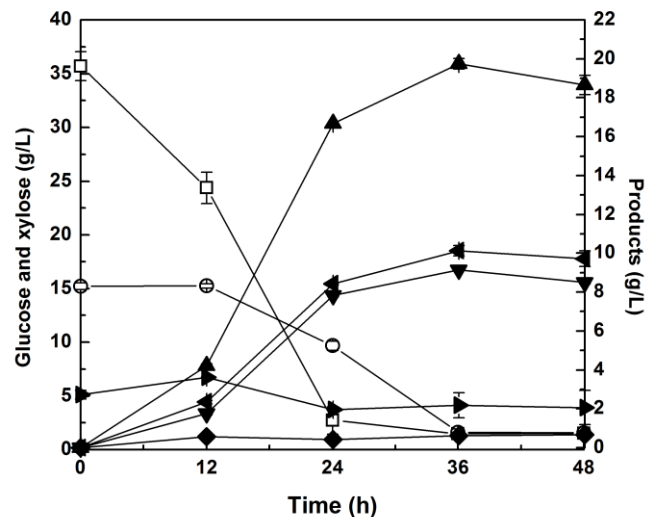


Figure 2.10 ABE fermentation from enzymatic hydrolysates of alkali pretreated phragmites(-□-, glucose; -○-, xylose; -▶-, acetic acid; -◆-, butyric acid; -▼-, acetone; -◀-, butanol; -▲-, ABE).

Attempts of bioethanol production from phragmites have been reported previously (Szijártó et al., 2009). However, there are, to the author's best knowledge, no report about butanol production from phragmites thus far. In the present study, enzymatic hydrolysates of alkali-pretreated phragmites could be successfully fermented by *C. saccharobutylicum* DSM 13864 and ABE yield and productivity comparable to glucose fermentation were obtained. One of the major advantages of alkali-pretreated lignocellulosic materials for butanol production is that no detoxification is required before the enzymatic hydrolysates can be subjected to fermentation. It has been reported that extra detoxification not only complicate the process but also cause undesirable sugar loss (T. Ezeji et al., 2007). According to previous research, there is no need to remove the sediments present in the hydrolysate as well (Gao and Rehmann, 2014; Gao et al., 2012), which potentially simplify the lignocellulosic butanol process and bring it closer to commercialization.

A closer look at the carbon balance between each conversion step in Table 2.4 shows that significant differences are observed between the two lignocellulosic materials in each step of conversion even though the final ABE yields are similar (14.6 g/100 g from raw switchgrass compared to 15.0 g/100 g from raw phragmites). For example, after the pretreatment, less carbohydrates (glucan plus xylan) were preserved in the case of switchgrass compared to phragmites (79.5% vs 91.4%), indicating that switchgrass is more sensitive to alkali pretreatment. However, in the following enzymatic hydrolysis step, a very high hydrolysis rate of 92.6% was achieved for switchgrass compared to 76.8% for phragmites. The reason for this is still unclear, but one of the possible explanations could be that the cellulose component of phragmites contains higher crystallinity compared to switchgrass, thus making the enzymes less accessible to their substrates; the other possible reason could be the higher lignin content of pretreated phragmites than that of pretreated switchgrass. These results indicate that optimization of the respective aspects for each feedstock might further increase the overall product yield.

Table 2.4 Carbon mass balance for ABE fermentation from NaOH pretreated switchgrass (first value) and phragmites (second value).

Compound	Conversion step	Theoretical maximum (g)	Achieved value (g)	Theoretical maximum based on last conversion only (g)
Switchgrass/Phragmites	Raw Material	100.0/100.0	100.0/100.0	100.0/100.0
Glucan and Xylan	Pretreatment	44.4/49.0	35.3/44.8	35.3/44.8
Total Reducing Sugar	Enzymatic Hydrolysis	49.7 ^a /54.8 ^a	36.5/38.5	39.4 ^a /50.1 ^a
ABE	Fermentation	18.9 ^b /20.8 ^b	14.6/15.0	15.0 ^b /19.0 ^b
ABE% of theoretical max		77.2/72.1		

^aAglucan to glucose conversion ratio of 1.111 and xylan to xylose ratio of 1.136 was used;

^bBased on the ABE yield from glucose in the present study

2.5.4. Conclusions

The invasive phragmites, as well as the potential energy crop switchgrass, both exhibit potential as feedstocks for lignocellulosic butanol production. NaOH pretreatment is a suitable method for both grasses, however the two crops react differently to the proposed pretreatment method, despite similar chemical composition leading to the conclusion that a better understand of the underlying mechanism might result in overall solvent yields larger than the 77 and 72 % of the theoretical maximum reported in this study.

2.6 Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction

Kai Gao, Valerie Orr, Lars Rehmann

Preface

The information presented in section 2.6 is based on the paper "Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction", published in *Bioresource Technology*, January 2016, Vol 206, Pages 77-85. This subsection presents the results towards the completion of objective 3 described in section 2.3.

In the last two sub-sections, lignocellulosic biomass including corn cobs, switchgrass, and phragmites were investigated as potential substrates for ABE fermentation mainly due to their abundance. However, substantial challenges remain to be overcome when trying to implement lignocellulosic biomass conversion strategies as discussed in the previous two sub-sections on a commercial scale. For example, to overcome the recalcitrance of lignocellulosic biomass, an extra pretreatment step is necessary, which may compromise the potentially low feedstock cost as it is very energy and chemical intensive. Besides, the cultivation of energy crops also requires land and water use, thus directly or indirectly leading to competition of resources with food-crop production.

In section 2.6, a non terrestrial substrate, microalgae, will be studied as potential feedstock for ABE fermentation. Microalgae have been receiving increasingly attention in recent years mainly due to their ability to accumulate lipids intracellularly, which can be further processed into biodiesel. On the other hand, certain species of microalgae has also been found to accumulate significant amount of starch, which is an ideal carbohydrates for the amylolytic solventogenic clostridia. For this reason, microalgae *Chlorella vulgaris* was cultivated in a pilot scale fermentor (100 L). The collected biomass was dried and stored for further studies. Prior to the utilization of algal carbohydrates for ABE fermentation, lipids were first removed from the cells by two methods, one is the traditional hexane/propanol method, the other is a novel ionic liquid assisted method (experiments carried out by my colleague Valerie Orr). The lipid-extracted algae resulted from both extraction methods were used as substrate for ABE fermentation studies.

2.6.1. Introduction

Concerns as to the sustainability of first generation biofuel production from edible crops has led to the investigation of alternative feedstocks for ethanol and butanol production. Lignocellulosic residues primarily derived from agricultural or forestry wastes have received a great deal of attention in recent years, however, economical lignocellulose conversion to free sugars remains a challenge due to the recalcitrant nature of cellulose, hemi-cellulose, and lignin (John et al., 2011). Microalgae have been primarily investigated for their ability to accumulate a significant portion of their dry weight as lipids suitable for biodiesel production. However, in order to advance the sustainability of microalgae biofuel production, the carbohydrate and protein rich residues remaining after lipid extraction should also be utilized. While a lot of attention has been focused on the use of lipid extracted algae (LEA) for high protein animal and fish feeds (Brennan and Owende, 2010), only a few studies have focused on the fermentation of algal biomass to other biofuels such as ethanol (Brennan and Owende, 2010; Daroch et al., 2013; John et al., 2011), butanol (Cheng et al., 2015; Ellis et al., 2012b; Jernigan et al., 2009; Potts et al., 2012; van der Wal et al., 2013a; Wang et al., 2016), as well as methane and hydrogen (Lakaniemi et al., 2013; Nguyen et al., 2010).

Microalgae residues are an attractive feedstock for fermentation for several reasons. While the composition of algal carbohydrates varies considerably with species, many industrially relevant species of green microalgae such as *Chlorella vulgaris* can accumulate a significant portion of their dry weight as readily digestible glucans such as starch (John et al., 2011). Unlike lignocellulosic biomass, other less readily fermented sugars such as xylose, mannose, and galactose are typically present in only small amounts (Foley et al., 2011). In contrast, macroalgae species such as *Ulva lactuca* use alternative storage molecules like alginate and agarose which contain significant proportions of xylose and rhamnose (van der Wal et al., 2013a). Microalgae carbohydrates present some further advantages as they are not associated with lignin (Foley et al., 2011). Lignin plays a major part in preventing catalysts and enzymes from accessing cellulose and hemicellulose and in the formation of inhibitors during hydrolysis (Ding et al., 2012). Furthermore, lignin is a major source of phenolic inhibitory compounds which can affect

the growth and production formation of microorganisms (T. Ezeji et al., 2007). Finally, microalgae have significant potential as a sustainable feedstock as they can be cultivated in a potentially carbon negative process, they do not require agricultural lands, they are not eaten in any substantial quantity, and they can be cultivated year-round with continuous harvesting (climate dependent) (Foley et al., 2011; John et al., 2011).

Butanol has been proposed as a superior alternative to ethanol as it has a heating value closer to gasoline, is less volatile, and is less corrosive to distribution and storage infrastructure (Dürre, 2007). Historically, high substrate cost combined with the development of petroleum based synthesis pathways resulted in the permanent decommissioning of commercial scale acetone, butanol, ethanol (ABE) fermentation plants (Jones and Woods, 1986). However, increasing concerns over carbon emissions and the environmental impacts of petroleum based fuel production has renewed interest in butanol production from inexpensive sustainable feedstocks (Gao and Rehm, 2014; Gao et al., 2014). Some progress has been made in the characterization of microalgae derived substrates for ABE fermentation using whole waste water algae (Ellis et al., 2012b; Jernigan et al., 2009), macroalgae (Potts et al., 2012; van der Wal et al., 2013a), and lipid extracted algae (Cheng et al., 2015). With the exception of Wang et al. (2016); who used whole algae, these studies have observed poor butanol productivity or conversions.

Finally, one of the greatest challenges in the advancement of the economic feasibility of a microalgae based biofuel process is reducing the cost of harvesting, drying, and lipid extraction. Traditional methods of lipid extraction such as hexane refluxing or extraction with a mixture of a polar solvent and an apolar solvent like hexane/2-propanol are time and energy consuming, use copious amounts of flammable or toxic solvents whose volatile organic compounds contribute to growing air pollution problems, and require well dried algae (<5% moisture). In contrast, in our recent work, the ionic liquid; 1-ethyl-3-methylimidazolium ethylsulfate ($C_2mim EtSO_4$), was used to develop a novel extraction process which is fast and water compatible. The final conditions investigated found fresh wet microalgae treated with $C_2mim EtSO_4$ at a mass ratio of 1:2 with methanol for 1h at ambient temperature, disrupted the microalgae cellular structure and the process was

compatible with water contents up to 82 wt% (Orr et al., 2015). This allowed the facile extraction of the lipid using a small amount of hexane for 15 min. Hexane was used to facilitate the extraction of the lipids for analytical purposes which will auto-partition from the polar hydrophilic ionic liquid microalgae mixture. The ionic liquid could be recycled up to 5 times without any decrease in performance which will aid in offsetting the high cost of ionic liquids (Orr et al., 2015). However, it should be noted that the cost of ionic liquid synthesis has been known to decrease by over 10 fold when scaled up to commercial scale production indicating that the high cost of ionic liquids during the research and development stage is due to their custom synthesis (Wagner and Hilgers, 2008).

In this study, *Chlorella vulgaris* was subjected to two lipid extraction processes and tested for its subsequent digestibility by *Clostridium saccharobutylicum*. Traditional solvent extraction which uses a mechanism of diffusion and is therefore limited by long residence times or high temperatures was contrasted with a previously developed low energy ionic liquid extraction process (Orr et al., 2015). Gross chemical composition of the raw and LEA was quantified. LEAs were subsequently hydrolysed to glucose using acid hydrolysis and detoxified using the resin L-493 as shown in Fig 2.11. HEA and LEA, acid hydrolysates, and detoxified hydrolysates were tested for substrate performance during ABE fermentation.

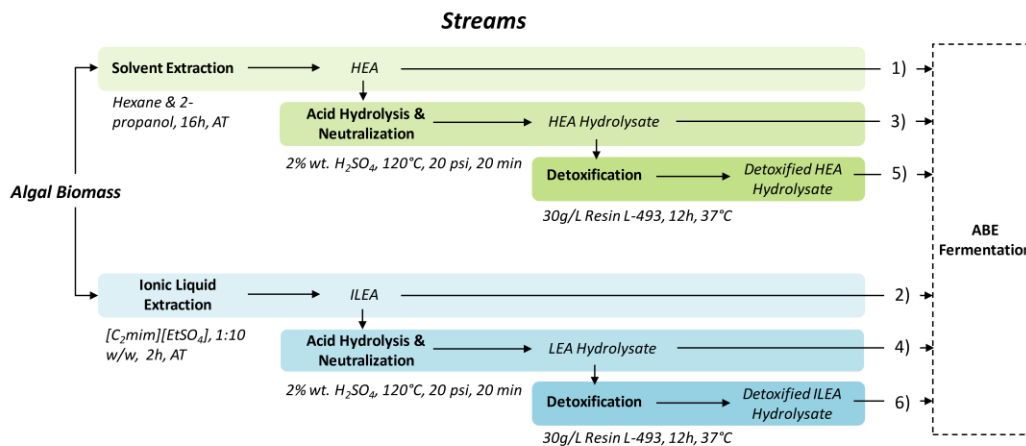


Figure 2.11 Schematic diagram of the experimental design used in this study

(Substrate used for subsequent fermentation are numbered 1-6.)

2.6.2. Materials and methods

Microalgae cultivation and Fractionation

Microalgae strain and cultivation conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained as an actively growing cultures in liquid media using aseptic technique in 150 mL Tris-acetate-phosphate (TAP) media pH 7.0 in 500 mL shaker flasks. Cultures were grown and maintained at 25 °C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The TAP medium used consisted of 20 mM Tris base, 1.58 mM K₂HPO₄, 2.4 mM KH₂PO₄, 7.0 mM NH₄Cl, 0.83 mM MgSO₄, 0.34 mM CaCl₂, 1 mL L⁻¹ glacial acetic acid, and 1 mL L⁻¹ of Hutner's trace elements solution (Hutner et al., 1950). After 48 h, an exponentially growing seed culture was inoculated into either 200 mL of media or a 5 L Labfors bioreactor (Infors HT) at 10% (v/v) and cultured for 5 d at 25°C and 400 rpm in TAP media with reduced NH₄Cl (5 mM) and supplemented with 1% (w/v) glucose in order to induce lipid production.

Harvesting and freeze drying of algal cultures

C. vulgaris cultures were harvested by centrifugation at 3,500 rpm in a Sorvall RT centrifuge (Fisher Scientific) for 20 min. Cell pellets were resuspended in deionised water and washed three times via centrifugation and resuspension to remove residual salts. The washed cells were frozen at -86 °C for a minimum of 8 h and lyophilised using a 4.5 L freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated then stored in a desiccator for further use. Dry weight was determined by overnight drying in an oven at 80 °C.

Analytical determination of total lipid content

The total lipid content was determined as fatty acid methyl ester content in triplicate using a slightly modified protocol from the National Renewable Energy Laboratory (NREL/TP-5100-60958). Briefly, approximately 10 mg of dried algae was mixed with 20 µL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg mL⁻¹), 300 µL of 0.6 M HCl, and 200 µL of a trichloromethane methanol mixture (2:1 v/v) and subsequently incubated for 1h at 85 °C in a water bath with stirring on a magnetic hot plate at 1,000 rpm. After cooling, 1 mL of hexane was added to each sample and mixed at ambient temperature at 1,000 rpm. Samples were centrifuged and 450 µL of the clear top hexane phase was spiked with 50 µL of the internal standard undecanoic acid methyl ester (C11:0Me) to have a final concentration of 100 µg mL⁻¹. FAME was separated and analysed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30m, 0.25 mm, 0.25 µm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min⁻¹ to 200°C, 3°C min⁻¹ 220°C, 10 min. Individual FAMES were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C15:0Me and dividing the total by the weight of cells used in the assay.

Hexane/2-propanol and Ionic liquid extraction

Hexane extractions were performed in triplicate by mixing 0.250 g of freeze-dried algae in 5 mL of hexane:2-propanol solution (H2P; 3:2 v/v) (Hara and Radin, 1978) for 16 h at ambient temperature with agitation by a magnetic stirrer. Ionic liquid extractions were performed by mixing 0.250 g of algae with 2.5 g of [C₂mim][EtSO₄] (Sigma-Aldrich) and incubating at ambient temperature for 2 h with agitation. Hexane extractions were filtered through a Buchner funnel fitted with a fine porosity fritted disc into a separating funnel. The remaining solids were washed with solvent until they were colourless (typically 3 times with 5 mL of H2P). The lipids were recovered from the ionic liquid extractions by the addition of 5 mL of hexane followed by agitation by hand. The extraction was repeated three times and the pooled hexane phase was transferred into a separating funnel. The organic extracts were washed with 0.1 M NaCl and the organic phase was transferred to a pre-weighed vessel. The solvent was evaporated and the mass of extractable lipids was measured using an analytical balance after the solvent was fully removed and the weight no longer fluctuated. Residual HEA was recovered from the funnel by resuspension in hexane and evaporating the solvent at ambient temperature using an evaporation dish. Residual ILEA was recovered by adding 10 mL of water or ethanol to the ionic liquid mixture to precipitate the residual solids. This solution was filtered through a fine porosity Buchner funnel and washed with additional solvent to remove the ionic liquid. The solids were dried using the same method as the HEA. Dried solids were suspended in water at a concentration of 1 mg mL⁻¹ and mounted on a slide for observation under a light microscope (Zeiss, Canada).

Gross compositional analysis

The glucan, starch, and protein compositions of the freeze-dried algae, ILEA, and HEA were determined in triplicate by following the procedures outlined by the National Renewable Energy Laboratory (Laurens, 2013; Sluiter and Sluiter, 2008; Sluiter et al., 2004). Protein content was measured by elemental analysis and a conversion factor of 6.35 was used as previously determined for *Chlorella vulgaris* (Safi et al., 2013).

Acid hydrolysis and detoxification

The LEA was subjected to acid hydrolysis to obtain monomeric sugars. Eight grams of LEA was mixed with 100 mL of 2% (v/v) H₂SO₄ and autoclaved at 121°C for 20 min.

After cooling to ambient temperature, the slurry was neutralized to a pH of 6.0 with 4 M NaOH, followed by centrifugation at 3,500 rpm to remove the sediments. The supernatant was either used directly for fermentation or detoxified by mixing with 30 g L⁻¹ oven-dried resin L-493 (Sigma-Aldrich) for 12 h. To investigate the effect of higher substrate loading, 12 grams of LEA (both HEA and ILEA) was acid hydrolyzed with 100 mL of 2% (v/v) H₂SO₄ and detoxified following the same procedures as described above.

Fermentation of LEA

Clostridium strain and cultivation conditions

Clostridium saccharobutylicum DSM 13864 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was stored as a spore suspension at 4 °C. The spore suspension was inoculated at 10 % (v/v) into seed media (3 g L⁻¹ yeast extract, 5 g L⁻¹ peptone, 5 g L⁻¹ soluble starch, 5 g L⁻¹ glucose, 2 g L⁻¹ ammonium acetate, 2 g L⁻¹ NaCl, 3 g L⁻¹ MgSO₄ 7H₂O, 1 g L⁻¹ of K₂HPO₄, 1 g L⁻¹ of K₂HPO₄, and 0.1 g L⁻¹ FeSO₄ 7H₂O) adjusted to a pH of 6.0, autoclaved, and transferred into an anaerobic chamber (Plas-Labs, Inc., Lansing, MI) for anaerobiosis. The seed culture was incubated for 12 h at 37 °C in order to generate an actively growing culture. Exponentially growing cells were transferred again to fresh media at 10 % (v/v) and this second seed culture was used to inoculate shaker flask experiments in this study.

Batch fermentation

Direct fermentation of LEAs were carried out in shaker flasks in triplicate with LEA at 80 g L⁻¹ substituted for glucose. LEA media was boiled on a hot plate to remove dissolved oxygen and transferred into an anaerobic chamber for anaerobiosis. Acid hydrolysates were prepared by dissolving solid media components into the liquid hydrolysate. For the investigation of the role of algal peptides and amino acids in supporting fermentation, yeast extract and peptone were not added to the media, however, NH₄CH₃COO remained as an inorganic nitrogen source. Samples were taken periodically for ABE and sugar analysis. Biomass density was not monitored due to the turbidity of the media.

To investigate the effect of high substrate loading during direct fermentation of LEA, a fed-batch mode has to be used as media viscosity was significantly increased when LEA was supplemented at higher initial concentrations (>8% w/v, data not shown). Briefly,

fed-batch fermentation was initiated with 8% solid loading of LEA; pre-weighed freeze-dried LEA in grinded powder was directly added to the fermentation broth to provide more substrates for ABE fermentation when the media viscosity was significantly decreased. For Fed-batch HEA, a total solid loading of 16% (w/v) was reached after the fermentation was fed twice at 4% (w/v) each time; For Fed-batch ILEA, a total solid loading of 12% was reached after the fermentation was fed once at 4% due to higher starch content in ILEA.

Sugar and ABE quantification

Concentrations of glucose, acetic acid, butyric acid, acetone, ethanol, and butanol was measured by HPLC as described previously (Gao and Rehmann, 2014). Briefly, an Agilent 1260 HPLC system equipped with a Hi-Plex H column and a refractive index detector (RID) was operated at 15°C with 5 mM H₂SO₄ as mobile phase and a flow rate of 0.5 mL min⁻¹.

2.6.3. Results and Discussion

Lipid extraction and compositional analysis

The results of the gross compositional analysis of untreated *C. vulgaris*, hexane extracted algae (HEA), and ionic liquid extracted algae (ILEA) are presented in Table 2.5. All compositional methodology was based on the validated standard procedures for biomass compositional analysis available from the National Renewable Energy Laboratory (NREL). Total available lipids were quantified by direct transesterification as FAME using a protocol recently developed by NREL (Wychen and Laurens, 2013). Gravimetric lipid recovery is reported for both solvent (13.6 ± 0.1 % wt, $n=3$) and ionic liquid extraction (12.4 % wt ± 0.6 , $n=3$) processes and were not found to be significantly different than the theoretical maximum recovery (13.8 ± 1.7 % wt, $n=3$). Ionic liquid extraction offers several advantages over the traditional solvent extraction processes; namely, greater compatibility with wet biomass (up to 82% wt water content), shorter processing times, and lower process temperatures (Orr et al., 2015; Teixeira, 2012). The residual biomass was recovered from the hexane extraction by filtration, however, during ionic liquid extraction the ILEA was recovered by a two-step process. Firstly, an anti-solvent (water or alcohol) is added to the mixture in order to dissolve the ionic liquid but

precipitate the residual carbohydrates and proteins. Secondly, this mixture is separated by filtration and the ionic liquid is recovered for reuse from the anti-solvent. Recovered ionic liquid can be reused at least 5 times without any decrease in performance (Orr et al., 2015). The composition of the residual algae in terms of total glucan, starch, and protein content is summarized in Table 2.5. Interestingly, protein recovered after the hexane extraction process was higher than the ionic liquid process. Ionic liquids hypothetically aid lipid extraction by disrupting the cellular structure of the algae, which may allow water soluble proteins to be removed during the washing step when water is used as the anti-solvent. Indeed, the use of ethanol as an alternative anti-solvent resulted in the exact same amount of protein recovered as the hexane extraction (27.4 ± 1.6 % wt). Thus, water was subsequently used for a scaled up ionic liquid extraction to produce adequate amounts of ILEA for fermentation. More than 80% of the carbohydrates present in the LEAs were in the form of starch for both HEA and ILEA. The lipid extraction method did not significantly alter the total recovery of sugars with 102.1% recovered after hexane extraction and 96.7% recovered after ionic liquid extraction, however, the ionic liquid extraction recovered a higher proportion of starch than the hexane extraction and resulted in higher total sugar content in the subsequent fermentation of the ILEA hydrolysate (A schematic of the process is shown in Fig 2.11). The compositional analysis is consistent with previously reported values for *C. vulgaris* (Illman et al., 2000). As the extraction of lipids did not significantly differ between hexane and ionic liquid extractions and the processing time is much shorter for the ionic liquid process, compositional analysis indicates this to be the preferable lipid extraction process.

Table 2.5 Gross chemical composition of untreated and lipid extracted *C. vulgaris*

	Lipid [% wt]	Glucan (Starch) [% wt]	Protein [% wt]
Unprocessed Algae	13.8 ± 1.7^a	51.4 ± 1.0 (41.3 ± 1.4)	13.6 ± 0.8
HEA	n.a	59.9 ± 0.0 (36.1 ± 1.6)	27.4 ± 1.6
ILEA	n.a	57.5 ± 0.4 (45.1 ± 2.3)	13.2 ± 0.8

^a, determined as total FAME using NREL LAP (van Wychen and Laurens, 2013)
n.a, not applicable; mean values of triplicate experiments were shown \pm standard error.

2.6.4. Direct fermentation of HEA and ILEA

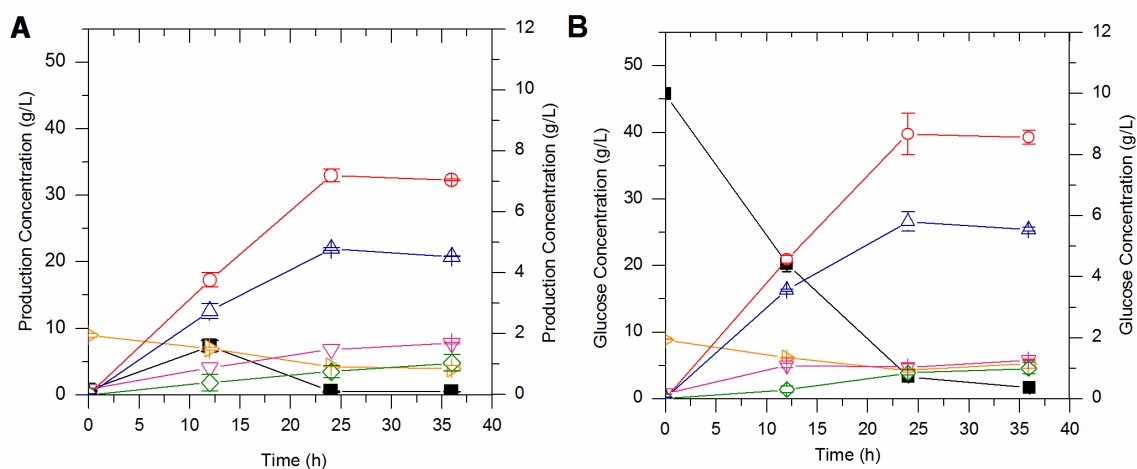


Figure 2.12 Control fermentations with (A) 50 g/L soluble starch or (B) 50 g/L glucose by *C. saccharobutylicum* 13864 at 37 °C, 150 rpm

Some species of *Clostridia* are capable of direct fermentation of starch through the excretion of amylolytic enzymes which degrade the extracellular starch into its glucose monomers (Paquet et al., 1991). Direct fermentation of starch has several advantages over the fermentation of hydrolysates; starch substrates are inexpensive as they require less processing steps and they potentially can be supplemented at higher concentrations than monosaccharides without causing substrate inhibition (Thang et al., 2010). Thang et al. (2011) observed that increasing the starch concentration above 50 g L⁻¹ had neither a negative nor positive effect on solvent production. The ability of *C. saccharobutylicum* to consume starch was first confirmed through a control fermentation using 50 g L⁻¹ soluble starch (Fig 2.12A) and compared to a fermentation using 50 g L⁻¹ of glucose (Fig 2.12B). Glucose was detected within the first 12 h of the starch fermentation indicating *C. saccharobutylicum* is capable of extracellular starch degradation. Fermentations of both starch and glucose were complete within 24 h of inoculation, however, the final ABE production was higher in the glucose fermentations than the starch fermentations (15.33 g L⁻¹ and 12.72 g L⁻¹). Similar fermentation profiles were observed with both sago and cassava starch using *C. saccharoperbutylacetonicum* (Thang et al., 2010). HEA (Stream 1 – Fig 2.11) and ILEA (Stream 2 – Fig 2.11) were assessed as substrates for direct

fermentation. The starch present in HEA (Fig 2.13A) and ILEA (Fig 2.13B) containing 28.88 g L^{-1} and 36.08 g L^{-1} of starch respectively was readily consumed by *C. saccharobutylicum* in both cultures. In both cases the available carbohydrates could directly be fermented reaching final butanol concentrations of 6.63 and 4.99 g L^{-1} respectively. Butanol formation started earlier in case of ILEA. Notably, HEA was also found to contain a higher starting concentration of acetate, presumably derived from the HEA solids. Acetate was likely removed during the washing step of the ionic liquid extraction and thus accounts for the higher starting concentration of acetate found in the HEA fermentations.

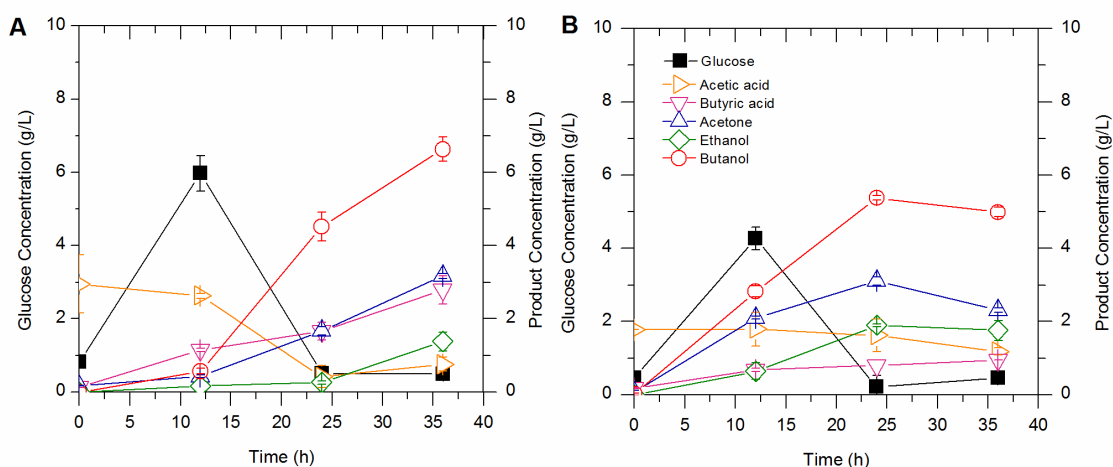


Figure 2.13 Direct fermentation of lipid-extracted algae with 8% solid loading (w/v) at 37 °C, 200 rpm. (A) hexane extracted algae (HEA) and (B) ionic liquid extracted algae (ILEA).

In order to further increase the solvent titer from direct LEA fermentation, fed-batch fermentation was carried out to reach higher total solid loading as shown in Fig 2.14. In Fig 2.14 (A) and (B), 4% HEA was fed once or twice with an initial solid loading at 8%, respectively, thus reaching a final solid loading at 12% and 16%. By comparing Fig 2.14 (A) and 2.14 (B), higher glucose accumulation was found in the latter which is mainly due to higher solid loading. In the end of both fermentations, glucose was almost completely consumed by the strain, and 17.53 g/L ABE including 12.61 g/L butanol, 4.51 g/L acetone and 0.41 g/L ethanol was produced with fed-batch HEA fermentation (twice)

compared to 13.03 g/L ABE including 9.48 g/L butanol, 3.1 g/L acetone and 0.45 g/L ethanol obtained with fed-batch HEA fermentation (once).

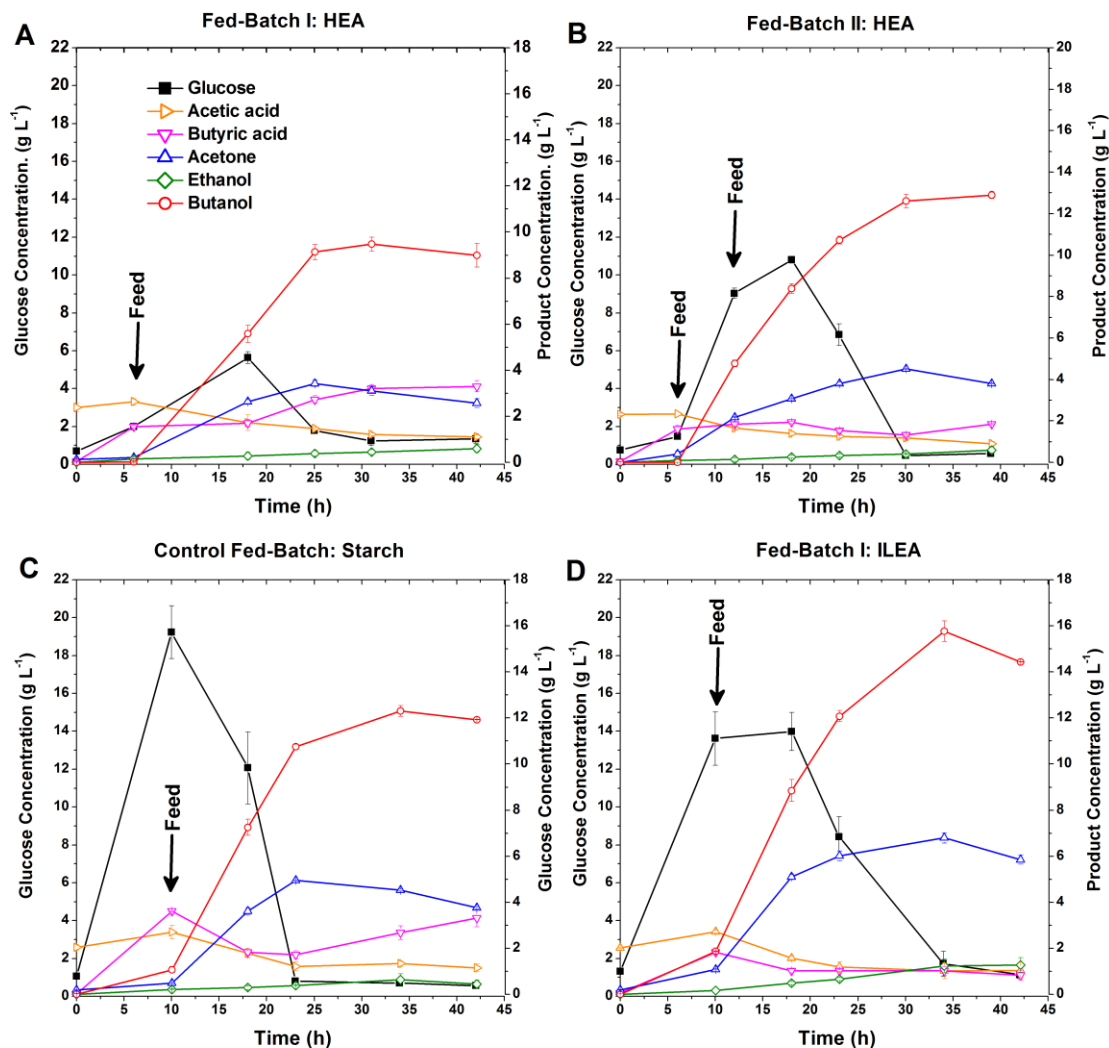


Figure 2.14 Fed-batch ABE fermentation of HEA and ILEA (arrows indicate the time point when grinded LEA was added to the fermentation broth; initial solid loading for all experiments was 8% (weight), grinded LEA powder 4% (weight) was directly added to the fermentation; A, HEA fed once; B, HEA fed twice; C, starch fed once; D, ILEA fed once)

Fed-batch fermentation of ILEA (fed only once) was also carried out to further increase the solvent production as shown in Fig 2.14 (D). Interestingly, glucose accumulation was found at a higher concentration even compared with twice-fed HEA fermentation. This

was probably due to the disruption of microalgae structure by ionic liquid that made the starch present in the ILEA more accessible to the bacterial amylolytic enzymes. In the end of the fermentation, 1.72 g/L glucose was left unutilized by the bacteria, a total solvent production of 23.79 g/L was produced, including 15.77 g/L butanol, 6.79 g/L acetone and 1.23 g/L ethanol. It was interesting to find that solvent productions from fed-batch fermentation of ILEA was even higher than that from the control starch fermentation. In Fig 2.14 (C), fed-batch starch fermentation resulted in an ABE production of 17.46 g/L, including 4.52 g/L acetone, 0.64 g/L ethanol and 12.30 g/L butanol. The reason for a better performance of ILEA as substrate compared to fed-batch fermentation of HEA and soluble starch is not clear yet, which could be due to the increased accessibility of the starch present in the ILEA compared to HEA and soluble starch as discussed earlier. On the other hand, least amount of butyric acid was accumulated during fermentation with ILEA, which may indicate that the strain was more capable of reassimilating butyric acids when ILEA was used as substrates.

Acid hydrolysis and detoxification of LEA

Dilute acid hydrolysis is known as an efficient method for starch hydrolysis and sugar yields greater than 90% are readily obtained using low solid loading concentration (Ho et al., 2013). In this study, LEA was hydrolysed using a final concentration of 2% (v/v) H₂SO₄ and a solid loading of 8% (w/v). The dilute acid hydrolysis used in this study is too mild for the complete hydrolysis of cellulose and therefore it is expected that glucose was predominately derived from the starch present in the LEA (Tsoutsos and Bethanis, 2011). Harsher hydrolysis conditions were avoided in order to limit the formation of fermentation inhibiting by-products (Tsoutsos and Bethanis, 2011). Consequently, ILEA generated a higher glucose concentration from the same solid loading than HEA (46 g L⁻¹ vs. 37 g L⁻¹ from 80 g L⁻¹ solid loading) which corresponded to the higher starch recovery of the ionic liquid based process as determined by enzymatic hydrolysis (45% and 36% by weight respectively, Table 2.5). Both samples were readily hydrolysed and the glucose recovery was between 115-119% based on the available starch, indicating that a small portion of the glucose was derived from non-starch carbohydrates present in the algae.

Acid hydrolysates were fermented after neutralization and centrifugation (Fig 2.15 A and B) or subjected to an additional detoxification step for 12 h using the resin L-493 (Fig 2.15 C and D). Partial glucose consumption (42.0%) was detected during the fermentation of the acid hydrolysate of the HEA (Stream 3 – Fig 2.11, Fig 2.15A) but fermentation was arrested in acidogenesis with unexpectedly high organic acid production (4.34 g L⁻¹ acetic acid and 4.11 g L⁻¹ butyric acid at 12h). This phenomenon, often called “acid crash” or “acidogenesis fermentation”, can be caused by an accumulation of undissociated acids or the presence of formic acid (Wang et al., 2011). However, the reason for this occurring with HEA hydrolysates is unclear as ILEA acid hydrolysate (Stream 4 – Fig 2.11, Fig 2.15B) was readily consumed after a short delay of 24 h. In order to reduce the lag phase, hydrolysates were detoxified using the resin L-493 prior to fermentation. Previous results indicate greater than 95% of total phenolic compounds are removed by the resin L-493 from the hydrolysates of lignocellulosic materials (data not shown). The delay was eliminated for both HEA (Stream 5 – Fig 2.11, Fig 2.15C) and ILEA (Stream 6 – Fig 2.11, Fig 2.15D). Detoxification allowed fermentation of HEA hydrolysates indicating that acid hydrolysis of HEA may create inhibitor compounds which acid hydrolysis of ILEA does not. As HEA was readily consumed in the direct fermentation scheme, it is unlikely that residual solvents (hexane/2-propanol) trapped within the biomass could effect this phenomenon. Detoxified hydrolysates further differed in their organic acid production with ILEA accumulating a lower concentration of butyric acid and resulted in lower final butanol concentrations (6.32 g L⁻¹ compared to 8.05 g L⁻¹ for HEA at 36 h). Butyric acid supplementation is known to effect higher butanol yields during ABE fermentation (Cheng et al., 2015; Regestein et al., 2015). These results combined with the results from undetoxified hydrolysates indicate that the lipid extraction process can significantly affect their subsequent fermentation.

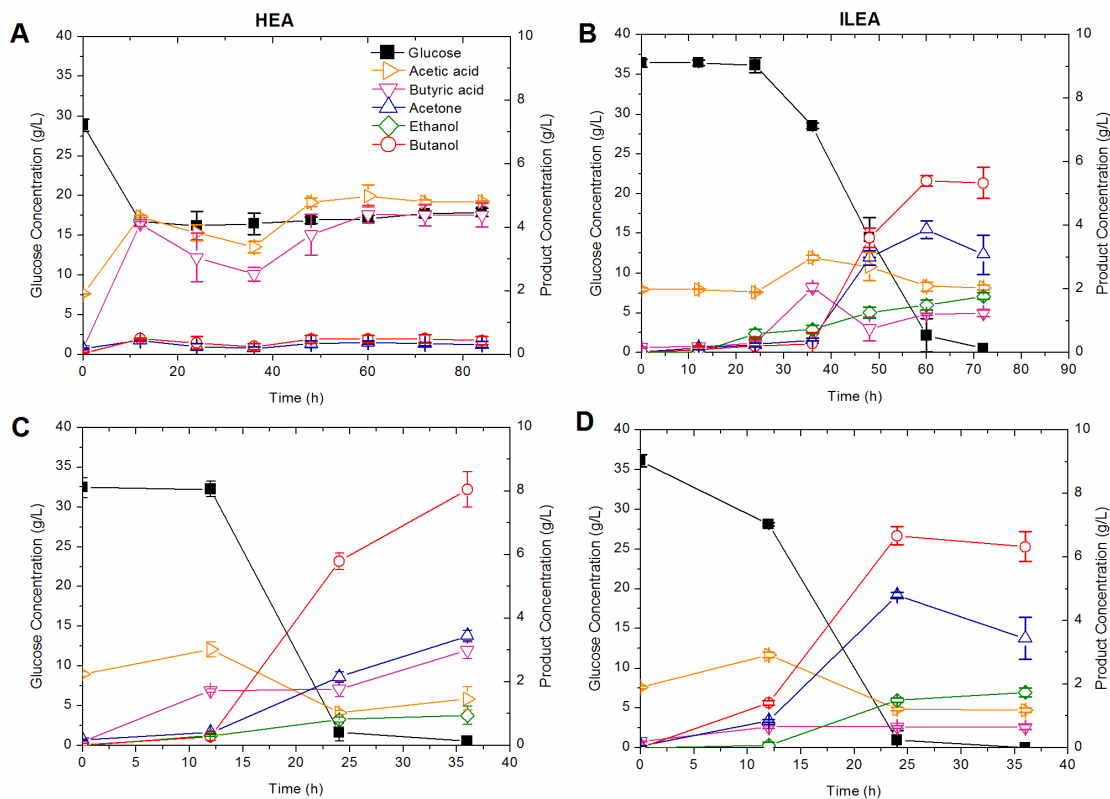


Figure 2.15 Fermentation of acid hydrolysates of lipid extracted algae (A and B, undetoxified; C and D, detoxified by resin adsorption; A and C, HEA; B and D, ILEA)

Subsequently, acid hydrolysis of LEA was carried out at higher solid loading (12%) using the same procedures and the resulted detoxified hydrolysate was fermented to obtain higher solvent productions. At higher substrate loading, the efficiency of acid hydrolysis was not compromised as proportional increase in glucose concentration was observed compared to results at lower solid loading. As shown in Fig 2.16 (A), for HEA hydrolysate, the fermentation began with an initial glucose level of 49.78 g/L glucose. All the glucose was utilized by the strain at 17 hrs and 15.13 g/L ABE was produced, including 5.14 g/L acetone, 0.29 g/L ethanol and 9.7 g/L butanol. For fermentation of ILEA hydrolysate (Fig 2.16B), higher initial glucose concentration was obtained (66.77 g/L), which was mainly due to the higher starch content of ILEA. After 38 h of fermentation, 14.46 g/L glucose was left unutilized, 17.24 g/L solvent was produced, including 3.97 g/L acetone, 0.93 g/L ethanol, and 12.34 g/L butanol. The partial utilization of glucose was probably due to product inhibition mainly by butanol toxicity.

Interestingly, this phenomenon was not observed during fed-batch ILEA fermentation (Fig 2.14D), and glucose utilization was still observed at similar butanol level. It was speculated that certain compounds present in the ILEA has stimulating impact on butanol fermentation, however, these compounds may be degraded after acid hydrolysis.

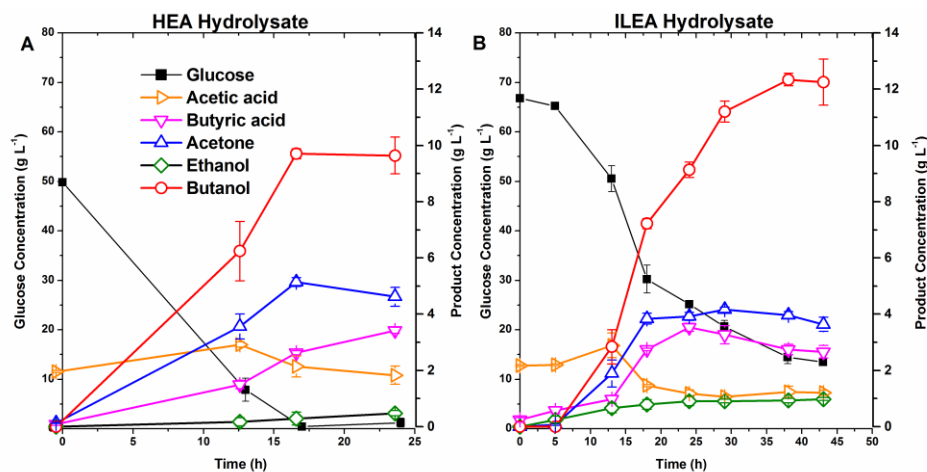


Figure 2.16 ABE fermentation of detoxified acid hydrolysates of LEA at 12% solid loading(A, HEA hydrolysate, B, ILEA hydrolysate; 37°C, 200 rpm).

Effects of algal derived amino acids on ABE fermentation

Compositional analysis of HEA indicated a considerable portion of protein recovered after lipid extraction. While *Clostridia* are unlikely to consume whole proteins in HEA, acid hydrolysis is also commonly employed for the degradation of proteins. Therefore, the effect of peptide and amino acids present in the acid hydrolysate of HEA were studied in order to minimize the cost of media components. Acid hydrolysate of HEA containing media with yeast extract and peptone and without yeast extract and peptone were compared to controls using pure glucose (Table 2.6). The acid hydrolysate of ILEA was not tested due to the low protein recovery. As seen in Fig 2.17 A and B, yeast extract and peptone do not contribute to higher alcohol production when glucose is used as a control, however, it does reduce total culture time and consequently increased ABE productivity (Table 2.6). When the detoxified hydrolysate is used culture time increases. However, in this case, supplementation with yeast extract and peptone does increase the butanol yield

from 5.42 to 8.05 g L⁻¹ (Fig 2.17C) and does not alter the culture time. It should also be noted that for detoxified hydrolysates of HEA, 23% of the glucose was not consumed in the absence of yeast extract and peptone whereas glucose was completely depleted when yeast extract and peptone were used. A higher butanol titer was achieved during fermentation when using detoxified acid hydrolysate supplement with yeast extract and peptone compared to the glucose controls (Table 2.6). This was also associated with higher butyric acid production during the fermentation of the detoxified hydrolysate than the glucose control. Interestingly, Wang et al (2016) found that protein concentrations greater than 500 mg L⁻¹ decreased butanol production and yield when fermenting lipid extracted *C. vulgaris* using *C. acetobutylicum* and included a NaOH wash step prior to acid hydrolysis in order to remove protein. This effect was not seen in this work.

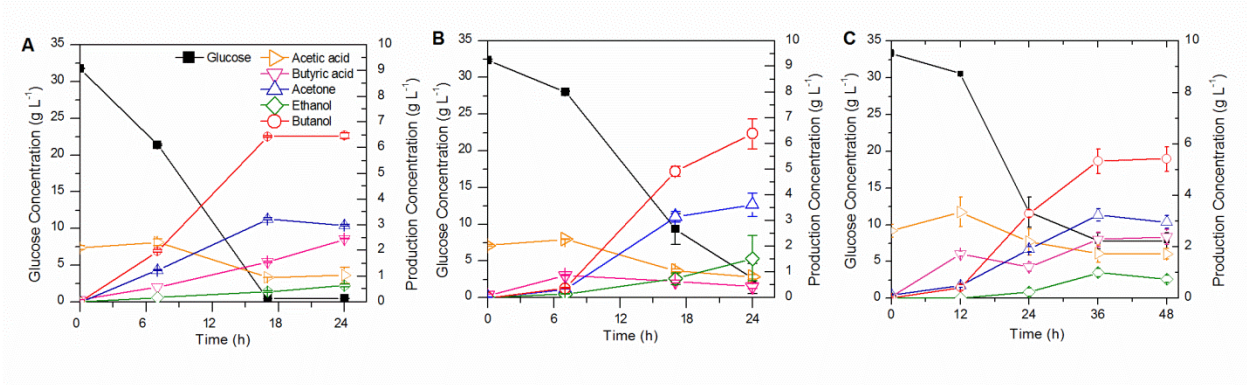


Figure 2.17 The effect of supplementation of yeast extract and peptone on fermentation at 37 °C, 200 rpm. (A) Glucose control with yeast extract and peptone. (B) glucose control without yeast extract and peptone. (C) detoxified acid hydrolysate of HEA without yeast extract or peptone.

Table 2.6 End point fermentation results from glucose or acid hydrolysate of HEA with and without YEP (yeast extract and peptone)

Media Composition	Glucose (Controls)		Detoxified Hydrolysate of HEA	
	+YEP	-YEP	+YEP	-YEP
Substrate Concentration				
Initial glucose Conc. [g L ⁻¹]	31.78 ± 0.08	32.33 ± 0.15	32.44 ± 1.25	33.35 ± 0.46
Final glucose Conc. [g L ⁻¹]	0.44 ± 0.01	2.63 ± 2.05	0.5 ± 0.00	7.83 ± 1.07
Organic Acids				
Acetic [g L ⁻¹]	0.96 ± 0.01	0.81 ± 0.08	1.47 ± 0.37	1.72 ± 0.21
Butyric [g L ⁻¹]	1.55 ± 0.03	0.44 ± 0.10	2.99 ± 0.26	2.38 ± 0.3
Organic Solvent				
Acetone [g L ⁻¹]	3.23 ± 0.05	3.61 ± 0.47	3.45 ± 0.16	2.96 ± 0.25
Ethanol [g L ⁻¹]	0.41 ± 0.04	1.52 ± 0.88	0.94 ± 0.29	0.75 ± 0.13
Butanol [g L ⁻¹]	6.45 ± 0.04	6.38 ± 0.59	8.05 ± 0.56	5.42 ± 0.48
Culture Performance				
Total Time [h]	17	24	36	36
Butanol [g g ⁻¹]	0.21	0.21	0.25	0.21
ABE [g L ⁻¹ h ⁻¹]	0.59	0.48	0.35	0.25

Batch fermentation of algal substrates

Recently, several attempts at the batch ABE fermentation of both micro- and macroalgae have been reported and are summarized and compared to the yields obtained in this study in Table 2.7. Only a small number of studies have begun to explore the use of algae biomass as a substrate for ABE fermentation. Only one recent study explored the use of lipid extracted algae for ABE fermentation. In this work, lipids were extracted from *C. sorokiniana* using microwave assisted solvent extraction. Several methods of hydrolysis were tested including enzymatic hydrolysis, however, no detoxification schemes were employed. Interestingly, the enzymatic hydrolysis using cellulase which should contain

the lowest proportion of inhibitory compounds was amongst the worst hydrolysis strategies for subsequent butanol production (Cheng et al., 2015). *C. acetobutylicum* ATCC 824 used in this study is known to be capable of producing amylolytic enzymes (Paquet et al., 1991) which should enable them to digest starch, the major carbohydrate present in *C. sorokiniana* (Choix et al., 2012). This suggests that perhaps residual solvents were not removed from the biomass prior to hydrolysis. In fact, increasing the proportion of hydrolysate resulted in poorer butanol yields (Cheng et al., 2015) which would be expected if toxic solvents were not completely removed.

The butanol concentrations and ABE productivities achieved in this study are amongst the highest reported thus far. This is partly due to the use of *C. saccharobutylicum* which can achieve complete fermentation in less time as well as the inclusion of a detoxification step. Wang et al. (2016) reported the fermentation of the whole biomass of *C. vulgaris* to butanol using *C. acetobutylicum* resulting in a higher butanol production 13.1 g L^{-1} but a similar conversion of 0.24 g g^{-1} (Wang et al., 2016) which is in line with the results presented in this study. The differences in butanol production is due to the lower sugar concentration used in this study ($33\text{-}36 \text{ g L}^{-1}$ vs. 55.6 g L^{-1}) (Wang et al., 2016).

Finally, feedstock cost is currently one of the biggest challenges in butanol production and by producing multiple biofuel productions from a single feedstock the feedstock cost can be split between each process. Previous work found low butanol conversion from microalgal sugars or very low productivity. The current study work demonstrates that production of biodiesel and butanol from a single feedstock is definitely feasible.

Table 2.7 Summary of ABE fermentation from algae-based substrates

Algal Species	Species (Strain)	Pretreatment	Substrate Concentration (Composition)*	Butanol Conc. (ABE) [g L ⁻¹]	ABE Productivity [g L ⁻¹ h ⁻¹]	Butanol yield or (Total ABE yield) [g g ⁻¹ glucose ¹ , starch ² total sugars ³]	Reference
<i>C. vulgaris</i> (Ionic liquid extracted algae)	<i>C. saccharobutylicum</i> (DSM 13864)	Untreated	8% (w/v)	4.99 (9.06)	0.25	0.15 ² (0.29 ²)	This study
		2% H ₂ SO ₄	36.45 g L ⁻¹ Glc	5.34 (10.19)	0.14	0.15 ¹ (0.28 ¹)	
		2% H ₂ SO ₄ & L-493	36.14 g L ⁻¹ Glc	6.32 (11.50)	0.32	0.17 ¹ (0.32 ¹)	
<i>C. vulgaris</i> (Hexane/2-propanol extracted algae)	<i>C. saccharobutylicum</i> (DSM 13864)	Untreated	8% (w/v)	6.63 (11.18)	0.31	0.23 ² (0.31 ²)	This study
		2% H ₂ SO ₄	28.88 g L ⁻¹ Glc	0.44 (1.52)	0.02	0.04 ¹ (0.14 ¹)	
		2% H ₂ SO ₄ & L-493	32.44 g L ⁻¹ Glc	8.05 (12.44)	0.35	0.25 ¹ (0.39 ¹)	
<i>C. vulgaris</i>	<i>C. acetobutylicum</i> (ATCC 824)	Enzymes	38.6 g L ⁻¹ TS	n.d	n.a	0.00 (n.a)	(Wang et al., 2016)
		3% H ₂ SO ₄	32.7 g L ⁻¹ TS	1.5	n.a	0.05 (n.a)	
		3% H ₂ SO ₄	69.4 g L ⁻¹ TS	0.5	n.a	0.01 ¹ (n.a)	
		1% NaOH + 3% H ₂ SO ₄	55.6 g L ⁻¹	13.1 (19.9)	0.27	0.24 ¹ (n.a)	
		2% H ₂ SO ₄ & 2% NaOH	37.93 g L ⁻¹ TS	3.35 (4.97)	0.07	0.13 ¹ (0.19 ¹)	
<i>C. sorokiniana</i> (Hexane/Methanol extracted algae)	<i>C. acetobutylicum</i> (ATCC 824)	2% H ₂ SO ₄ & 2% NaOH	60.81 g L ⁻¹ TS	2.75 (4.09)	0.01	0.10 ¹ (0.15 ¹)	(Cheng et al., 2015)
		2% H ₂ SO ₄ & 2% NaOH	71.21 g L ⁻¹ TS	2.42 (3.16)	0.02	0.12 ¹ (0.13 ¹)	
		2% H ₂ SO ₄ & 2% NaOH	89.08 g L ⁻¹ TS	3.86 (6.32)	0.02	0.09 ¹ (0.14 ¹)	
		Untreated	10% (w/v)	0.52 (0.73)	0.01	0.17 ¹ (0.26 ¹)	
Mixed culture waste water	<i>C. saccharoperbutyl-acetonicum</i> (ATCC 27021)	1 M H ₂ SO ₄ & 5 M NaOH	10% (w/v)	2.26 (2.74)	0.03	0.20 ¹ (0.24 ¹)	(Ellis et al., 2012b)
		1 M H ₂ SO ₄ & 5 M NaOH	10% (w/v) + 1% (w/v) Glc	5.61 (7.27)	0.08	0.21 ¹ (0.27 ¹)	
		1 M H ₂ SO ₄ & 5 M NaOH + cellulase, xylanase	10% (w/v)	7.79 (9.74)	0.10	0.25 ¹ (0.31 ¹)	
		Untreated	10% (w/v)	0.52 (0.73)	0.01	0.17 ¹ (0.26 ¹)	
Mixed culture waste water	<i>C. saccharoperbutyl-acetonicum</i> (ATCC 27021)	4% H ₂ SO ₄ , 3 h, 120°C	n.a	n.a	n.a	0.13 ³ (n.a)	(Jernigan et al., 2009)
<i>Ulva lactuca</i> (Macroalgae)	<i>C. beijerinckii</i> (NCIMB 8052)	10 min 150°C + cellulase	15.8 g L ⁻¹ TS (50% Glc, 35% Rha, 15% Xly)	3.3 (5.5)	0.02	0.21 ³ (0.35 ³)	(van der Wal et al., 2013b)
	<i>C. acetobutylicum</i> (ATCC 824)	10 min 150°C + cellulase	15.6 g L ⁻¹ TS (50% Glc, 35% Rha, 15% Xly)	0.3 (0.3)	0.00	0.03 ³ (0.03 ³)	
<i>Ulva lactuca</i> (Macroalgae)	<i>C. saccharoperbutyl-acetonicum</i> (ATCC 27021)	1% H ₂ SO ₄	15.2 g L ⁻¹ TS (27% Glc, 57% Ara, 16% Xyl)	4.51 (n.a)	n.a.	0.29 ³ (n.a)	(Potts et al., 2012)

-, no pretreatment or hydrolysis for the substrate; na, not available; *TS, Total sugar concentration; Glc, Glucose; Ara, arabinose; Xly, xylose; Rha, rhamnose

2.6.4. Conclusions

Algal residues were assessed as ABE fermentation substrates. Fermentation results differed significantly between HEA and ILEA indicating the extraction method affects the subsequent fermentation. Substrate utilization and solvent yield was significantly improved after resin adsorption demonstrating the need for hydrolysate detoxification prior to fermentation. Amino acids and peptides released by acid hydrolysis were found to have little effect on fermentation. Direct fermentation of starch was possible using the LEAs, and solvent titer comparable to or even better than control starch fermentation was obtained at increased solid loadings. Direct fermentation reduced the number of preparatory steps and accordingly would be a faster and lower cost option.

2.7 Combined Detoxification and In-situ Product Removal by a Single Resin During Lignocellulosic Butanol Production

Kai Gao and Lars Rehamann

Preface

The information presented in section 2.7 is based on the paper " Combined Detoxification and In-situ Product Removal by a Single Resin During Lignocellulosic Butanol Production", published in Scientific Reports, 6:30533(DOI: 10.1038/srep30533). This subsection presents the results towards the completion of objective 4 described in section 2.3.

Despite algae being a highly promising feedstock for biofuel production, the current availability of lignocellulosic biomass renders it the most likely feedstock for the next generation of biofuel plants. One of the major problems associated with biofuel production from lignocellulosic biomass is the generation of fermentative inhibitors during pretreatment (Jönsson et al., 2013). In sections 2.4 and 2.5, alkali-pretreated lignocellulosic feedstocks including corn cobs, switchgrass, and phragmites are successfully fermented for butanol production with solvent yield comparable to glucose fermentation. One of the major contributing factors to these success could be the less inhibiting compounds generated compared to other pretreatment methods and a post pretreatment washing, however, the cost of alkali pretreatment is a serious limitation (Jönsson and Martín, 2015).

Dilute sulfuric acid pretreatment is considered as one of the most promising pretreatment methods in terms of industrial application and it has been studied for a wide range of lignocellulosic biomass (Hu and Ragauskas, 2012; Jönsson and Martín, 2015). In general, after dilute acid pretreatment, the majority of hemicellulose of the original biomass is hydrolysed in the pretreatment liquid, and the cellulose part remains in the form of solids but with enhanced enzymatic digestibility. A side effect of acid pretreatment is the formation of lignocellulose-derived by-products that inhibit the fermentative organisms (Jönsson and Martín, 2015), and different techniques including chemical additives (such

as alkali and reducing agents), enzymatic treatment (laccase and peroxidase), heating and vaporization, liquid-liquid extraction (e.g. ethyl acetate), liquid-solid extraction (e.g. activated carbon, ion-exchange resin, etc) as well as microbial treatment have been developed to overcome this issue as summarized by Jönsson et al (2013).

Another major problem associated with traditional ABE fermentation is butanol toxicity. Butanol, the product of interest, can be severely inhibiting to the fermentation organism once accumulated to a certain amount (~10 g/L) in fermentation broth, which results in not only expensive downstream cost from distillation of a highly diluted solvent stream, but limit the initial substrate concentration that can be utilized by the bacteria (Kujawska et al., 2015).

In this section, dilute sulfuric acid pretreated phragmites will be used as substrates for ABE fermentation. To overcome the issue of fermentative inhibitors, a commercially available polymeric resins (Optipore Dowex[®] L-493) was used to remove the toxic compounds present in the hydrolysates. The resin was found to be efficient in removing phenolic compounds and the fermentation of detoxified phragmites hydrolysate was significantly improved compared to the undetoxified hydrolysate. Furthermore, the role of resin L-493 as an in-situ butanol removal device was also explored with phragmites hydrolysate. Resin L-493 has been reported to have a high affinity and selectivity to butanol, and has been used in glucose-based ABE fermentation for butanol removal (Nielsen and Prather, 2009; Wiehn et al., 2013). In the present study, phragmites hydrolysate (supplemented with glucose) was first detoxified with resin L-493, and fermentation was carried out without removing the resin. Results showed that the clostridium cells were able to convert all the sugars present initially in the hydrolysates since the butanol toxicity was significantly reduced in the help of butanol-absorbing resin. Butanol was efficiently removed by methanol from the resin after the fermentation was finished.

The research described in section 2.7 provides a novel solution to remove fermentative inhibitors by polymeric resins generated during pretreatment of lignocellulosic biomass

under acidic conditions. Furthermore, dual functionality of the resin was realized by also using it as an in-situ product removal agent.

2.7.1. Introduction

Butanol, a major product of the acetone-butanol-ethanol (ABE) fermentation, is an important bulk chemical with applications in the productions of solvents, cosmetics and pharmaceuticals. It has also been considered as a renewable liquid transportation fuel with advantages over ethanol (Dürre, 2007; Jones and Woods, 1986). Traditionally, agriculturally produced substrates such as corn (Nasib Qureshi and Blaschek, 2000), molasses (Qureshi and Maddox, 1992), and whey permeate (Ennis and Maddox, 1989) were used for industrial production of ABE through fermentation processes. However, ABE fermentation based on these conventional substrates suffered from increasing prices of feedstock particularly after the second world war and was considered economically unfavourable compared to synthetic routes using petrochemical feedstock (Jones and Woods, 1986). To reduce the cost of fermentative butanol production, alternative low cost substrates such as Jerusalem artichokes (Sarchami and Rehm, 2015, 2014), lignocellulosic materials such as corn cobs, corn stover, switchgrass and phragmites (Gao and Rehm, 2014; Gao et al., 2014; Napoli et al., 2011; Raganati et al., 2012) have been investigated as potential substrates for ABE fermentation. The costs of abundant lignocellulosic biomass tends to be low (King et al., 2010), however extra process steps such as pretreatment and hydrolysis are required prior to fermentation (Hu and Ragauskas, 2012). Additionally, fermentation inhibitors are often generated during the pretreatment process which either halt or slow down reaction rates of the fermentation (Qureshi et al., 2013). Thus an extra detoxification step is often required to achieve successful fermentation.

Another major problem associated with ABE fermentation is end-product inhibition. For example, butanol titers in a batch fermentation by *C. acetobutylicum* ATCC 824 (most commonly studied butanol producing strain) rarely exceed 13 g/L (Jones and Woods, 1986). Such dilute product streams will cause high energy costs during downstream processing (Wiehn et al., 2013). In fact, economic analyses have suggested that if the butanol titers were raised from 12 g/L to 19 g/L, the separation costs would be cut in half

(Papoutsakis, 2008). One of the approaches to avoid inhibitory concentrations is to remove butanol from the cultures while it is being produced. One of the many in-situ product removal techniques is through adsorption by a polymeric resin. Compared to other types of in-situ butanol recovery techniques, adsorption shows superior properties in the stability of extraction phase, biocompatibility, phase immiscibility, reusability, and overall energy efficiency (Amrita and Vijayanand S., 2012; Liu et al., 2014; Prpich et al., 2006; Rehmann et al., 2007). Dowex®Optipore L-493 (poly styrene-*co*-DVB derived resin) has been identified to have high butanol affinity and partitioning coefficient from a pool of commercial resins available (Nielsen and Prather, 2009) and was recently used in expanded bed adsorption for a fed-batch ABE fermentation from pure glucose (Wiehn et al., 2013). On the other hand, L-493 has been reported to be able to remove various organic compounds from water including phenol (Ipek et al., 2012) and endocrine disrupting compounds (EDCs) (Solak et al., 2014). However, to the author's best knowledge, this resin has not been used for detoxification of lignocellulosic hydrolysate so far.

A potential lignocellulosic feedstock is *Phragmites australis*, also known as common reed. It is regarded as an invasive species in North America (Saltonstall, 2002) and has recently been considered as a promising bioenergy crop due to many attractive features such as high biomass productivity (C4 photosynthesis), low requirement on irrigation and fertilizer, easy large-scale planting, and less drying costs due to winter standing (Sathitsuksanoh et al., 2009). Alkali-pretreated phragmites hydrolysate was successfully fermented to butanol (Gao et al., 2014), however, ABE fermentation from acid pretreated phragmites has not yet been reported.

The objectives of the present study are therefore three-fold. The overall goal is the utilization of phragmites with dilute sulphuric acid pretreatment as substrate for ABE fermentation, where detoxification of the feedstock (removal of phenolix, furans, etc.) and detoxification of the fermentation broth (in-situ product removal of butanol) are realized with a single sorption resin.

2.7.2. Materials and methods

Raw materials

Raw phragmites were collected near London, Ontario, Canada. Air-dried biomass (moisture content ~5%) was first cut into small pieces (~5 cm × 2 cm) and then grounded by a grinder (IKA® MF10, Sigma Aldrich) to pass a 2 mm sieve and stored in sealed plastic bags at room temperature until used for pretreatment. *C. saccharobutylicum* DSM 13864 was purchased from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. Cultures of this strain were routinely maintained as spore suspensions at 4°C in seed cultures. Preparation of inoculums has been reported elsewhere (Sarchami and Rehm, 2014).

Dilute sulfuric acid pretreatment and enzymatic hydrolysis

Biomass (20 grams of phragmites) was mixed well with 180 ml 0.5%, 1% and 2% (v/v) sulphuric acid in 500 ml screw-capped bottles, and soaked for 15 min before thermal treatment for 60 min at 121°C (AMSCO Eagle Series 2041 Autoclave, Steris, Mentor, OH). After the pretreatment, the slurries (SAH) were cooled to room temperature, and samples were taken to measure the sugar concentration and inhibitor levels in the hydrolysate.

Before enzymatic hydrolysis, the pH of the SAH was adjusted to 5 by adding 10 M NaOH. Cellic CTec2 (kindly donated by Novozyme) was dosed to give a filter paper activity of 15 FPU/g biomass. Hydrolysis was performed in an orbital incubator (InforsMulitron, Infors Switzerland) at 50 °C, 150 rpm for 72 h. Samples were periodically taken for sugar analysis. The enzymatic hydrolysates of dilute sulfuric acid pretreated phragmites (SAEH) was transferred to 50 ml falcon tubes and centrifuged at 3,500 rpm (ST40R, Thermo Scientific) to remove sediments. The supernatant was stored in pre-sterilised flasks at 4 °C for detoxification and fermentation studies.

Detoxification and fermentation

Dowex® Optipore L-493 (a commercial polymer resin, chemical name poly (styrene-co-DVB)) was used for detoxification studies. It has a particle size ranging from 20 to 50

mesh, a pore size of 1.16 ml/g pore volume, and the bulk density is 680 g/L. The resin has a high surface area of 1100 m²/g. Before use, the resin was dried at 50 °C in a convection oven for 24 h. Approximately 0.9 g of resin was added to 50 ml serum bottles, then 30 ml of the SAEH (supernatant collected from previous step) was transferred to the same bottle, corresponding to a resin concentration of 30 g/L. The bottle was capped air tight and placed in an incubator (InforsMultitron, Infors Switzerland) at 37°C, shaking (200 rpm) for 12 hours to allow detoxification. Control experiments were carried out without the addition of polymer resin as a comparison. Samples were taken at the end of experiments to measure the concentration of sugars, acids, furfural, HMF and total phenolic compounds.

About 25 ml of detoxified SAEH (without resin) was transferred to a pre-sterilized 125 ml Erlenmeyer flask by aspiration through syringe. Nutrients for bacteria growth were added as reported previously (Gao and Rehmann, 2014); the media contained (per litre) 3 g yeast extract, 5 g peptone, 2 g ammonium acetate, 2 g NaCl, 3 g MgSO₄, 1 g KH₂PO₄, 1 g K₂HPO₄, and 0.1 g FeSO₄. No extra carbon source was supplemented. The pH of the media was adjusted to around 6.5 by 1 M NaOH/H₂SO₄ before fermentation started. The flasks containing the media were heat shocked in a water bath (80°C) for 10 min to remove dissolved oxygen and transferred to an anaerobic chamber (Model 855-ACB, Plas Labs, Lansing, MI) for anaerobiosis. *Clostridium* cells cultured at a temperature of 37 °C for 10–12 h without any agitation were used as the inoculum for fermentation studies at an inoculum size of 10% (v/v). Samples were taken every 12 h until fermentation stopped. Fermentation with undetoxified hydrolysate was also carried out as control experiments.

Fermentation with in-situ butanol removal

Supernatant of SAEH (30 ml) was transferred to pre-sterilised shaking flasks (125 mL). After nutrients were added, pure glucose was supplemented to adjust the total sugar concentration in fermentation media to 80 g/L. 1.5 g polymer resin was added to the media before the pH was adjusted to 6.5. The flasks were heat shocked and transferred to anaerobic chamber to equilibrate overnight (37 °C, 200 rpm). The fermentation media

containing polymer resins was inoculated with 3 ml seed cultures to initiate fermentation. Samples were taken every 12 h for measurements of sugars, acids, and solvents.

Equilibrium adsorption isotherm and desorption

Butanol adsorption experiments were performed using 30 ml serum bottles containing 0.5 g L-493 resin(oven dried) and 10 mL aqueous butanol solution with concentrations of 4 g/L, 8 g/L, 16 g/L and 20 g/L. Butanol equilibrium data between methanol and resin were generated similarly (1 gram of resin was immersed in 3 ml methanol containing same concentrations of butanol as mentioned above). The bottles were capped tightly and agitated for 12 h (37 °C, 220 rpm). At the end of the adsorption experiments, samples were taken by aspiration through a syringe to measure the butanol concentration in the liquid phase. The amount of butanol adsorbed by the resin (mg/g) was calculated via the mass balance as reported elsewhere (Lin et al., 2012).

Desorption of butanol from equilibrated resins (both aqueous phase and fermentation with in-situ product removal) was performed by first removing free bulk liquid (fermentation media) by syringe aspiration. Then methanol (20 ml pure methanol/g oven-dried resin) was injected. The bottle was sealed properly and placed into an air-bath incubator (InforsMultitron, Infors Switzerland) at 37°C (220 rpm) for 12 h. Samples were taken to measure the concentrations of butanol and other components desorbed from the resin via HPLC.

Analytical methods

Concentrations of glucose, xylose, acetic acid, butyric acid, and ABE were determined by high pressure liquid chromatography (HPLC) using an Agilent 1260 liquid chromatography system (Agilent Technologies, Inc., CA, USA) equipped with a Hi-plex H column (7.7×300mm) at 15°C and a refractive index detector. 5 mM H₂SO₄ was used as mobile phase with a flow rate of 0.5 ml/min. Furfural and hydroxymethylfurfural (HMF) were measured by a diode array detector (DAD, G1315C, Agilent) with a wavelength of 280 nm. An Agilent Poroshell 120 EC-C18 column (4.6×100 mm) was used with the same HPLC system to measure individual phenolic compounds. The signal of DAD was set to 270 nm and a gradient method was used. Briefly, the gradient started

and held at 95% mobile phase A (0.1% Formic acid) and 5% mobile phase B (Acetonitrile); then ramped to 40% B, and then re-equilibrated to the initial condition. The flow rate was kept at 1 ml/min. All samples collected were centrifuged at 12,000 rpm for 10 min and filtered through 0.2 μm filters before analysis.

Total phenolic compounds were semi-quantitatively estimated based on a slightly modified method reported elsewhere (Li et al., 2014). In brief, the hydrolysate was diluted with distilled water and the absorbance was determined at 280 nm by an UV-Visible spectrophotometer (Evolution 60S, Thermo Scientific) with distilled water as reference. The OD value is presented in this paper as a relative measure of the total phenolic compounds in solution.

ABE productivity (g/L/h) was calculated as the maximum ABE concentration achieved (g/L) divided by the fermentation time (h). ABE yield was calculated as the maximum amounts of solvents (ABE) produced divided by the amount of sugar available initially in the fermentation media and are expressed as g/g.

Triplicates were carried out for all experiments and the average numbers and standard errors are shown in tables and figures.

2.7.3. Results and discussion

The experimental design is schematically illustrated in Figure 2.18. *Phragmites australis* was treated with dilute sulphuric acid to generate sulfuric acid pretreated hydrolysates (SAH), which was further hydrolyzed enzymatically (CellicCTec 2, Novozyme, Denmark) to obtain fermentable sugars (SAEH). The effect of inhibitor removal was investigated by fermenting the SAEH directly (stream 1) and after equilibrating with resin L-493 (Dow Canada, Calgary, AB), which was removed prior to fermentation (stream 2). The combined effect of inhibitor removal and in-situ product removal was investigated similarly, however the initial glucose concentration was adjusted to 80 g/L (resulting in inhibitory butanol concentrations) and the resin was left in the fermentation vessel (stream 4).

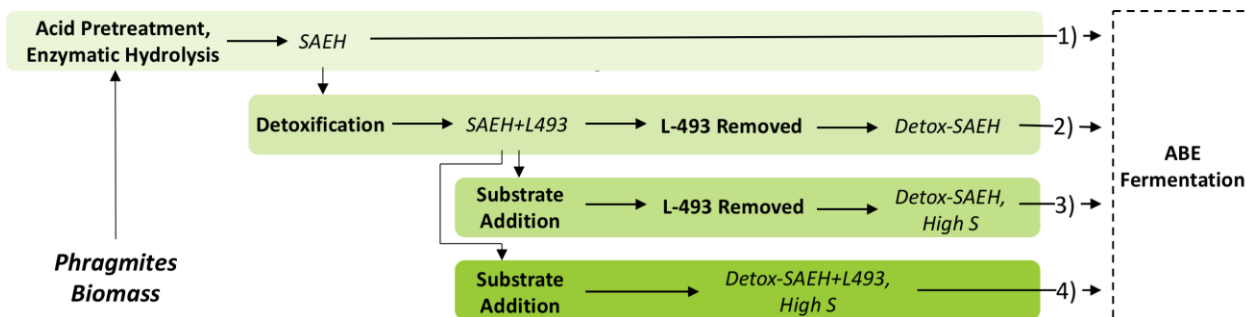


Figure 2.18 Schematic of the experimental procedures. Stream 1 represent the direct fermentation of the hydrolysate, Stream 2 the fermentation after detoxification, Stream 3 the fermentation with high initial substrate concentration and Stream 4 the fermentation with high substrate and in-situ product removal.

Dilute sulfuric acid pretreatment and enzymatic hydrolysis

Sulfuric acid pretreatment of the biomass resulted in the typical formation of HMF and furfural, as shown in Table 2.8. The table also shows the sugar profiles before and after enzymatic hydrolysis. It can be seen that during the dilute sulfuric acid pretreatments, considerable amounts of xylose (~16 g/L) were released whereas glucose formation (1.75 to 2.60 g/L) was low, indicating that most of the cellulose was not hydrolysed. As the concentrations of sulphuric acid increased from 0.5% to 2%, HMF levels in the SAH also rose from 0.09 g/L to 0.36 g/L, and the relative content of total phenolic compounds showed significant increase as well; interestingly, furfural concentrations remained stable between 0.09 and 0.12 g/L.

Table 2.8 Profiles of sugars and inhibitors after pretreatment at different acid concentrations (SAH) and subsequent enzymatic hydrolysis (SAEH).

Substrate	H ₂ SO ₄ conc. % (v/v)	Glucose (g/L)	Xylose (g/L)	Acetic acid (g/L)	HMF (g/L)	Fufural (g/L)	Total phenolic compounds (OD)
SAH	0.5	1.75 (0.04)	16.03 (0.27)	4.53 (0.05)	0.09 (0.01)	0.10 (0.00)	92±2
	1.0	2.20 (0.03)	16.61 (0.05)	4.82 (0.02)	0.20 (0.00)	0.12 (0.00)	144±6
	2.0	2.60 (0.06)	16.40 (0.55)	5.32 (0.56)	0.36 (0.06)	0.09 (0.01)	263±4
SAEH	0.5	15.67 (0.62)	17.22 (0.28)	5.33 (0.49)	0.09 (0.01)	0.08 (0.00)	85±3
	1.0	17.42 (0.74)	17.75 (0.09)	4.83 (0.08)	0.18 (0.01)	0.11 (0.01)	135±5
	2.0	16.81 (1.10)	16.82 (0.05)	4.85 (0.00)	0.28 (0.02)	0.09 (0.00)	260±4

Average values of triplicate experiments with standard error in brackets

All SAH was subjected to enzymatic hydrolysis in order to obtain fermentable sugars. As shown in Table 2.8, glucose levels in SAEH increased from 1.75-2.60 g/L to 15.67-17.42 g/L; however, the xylose concentrations showed no significant change after enzymatic hydrolysis. In terms of sugar yield, 296~317 g sugars was obtained from 1 kg of raw phragmites subjected to dilute H₂SO₄ pretreatment, compared to 385 g of sugars from phragmites pretreated with 1% NaOH that has been reported previously (Gao et al., 2014). The relatively lower sugar yield from acid pretreated phragmites was probably due to multiple reasons. First of all, composition analysis showed that phragmites has a high lignin content (~29%) (Gao et al., 2014), and lignin is considered as the major barrier that prevent enzymes from binding to their cellulose targets (Vermaas et al., 2015). In this regard, alkali pretreatment was shown to be effective in lignin removal, thus resulting in more efficient enzymatic hydrolysis; however, it is well known that acid pretreatment has no significant effect on lignin removal, hence a lower sugar yield is expected (Bensah and Mensah, 2013). Second, in the present study, for the purpose of testing the resin's function as a detoxification agents, acid pretreated phragmites was directly subjected to enzymatic hydrolysis after neutralization without including an extra washing step to remove degradation products derived from lignocellulose, which may have reduced the efficiency of hydrolysis by enzyme deactivation and precipitation (Qin et al., 2016).

These results suggest that increasing the concentrations of sulphuric acid from 0.5% to 2% does not necessarily result in higher sugar production in both pretreatment stage and the following enzymatic hydrolysis, but generated higher levels of inhibitors such as HMF and phenolic compounds. Therefore, phragmites hydrolysates pretreated with 0.5% (v/v) H₂SO₄ was used for further studies.

Detoxification and fermentation

The ability of *C. saccharobutylicum* to ferment the hydrolysate to butanol was evaluated with and without the detoxification step. Time profiles of the fermentation of undetoxified SAEH are shown in Fig 2.19(a). It can be seen that *C. saccharobutylicum* was able to utilize the glucose present in the undetoxified hydrolysate. Initially, 13.96 g/L glucose and 16.12 g/L xylose were present in the hydrolysate. After 24 h, 9.88 g/L glucose was consumed, corresponding to 71% of the initial glucose concentration. However, xylose utilization by the bacteria was severely inhibited. The fermentation resulted in a total ABE production of 3.53 g/L, including 2.63 g/L acetone, 0.17 g/L ethanol, and 0.73 g/L butanol, as well as 7.44 g/L acetic acid (most of it a product of the pretreatment) and 3.70 g/L butyric acid. Interestingly, the initial 0.04 g/L HMF and 0.07 g/L furfural present in the undetoxified hydrolysate were not detected after 12 h of fermentation (data not shown), indicating that the strain has the ability to metabolize HMF and furfural, which could possibly explain results from previous studies showing that supplementation of HMF and furfural (0.3 to 2 g/L) can actually help improve ABE fermentation in terms of cell concentration and solvents production (T. Ezeji et al., 2007). However, the overall sugar utilization and solvent production was low and other toxic compounds present in the untreated hydrolysate likely inhibit the bacteria's growth and ability to utilize the sugars especially xylose. This is likely caused by phenolic compounds, as it was reported that ferulic acid (a phenolic acid) as low as 0.3 g/L was a strong inhibitor to cell growth and solvent production (T. Ezeji et al., 2007). Other toxic compounds also include phenol, p-coumaric acids, and syringaldehyde, etc. Therefore, to facilitate better sugar consumption and ABE yield, the hydrolysate likely needs to be detoxified to improve its fermentability.

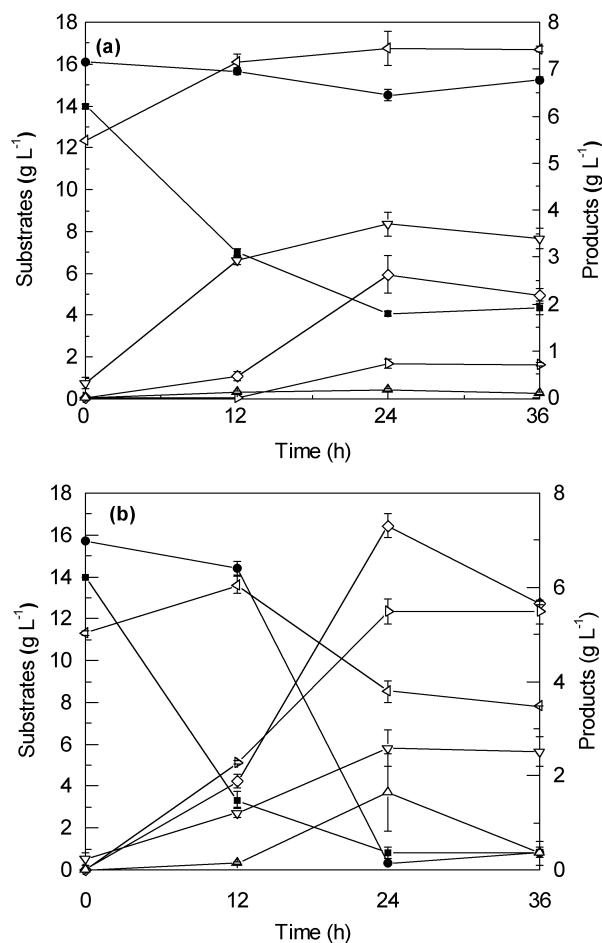


Figure 2.19 Profiles of ABE fermentation from (a) un-detoxified or (b) detoxified phragmites hydrolysates at 37°C, 150 rpm (-■-, glucose; -●-, xylose; -◀-, acetic acid; -▼-, butyric acid; -◆-, acetone; -▶-, butanol; -▲-, ethanol)

The effect of detoxification by resin L-493 on phragmites hydrolysate (pretreated with 0.5% H₂SO₄) was investigated. As shown in Table 2.9, the level of glucose, xylose, acetic acid and HMF remained almost unchanged after detoxification with the resin (30 g/L). However, the detoxified hydrolysate showed remarkable decrease in furfural content and total phenolic compounds, a 95% decrease in relative content of total phenolic compounds was observed after detoxification. These results indicated the polymer resin L-493 has a low affinity for the fermentation substrate such as glucose and xylose, but a much higher affinity for the phenolic compounds in the hydrolysate. In comparison, significant sugar reduction (15%) was observed when sulphuric acid treated corn fiber

was detoxified with resin XAD-4 (Qureshi et al., 2008), rendering L-493 more suitable for this purpose.

Table 2.9 Composition analysis of phragmites hydrolysate (pretreated with 0.5% H₂SO₄) before and after resin detoxification

	Glucose (g/L)	Xylose (g/L)	Acetic acid (g/L)	HMF (g/L)	Furfural (g/L)	Total phenolic compounds (OD)
Undetoxified	15.46±0.09	17.93±0.06	4.08±0.00	0.06±0.01	0.09±0.00	82±4
Detoxified	15.00±0.17	17.06±0.08	3.76±0.06	0.04±0.02	0.02±0.01	4±0

Although it is difficult to fully characterize the many types of phenolic compounds present in the phragmites hydrolysates, some of the most representative phenolic compounds have been measured by HPLC and summarized in Table 2.10. As can be seen from Table 2.10, a series of phenolic compounds have been generated during dilute H₂SO₄ pretreatment at different acid concentrations (0.5%-2%). It was interesting to find that less p-coumaric acid, syringaldehyde, and ferulic acid were generated when acid concentration was increased from 0.5% to 2%; however, for phenol, a slight increase was observed when higher acid concentrations was used. Nonetheless, no phenolic compounds identified in the present study was detected after detoxification, unidentified peaks that are also considered as potential phenolic compounds (due to similar elution times compared to identified phenolic compounds) were also not observed after detoxification (data not shown), which indicates resin L-493 can be effectively used for removal of phenolic compounds. Although the profiles of phenolic compounds present in lignocellulose hydrolysates are highly dependent on the types of feedstocks and pretreatment conditions, results obtained in this study compared well to what is available in the literature (Mitchell et al., 2014).

Table 2.10 Concentrations of selective phenolic compounds present in the acid hydrolysates of phragmites (before and after resin detoxification)

Compounds (mg/L)	Undetoxified			Detoxified		
	0.5% H ₂ SO ₄	1% H ₂ SO ₄	2% H ₂ SO ₄	0.5% H ₂ SO ₄	1% H ₂ SO ₄	2% H ₂ SO ₄
4-Hydroxybenzoic acid	3.8	5.7	5.3	-	-	-
Phenol	35.1	38.2	42.4	-	-	-
p-Coumaric acid	79.1	31.2	10.8	-	-	-
Syringaldehyde	9.7	6.4	5.5	-	-	-
Ferulic acid	70.5	36.4	13.4	-	-	-
2,6-Dimethoxyphenol	18.1	15.0	20.2	-	-	-

-, undetected; values presented are average of triplicate experiments.

The fermentation results with detoxified phragmites hydrolysate are shown in Fig 2.19 (b). Initially 13.98 g/L glucose and 15.72 g/L xylose was present in the hydrolysate. After 24 h of fermentation, the culture was able to utilize 13.17 g/L glucose and 15.37 g/L xylose, leaving 0.81 g/L glucose and 0.35 g/L xylose unutilized. The fermentation resulted in an ABE production of 14.44 g/L, including 7.31 g/L acetone, 1.64 g/L ethanol, and 5.49 g/L butanol, corresponding to an ABE yield and productivity of 0.49 g/g and 0.60 g/L/h, respectively. At 36 h, the culture accumulated 3.48 g/L acetic acid and 2.51 g/L butyric acid. Much less acetic acid was remained from detoxified SAEH compared to 7.44 g/L from original SAEH, which may indicate that toxic compounds in original SAEH might reduce the strain's ability to assimilate acetic acid. Only trace amounts of HMF and furfural were detected at the beginning of fermentation, and both compounds were not detected after 12 hrs. These results suggested that detoxification by resin L-493 is an efficient way to improve the fermentability of acid hydrolysate of lignocellulosic biomass. The ABE yield was improved from 0.12 g/g without detoxification to 0.49 g/g with detoxification by polymer resin, which compared well to previous reported solvent yields from mixed sugars (glucose and xylose) and corn cobs hydrolysates by the same strain (Gao and Rehmann, 2014).

Adsorption and desorption of butanol by resin L-493

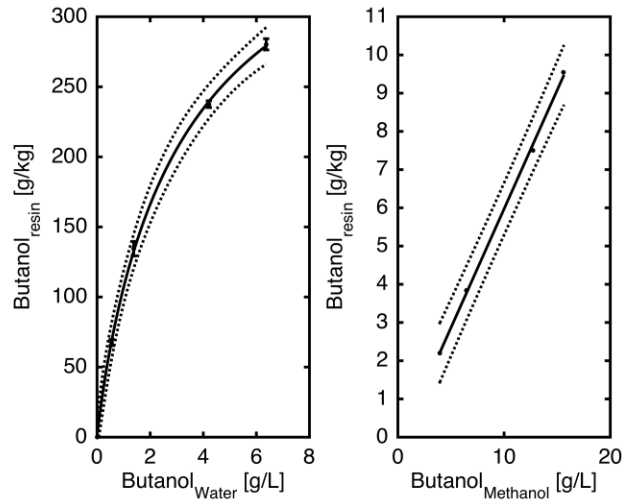


Figure 2.20 Butanol adsorbed to resin as a function of the equilibrium butanol concentrations in (a) aqueous phase (50 g/L resin) and (b) methanol phase (333 g/L resin) with various initial butanol concentrations (4, 8, 16 and 20 g/L) at 37°C after shaking for 12 h (220 rpm). Solid squares represent butanol adsorbed by the resin (g/kg); the solid lines represent the fitted Langmuir isotherm (water) and linear isotherm (methanol) while the dotted lines represent the 95% prediction limits

The selected resin was capable of removing fermentation inhibiting phenolic compounds from the hydrolysate, as discussed above. To exploit the material to its fullest it was investigated for its ability to additionally function as an in-situ product removal agent. The equilibrium distribution of butanol between water and the resin is shown in Fig 2.20(a). The equilibrium favors sorption to the resin and the data is in good agreement with values reported elsewhere (Nielsen and Prather, 2009). The isotherm data could be fitted with the Langmuir model and the following relationship was found:

Equation 3

$$Butanol_{Resin} \left[\frac{g}{kg} \right] = \left(0.0023 \pm 0.0008 + \left((0.007 \pm 0.001) Butanol_{Water} \left[\frac{g}{L} \right] \right)^{-0.922 \pm 0.401} \right)^{-1}$$

Butanol was re-suspended from the resin via equilibration in methanol. This step was used for analytical purposes to quantify the total amount of butanol produced during fermentation with in-situ product removal. An isotherm is shown in Fig 2.20 (b) and over the tested range a simple linear partitioning coefficient of $K_{R/Me} = 0.6214 \pm 0.0651$ $\frac{g_{butanol}}{kg_{resin}} / \frac{g_{butanol}}{L_{methanol}}$ could be determined for the equilibrium data. The relationship was used to determine the amount of butanol in the resin at the end of the fermentation after desorption of butanol into methanol followed by HPLC analysis. This method was validated by equilibrating target butanol concentrations in fermentation media (16 and 20 g/L) with the resin (50 g/L). The aqueous butanol concentration was effectively reduced to sub-inhibitory levels (Table 2.11) and removal of the resin from the aqueous phase and submersion in methanol result in high butanol recovery.

Table 2.11 Sorption and desorption of butanol by resin L-493.

Initial [Butanol] _{Aq} (g/L)	Equilibrated [Butanol] _{Aq} (g/L)	[Butanol] _{methanol} (g/L)	Butanol Recovery %
16	4.21	11.14	96
20	6.37	13.61	99

Integration of Hydrolysate Detoxification and Fermentation with In-situ Butanol Removal

One of the major drawbacks of the ABE fermentation is low product titers due to the toxicity of butanol to the producing organisms. The tested hydrolysis conditions did not yield high enough sugar concentrations to result in product inhibition at the end of the fermentation. Therefore, the hydrolysate was supplemented with pure sugars in order to achieve higher product titers. A control fermentation (Fig 2.21a) with mixed sugar (53.31±0.45 g/L glucose and 19.93±0.06 g/L xylose) was performed to compare the results of fermentation with in-situ butanol removal (Fig 2.21b). During the control fermentation, 48.30±0.28 g/L glucose and 16.78±0.08 g/L xylose were consumed after 24 h of fermentation, leaving behind 8.16±0.76 g/L sugar unutilized. In the end of fermentation, 21.45±0.42 g/L ABE was produced (including 6.73±0.72 g/L acetone, 13.45±0.31 g/L butanol and 1.27±0.05 g/L ethanol), corresponding to an ABE yield and productivity of 0.33 g/g and 0.89 g/L/h, respectively. The results from control

fermentation clearly show that the strain is not able to completely utilize all the sugars available in the fermentation broth with an initial sugar concentration of ~ 70 g/L, likely due to product inhibition (Moreira et al., 1981).

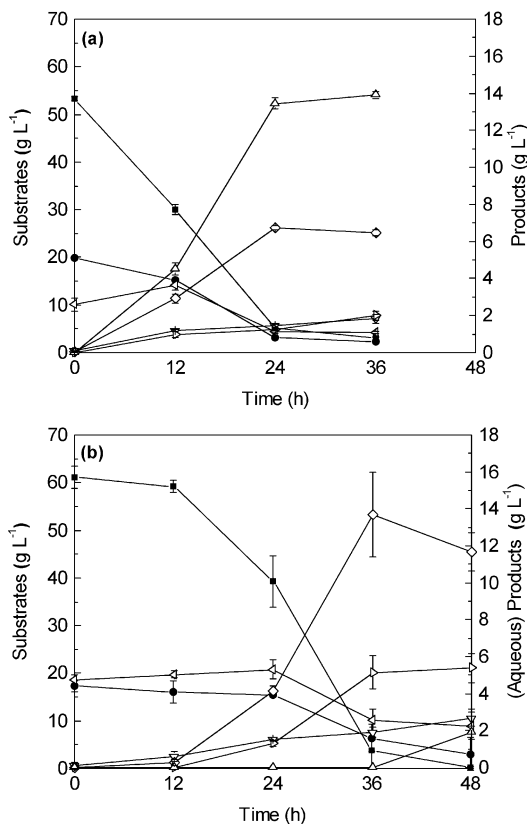


Figure 2.21 Profiles of ABE fermentation from (a) mixed sugar and (b) phragmites hydrolysate with in-situ butanol removal at 37°C, 150 rpm (-■-, glucose; -●-, xylose; -◁-, acetic acid; -▽-, butyric acid; -◇-, acetone; -▷-, butanol; -△-, ethanol).

In order to maintain sub-inhibitory product concentrations an ABE fermentation from SAEH was carried out with in-situ butanol removal by resin L-493 (50 g/L resin dosage) (glucose supplemented, Fig 2.21b). Before the fermentation started, SAEH was detoxified with the same resin. Initially, ~ 60 g/L glucose (15 g/L from SAEH and 45 g/L from pure glucose) and 17 g/L xylose (from SAEH) were present in the fermentation media. Slow initial utilization of sugars (both glucose and xylose) was observed within the first 12 h of fermentation, compared to a rapid glucose utilization immediately after inoculation with detoxified SAEH at lower initial sugar concentrations (Figure 2.19b). The only difference

here is the initial sugar concentration (80 vs 30 g/L), therefore, substrate inhibition in combination with slight toxicity caused by non-removed inhibitors is likely the major reason to cause the delay in sugar utilization, which was not observed with pure sugars. The strain started to use glucose rapidly after 12 h; however, xylose utilization was not obvious until 24 h. Similar mixed sugar (mainly glucose and xylose) usage pattern was reported previously (Gao and Rehmann, 2014; Raganati et al., 2014). After 48 h of fermentation, 0.19 g/L glucose and 3.05 g/L xylose was left unutilized in the fermentation media (Table 2.12).

Table 2.12 Profiles of fermentation of detoxified SAEH with in-situ butanol removal

Glucose (g/L)	Xylose (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Acetone (g/L)	Ethanol (g/L)	Butanol (aq) (g/L)	Effective Butanol (g/L)
0.19±0.26	3.05±3.47	2.25±0.73	2.7±0.46	11.66±1.05	1.93±0.00	5.38±0.80	16.4*

* The effective butanol concentration was estimated as the total amount of butanol in the system (aqueous and in resin) divided by the total volume of aqueous phase.

During the first 24 h, acetic acid was produced by the cultures and a maximum concentration of 5.31 g/L was obtained at 24 h, indicating that the cultures was going through acidogenesis. After 24 hrs, a significant acetic acid re-assimilation was observed as the acetic acid level was reduced to 2.17 g/L after 60 h. In comparison, no significant re-assimilation of butyric acids was observed during the fermentation.

Solvent production was observed after 24 hrs. The butanol concentration in the aqueous phase reached 5.38 g/L after 48 hrs (Table 2.12), which is well below the butanol threshold level in a batch fermentation (~12-13 g/L) (Papoutsakis, 2008). Additional butanol was sorbed to the resin, up to 263 mg/g can be estimated, assuming equation 1 is adequate for the system also containing additional fermentation products as well as the by-products from the acid hydrolysis. As shown in Fig 2.21(b), selective adsorption of butanol by resin L-493 makes acetone a predominant product in the aqueous phase and a maximum acetone concentration of 13.70 g/L was achieved at 36 h.

After the fermentation was stopped, desorption experiments with methanol were carried out to recover the butanol that had been absorbed by the resin. Substantial amount of butanol (11.02 g/L), acetone (2.97 g/L) and butyric acid (1.92 g/L) were recovered in the

methanol phase (30 ml), while only trace amounts of glucose, xylose and ethanol were detected. The total butanol produced (161.4 mg in aqueous phase and 330.6 mg in the resin) is equivalent to an effective butanol titer of 16.4 g/L. The measured value is slightly below what would have been expected based on the the aqueous phase concentration and the resulting equilibrium concentration in the resin based on equation 3. However, the isotherms shown in Fig 2.20 were measured for a system containing only water and butanol. In the case of the fermentation, acetone and ethanol were also present, and the resin was loaded with the phenolic by-product from the acid hydrolysis, which were adsorbed during the detoxification stage. In the end, an effective ABE production of 33 g/L was obtained, corresponding to an ABE yield and productivity of 0.41 g/g and 0.69 g/L/h, respectively.

The major contribution of the current study is to use Optipore L-493 for both hydrolysate detoxification and in-situ butanol removal in a single process. It was recently reported that Optipore L-493 has been considered as effective in-situ butanol absorbent and used in expanded bed adsorption for a fed-batch ABE fermentation (Nielsen and Prather, 2009; Wiehn et al., 2013). However, this resin has not been used for detoxification of lignocellulosic hydrolysate yet to the authors' knowledge.

The inhibiting effect of dilute sulfuric acid pretreatment on ABE fermentation has been observed among a wide range of substrates, such as dried distillers' grains and solubles (DDGS), corn fiber, barley straw, corn stover and switchgrass (Ezeji and Blaschek, 2008; Qureshi et al., 2010a, 2010b, 2008). Detoxification techniques such as overliming and inhibitor removal by resin XAD-4 have been employed but unsatisfied results were obtained (Table 2.13), and the cost of chemicals during overliming is also making the process less economic (Qureshi et al., 2008). Results presented in this study shows that a previous contact with resin L-493 alone can efficiently remove inhibitors present in the phragmites hydrolysate. Phragmites used in this study is a typical herbaceous plant and has similar compositions compared to other cellulosic feedstocks studied before (Lee et al., 2007); levels of toxic compounds generated in the present study are also comparable to those found in other feedstocks such as wheat straw, barley straw and switchgrass (Qureshi et al., 2010a). Therefore, it is reasonable to conclude that resin L-493 has potential for inhibitor removal for other lignocellulosic hydrolysates as well. The

application of resin L-493 in both detoxification and in-situ product removal fermentation makes it possible to simplify the process for cellulosic butanol production while increasing the process's production efficiency.

Table 2.13 Performance of different types of resin used as inhibitor and butanol absorbents

Substrate	Resin	Detoxification/In-situ butanol removal	Performance			Ref.
			Effective butanol (ABE) concentration (g/L)	ABE productivity (g/L/h)	ABE (butanol) yield (g/g)	
*SACFH (54.3 g/L sugars)	-	-/-	(1.4)	-	-	
SACFH + overliming (46.3 g/L sugars)	XAD-4 resin	+/-	6.4 (9.3)	0.10	0.21	(2008)
SAEH (30 g/L sugars)	-	-/-	0.7 (3.5)	0.15	0.12	
SAEH (30 g/L sugars)	Optipore L-493	+/-	5.5 (14.4)	0.60	0.49	this study
SAEH +45 g/L glucose	Optipore L-493	+/+	16.4 (33.0)	0.69	0.41	
8% glucose	Optipore SD-2	-/+	19.5	N/A	(0.24)	(2009)
Control mixed sugar	-	-/-	13.5 (21.5)	0.89	0.33	this study

*SACFH, dilute sulfuric acid pretreated corn fiber hydrolysate followed by enzymatic hydrolysis.
+, positive; -, negative or not available.

2.7.4. Conclusions

In the present study, *Phragmites australis* hydrolysate (detoxified by resin L-493) was efficiently fermented for butanol production. Resin L-493 showed great potential in hydrolysate detoxification along with its role as an in-situ butanol recovery device. The dual function of resin L-493 makes it possible to simplify the process for cellulosic butanol production while increasing the process's production efficiency.

Section 3 - Summary and Conclusions

3.1 Summary

3.1.1. Bio-butanol production from NaOH-pretreated corn cobs, switchgrass and phragmites

In section 2.3 and 2.4, biomass including corn cobs, switchgrass, and phragmites was studied as potential substrates for ABE fermentation. To reduce the recalcitrance of lignocellulosic biomass and facilitate sugar yield during enzymatic hydrolysis, alkali pretreatment was employed. The pretreated biomass showed significant reduction in lignin content, meanwhile carbohydrate content was enriched. Results of enzymatic hydrolysis showed significant increase in sugar yield compared to untreated biomass. It was also found that a post-pretreatment washing of pretreated biomass with water helps remove toxic compounds that inhibit both cellulolytic enzymes and the subsequent fermentation. Finally, solvent yields that are comparable to control glucose fermentation was obtained from all biomass investigated. These results clearly showed that by using the process developed in the thesis, the abundant lignocellulosic biomass can be efficiently used as substrates for butanol production, thus potentially lowering the cost of ABE fermentation.

3.1.2 Lipid extracted algae for butanol fermentation

Although biofuel production from lignocellulosic biomass is considered as more sustainable compared to the first generation biofuel produced from food crops, it still faces many limitations such as the chemical and energy intensive pretreatment step, competition with resources including land and water, etc. Alternatively, carbohydrates from microalgae is a promising choice for biofuel production as they are much more accessible compared to cellulose and hemicellulose from lignocellulosic biomass. In section 2.5, algal carbohydrates was investigated as substrates for bio-butanol production. Lipids was removed from microalgae by either traditional solvent method or novel ionic liquid assisted method. Algae residues resulted from both extraction methods (HEA and ILEA) was investigated as feedstock for ABE fermentation. It has been found that both LEAs can be directly fermented for solvent production, the highest butanol/ABE titer

obtained was 15.77/23.79 g/L during fed-batch fermentation of ILEA with a total solid loading at 12%. On the other hand, acid hydrolysates of LEAs was also investigated as substrates for ABE fermentation due to higher solvent yield from glucose compared to direct starch fermentation. Different extent of inhibitions was found when acid hydrolysates of LEAs was subjected to ABE fermentation, which can be alleviated by resin adsorption (L-493), and the fermentation results was significantly improved. Acid hydrolysis was found effective for conversion of algal starch into glucose at increased solid loading (12%), and 12.34 g/L butanol was produced from ILEA hydrolysates in comparison to 9.7 g/L butanol from HEA hydrolysates.

3.1.3 Detoxification and in-situ butanol removal by single resin L-493

Degradation products are one of the major factors limiting the utilization of lignocellulosic biomass, as they can inhibit cell growth and solvent productions. In section 2.5, acid hydrolysates of LEA was detoxified by resin L-493 and the fermentation performance was significantly improved. Hence, in section 2.6, one purpose is to use the same resin to detoxify lignocellulosic hydrolysates. Dilute sulfuric acid pretreatment is one of the most studied pretreatment method, but degradation products such as phenolic compounds are also generated, which could have negative effect on the following fermentation. In section 2.6, dilute sulfuric acid pretreated phragmites was investigated as substrates for butanol fermentation. Direct fermentation of enzymatic hydrolysate of dilute sulfuric acid pretreated phragmites showed weak solvent productions and sugar utilization. HPLC analysis showed that a number of typical phenolic compounds at different concentrations was detected in the phragmites hydrolysates. However, after detoxification by resin L-493, the phenolic compounds were no longer detected, and good solvent yield was obtained, which indicates that resin L-493 is suitable for detoxification of lignocellulosic hydrolysates by efficient removal of phenolic compounds. Furthermore, the same resin was investigated as an in-situ butanol recovery device to reduce the butanol toxicity, which enabled the strain to utilize a higher initial substrate concentration and subsequently a higher butanol titer was obtained.

3.2 Conclusions

The lignocellulosic biomass studied in this work can all be converted efficiently to butanol after NaOH pretreatment, which indicates the process developed in the current study has the potential to be applied to a wide range of lignocellulosic biomass species. Under the selected conditions, corn cobs give the highest sugar and solvent yield. The carbohydrate fraction of lipid extracted microalgae can also be used as a feedstock for ABE fermentation and no hydrolysis is necessary when employing starch converting chlostridia. However, at this point commercial biofuel production will still likely use the cheaply available lignocellulosic biomass and the resin L-493 can be used to detoxify the biomass hydrolysate and to remove butanol in-situ from the fermentation broth, thereby solving two major problems in lignocellulosic butanol production.

3.3 Future work and recommendation

The results presented in this work indicated that herbaceous lignocellulosic biomass are suitable substrates for ABE fermentation using the process developed in the present study. A preliminary scale-up fermentation with corn cobs resulted in lower sugar yield after enzymatic hydrolysis and incomplete xylose utilization during the fermentation. The lower sugar yield was probably due to less efficient heat transfer during the pretreatment by autoclave as higher solid recovery was obtained in scale-up pretreatment. Hence the pretreatment time should be extended to reach similar pretreatment efficiency obtained during small-scale study. Although partial utilization of xylose was observed in both small scale and large scale fermentation, xylose utilization in small scale was significantly higher compared to large scale study. In small scale, the incomplete xylose utilization was probably due to butanol toxicity (toxic level of butanol was reached before the bacteria can utilize the remaining xylose) as relatively high glucose concentration was present in the beginning of the fermentation. In comparison, sugar concentration was relatively low compared to small scale study due to less efficient hydrolysis, and the partial xylose utilization was probably mainly as a result of less efficient mixing. Therefore, studies on optimization of mixing is suggested to achieve a better sugar utilization and hence higher solvent yield at large scale.

High solvent flux and recovery was obtained during the pervaporation studies with spent corn cob and glucose fermentation broth, indicating that the pervaporation system (based on a commercial membrane Pervap 4060) has a great potential to be utilized for in-situ solvent recovery (data not shown). In order to fully realize the potential of the pervaporation system, batch, fed-batch and continuous fermentation integrated with pervaporation are proposed for future experiments. In batch fermentation, the effect of pervaporation on substrate utilization will be mainly evaluated. Theoretically, the in-situ removal of butanol from the fermentation broth will lower the butanol toxicity toward the bacteria, thus enabling the strain to be able to utilize higher initial concentrations of substrate. In the fed-batch fermentation, concentrated lignocellulose hydrolysate will be fed into the fermentor when substrate level is low; the pervaporation unit will be turned on and off to control the butanol level in the fermentation broth below the toxic level but also guarantee a continuous permeate phase enriched in solvent. In a continuous fermentation, steady state of fermentation will be reached by continuously feeding concentrated biomass hydrolysate into the fermentor at certain dilution rate. The spent fermentation broth will be fed into pervaporation unit for further separation of solvent. The effect of different dilution rate will be investigated in this part of research. A complete mass balance will be presented to validate the results.

In the present study, carbohydrates derived from microalgae was shown to be a superior substrate for ABE fermentation compared to those from lignocellulosic biomass mainly due to their higher accessibility. However, one of the major problems associated with utilization of microalgae is the high drying cost. For example, in the current study, microalgae collected was first freeze-hydrolyzed prior to the following experiments. In a recent study, the same ionic liquid assisted lipid extraction method was shown to be compatible to wet algae (water content 0-82%) (Orr et al., 2016), hence the resulting wet algae residues could be further investigated as potential substrates for ABE fermentation.

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