Bioconversion of lignocellulose into acetone-butanol-ethanol (ABE): pretreatment, enzymatic hydrolysis and fermentation

by

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Abstract

The objective of the present dissertation is to investigate the bioprocess for the production of acetone-butanol-ethanol (ABE) from lignocellulosic feedstocks via ABE fermentation with solvent-producing *Clostridium acetobutylicum* ATCC 824. The first part of this dissertation focuses on the use of natural herbaceous feedstocks (corn stover, switchgrass, etc.) as the starting materials. To serve as carbon source for *Clostridium*, pretreatment and enzymatic hydrolysis, as prerequisites for downstream fermentation, are necessarily required to degrade the polymeric carbohydrate into monomer sugars. In this dissertation, alkaline pretreatment is chosen as the pretreatment method among various strategies as it is capable of preserving most of the hexose and pentose in the feedstocks, both of which can be catabolized by the culture for solvent production. It was found that supplementation minute quantity of poly-diallyl-dimethyl-ammonium chloride (polyDADMAC) in alkaline pretreatment led to slightly negative effect on delignification but enhanced enzymatic hydrolysis of pretreated solids quite significantly (e.g., 14-25% increase in glucan digestibility). Structural characterization of pretreated solids with SEM, XRD and BET indicated that polyDADMAC addition substantially disrupted and swelled the crystallized cellulose fibers. As supportive control, lignin-free cellulose was treated with hot water with and without addition of polyDADMAC. It was shown that addition of polyDADMAC in hot-water treatment enhanced enzymatic hydrolysis of lignin-free cellulose likewise. Therefore, the effect of polyDADMAC on alkaline pretreatment was

speculated to be by direct interaction with cellulosic fiber, rather than with lignin or other extraneous components. The possible mechanism behind such a disruption was also discussed.

Following the alkaline pretreatment, the acquired solid was found to be inhibitory to ABE fermentation. An *in situ* detoxification method with the addition of non-ionic surfactant Tween 80 was therefore applied to improve the fermentability of the solids. Both simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) were applied for solvent production with Clostridium acetobutylicum (ATCC-824). Fermentation with Avicel and pure sugars as substrate was also carried out control. The results from SSF of Avicel indicated that enzyme loading played a key role in the bioconversion yields. SSF of alkali-pretreated switchgrass could be as efficient as SSF of Avicel and addition of Tween 80 was found to enhance the bioconversion yield quite significantly. However, attempts at SSF were strongly limited by solid loading with 5 wt. % as maximum level. With an enzyme loading of 15 FPU/g glucan, SSF of 5% well-pretreated switchgrass produced 12.2 g/L of total solvents, the level of which was far from the inhibition threshold for this specific culture. Therefore, SHF was further applied and produced 14.3 g/L of solvents. The bioconversion features of SSF and SHF are also compared in conjunction with the overall bioconversion yield and productivity.

The second part of this dissertation aims to investigate the technical feasibility of utilizing the waste materials in the pulp and paper industry as feedstock for bio-butanol production. The concept of integrating of the biorefinery to the pulping industry has been proposed to diversify the product portfolio and generate extra revenue for the pulp mills. In a Kraft pulping process, woodchips are fractionated into major product of pulp and a number of byproducts (extractives, hemicellulose-degraded carbohydrates and lignin) ended up as a complex mixture in the black liquor. The hemicellulose and lignin in the black liquor is usually

incinerated in the recovery boiler for power generation. In consideration of the heating value of hemicellulose (13.6 MJ/kg) is rather low in comparison with that of lignin (27.0 MJ/kg), the hemicellulose represents a highly underutilized sugar source. In this part of the dissertation, the hemicellulose pre-hydrolysate is investigated as liquid sugar feedstock for production of acetone, butanol and ethanol by ABE fermentation. For this purpose, hot-water treatment is applied to Poplar (hardwood) and Southern pine (softwood) to extract the hemicellulose portion. The acquired hemicellulose prehydrolysate was analyzed to contain, in addition to the carbohydrates mostly in the form of oligosaccharides, various degradation compounds. The toxicity test with model degradation compounds indicates phenolic compounds exert tremendous inhibition on the cell growth. Therefore, detoxification is required prior to fermentation. Adsorption with activated carbon is found to greatly reduce the phenolic content. Upon detoxification, simultaneous saccharification and fermentation (SSF) of concentrated poplar prehydrolysate with 43.3 g/L of sugar produced a total of 10.8 g/L ABE giving a solvent yield of 0.25 (g-solvent/g-sugar). Comparatively, SSF of concentrated southern pine prehydrolysate resulted an ABE production of 13.2 g/L and productivity of 0.28 (g-solvent/g-sugar). The details of hot-water extraction conditions, performance of detoxification as well as the fermentation profiles are discussed.

In the paper making process, the poor-quality and short fibers in the wood pulp, which are hardly to be retained on the fiber screens and paper machines, are discharged into the waste stream as paper mill sludge (PS). PS is investigated as a feedstock for acetone-butanol-ethanol (ABE) production by simultaneous saccharification and fermentation (SSF). As a waste material in the pulp mills, sludge typically contains, in addition to the carbohydrates, considerable portion of inorganic ashes originated from the fillers (clay, TiO_2 and $CaCO_3$) added in the paper making process, exhibiting tremendous inhibition effects on enzymatic hydrolysis. Therefore,

bioconversion of sludge requires partial removal of ash to enhance its enzymatic digestibility. Enzymatic hydrolysis was found to be the rate-limiting step in the ABE production by SSF. A total of 16.4-18.0 g/L of ABE solvents were produced in the SSF of de-ashed PS with solid loading of 6.3-7.4% and enzyme loading of 10-15 FPU/g-glucan, and the final solvent yield reached 0.27 g/g sugars. These levels of solvent in SSF are comparable to those of glucose and cellulose controls, approaching to the upper limits. No pretreatment and pH control are needed in ABE fermentation of paper mill sludge, which makes it an attractive feedstock for production of butanol.

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List of Abbreviations

ABE	acetone-butanol-ethanol
SAA	soaking in aqueous ammonia
CS	corn stover
SG	switchgrass
CrI	crystallinity index
DP	degree of polymerization
PS	paper mill sludge
SSF	simultaneous saccharification and fermentation
SHF	separate hydrolysis and fermentation

I. Introduction

N-butanol (IUPAC nomenclature 1-butanol) is four-carbon, colorless and flammable liquid alcohol. Currently, butanol is primarily produced from propylene in the petro-chemical industry. In 2014, n-butanol was reported to have a global market of 3 million tons, which is worth $5 billion. The market of n-butanol was expected to increase 120,000 metric tons/yr (http://www.cobalttech.com/biobutanol.html). Butanol itself could be directly used as an excellent solvent, paint thinner, hydraulic and brake fluid and as the base for the production of perfumes and cosmetics (Duerre, 2008). Additionally, as a great platform for chemical synthesis, butanol among the worldwide production is primarily used for synthesis of a wide range of industrial chemicals. The derivatives of butanol (e.g., acrylate/methacrylate esters, glycol ethers and butyl acetate) have versatile industrial applications (Duerre, 2008; Jones & Woods, 1986). Acrylate/methacrylate ester, for example, is a distinctive monomer for the production of polymeric plastics, surface coatings/paintings and elastomers, while butyl glycol and butyl acetate are necessarily required for the production of lacquers and surface cleansers (Duerre, 2008; Jones & Woods, 1986). Other butanol derivatives (e.g., butylamine and amino resins) are involved in the production of pesticides, emulsifier, plasticizer, etc. (Jones & Woods, 1986; Lee et al., 2008).

Butanol has been traditionally produced via the catalytic OXO-pathway in the petro-chemical industry. In addition to the chemical synthesis route, fermentation (ABE fermentation) offers an alternative option for butanol production. In fact, fermentative

butanol production had been the prevalent way until the boom of petrochemical industry in 1960s (Jones & Woods, 1986). Solvent-producing *Clostridium* was the primary microbe for this bio-process, which produced, in addition to the acetate, butyrate, carbon dioxide and hydrogen, the solvent product of acetone-butanol-ethanol at a ratio of 3:6:1. Conventional feedstock for this commercial bioprocess was exclusively food-based sugar or starch, occupying 60-80% of overall production cost, which made this bioprocess failed to compete with the chemical synthesis route (Jones & Woods, 1986; Taconi et al., 2009).

Recently, this bioprocess has regained interests for lignocellulosic-based rather than the food-based bio-butanol production. Lignocellulosic feedstock represents a cheap, abundant and renewable carbon source suitable as raw material for bio-butanol production. Natural lignocellulosic feedstock is primarily composed of cellulose, hemicellulose and lignin. Lignin, cross-linking with hemicellulose through covalent and hydrogen bond, forms a three-dimensional matrix, which embraces the crystallized cellulose fibers and protects the carbohydrate from external degradation.

Bioconversion of lignocellulose generally involves four major unit processes: pretreatment, enzymatic hydrolysis, fermentation and product recovery. Pretreatment is the prerequisite for downstream enzymatic hydrolysis. Major role of pretreatment is to necessarily disrupt the compact structure of lignocellulose and to enhance enzyme access to cellulose and hemicellulose in enzymatic hydrolysis. Enzymatic hydrolysis is then applied to degrade the cellulose and hemicellulose into fermentable sugars. The acquired hydrolysate is then fermented into acetone-butanol-ethanol with the solvent-producing Clostridium culture. Additionally, integration of enzymatic hydrolysis and fermentation

into one single unit process, i.e., simultaneous saccharification and fermentation (SSF), has also been widely applied as a counterpart for separate hydrolysis and fermentation (SHF). Downstream products recovery from the fermentation broth was performed via distillation, extraction, adsorption, etc.

The primary goal of the present dissertation investigated the use of various sources of non-food feedstock for bio-butanol production with the solvent-producing culture of *Clostridium acetobutylicum* ATCC 824. A total of five tasks are proposed in this study:

i. Investigated the effects of polyDADMAC addition on the alkali-pretreatment of lignocellulose (corn stover and switchgrass)
ii. Bioconversion of alkali-pretreated switchgrass into acetone-butanol-ethanol via simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF)
iii. Investigated the use of hot-water extracted hemicellulose pre-hydrolysate of pulping woods as liquid sugar feedstock for fermentative bio-butanol production
iv. Acetone-butanol-ethanol (ABE) production from Kraft pulp mill sludge by simultaneous saccharification and fermentation (SSF)
v. Integration of in situ product recovery into the fermentation process

II. Literature Review

II.1 ABE fermentation

II.1.1 Background for the ABE fermentation

Butanol was first discovered as a fermentation product in 1861 by Louis Pasteur and recognized as an excellent building block for synthetic rubber (Duerre, 2008; Jones & Woods, 1986). Driven by the need of synthetic rubber, the first commercial plant for butanol production was erected in 1912 with a Fernbach's bacillus culture by Strange & Graham, Ltd (Jones & Woods, 1986). Shortly after the launch of the plant, Chaim Weizmann isolated the strain of Clostridium *acetobutylicum* exhibiting better performance on production of both acetone and butanol. At the beginning of World War I in 1914, Clostridium *acetobutylicum* was introduced into the commercial fermentation process to meet the expanding market for acetone, which was a necessary solvent for the large-scale production of cordite (Duerre, 2008; Durre, 1998; Jones & Woods, 1986). During the war, the Weizmann process successfully supplied adequate amounts of acetone to the allies, directly affected the outcome of the war.

After the war, as a result of rapid development of the automobile industry, the lack of industrial solvents for the manufacture of quick-drying lacquers continued driving this bioprocess in operation. At that time, butanol was primarily used to synthesize butylacetate, which was an excellent solvent for the production of quick-drying lacquers (Jones & Woods, 1986). In USA, the Commercial Solvents Corporation (CSC) was the first company that ventured ABE fermentation process for butanol production in 1919 (http://en.wikipedia.org/wiki/Commercial_Solvents_Corporation). The demand for butanol rapidly expanded and the plants for ABE fermentation extensively spread among the world by 1930s, including England, United States, Soviet Union, Japan, India,

Austrilia, and South Africa, Egypt, Brazil and China. The largest plant (Peoria, Illinois, USA) with a total volume of 18,168 m^3 including 96 parallel fermenters could produce approximately 100 tons of solvents per day (Duerre, 2008; Gabriel, 1928). The feedstocks for this process were exclusively food-based maizes or potatoes. Although the economic issues had been argued due to the shortage or high cost of these food-based feedstocks, particularly during the war time, this bioprocess remained as one of the largest industrial fermentation process, only second to the ethanol fermentation process until 1950S (Jones & Woods, 1986).

Most of the plants in the United States were shut down by 1960. There are several reasons accountable for the cessation of this bioprocess. The direct one is that biological production of butanol hardly competed with the chemical synthesis route (Green, 2011; Jones & Woods, 1986; Tashiro et al., 2013). With the boom of petroleum industry, butanol has been dominantly synthesized from propylene via the OXO-pathways, as indicated: (http://en.wiki-pedia.org/wiki/Butanol)

$$Propylene + Syngas \xrightarrow{Hydroformylation} Butyralddehyde \xrightarrow{Hydrogenation} 1-butanol$$

Additionally, food-based feedstocks resulted to prohibitive cost. And extensively utilizing those feedstocks for solvents production potentially disrupted the delicate food supply chain (Green, 2011; Jones & Woods, 1986; Tashiro et al., 2013). Finally, strong end-product inhibition on the culture severely limited the solvents titer and productivity, which makes downstream solvent recovery considerably difficult (Anbarasan et al., 2012; Groot et al., 1990; Nielsen & Prather, 2009). Albeit many new solvents-producing strains were isolated, few were able to dramatically alleviate the inhibition issue.

Recently, this bioprocess has been drawing resurgent attention for the production of bio-butanol as the next generation biofuel due to its superior physical properties over ethanol. First, the energy density of butanol is close to that of gasoline, significantly higher than that of ethanol (Harvey & Meylemans, 2011). The net heat combustion value (NHOC) for butanol/gasoline/ethanol was reported to be 26.8/32.3/21.1MJL^{-1} and the anti-knock index for butanol/gasoline/ethanol were reported to be 87/87/113 (Harvey & Meylemans, 2011; Jang et al., 2012). As a fuel extender, butanol is capable of being blended into the gasoline at any ratio and substantially less soluble in water as compared to ethanol (Green, 2011; Harvey & Meylemans, 2011; Qureshi et al., 2010a). Finally, butanol is less corrosive than ethanol, allowing butanol can be directly used as fuel without modification of the existing engine (Harvey & Meylemans, 2011; Jang et al., 2012). For example, David Ramey, the founder of Butylfuel™ Inc., travelled across the United States with his Buick truck entirely powered with butanol in 2005 (http://nabc.cals.cornell.edu/Publications/Reports/nabc_19/19_4_6_Ramey.pdf). In that trip, butanol consumption was averaged at 24 miles per gallon, comparable to that of gasoline. As BP and DuPont announced a joint venture to revive this bio-process for butanol production as biofuel in 2007, a number of companies also claimed their attempts to re-commercialize this bio-process, including Sovert (UK), Butamax, Gevo, Cathay Indus-trial Biotech (China), Cobalt Technologies, etc..

II.1.2 Microorganisms & metabolism

The general biocatalyst for the ABE fermentation belongs to the Clostridia family, which are strictly anaerobic and Gram-positive microbes. Several species of Clostridia are capable of producing solvents (acetone/isopropanol-butanol-ethanol), including *acetobutylicum*, *beijerinckii*, *saccharobutylicum*, *saccharoperbutylacetonicum*,

pasteurianum, sporogenes (Jones & Woods, 1986; Kumar & Gayen, 2011). Among those species, C. *acetobutylicum* was the one applied in the industrial Weizmann process with acetone, butanol and ethanol as solvents products. This culture produces the solvents of ABE at ratios of 3:6:1. C. *beijerinckii* is another widely used one with better performance on solvents production than C. *acetobutylicum* (Ezeji & Blaschek, 2008b; Jones & Woods, 1986). The representative strains of these species are C. *acetobutylicum* ATCC824 and P260, *beijerinckii* NCIMB8052 and BA101, *saccharobutylicum* P262, *saccharoperbutyl-acetonicum* N1-4, which are extensively used in many studies (Ezeji & Blaschek, 2008b; Ezeji et al., 2003; Kumar & Gayen, 2011; Madihah et al., 2001; Thang et al., 2010). Most of the Clostridium strains are capable of digesting both monosaccharides and low-molecular-weight polysaccharides for solvents production, including most of the prevalent hexoses, pentoses, starch, molasses, lactoses, etc. (Ezeji & Blaschek, 2008b; Jones & Woods, 1986). For example, (Thang et al., 2010) reported that C. *saccharoperbutyl-acetonicum* N1-4 directly utilized cassava starch and produced comparable level of solvents as that from glucose.

A typical pattern of ABE fermentation in batch mode involved a biphasic metabolism. The first stage of fermentation is known as the acidogenic phase, which usually occurs in the exponential growth phase. With rapid consumption of carbon source, the culture primarily produced hydrogen, carbon dioxide, acetate and butyrate, resulting in a decrease of the broth pH (Duerre, 2008; Jones & Woods, 1986; Lee et al., 2008). In response to the decreased external pH endangering the cytoplasm of the microbes, the culture forms self-protecting endospores and initiates the solventogenic phase, during which the acids were re-assimilated and converted into the solvents with continuing

carbon source consumption (Duerre, 2008; Jones & Woods, 1986; Lee et al., 2008). Studies have shown that maintenance of proper broth pH throughout the fermentation played an essential role on the rate of cell growth and the performance of solvents production otherwise the fermentation would become premature with acetate and butyrate as major fermentation products (Holt et al., 1984; Monot et al., 1984).

The culture is known to be severely inhibited by the end-products at the later stage of solventogenesis. Butanol, as the most toxic end-product in ABE fermentation, affects the integrity of cell membranes and thus inhibits the cell in a manner dramatically disrupting the membrane-associated functions, such as sugar uptake and solvents synthesis, when its concentration reached to the inhibitory level at approximately 8g/L (Harvey & Meylemans, 2011; Jones & Woods, 1986; Ounine et al., 1985; Tomas et al., 2004; Vanderwesthuizen et al., 1982). (Ounine et al., 1985) observed that the uptake of glucose and xylose was significantly decreased in the presence of butanol at inhibitory levels. The maximum of butanol concentration that wild type cultures can tolerate has been reported to be at 13 g/L, such as C. *acetobutylicum* ATCC 824 and C. *beijerinckii* NCIMB 8052 (Tashiro et al., 2013; Tomas et al., 2004). The cell was completely degenerated by the autolysin excreted by the cell itself when exposing to the butanol solution at the threshold level of 16 g/L (Vanderwesthuizen et al., 1982).

Strong end-products inhibition has been known as one of the inherent issues confronting the resurgence of this bioprocess. The practical maximum levels of solvents for most of the wild-type Clostridia strains have been limited in the range of 17-21 g/L with 10-12 g/L of butanol as a result of end-product inhibition (Ezeji & Blaschek, 2008b; Ezeji et al., 2003; Kumar & Gayen, 2011; Madihah et al., 2001; Thang et al., 2010). A

number of studies focusing on genetic modification of the wild type strain to enhance butanol tolerance were reported (Chen & Blaschek, 1999; Harvey & Meylemans, 2011; Jones & Woods, 1986; Tomas et al., 2003). For example, *pGROE1* mutants of the wild type C. *acetobutylicum* ATCC 824 improved the butanol tolerance and subsequent butanol concentration from the practical maximum of 13 g/L to 18.5 g/L, while the mutant BA 101 for C. *beijerinckii* NCIMB 8052, was able to produce 19 g/L of butanol with a butanol tolerance up to 21 g/L in a batch fermentation (Jones & Woods, 1986; Tomas et al., 2003). The mutant C. *beijerinckii* BA 101 was identified as the best butanol-producing strain (Chen & Blaschek, 1999; Ezeji et al., 2010; Lee et al., 2008; Qureshi & Blaschek, 2001a; Tomas et al., 2003). In order to avoid the mixed-product fermentation, several bioethanol-producing cultures, such as *Escherichia coli* and *Saccharomyces cerevisiae*, have also been targeted for butanol production by introducing butanol metabolic genes (Atsumi et al., 2008; Balan, 2014; Si et al., 2014). However, those attempts achieved limited improvement on solvent production, hardly making any great progress for this bioprocess.

II.2 Feedstock for ABE fermentation

II.2.1 Feedstock sources

The feedstocks the commercial ABE fermentation in the early twentieth century were primarily food-based starches from maize, potatoes and wheat, or sugars from molasses, projecting intensive production cost from the competitive utilization of those substrates for solvents production versus the feeding purpose (Jones & Woods, 1986; Lee et al., 2008; Sun & Liu, 2012). The cost of food-based feedstock had been estimated to account for 60-80% of the total production cost in the commercial process (Taconi et al.,

2009). Therefore, the resurgence of this bioprocess for bio-butanol production depends largely on the availability of low-cost and abundant feedstocks. The fact that solvents-producing Clostridia are able to utilize many types of carbohydrates has granted a broad range of feedstocks options, such as cheap sugar sources, lignocellulosic biomass, glycerol and algal biomass (Jang et al., 2012; Jones & Woods, 1986). Glycerol as a substrate for solvents production has been an attractive idea in consideration of the abundance of glycerol as the major byproduct derived from the rapidly expanding market of biodiesel (Biebl, 2001; Jang et al., 2012; Taconi et al., 2009). The wild-type strain of C. *pasteurianum* was the microbe capable of bioconversion of glycerol into solvents. The production cost of butanol could be significantly reduced if co-locating biodiesel plant with a fermentation plant (Taconi et al., 2009). Fermentation of glycerol was reported to achieve the butanol yield of 15-20 % (g-butanol/g glycerol), which is comparable to that from pure glucose (Biebl, 2001; Taconi et al., 2009). But the process was limited by its rather long fermentation time.

As a cheap, abundant and renewable carbon source, lignocellulosic feedstock has great potential to be the substrate of choice for the bio-butanol production. Common types of lignocellulose include woody biomass, agricultural residues, and industrial wastes. Cellulose, hemicellulose and lignin project to be the major components of lignocellulose, which could account for approximately 80wt. %. As reported in many studies, various sources of lignocellulose have been proven to be suitable feedstocks for ABE production, including cheese whey, distiller's grains and solubles, wheat bran, barley straw, corn stover, switchgrass, bagasse, wood chips, etc. (Ezeji & Blaschek, 2008b; Liu et al., 2010; Lu et al., 2013; Lu et al., 2012; Qureshi et al., 2010a; Qureshi et

al., 2010b; Raganati et al., 2013; Shah et al., 1991a; Thang et al., 2010). Results from these studies indicated that fermentation of the hydrolysate from those lignocellulosic feedstocks could produce comparable levels of solvents as that from pure sugars (Ezeji & Blaschek, 2008b; Qureshi et al., 2010a; Qureshi et al., 2010b; Thang et al., 2010). In the case of lignocellulosic feedstock, substrate processing, however, was necessarily required (e.g., pretreatment and enzymatic hydrolysis).

Lignocellulosic feedstock

Biofuel has been envisioned to replace 30% market share in current petroleum industry by 2030 and thus to reduce the US dependence on foreign energy (Limayem & Ricke, 2012; Perlack et al., 2005). The availability of low-cost and abundant raw materials to be translated into biofuel is the fundamental premise for that goal. In USA, lignocellulosic biomass is the most abundant and renewable feedstock of choice with an anticipated annual production of 1.4 billion dry-tons (Limayem & Ricke, 2012; Perlack et al., 2005). Depending on the origins, lignocellulose could be basically classified as agricultural residues, forestry resources, herbaceous biomass and municipal and industrial wastes.

II.2.1.1 Agricultural residues

DOE predicted that a total of 350-450 million tons per year of the crop waste were available for being utilized as feedstock for bioenergy production (Hess et al., 2007; Perlack et al., 2005; Sokhansanj & Hess, 2009). Common examples of crops wastes include corn stover, wheat and barley straw and sugarcane bagasse, which are vastly produced after the crops harvest. The agricultural residues can be collected with the

existing harvesting equipment. Dust contamination of agricultural residues appears to be a huge challenge during collection, requiring intensive energy to clear away (Balan, 2014). Before baling the agricultural wastes, the moisture of residues needs to be reduced below 15% so as to prevent microbial degradation during storage (Balan, 2014; Board, 2010; BRDB, 2010).

II.2.1.2 Forestry feedstock

In USA, woody biomass is another substantially available source of feedstocks with estimated production of 370 million dry tons per year. Major source of forestry materials is directly harvested from natural trees; minor is collected from forestry wastes, such as sawdust, bark, wood chips and shavings (Hadar, 2010). Based on the difference of seed-reproduction, woody plants are divided into two types: hardwood and softwood. Softwood trees primarily refer to the gymnosperm conifers whereas hardwood belongs to the angiosperm species. Examples of hardwood include poplar, oak and aspen, etc. while most of the evergreen tree, such as pine and spruce, belong to the softwood species (Hadar, 2010). The average fiber length of hardwood (1mm) is 3 times shorter than that of softwood (3mm) (Sjostorm, 2010). The difference of inner cell structure could partially explain the more recalcitrance of softwood as compared to hardwood.

II.2.1.3 Herbaceous biomass

Perennial grasses (so-called energy crops) could also serve as a great feedstock source for bioenergy production. Well-studied species of energy crops include switchgrass, sorghum and *Miscanthus*. These grass species are vastly available on the non-agricultural land in the Midwest, Southeast, and Great Plains. Fast-growing energy crops have similar carbohydrate contents as the agricultural biomass but appear to be a

cheaper option due to its vast abundance on non-agricultural land (BRDB, 2010). In comparison with the forestry biomass, grassy biomass is less recalcitrant to release the carbohydrate and more environmentally friendly in consideration of the deforestation concerns (Limayem & Ricke, 2012). The grassy biomass can be collected with the existing harvesting system for the agricultural residues.

II.2.1.4 Municipal and industrial solid waste

Additionally, municipal and industrial solid waste could also contribute to a vastly available and attractive starting material for bioenergy production. Well-known examples of such solid wastes include waste papers and cardboard, paper mill sludge, newspaper, black liquors, cheese whey, distillers's grains (Ezeji & Blaschek, 2008b; Hadar, 2010; Holtzapple et al., 1992; Jones & Woods, 1986; Kudahettige-Nilsson et al., 2015; Limayem & Ricke, 2012; Zhao et al., 2012). These waste materials are usually discharged into the landfills, causing not only substantial expenses for disposal but also environmental concerns. Bioconversion of these municipal and industrial wastes appears to be a great option to alleviate the disposal problems and more importantly, generate value-added products. The organic portion of these wastes is in the form of either poor-quality fibers or low-DP polysaccharides and thus readily bio-convertible. As reported in many studies, separation of the organic components from the raw solid wastes is desirable toward an efficient bioconversion (Ezeji & Blaschek, 2008b; Jones & Woods, 1986; Kudahettige-Nilsson et al., 2015; Zhao et al., 2012).

II.2.2 Chemical compositions and physical structures of lignocellulose

II.2.2.1 Chemical Compositions

Generally, lignocellulosic biomass is composed of cellulose (30-50%), hemicellulose (20-35%), lignin (15-20%) and minor components (15-20%), such as ash and protein (Harmsen et al., 2010; Mood et al., 2013). The carbohydrate contents of cellulose and hemicellulose account for the major fraction (up to 75 wt.%) of entire lignocellulose. Table II-1 summarized general chemical compositions of the prevalent feedstocks. Depending on the species, the chemical compositions of lignocellulose could exhibit significant variations, especially on the hemicellulose and lignin content. Generally, the forestry biomass contains higher lignin content while the agricultural residues and herbaceous biomass are richer in the hemicellulose content. Studies have shown that the chemical composition within the same type of lignocellulose could also be different as determined by the age of plant, growth conditions, climate, etc. (Adler et al., 2006; Pordesimo et al., 2005).

Table II-1 Chemical Compositions of prevalent lignocellulosic feedstocks*

Feedstocks	Cellulose	Hemicellulose	lignin	Extractives	Ash
Hardwood	45-50	25-40	20-25	2-7	0.2-1.1
Softwood	40-45	25-30	25-35	2-4	0.2-0.5
Agricultural residues	37-45	25-50	10-20	4-15	4-10
Grasses	25-40	35-50	15-25	8-20	6-10
MSW	60-70	10-20	1-4	-	0-0.5

*. Data referenced from (Limayem & Ricke, 2012; Pordesimo et al., 2005; Zhao et al., 2012)

II.2.2.2 Cellulose

The microstructure of lignocellulose is presented in Fig.I-1 (Potters et al., 2010). Cellulose is a linear polymer (polysaccharide) of D-glucose linked through the β1-4 glycosidic bonds, as indicated in Fig.I-1. The DP of cellulose could stretch up to 17000 due to the intermolecular β1-4 linkage between glucose monomers (Harmsen et al., 2010). Micro-fibrils are bundled together through the hydrogen bonds between different polysaccharides chains. As primary structural components of cell walls, cellulosic fibers are formed by crystalizing the micro-fibrils due to the presence of highly stable and robust inter-and-intra molecular hydrogen bonds (Harmsen et al., 2010; Zhang et al., 2005). It is reported that the inter-and-intra molecular hydrogen bonds remained robustly stable until subjecting to severe conditions at 320 °C and 25 MPa, at which the fibers started to de-crystallized into amorphous form (Deguchi et al., 2006; Mood et al., 2013). Studies have also shown that the degree of crystallinity is one of the factors accountable for the recalcitrance of cellulose; highly crystallized cellulose fibers were more resistant

to the enzymatic hydrolysis than that in the amorphous form (Fan et al., 1980; Hall et al., 2010).

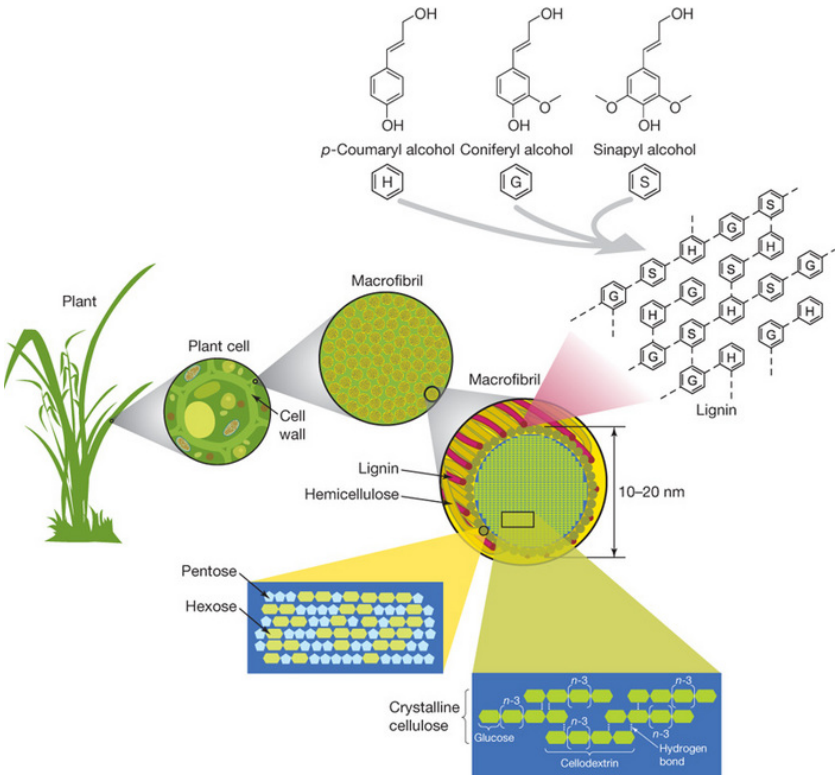


Figure II-1 Physical structures of lignocellulose (Potters et al., 2010)

II.2.2.3 Hemicellulose

In contrast to the highly crystallized structure of cellulose, hemicellulose is an amorphous and branched copolymer of hexose (D-glucose, D-galactose and D-mannose), pentose (D-xylose and L-arabinose), acetyl group and uronic acids (D-glucuronic acid and D-galacturonic acid), as shown in Fig. II-1 (Harmsen et al., 2010; Limayem & Ricke, 2012). Hemicellulose represents the secondary abundant sugar sources in the plant cell wall. For most of the feedstocks types, β1-4 linked xylose dominates the majority of monomer sugars in the backbone of hemicellulose structure whereas mannose is the

leading component in the hemicellulose of softwood (Harmsen et al., 2010). Due to the randomly amorphous and branched structure, hemicellulose is highly susceptible to be hydrolyzed by acid or cellulase enzyme.

II.2.2.4 Lignin

Lignin is a complex copolymer of three phenyl propionic alcohol monomers, including ρ-coumaryl, coniferyl and sinapyl alcohol, as indicated in Fig.II-1. Depending on the types of feedstock, lignin is composed of different ratios of these monomers. For example, major components of softwood lignin are primarily from coniferyl alcohol and ρ-coumaryl alcohol while coniferyl and sinapyl alcohols dominate the components for the hardwood lignin (Harmsen et al., 2010; Sjostorm, 2010). Lignin complex has a three-dimensional network cross-linking with hemicellulose through the covalent and hydrogenic bonds. This cross-linked network forms a matrix and embraces the crystallized cellulose fibers, protecting the fibers from being mechanically damaged. The carbohydrate-lignin matrix serves as a physical barrier to protect the carbohydrates from being enzymatically degraded into fermentable sugars, which is often referred as the recalcitrance of lignocellulose in the biofuel industry. To enhance the enzyme access to the cellulose and hemicellulose, pretreatment is generally required to disrupt the recalcitrance of biomass. As shown in Table II-1, lignin is the third abundant component in plant cell wall and is more abundant in the woody biomass than the agricultural residues or herbaceous biomass. This could partially explain the well-known notion that the woody biomass is more recalcitrant than the grass or agricultural residues and thus requires more harsh pretreatment conditions.

II.3 Bioconversion of lignocellulosic feedstock

II.3.1 Types of pretreatment

The complex structure of lignocellulose requires pretreatment to be a fundamental process in bioconversion of lignocellulose. Pretreatment aims to disrupt the recalcitrance of lignocellulose by altering the physical properties, such as particle size, porosity, pore size, cellulose crystallinity, degree of polymerization, accessible surface area, charge and chemical compositions (Kim et al., 2015; Limayem & Ricke, 2012). The effect of pretreatment on the physical structure of lignocellulose was illustrated in Fig.I-2. In addition to the disruption of recalcitrance of lignocellulose, an effective pretreatment method should be able to preserve most of the carbohydrates, minimize chemical and energy cost and lower the level of degradation products (Kumar et al., 2009; Mosier et al., 2005). Over the decades, various pretreatment technologies have been developed, including physical, biological and chemical methods (Hendriks & Zeeman, 2009; Kumar et al., 2009; Wyman et al., 2005).

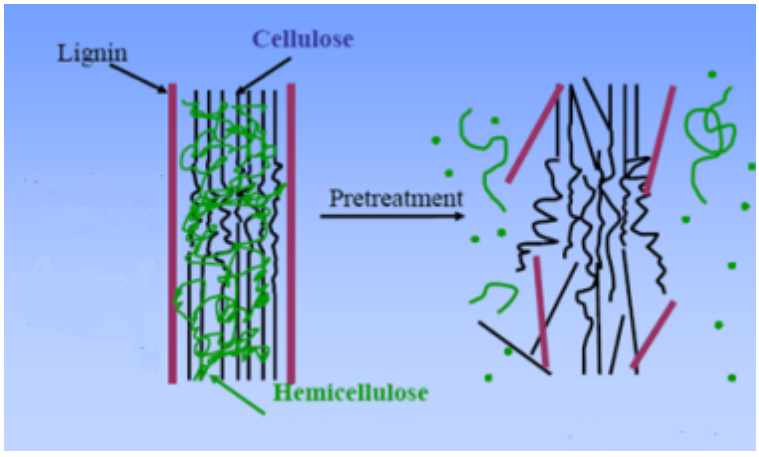


Figure II-2 Illustration of the effects of pretreatment on physical structure of lignocellulose (Mosier et al., 2005)

II.3.1.1 Physical Pretreatment

The effects of physical pretreatment involve the reduction in the particle size, the degree of crystallinity and polymerization and the increase of accessible surface area for the cellulase enzyme (Kumar et al., 2009; Mosier et al., 2005). Prevalent methods for physical pretreatment include mechanical milling, chipping, grinding or combination of them. Physical pretreatment is lightly used as a major industrial option due to high equipment and energy cost. The mechanical chipping is often adopted as the first step in bringing down the particle size of lignocellulose in the bio-refinery. The particle size of lignocellulose after mechanical chipping is usually in the range of 10-30 mm (Kumar et al., 2009). The finer the particle size is targeted, the more energy input is required (Mosier et al., 2005). And since woody biomass is more recalcitrant than the herbaceous biomass, the energy consumption for physical pretreatment of woody biomass is more intensive (Zhu et al., 2010a).

II.3.1.2 Biological Pretreatment

Biological pretreatments focus on utilizing lignin-degrading enzymes excreted from microorganisms to break down the matrix of lignin-carbohydrates. Biological pretreatment has been advocated as an energy-saving and environmental-friendly technology. Various species of fungi are capable of production lignin-lytic enzyme, including peroxidase, manganese peroxidase, and laccase, which could degrade the lignin into carbon dioxide and water. Of these microorganisms, the white fungi species, including Ceriporia lacerate, Stereum hirsutum, and Polyporus brumalis, has been extensively used to remove the lignin barrier in lignocellulose (Mosier et al., 2005; Zhang et al., 2007a). The low pretreatment rate is recognized as the major drawback of this

biotechnology, requiring at least 4 weeks degrading decent level of lignin (Kumar et al., 2009; Zhang et al., 2007a). Biological pretreatment is applied in conjunction with other pretreatment together as a means to reduce the energy input (Zhu et al., 2010a).

II.3.1.3 Chemical Pretreatment

Of the many different pretreatment technologies, chemical pretreatment appears to be the most prominent technology that significantly improves the saccharification yields. Depending on the pretreatment reagents, chemical pretreatment can be categorized as acid-, alkali-, organic solvent- based and different types of chemical pretreatments have different effects on the change of the structure of lignocellulose (Kumar et al., 2009; Limayem & Ricke, 2012; Mosier et al., 2005). For example, acid-catalyzed pretreatment extensively hydrolyzes hemicellulose and redistributes lignin on the surface of biomass while pretreatment under alkaline conditions are typically characterized as lignin degradation and removal (Kumar et al., 2009; Yang & Wyman, 2008). Organosolv pretreatments employ organic solvents to extract hemicellulose and lignin portion from biomass (Kumar et al., 2009). Ionic liquid pretreatment has been advocated as a green method to fractionate carbohydrates from lignin through dissolution cellulose portion of biomass (Brandt et al., 2013; Shill et al., 2011). The method selection is mainly based on the recalcitrance of the type of lignocellulose. The detailed features of leading chemical pretreatments methods are summarized below.

Acid-catalyzed pretreatment

Several acids, including sulfuric acid, hydrochloric acid, nitric acid and phosphoric acid, have been investigated as reagents for pretreatment of the lignocellulose (Kumar et al., 2009; Mosier et al., 2005; Yang & Wyman, 2008). As the cheapest acid

source, sulfuric acid remains the most popular reagents in pretreatment. Based on the acid concentrations, acid-catalyzed pretreatment can be divided into two categories: concentrated acid and dilute acid. Concentrated acid pretreatment is usually effective on most of the types of lignocellulose to directly saccharify the cellulose and hemicellulose portion into sugars without subsequent enzymatic hydrolysis. The intensive and non-recoverable chemical input appears to be a major limitation in this method. Additionally, due to the strong corrosive nature of sulfuric acid, this method requires extra cost on the acid-resistant reactors (Harmsen et al., 2010; Kumar et al., 2009).

In contrast to the concentrated acids, dilute acid pretreatment (less than 4%) appears to be a cost-effective option for most of the herbaceous and agricultural lignocellulose, such as switchgrass, corn stover, and rice/wheat straws. Dilute acid pretreatments are usually operated at high temperature (above 120 °C) with short residence time. It dramatically opens up the lignin-carbohydrate matrix and increases the accessibility of cellulose by hydrolyzing 75-90% of the hemicellulose (Kumar et al., 2009; Pordesimo et al., 2005). The lignin structure is also severely disrupted as characterized by the NMR (Moxley et al., 2012; Sannigrahi et al., 2008). Particularly notable is that high-cost phosphoric acid pretreatment could swell the cellulose structure and further enlarger the accessible surface area in addition to the common features of the acid pretreatment (Zhang et al., 2007b). Two primary concerns are associated with this method. First, the reactor needs to be resistant to the corrosive sulfuric acid especially at elevated temperature. Second, this method might potentially generate inhibitory products from the degradation of hemicellulose or lignin in the liquid stream, which introduces subsequent cost on the detoxification (Kumar et al., 2009; Yang & Wyman, 2008). Common

inhibitory compounds for the bio-ethanol producing culture (yeast or E. *coli)* include furfural, HMF, acetic acid while degraded phenolic compounds such as ferulic acid and coumaric aicd are known to be toxic for the butanol culture (C. *acetobutylicum*) (Ezeji & Blaschek, 2008b; Sun & Liu, 2012).

Alkali-based pretreatment

In comparison to the acid-catalyzed pretreatment, alkaline pretreatment is operated at mild temperatures and disrupts the recalcitrance of lignocellulose by selectively removing lignin and acetyl components while preserving most of the carbohydrates. In alkaline pretreatment, delignification is accomplished by cleaving the ester bond cross-linking the lignin and carbohydrate (Chakar & Ragauskas, 2004; Kim et al., 2016; Kumar et al., 2009). As a result of lignin removal, the surface area and porosity is substantially enlarged, extensively exposing the cellulose and hemicellulose (Yang & Wyman, 2008). The most popular alkaline reagents include ammonia, sodium hydroxide and lime (Kim & Lee, 2005; Kim et al., 2006). Sodium hydroxide has been traditionally used as a delignification reagent in the pulping industry. In pretreatment, the concentration of sodium hydroxide is usually applied in the range of 1-5% depending on the recalcitrance of the lignocellulose. Since sodium hydroxide is a strong alkali, pretreatment with sodium hydroxide is effective on most of the lignocellulose. But it might impose potential threat on the degradation of the cellulose and hemicellulose, especially for the less recalcitrant lignocellulose, such as corn stover and wheat straw (Gupta & Lee, 2010). The mechanisms involves in the degradation of cellulose and hemicellulose are often documented as “peeling reaction”, which occurs at the reducing end of the cellulose or hemicellulose (Gupta & Lee, 2010; Mozdyniewicz & Sixta, 2012).

As a cheap alkali, lime is another attractive option for alkaline pretreatment in that lime can be regenerated after pretreatment (Chang et al., 1997; Mosier et al., 2005). Although the delignification capacity of lime is less severe than that of sodium hydroxide operated, lime is proven to be an effective pretreatment reagent for a broad range of herbaceous and agricultural lignocellulose, resulting to substantial enhancement of the enzymatic digestibility (Chang et al., 1997; Chang et al., 1998). However, lime pretreatment has poor performance on the woody biomass. Pretreatment with aqueous ammonia (5-15%) has also been widely studied as a cost-effective delignification technology. The major advantage of this technology is that recovery of ammonia could be an easily accomplished through evaporation. Additionally, as a mild and non-corrosive alkali, aqueous ammonia-based pretreatment does not impose extra cost on the pretreatment reactor. Two ammonia-based pretreatment processes were developed: ammonia recycled percolation (ARP) and soaking in aqueous ammonia (SAA)(Kim et al., 2016; Kim & Lee, 2006; Kim & Lee, 2005; Kim & Lee, 2007; Kim et al., 2006). Each of them was proven to be effective in pretreatment of both herbaceous and woody biomass, resulting to substantial lignin removal but with negligible carbohydrates degradation (Gupta et al., 2008; Kim & Lee, 2006; Kim et al., 2006).

Solvent-based pretreatment

Solvent-based process disrupts the recalcitrance of lignocellulose by selectively dissolution certain component from lignocellulose. Two major types of solvents, including organic solvent and ionic liquid, are often employed as pretreatment reagents. Methanol, ethanol and acetone are typical organic solvents for the organosolv process. Organosolv pretreatment employs warm organic solvents to extensively dissolve lignin

and hemicellulose portion of lignocellulose (Kumar et al., 2009). After organosolv pretreatment, cellulose-rich solids are fractionated through washing firstly with solvents and then with water; solid lignin is precipitated from the aqueous solvents solution after the recovery of organic solvents through distillation; and hemicellulose sugars remain in the aqueous solution (Kumar et al., 2009; Pan et al., 2007; Zhao et al., 2009). In addition to the lignin and hemicellulose removal, organosolv pretreatment substantially swell the compact structure of cellulose as well, resulting increased accessible surface area (Zhao et al., 2012).

In contrast to the organosolv process, ionic liquid pretreatment employs certain species of ionic liquids to selectively solubilize the carbohydrates portion of lignocellulose (Brandt et al., 2013; Shill et al., 2011; Zhang et al., 2005). Dissolved carbohydrates potion of lignocellulose is then precipitated with the addition of anti-solvents, such as water and ethanol. Ionic liquids are room-temperature liquid salts based on several species of cations and anions (Brandt et al., 2013). Typical examples of ionic liquids include 1-butyl-3-methyl imidazolium chloride ([Bmim]Cl) and 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]), which are capable of effectively fractionating the carbohydrate portion from lignocellulose (Brandt et al., 2013; Shill et al., 2011; Zhang et al., 2005). The anions of ionic liquid substantially disrupt the inter-and-intra-molecular hydrogen bonds of cellulose, dramatically decreasing cellulose crystallinity (Brandt et al., 2013; Shill et al., 2011; Zhang et al., 2005). Although the solvent-based process has been advocated as green and effective pretreatment method to significantly increase the enzymatic hydrolysis of lignocellulose, it hardly becomes a leading

technology of first priority due to the expensive costs on the solvent itself as well as subsequent solvent recovery.

II.3.1.4 Physicochemical Pretreatments

Steam explosion is the most typical physicochemical method in combination of both physical and chemical process. Based on the catalyst, steam explosion can be generally divided into three categories: un-catalyzed steam explosion, acid-catalyzed steam explosion, and ammonia fiber expansion. Un-catalyzed steam explosion employs high-pressure saturated steam (160-260°C and 0.69-4.83 MPa) to impregnate the lignocellulose. During the impregnation, the hemicellulose and acetyl group are partially hydrolyzed due to the acidity of high-pressure steam (Harmsen et al., 2010; Kumar et al., 2009; Mosier et al., 2005). By suddenly discharging the high-pressure steam, the particle size of lignocellulose is substantially reduced due to explosion, dramatically disrupting the lignin matrix (Harmsen et al., 2010; Kumar et al., 2009; Mosier et al., 2005). Only limited portion of lignin is removed and most of the degraded lignin is re-precipitated on the surface of the pretreated solids. Steam explosion dramatically alters the structure of lignocellulose, such as the increase in the pore volume and surface area of biomass, reduction in particle size and the degree of crystallinity (Harmsen et al., 2010; Kumar et al., 2009). Major concerns associated to this method include intensive energy input, formation of inhibitors, and high equipment cost.

With the addition of acids (sulfuric acid or sulfur dioxide, carbon dioxide) into the high-pressure steam, acid-catalyzed steam explosion has better performance on enhancement of enzymatic digestibility than the un-catalyzed one. Particularly notable is that SO_2-catalyzed steam explosion has been known as the most effective method for the

recalcitrant softwood lignocellulose (Galbe & Zacchi, 2002). However, introducing SO_2 as catalyst in biomass pretreatment also creates environmental concerns. Ammonia fiber explosion (AFEX) is one of the proven technology that significantly improves the enzymatic digestibility while preserve most of the carbohydrates (Kim et al., 2011; Yang & Wyman, 2008). But the efficacy of AFEX is only limited to the herbaceous and agricultural biomass, not to the recalcitrant woody lignocellulose (Yang & Wyman, 2008).

II.3.2 Enzymatic hydrolysis

In comparison to the chemical process (concentrated acid hydrolysis), enzymatic degradation of lignocellulose into fermentable sugars has several key advantages: higher sugar yields, lower energy input and lower level of inhibitory products (Yang et al., 2011). Enzymatic hydrolysis is a typical heterogeneous catalysis process involving insoluble cellulose and soluble enzyme. The catalysis occurs at the solid-liquid interface, where the enzyme cuts off the intra-and-inter-molecular hydrogen bond of cellulose with the addition of one water molecular, as indicated in Fig. I-3. Due to the complexity of the heterogeneous system, enzymatic hydrolysis is jointly affected by both the substrate properties and the enzyme-related factors. Enzymatic digestibility is often correlated with a number of key characteristics of lignocellulose, including chemical composition, porosity, pore size, degree of crystallinity and polymerization, accessible surface area, surface charge, etc., whereas enzyme-related factors include specific activity, non-specific binding, end-products inhibition, reaction conditions (pH, temperature and speed), etc., (Arantes & Saddler, 2011; Del Rio et al., 2011; Eriksson et al., 2002; Kim & Um, 2015; Kim et al., 2015; Yang et al., 2011; Yang & Wyman, 2006).

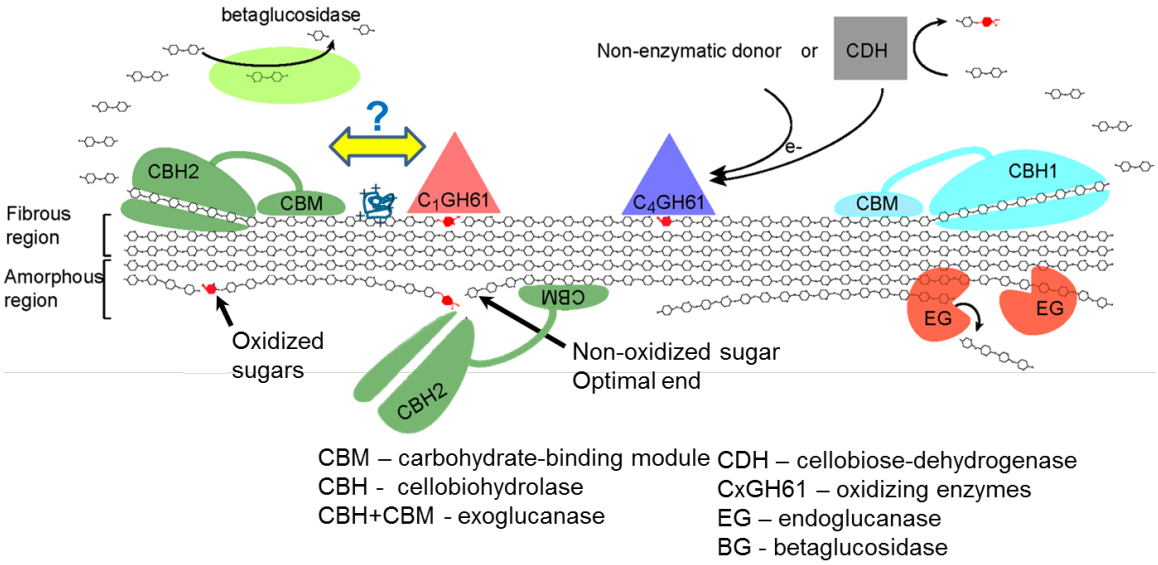


Figure II-3 Synergistic effects of enzyme complex on enzymatic hydrolysis of cellulose (Horn et al., 2012)

II.3.2.1 Enzyme complex

Cellulase enzyme, capable of degrading the polysaccharides in the lignocellulose, is a complex of glycoside hydrolases with endoglucanases (EG), exocellulases or cellobiohydrolases(CBH), and β-glucosidase(BG) as major components. In enzymatic hydrolysis, these individual component acts synergistically on the insoluble cellulose, as indicated in Fig.I-3. Individual role of EG on the synergism involves the generation of new end of polysaccharide chain by randomly cutting off the internal β1-4 glycosidic bonds. The new polysaccharide chain is then attacked by CBH, cleaving off the oligosaccharides or cellobiose from the end of the chain. There are two types of CBHs: CBH I and CBH II. CBH I processes the polysaccharide from the reducing end of polysacchride while CBH II generates the oligosaccharides from the non-reducing end.

BG finally hydrolyzes these oligosaccharide or cellobiose into monosaccharides. Additionally, CBH and EG possess typical carbohydrates binding modules (CBM) that are crucial for the cellulase attacking highly crystallized regions of cellulose (Horn et al., 2012; Yang et al., 2011). Many different wild-type species of microorganisms can produce this kind of enzyme cocktails (Horn et al., 2012; Yang et al., 2011). Of these microbes, Filamentous fungi, particularly the strains of *Trichoderma* and *Aspergillus*, have been widely developed for the commercial enzyme production (Yang et al., 2011). *T. reesei*, for example, produces enzyme complex with CBH and EG, BG as major components and xylanases, hemicellulases as minor components. Among the major components of *T. reesei,* BG is often produced at low proportion compared to CBH and EG, leading to the accumulation of high level of cellobiose, which is inhibitory to the the activity of CBH, compromising the synergistic action of the cellulase complex (Reczey et al., 1998; Yang et al., 2011). In comparison to the strains of *T. reesei, Aspergillus* strains are capable of producing high levels of BG (Horn et al., 2012; Sternberg et al., 1977). Therefore, commercial cellulase complex is usually used with the addition extra BG to optimize the proportion of individual components and maximize the activity of cellulase enzyme.

II.3.2.2 Cellulase Activity enhancer

Although enzymatically degradation of lignocellulose offers high sugar yield and low energy cost, the rate of sugar releases is quite slow, requiring substantially high dosages of expensive enzyme, typically more than 15 FPU/g glucan, to ensure an efficient conversion (Horn et al., 2012; Yang et al., 2011). Application of cellulase activity enhancers offers the promise for dramatic reduction in cellulase loadings and thus

the production cost. Many different types of enzyme additives have been shown to improve the cellulase activity, including protein-based (bovine serum albumin (BSA), expansins, and swollenim), non-ionic surfactant-based (polyethylene glycol (PEG), Tween-20, Tween-80) and cationic polyelectrolytes-based (C-PAM and polyDADMAC) (Eriksson et al., 2002; Kim et al., 2007; Kim & Lee, 2007; Li et al., 2012a; Reye et al., 2011b; Yang & Wyman, 2006). The specific mechanisms behind the activity enhancers have remained elusive. Depending on the type of additives and substrate, various explanations are concluded (Kim et al., 2007; Li et al., 2012a; Reye et al., 2011b). For the BSA and surfactant-based additives, it was concluded that the additives interact with lignin and reduce the non-productive binding between cellulase and lignin and thus increase the enzyme adsorption to the substrate (Kim et al., 2007; Li et al., 2012a). Therefore, they are reported to be only effective for the lignocellulosic feedstocks, not for the lignin-free substrate (Eriksson et al., 2002; Yang & Wyman, 2006). Additionally, these types of enhancers prevent enzyme deactivation of during long-term enzymatic hydrolysis (Eriksson et al., 2002). For example, BSA was found to readily adsorb to the dilute acid pretreated corn stover, resulting a 50% reduction in the enzyme dosage while maintaining the same level of enzymatic digestibility. Other types of activity enhancers, such as expansins and cationic polyelectrolytes, are proven to alter the physical structures of substrate and thus increase enzyme access to the substrate (Kim et al., 2009; Reye et al., 2011b). C-PAM was reported to enhance the activity of cellulase by a factor of two by neutralizing the surface charge of substrate (Reye et al., 2011b). It is of great significance to reduce the production cost by partially replacing the costly enzyme with cheap additives.

II.3.2.3 Enzymatic hydrolysis at high solid loading

Due to water-insoluble property of lignocellulose, bioconversion of lignocellulose at high solid consistency is still a challenging technical issue. Processing lignocellulose at high solid consistency would improve plant productivity with a more concentrated product syrup, saving both capital and operation cost, especially in product recovery (Jorgensen et al., 2007; Kristensen et al., 2009; Lynd, 1996; Wang et al., 2011). It has been estimated that in the case of commercial bioethanol process, it requires at least 15% of solid loading to achieve 4% ethanol in the fermentation broth, the level of which makes the ethanol recovery through distillation economically viable (Lynd, 1996; Tolan, 2002). In the corn ethanol industry, the concentration after fermentation is between 10 to 12 wt.% and it is achieved by simultaneous saccharification and fermentation with gradual feeding of partially hydrolyzed corn (fed-batch operation) (Bothast & Schlicher, 2005; Yasuda et al., 2014). Similar technology has been applied to lignocellulosic feedstock with total solid feeding up to 20 wt. % at pilot scale (Jorgensen et al., 2007). In straight batch hydrolysis with single feed, the maximum solid loading appears to be about 10 wt. %.

Another undesirable challenge for processing lignocellulose at high solids consistency is that the bioconversion yield exhibits a decreased pattern with increasing solid loading even with fixed enzyme-to-substrate ratio, which is often documented as the "solids effect" associated with bioconversion of lignocellulose (Kristensen et al., 2009; Wang et al., 2011). Although specific mechanism behind the solids effect remains unknown, several proven causes might partially account for it. Of the causes, end-product inhibition on the effectiveness of cellulase appears to be a major cause (Kristensen et al.,

2009). Other plausible causes include mass transfer limitation and the decrease in rate of enzyme-substrate adsorption (Kristensen et al., 2009; Wang et al., 2011). Studies have shown the solids effect cannot be avoided by simply increasing the enzyme dosage (Kristensen et al., 2009). The reductions in the product yield somewhat tamper the advantages of bioconversion at high solid loadings.

II.3.3 Fermentation

In terms of the methods for fermentation of lignocellulosic feedstocks into value-added products, three process concepts have been developed: consolidated bioprocessing (CBP), separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF). CBP is a relatively new bioconversion strategy that incorporates cellulase production, enzymatic hydrolysis and fermentation into one single process without enzyme addition. Few wild-type microorganisms are able to fulfill all these functions. Therefore, most of the cultures used in the CBP are recombinant strains. CBP is expected to be the ultimate bioconversion strategy with lowest capital cost (Olson et al., 2012). But this bioprocess suffers from low bioconversion yield and excessive by-products (Olson et al., 2012).

Traditionally, fermentation is performed as an independent unit process following the enzymatic hydrolysis, which is known as SHF. In this case, both enzymatic hydrolysis and fermentation are operated at their optimal conditions (pH and temperature). The optimal conditions for most of commercial enzyme (SPEZYME® CP or Cellic® CTEC-2) are at the temperature of 50 ±5°C and pH of 4.0–5.0, whereas common types of solvent-producing microbes have better metabolic performance at temperature of 36±1°C and broth pH of 6.5±1. Major drawback of SHF is that the end-

product inhibition on the activity of cellulase is prominent as evidenced by the low enzymatic digestibility and slow rate of enzymatic hydrolysis, which largely compromises the overall bioconversion yields (Olofsson et al., 2008; Sternberg et al., 1977; Yasuda et al., 2014).

To reduce the end-products inhibition, combination of the two separate unit processes into a single one at compromised conditions (usually around 37 °C) was introduced by (Gauss et al., 1976), which was later named as SSF. In SSF, end-products inhibition on enzymatic hydrolysis is greatly reduced since the sugar produced by the enzyme is simultaneously consumed by the microbes, resulting to a higher overall bioconversion yield as compared to the SHF although applied comprised condition (Gauss et al., 1976). Additionally, it would save substantial equipment and operation cost by combination of the enzymatic hydrolysis and fermentation into one process (Olofsson et al., 2008).

Depending on the microbes' capability of utilizing pentoses as carbon source, SSF can be further upgraded into simultaneous saccharification and co-fermentation (SSCF). In SSCF, in addition to the hexose fermentation, pentoses, as the second abundant yet highly underutilized sugar source in lignocellulose, are co-fermented in one reactor (Gauss et al., 1976). Most of the ABE-producing microorganisms are able to utilize pentose as carbon source. However, the limitation of SSF or SSCF is that due to the water-insoluble property of lignocellulose, it is hard to work under high solid loadings, resulting to a dilute product concentration, which may potentially increase the difficulty for products recovery. Therefore, SSF or SSCF with fed-batch mode appears to be an industrially viable option.

II.3.4 Solvents recovery

Multistage distillation has been traditionally applied as the methods for solvents recovery in ABE fermentation. Since butanol has a higher boiling point than water (118 °C), employing distillation to recover solvents products from the broth projects intensive energy cost, allowing the water to be totally evaporated (Jones & Woods, 1986; Nielsen & Prather, 2009). Additionally, due to the inhibition problems, the solvents are usually produced at relatively low levels (typically 18-25 g/L), which make the solvents recovery via distillation to be more difficult (Jones & Woods, 1986; Nielsen & Prather, 2009). To reduce the energy cost on solvents recovery, many different recovery technologies have been investigated, including adsorption, gas stripping, liquid-liquid extraction, and membrane-based pervaporation.

The hydrophobic property of n-butanol allows adsorption to be a method for products recovery. A number of hydrophobic adsorbents exhibit high affinity to n-butanol, including polystyrene-based resins, zeolite with high SiO_2/Al_2O_3 ratio, activated carbon, silicalite (Nielsen & Prather, 2009; Saravanan et al., 2010; Vane, 2008). The recovery process can be performed in either batch or continuous mode. Separation of the solvents from the adsorbents and regeneration of adsorbents can be achieved via thermal desorption (Nielsen & Prather, 2009; Vane, 2008). Most of the adsorbents are completely immiscible with fermentation broth and thus biocompatible with the microbes. Adsorbent fouling appears to be a huge issue, especially for the lignocellulose-based fermentation process, in which non-hydrolyzed solid residue may block the surface area of adsorbents. Additionally, in some cases, fermentation by-products, including microbe cells, sugars,

and organic acids, may also attached to the adsorbents limiting the effectiveness of this technology (Vane, 2008).

Gas stripping is a simple and effective method to recover solvents from fermentation broth. It typically utilizes inert gas to selectively capture the solvents out the fermentation broth. The mechanism behind gas stripping is based on the mass transfer theory, in which solvents in the aqueous phase transport across the liquid-gas interface into the gas phase (Lu et al., 2012; Qureshi & Blaschek, 2001b; Vane, 2008). Therefore, the performance of gas stripping is dictated by the temperature, interface area, mass transfer coefficient, flow rate, etc. (Vane, 2008). A number of studies demonstrated the feasibility of utilization gas stripping to recover solvents from the ABE fermentation broth employing the gas products in the ABE fermentation (carbon dioxide and hydrogen) (Lu et al., 2012; Qureshi & Blaschek, 2001b). However, subsequent solvents purification and stripper gas regeneration are proven to be difficult and energy-intensive, making this technology less likely to be an industrial option (Vane, 2008).

Membrane-based pervaporation is another well-known separation technology. A vacuum pump is required in this process to create vacuum at one side of the membrane. Under the pressure difference, semipermeable membrane allows the solvents to diffuse through the membrane and to evaporate as vapor on the vacuum side of membrane. In the case of ABE fermentation, a hydrophobic and solvents-permeable membrane is preferred. Examples of this type of membrane include silicalite-silicone based membrane, Polydinethylsiloxane (PDMS) (Liu et al., 2011; Vane, 2008). Despite its proven separation capacity, this technology is not recognized as an economically viable option due to the substantial energy input as well as high cost for this type of membrane (Liu et

al., 2011; Vane, 2008). Additionally, similar to the adsorption technology, membrane fouling appears to be a huge concern that makes this process unsuitable for large-scale application.

Liquid-liquid extraction is recognized as the most promising solvent recovery strategy for ABE fermentation (Vane, 2008). Selection of extractants must meet the following criteria:

1. higher selectivity of solvents relative to the aqueous phase
2. higher distribution coefficient of solvents
3. readily to be separated from the aqueous phase
4. low volatility if extractant is regenerated by evaporation
5. immiscible with aqueous phase, low cost

Depending on the toxicity of the extractant, the extraction process can be performed either in a direct contact way between non-toxic extractant and the broth or under a hydrophobic membrane-assisted manner for a toxic extractant. For a toxic but effective extractant, an additional cell recycle process is proposed to avoid the cell contamination prior to the extraction process (Barton & Daugulis, 1992; Vane, 2008). Common examples of effective extractants for ABE fermentation include oleyl alcohol, vegetable and miner-based oils, dodecanol (Barton & Daugulis, 1992; Liu et al., 2011; Vane, 2008). Extractant regeneration and solvents purification has been typically accomplished by vacuum flash vaporization, distillation or gas stripping, contributing the primary energy cost for this process (Vane, 2008).

III. Effects of PolyDADMAC on pretreatment of lignocellulosic and lignin-free biomass

Abstract

Pretreatment is a necessary unit-process in bioconversion of lignocellulosic biomass. It was found that supplementation minute quantity of poly-diallyl-dimethyl-ammonium chloride (polyDADMAC) in alkaline pretreatment of corn stover led to slightly negative effect on delignification but surprisingly enhanced enzymatic hydrolysis of pretreated solids. SEM images of pretreated solids revealed that polyDADMAC significantly disrupted and swelled cellulose fibers in pretreatment. As supportive control, lignin-free cellulose was treated with hot water with and without addition of polyDADMAC. It was shown that addition of polyDADMAC in hot-water treatment enhanced enzymatic hydrolysis of lignin-free cellulose likewise. It was then speculated that the effectiveness of polyDADMAC was appeared to be by direct interaction with cellulosic fiber, rather than with lignin or other extraneous components. A two-stage pretreatment was then performed to reaffirm this hypothesis; corn stover was first treated with alkali alone, and then treated with polyDADMAC.

Keywords: Pretreatment, polyDADMAC, enzymatic hydrolysis, digestibility, delignification.

III.1 Introduction

Production of biofuel from lignocellulosic biomass has been vastly investigated to improve the U.S. energy independence and alleviate environmental concerns resulted from fossil fuel combustion. Currently, bioconversion of lignocellulose basically involves three major processes: pretreatment, enzymatic hydrolysis and fermentation. Pretreatment is a prerequisite for downstream enzymatic hydrolysis; major role of pretreatment is to necessarily disrupt the recalcitrance of biomass and to enhance enzyme access to cellulose and hemicellulose in enzymatic hydrolysis (Kumar et al., 2009; Mosier et al., 2005). Over the decades, various pretreatment technologies have been developed, including biological, physical and chemical methods (Hendriks & Zeeman, 2009; Kumar et al., 2009; Wyman et al., 2005). Biological pretreatments utilize lignin-degrading enzymes secreted from microorganisms to degrade lignin and enhance enzymatic hydrolysis. Physical pretreatment basically reduces feedstock's particle size and crystallinity by mechanical forces. Acid, base and solvents are commonly used chemicals in chemical pretreatment. Acid-catalyzed pretreatment works to extensively hydrolyze hemicellulose and to redistribute lignin on the surface of biomass. Pretreatment under alkaline conditions are characterized as lignin degradation or removal. Organosolv pretreatment employs organic solvents to extract hemicellulose and lignin portion out of biomass. Expensive costs of chemicals and energy on those methods still challenge the economic issues of pretreatment and stimulate technical advance of pretreatment.

Various studies have focuses on supplementation of additives to enhance pretreatment performance. Those additives included non-ionic surfactants, oxidants, and even inorganic salts. (Qing et al., 2010) reported that supplementation of surfactant

Tween 80 in dilute acid or hot water pretreatment of corn stover contributes to lignin removal and increase sugar yields in enzymatic hydrolysis. Incorporation of hydrogen peroxide into alkaline pretreatment of hardwood or corn stover dramatically reduced pretreatment severity but maintained comparable delignification and enzymatic hydrolysis (Banerjee et al., 2011; Gupta & Lee, 2010). Addition of sodium sulfite in alkaline pretreatment improves carbohydrates recovery as well as enzymatic digestibility (Li et al., 2012b). The general effect of these additives on pretreatment is to enhance lignin removal and thus to reduce non-productive binding of enzyme to lignin (Kim & Holtzapple, 2006; Laureano-Perez et al., 2005).

It was recently found that supplementation of small amounts of poly-diallyl-dimethyl-ammonium chloride (polyDADMAC) in alkaline pretreatment significantly altered the structure of pretreated solids, which significantly improved enzymatic digestibility. PolyDADMAC is a cationic polymer, commonly used as flocculants in pulp and paper industry. It contains highly charged ionic groups: DADMAC cation and chloride anion in its chemical structure. One common example of its application is used for lignin precipitation from black liquor in pulping process by forming complex with negatively charged lignin (Lappan et al., 1997; Maximova et al., 2001; Razali et al., 2011).

In this study, polyDADMAC was tested as pretreatment additive to enhance enzymatic hydrolysis. Corn stover and switchgrass were pretreated by soaking in aqueous ammonia (SAA) with and without polyDADMAC addition. Pretreated solids were analyzed for composition paying special attention on delignification, and further examined for structural changes by scanning electron microscope (SEM), X-ray

diffraction (XRD) and BET surface area analysis. With addition of polyDADMAC, pretreated solids had slightly higher amount of residue lignin, but significantly enhanced enzymatic hydrolysis. With morphological analysis of pretreated solids, the possible mechanisms by which polyDADMAC interacted to the cellulose fibers and thus affected the enzymatic hydrolysis were also discussed.

III.2 Materials and methods

III.2.1 Materials

Corn stover and switchgrass were provided by National Renewable Energy Laboratory (Golden, Colorado) and Ceres Inc. (Thousand Oaks, CA). Avicel and solka floc, as lignin-free biomass, were purchased from Sigma-aldrich (St. Louis, Mo) and International Fiber Corporation (Urbana, OH). Novozymes (North America Inc.) kindly supplied cellulase enzyme: Cellic® CTec2 (Batch No. VCNI0001) with specific activity of 119 FPU/ml and protein number of 255.22 ± 4.24 mg protein/ml. PolyDADMAC with a molecular weight of 1.8 MDa and charge density of 20 mole percent was supplied from Eka Chemical Inc. (Marietta, GA).

III.2.2 Pretreatment

Corn stover and switchgrass were pretreated by soaking in 15 wt. % ammonia solutions while lignin-free cellulose was treated with hot water. Unless otherwise noted, the liquid-to-solid ratio for all the pretreatments in this study was 9; 10 g dry biomass was soaked into 90 g pretreatment reagent. Prior to pretreatment, polyDADMAC was mixed into pretreatment reagent at a dosage of 0.3 wt. %. Pretreatment was performed in thermostatic oven. Two pretreatment conditions were applied: 160 °C for 1 hour and 60 °C for 24 hours. Pretreatments with and without supplementation of polyDADMAC were

run in parallel. After pretreatment, pretreated solids were washed with tap water and air-dried on aluminum foil till the moisture was reduced below 10%.

III.2.3 Composition Analysis

The compositions of solids before and after pretreated were determined through a two-stage acid hydrolysis according to NREL (National Renewable Energy Laboratory) analytical procedure NREL/TP-510-42618. The carbohydrates in the solids were analyzed by high performance liquid chromatography (HPLC) equipped with Aminex HPX-87P column (Bio-Rad Laboratories, Inc.).

III.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated solids was performed on 2 and 5 (w/v)% solid loadings in 125 ml Erlenmeyer flask. Sodium citrate (pH 4.8) was used as buffer to stabilize optimal pH for enzyme and sodium azide was applied as antibiotic to prevent microbe contamination. For 2% solid loading, enzyme was added at dosage of 5 FPU/g-glucan whereas for 5% solid loading, 7.5 FPU/g-glucan was applied. Being loaded with buffer, pretreated solids, enzyme and antibiotics, Erlenmeyer flasks were placed on orbital shaker (MAXO 2000, Thermo Scientific) at 50 °C and 200 rpm to initiate enzymatic hydrolysis. Samples were taken at intervals of 24 until 120 hr. Enzymatic digestibility was defined as the percentage of sugar released over a period of hydrolysis on the basis of theoretical sugar concentration from pretreated biomass.

III.2.5 Surface morphology of pretreated solids

Before SEM examination, pretreated solids were mounted on aluminum stub via double-stick carbon tape and then gold-coated for 2 minutes with EMS 550X Sputter

Coating Device (Electron Microscopy Sciences, Hatfield, PA). Coated samples were then scanned and pictured at 1000X magnifications with Zeiss EVO 50 Scanning Electron Microscope (Carl Zesis Microscopy LLC, Thornwood, NY).

III.2.6 Crystallinity index

The crystallinity of pretreated solids was empirically determined by X-ray diffraction (XRD) according to the peak height method (Segal et al., 1959; Terinte et al., 2011). Pretreated solids were scanned with D8 Advance XRD (Bruker, Madison, WI) from 2θ of 10° to 35° at a speed of 1 °/min with a step size of 0.01°. The crystallinity index (CrI) was calculated as:

$$\text{CrI}=\frac{I_{002}-I_{non-cr}}{I_{002}}\times 100\%,$$

where, $I_{002}$is the peak height at 2θ = 22.5° representing the intensity of crystalline portion and I_{non-cr} is the valley height at 2θ=18.7° representing the non-crystalline part of cellulose.

III.3 Results and discussion

III.3.1 Chemical compositions of pretreated solids

Table III-1 lists pretreatment conditions, solid recovery, delignification and solid compositions of corn stover and switchgrass. The existence of extractives in biomass, and the loss of cellulose and hemicellulose during pretreatment might account for the solid loss after pretreatment, leading to mass closure less than 100% (Chen et al., 2010).

Corn stover and switchgrass were significantly de-lignified after SAA pretreatment. About 80% and 73% of lignin in corn stover were removed respectively corresponding to the conditions at 160 °C for 1 hour and 60 °C for 24 hours. Pretreatment

at 160°C led to more hemicellulose loss in spite of higher delignification. Further optimization of pretreatment conditions is needed to balance delignification and hemicellulose loss. Since this work mainly focuses on the effect of polyDADMAC on pretreatment, not for the optimization of pretreatment conditions, switchgrass was only pretreated at 160 °C for 1 hour, at which about 61% of lignin was removed.

Table III-1 Compositions of corn stover and switchgrass before and after pretreatment

Component%	Corn stover [a]					Switchgrass[a]		
	Untreated	160 °C 1 hour		60 °C 24 hour		Untreated	160 °C 1 hour	
		w/o[b]	w/[b]	w/o	w/		w/o	w/
Glucan%	34.7	57.2	58.4	53.4	53.7	32.1	56.7	56.0
Xylan%	21.3	21.2	21.3	26.0	25.4	18.5	20.4	19.5
K-Lignin %	21.7	7.8	10.1	9.4	11.8	19.5	13.9	14.8
Total%	77.6	86.1	89.9	88.8	90.9	70.1	91.0	90.3
Solid recovery[c]	ND	59.6%	56.3%	63.0%	64.0%	ND	55.0%	54.7%
Delignification[d]	ND	79.8%	73.9%	72.7%	65.2%	ND	61.0%	58.7%

Note:
a. Corn stover and switchgrass was pretreated with soaking in aqueous ammonian (15%).
b. w/o: without addition of PolyDADMAC in pretreatment; w/: with the addition of PolyDADMAC in pretreatment
c. Solid recovered after pretreatment

As shown in Table III-1, solids pretreated with polyDADMAC-SAA have slightly higher lignin content than that with SAA only. In alkaline pretreatment, lignin was peeled off from carbohydrate by cleaving ester bond between lignin and carbohydrate (Kumar et al., 2009). PolyDADMAC was known to form complex with lignin in alkaline solution (Lappan et al., 1997; Li & Pelton, 1992). The slightly negative effect on delignification was likely caused by lignin-polyDADMAC complex, protecting lignin from being peeled off in pretreatment.

Avicel consists of 98% of glucan and solka floc is characterized as 77.3% of glucan and 22.8% of xylan. By measuring sample weight before and after hot-water

treatment, there was no measurable weight loss, suggesting no carbohydrates were hydrolyzed into oligomer or monomer sugars during hot-water treatment.

III.3.2 Enzymatic hydrolysis of pretreated corn stover and switchgrass

Time-course profiles of enzymatic hydrolysis of pretreated corn stover were shown in Fig III-1 with A for solid pretreated at 160°C for 1 hour and B for that pretreated at 60°C for 24 hours. By mixing 0.3 wt. % polyDADMAC into ammonia solution prior to pretreatment, enzymatic digestibility were significantly improved. For example, 120-hr enzymatic digestibility of pretreated corn stover at 160 °C exhibited 18.4% of increase in glucan digestibility, from 58.4% to 76.8% and 21.8% of increase in xylan digestibility, from 51.4% to 73.2%, while for corn stover pretreated at 60°C, glucan and xylan digestibility achieved increases of 13.7% and 7%.

Addition of polyDADMAC in pretreatment has negative effect on lignin removal but enhance enzymatic hydrolysis of pretreated solids. This result contradicts the widely accepted notion that higher level of residue lignin always inhibits enzymatic hydrolysis (Eriksson et al., 2002; Kristensen et al., 2007). However, this controversial effect was reconfirmed in pretreatment of switchgrass (Fig. III-2). In this case, polyDADMAC supplementation resulted to approximately 3% less delignification but 18%/12% increase in glucan/xylan digestibility.

Similar results were recently reported in polyDADMAC-sodium hydroxide pretreatment (Ji & Lee, 2013). (Ji & Lee, 2013) claimed that with the addition of polyDADMAC in alkaline pretreatment, the residual lignin was modified to have less affinity to cellulase enzyme similar to that from dilute acid pretreatment. However, it is known that the property of residual lignin was highly dependent on pretreatment pH

(Kumar & Wyman, 2009). In this study, addition of minute amount of polyDADMAC into ammonia led to non-detectable pH variations on both before and after pretreatment. Therefore, the lignin modification was not likely to be the reason behind the enhanced enzymatic digestibility.

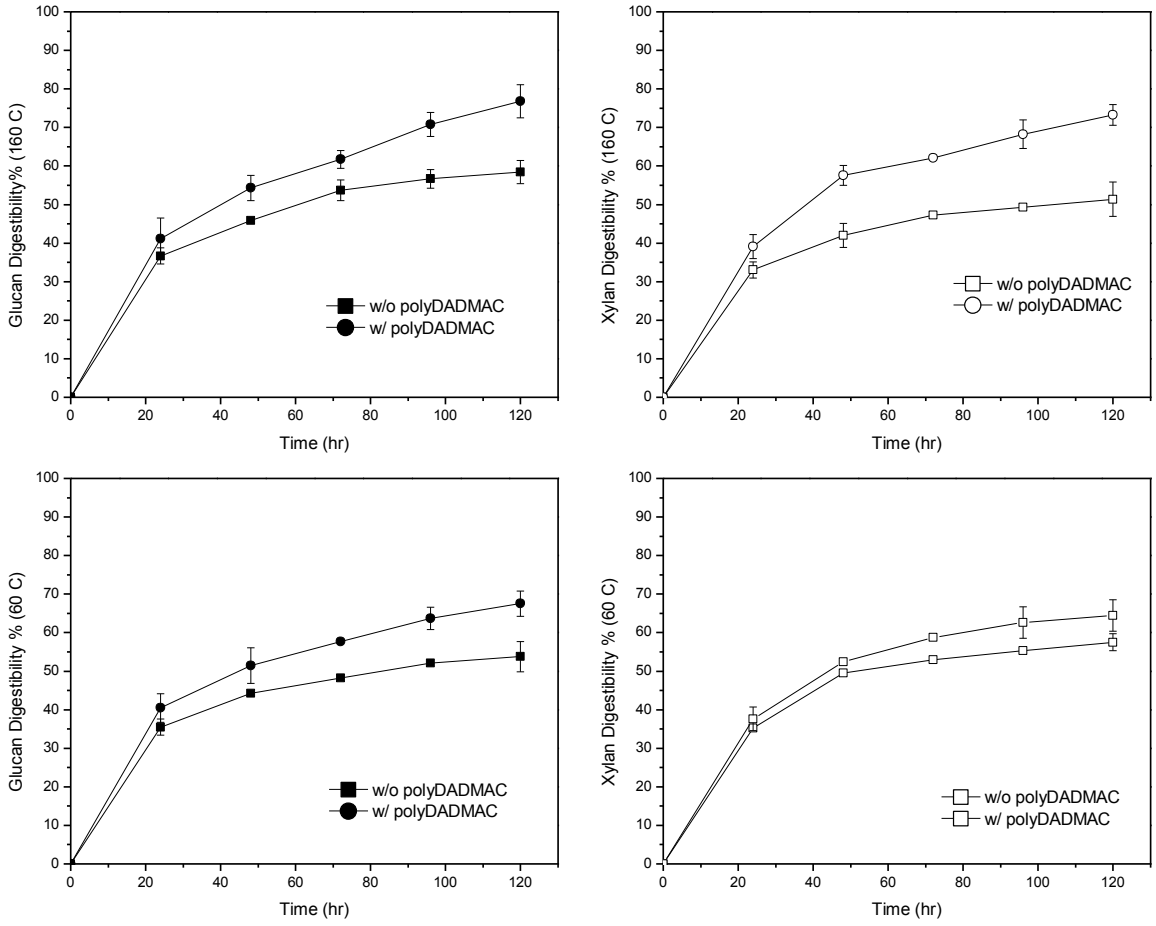


Figure III-1 Enzymatic hydrolysis of corn stover pretreated at 160 °C for 1 hour (top two) and 60 °C for 24 hours (bottom two) with and without addition of polyDADMAC

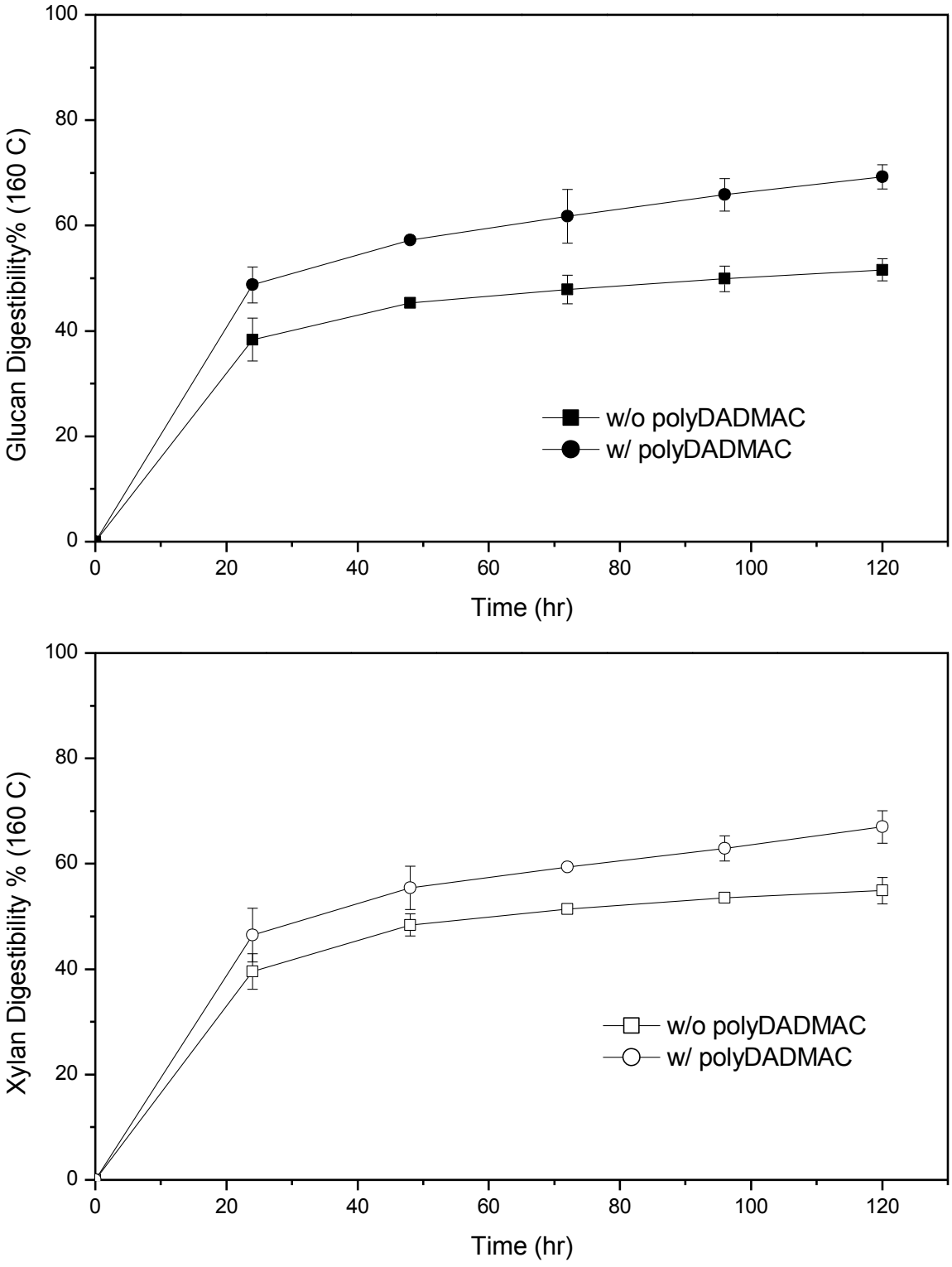


Figure III-2 Enzymatic hydrolysis of switchgrass pretreated at 160 °C for 1 hour with and without addition of polyDADMAC

III.3.3 SEM images of pretreated corn stover and switchgrass

The morphologies of pretreated solids were identified with SEM and shown in Fig. 3. Untreated corn stover (Fig. 3A) exhibited rigid and compact structure with lignin tightly covering the surface of corn stover. Alkaline pretreatment was known to remove considerable portion of lignin from lignocellulose. After pretreatment, more than 70% of lignin was removed from corn stover, extensively exposing cellulose fibers, as in Fig. 3B. Supplementation of polyDADMAC in pretreatment of corn stover disrupted the exposed cellulosic fibers quite significant, dramatically enlarging enzyme accessible surface area (Fig. 3C). The SEM images of pretreated switchgrass were listed in Fig. 3D-F. Untreated switchgrass (Fig. 3D) and SAA pretreated one (Fig. 3E) exhibited similar morphologies as untreated corn stover and SAA pretreated corn stover. The effects of polyDADMAC on pretreated solids were illustrated in Fig. 3F. The smooth bundle of exposed cellulose was densely etched, forming many caustic pits. Either the significantly disrupted cellulosic fibers as in pretreated corn stover or the caustic pits on the surface of pretreated switchgrass were believed to open up the crystalline structure of fibers, rendering the solids more susceptible to cellulase.

It was reported that when interacting to cationic polyelectrolytes, the overall physical properties of cellulose were heavily affected (Mosse et al., 2012; Reye et al., 2011a; Reye et al., 2011b; Wu et al., 2009). (Wu et al., 2009) summarized that polyelectrolytes with high charge densities were readily to penetrate into the pores of cellulose and adsorb onto the inner wall of cellulose. (Reye et al., 2011a) reported that cationic polyelectrolytes, when applied as additive in enzymatic hydrolysis, were able to modify the surface charge of wood fiber. As a result of surface charge modification, the

network of electrostatic balance and hydrogen bonds between cellulose inter-chain was disrupted, reducing the surface tension of cellulose (Mosse et al., 2012). The disrupted morphology of pretreated solids with polyDADMAC addition was believed to be associated with the reduced surface tension as a result of surface charge modification.

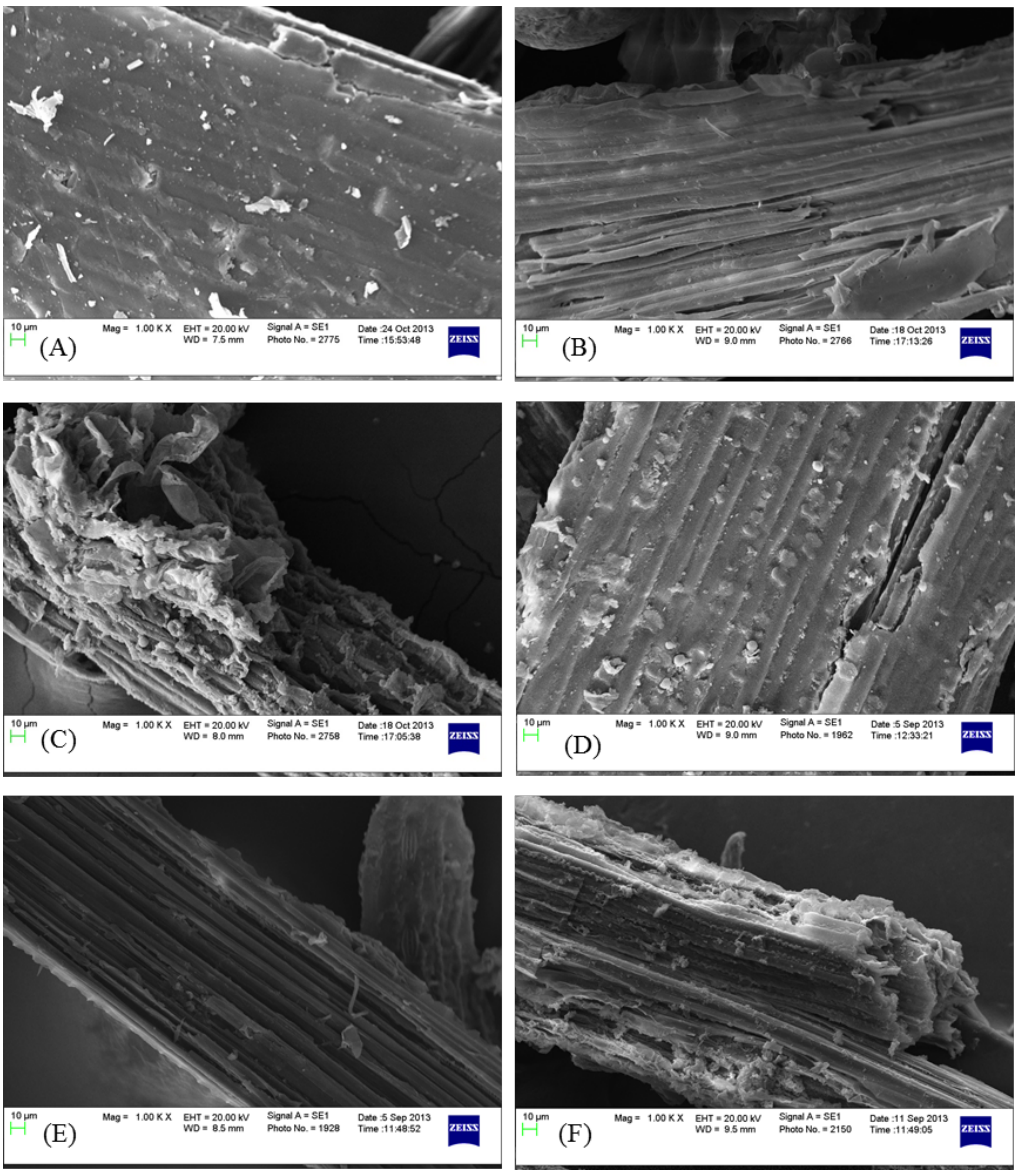


Figure III-3 SEM images of corn stover and switchgrass pretreated at 160 °C for 1 hour (A). untreated corn stover. (B). SAA pretreated corn stover w/o polyDADMAC. (C). SAA pretreated corn stover w/ polyDADMAC. (D). untreated switchgrass. (E). SAA pretreated switchgrass w/o polyDADMAC. (F). SAA pretreated switchgrass w/ polyDADMAC

III.3.4 XRD pattern of pretreated switchgrass

The XRD patterns of treated switchgrass and Avicel with and without polyDADMAC were presented in Fig.4. All the patterns exhibited typical lignocellulose

peaks; the highest peak at 2θ = 22.5° reflected the crystalline portion of lignocellulose and the valley at 2θ = 18.7° represented the amorphous portion, including hemicellulose and lignin. Corn stover pretreated by SAA alone had highest crystallinity (CrI=50.1%), followed by the SAA-polyDADMAC pretreated (CrI=46.0%) and the untreated (CrI=35.7%). Due to extensively removal of amorphous lignin in pretreatment, SAA pretreated corn stover had a higher crystallinity than the untreated one (Chang & Holtzapple, 2000; Kim & Lee, 2007). (Zhang & Lynd, 2004) reported that the reduction in crystallinity of lignocellulose was usually co-occurred with the disruption of surface structure and inter-and-intra molecular bond. In this case, not only the surface of pretreated corn stover was severely disrupted but the crystallinity of pretreated corn stover decreased by 4% as a result of polyDADMAC addition in pretreatment.

III.3.5 Effect of polyDADMAC on hot-water treatment of lignin-free substrate

To further elucidate the interaction between polyDADMAC and cellulose fibers, solka floc was treated by soaking in hot-water as control and polyDADMAC solution (0.3 wt. %) at the same conditions as in pretreatment of corn stover. The time-course profiles of enzymatic hydrolysis of regenerated solka floc were shown in Fig. 5 and 6, corresponding to the treatment conditions at 160 °C and 60 °C. For the solids treated with and without polyDADMAC addition, the saccharification profiles at the first 24 hours were comparable to each other. Whereas, for polyDADMAC-treated solka floc, the enzymatic hydrolysis rate was remarkably improved afterwards, compared to that for the hot-water treated solids. Over 120-hour enzymatic hydrolysis, for hot-water treated solka floc, glucan digestibility and xylan digestibility only reached to 41% and 27%, while

totally 67% of glucan digestibility and 45% of xylan digestibility was achieved for polyDADMAC-treated solka floc.

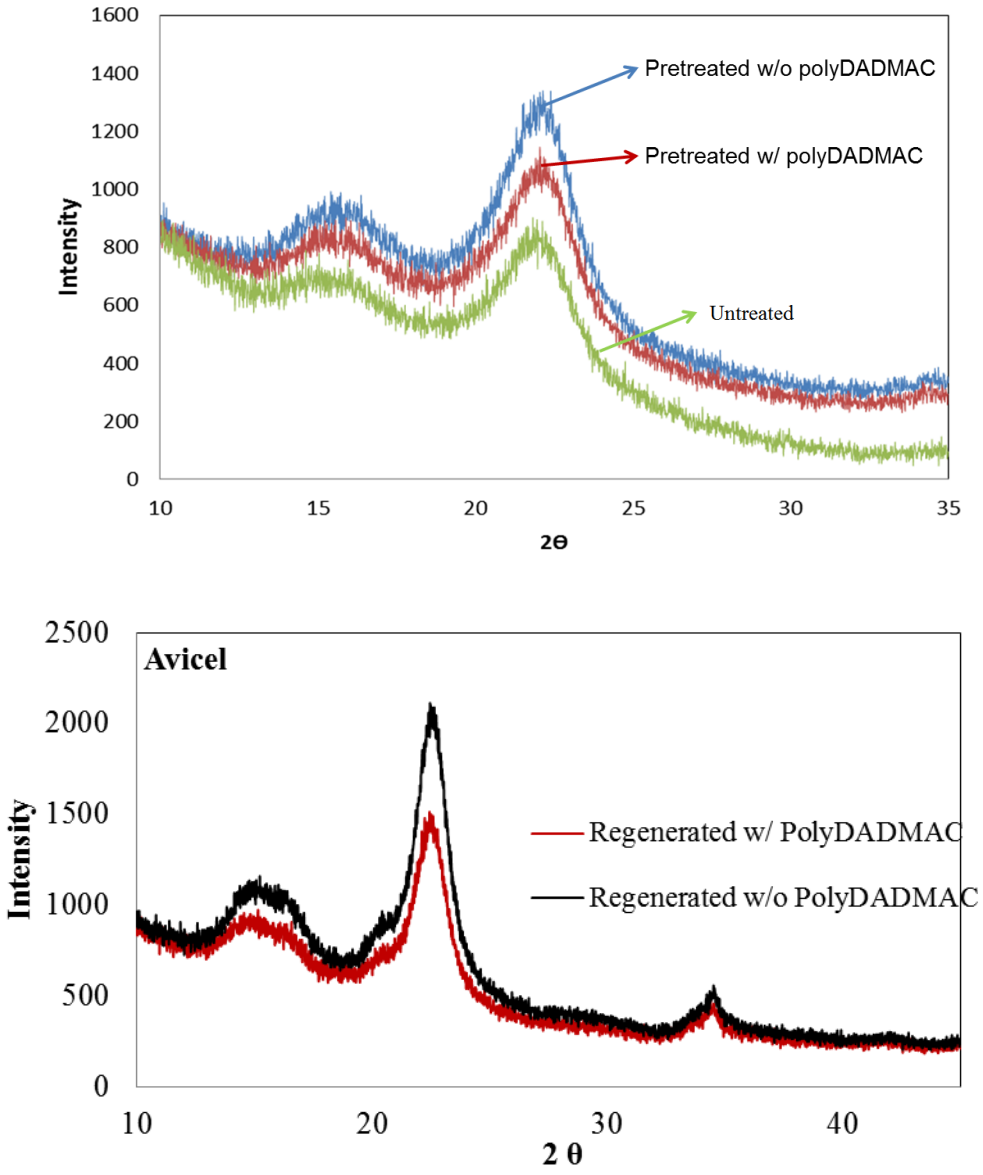


Figure III-4 XRD patterns of pretreated corn stover and Avicel

A notable observation was that as a result of polyDADMAC addition, enzymatic digestibility of regenerated solka floc exhibited similar levels of increase, suggesting that

high temperature was not a necessary requirement for the interaction between polyDADMAC and cellulose fibers.

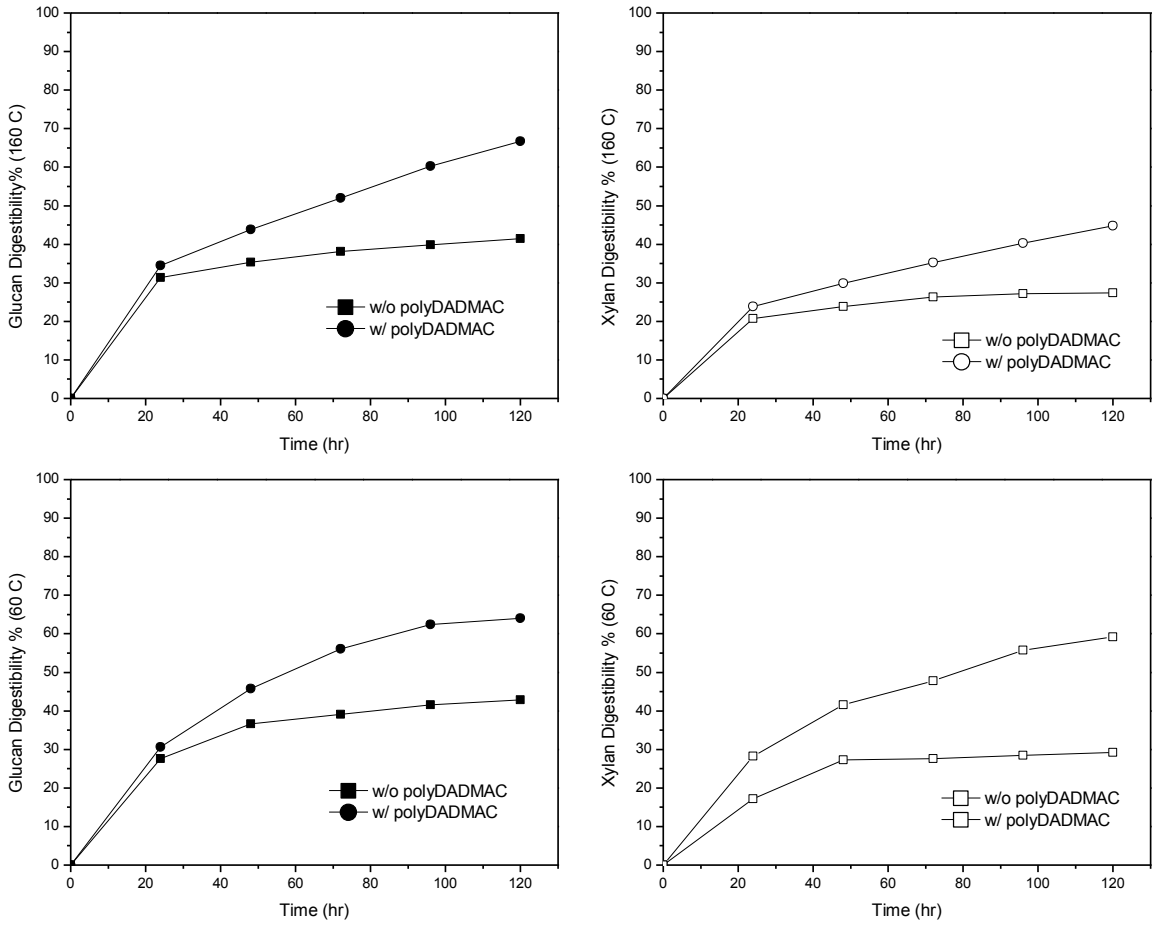


Figure III-5 Enzymatic hydrolysis of regenerated Solka floc at 160 °C for 1 hour (top two) and at 60 °C for 24 hours (bottom two)

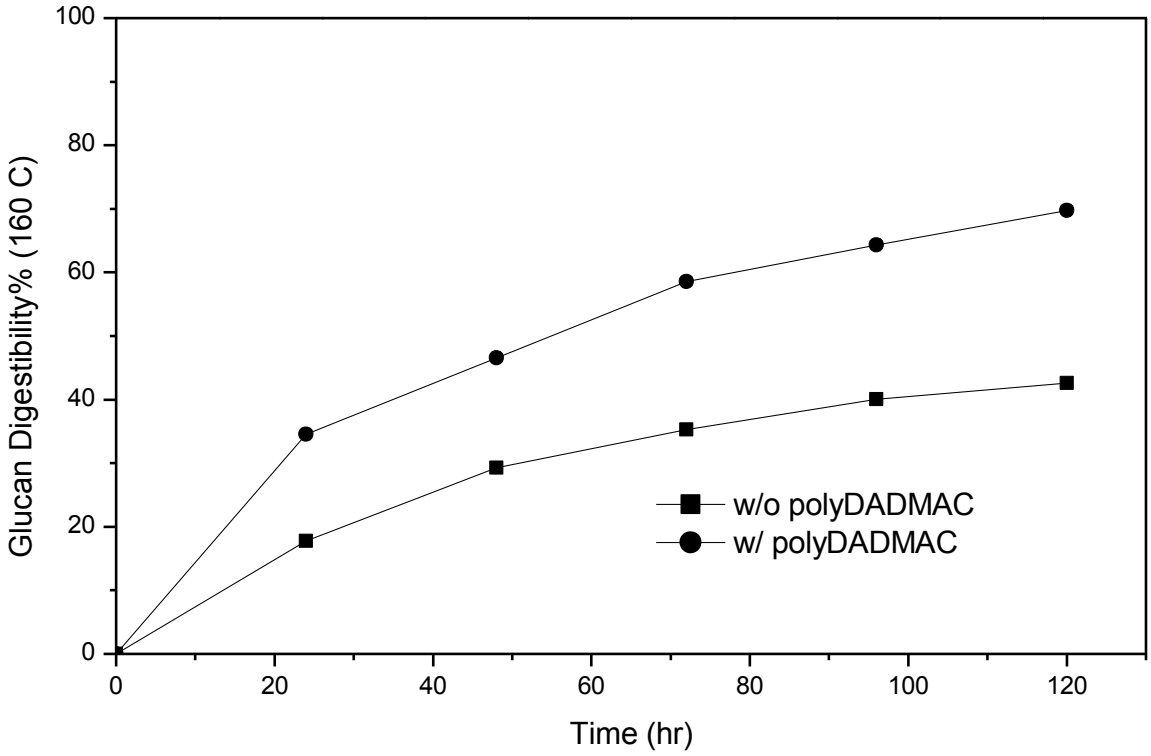


Figure III-6 Enzymatic hydrolysis of regenerated Avicel at 160 °C for 1 hour

Fig. 6 presented the time-course enzymatic digestibility of hot-water treated Avicel with and without polyDADMAC addition. A glucan digestibility of 45% over 120 hours was achieved for water-treated Avicel, whereas it only took about 48 hours for polyDADMAC-treated Avicel to reach this level of conversion. With polyDADMAC addition, the 120-hr glucan digestibility increased by 31%, from 45% for the control to 76%. As a result of polyDADMAC addition, not only the overall digestibility but the saccharification rate was dramatically increased. It has been documented that the kinetics of enzymatic hydrolysis was closely correlated to the accessible surface area (Arantes & Saddler, 2011; Kim et al., 2015; Zhang & Lynd, 2004; Zhu et al., 2010b). The surface

area and binding sites of pretreated solids substantially increased as a result of fiber disruption.

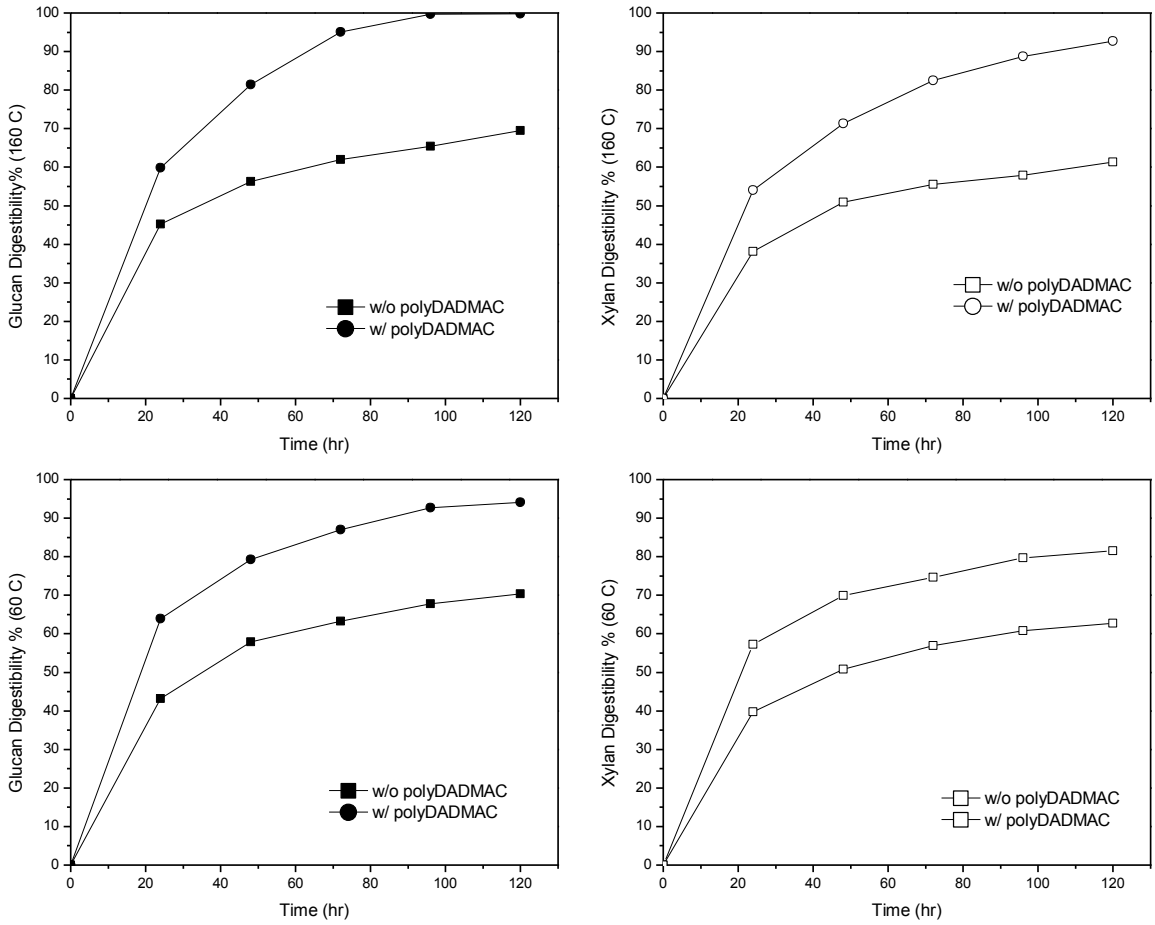


Figure III-7 Enzymatic hydrolysis of paper mill sludge regenerated at160 °C for 1 hour

PolyDADMAC exhibited the most distinctive effect on improvement of enzymatic hydrolysis of regenerated paper mill sludge. Fig. 7 presented the saccharification yield as a function of time in enzymatic hydrolysis of 2% paper mill sludge regenerated at 160 °C for 1 hour. As shown in Fig. 7, over 120 hours, in enzymatic hydrolysis of water-treated paper mill sludge, approximate 70% cellulose and 61% hemicellulose were converted into monomer sugars, whereas almost all the carbohydrates were saccharified into fermentable sugars for polyDADMAC-regenerated

sludge. Due to it has already undergone through severely mechanical refining in the pulping process, paper mill sludge contains large portion of short fibers with high surface area, facilitating the interaction between polyDADMAC and sludge fibers. Therefore, the effect of polyDADMAC on paper mill sludge was more significant than that on solka floc.

The results from regenerated lignin-free biomass indicate that polyDADMAC could directly interact with cellulose without the presence of alkali. And more importantly, the interaction between polyDADMAC and cellulose results to improvement of enzymatic hydrolysis over a wide range of feedstock, from lignin-free biomass to lignocellulose.

III.3.6 Enzymatic hydrolysis of two-step pretreated corn stover

A two-step pretreatment was then performed on corn stover, in which corn stover was first pretreated by soaking in aqueous ammonia at 160 °C for 1 hour. Then the pretreated solids were divided in two groups for the second-step treatment. One group was impregnated with polyDADMAC solution; the other one was running as control by impregnating with water. Both of them were treated for 1 more hour at 160 °C. Same amounts of chemicals were used in two-step pretreatment as that in polyDADMAC-SAA pretreatment. Since there was no significant weight loss after second step pretreatment, it was assumed the composition did not change in second-step pretreatment.

Pretreated solids were then subjected to enzymatic hydrolysis at 2% solid loading and the results were shown in Fig. 8. In this case, glucan and xylan digestibilities were increased by 21.3% and 25.4%, respectively. In terms of enhancement of enzymatic hydrolysis, the effects of polyDADMAC on two-step pretreatment were more remarkable as compared to polyDADMAC-SAA pretreatment in which, as shown in Fig. 1A, glucan

and xylan digestibilities were only increased by 18.4% and 21.8%. It should be noted that before impregnating SAA pretreated solids with polyDADMAC solution, about 80% of lignin has already been removed after first step pretreatment. Therefore, the existence of lignin is more likely to be an impediment for polyDADMAC being used as pretreatment additive due to complex formation between polyDADMAC and lignin, preventing the interaction between polyDADMAC and cellulose. The enhancement of enzymatic hydrolysis of pretreated corn stover was associated with the direct interaction between polyDADMAC and cellulosic fibers.

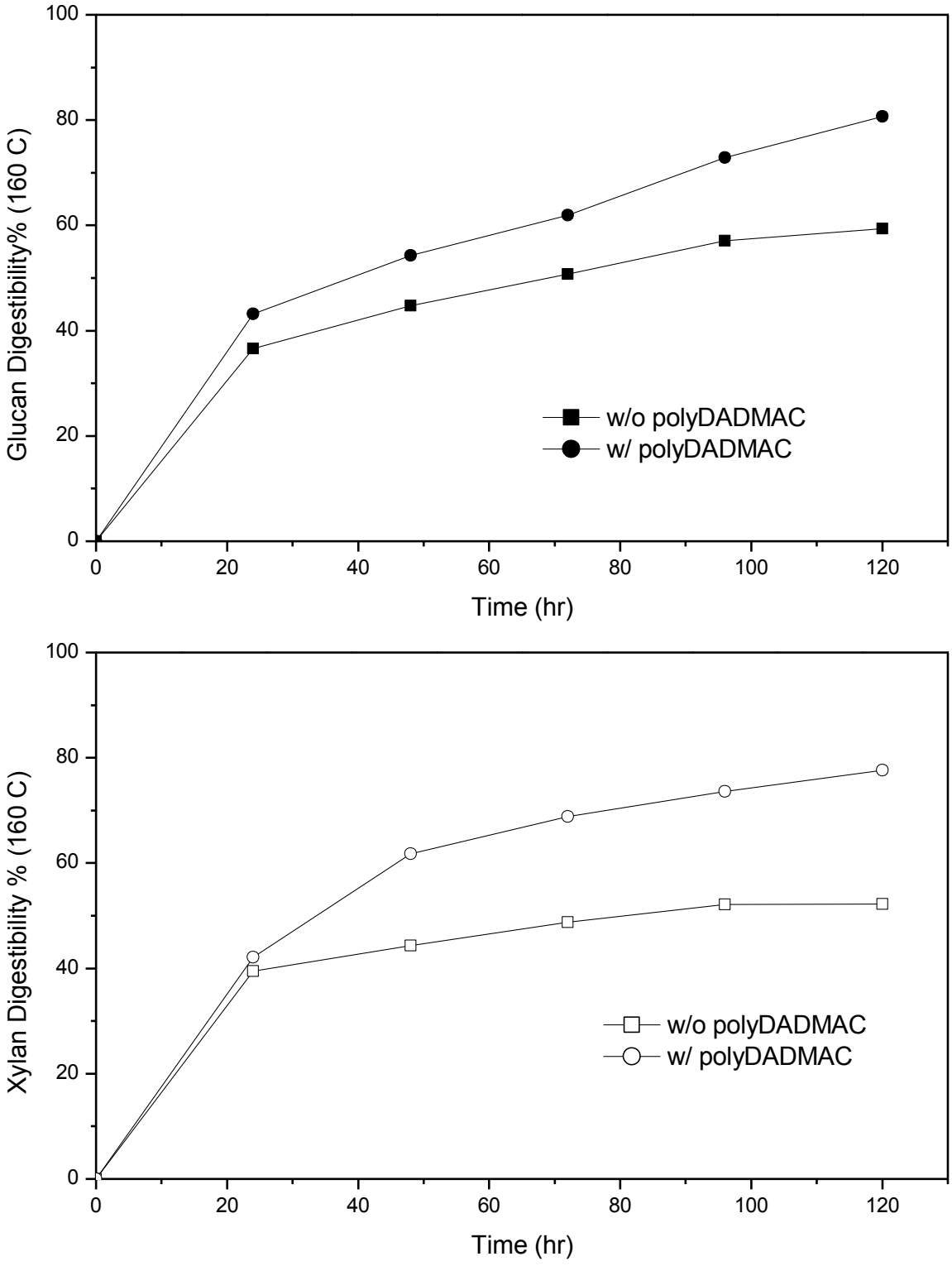


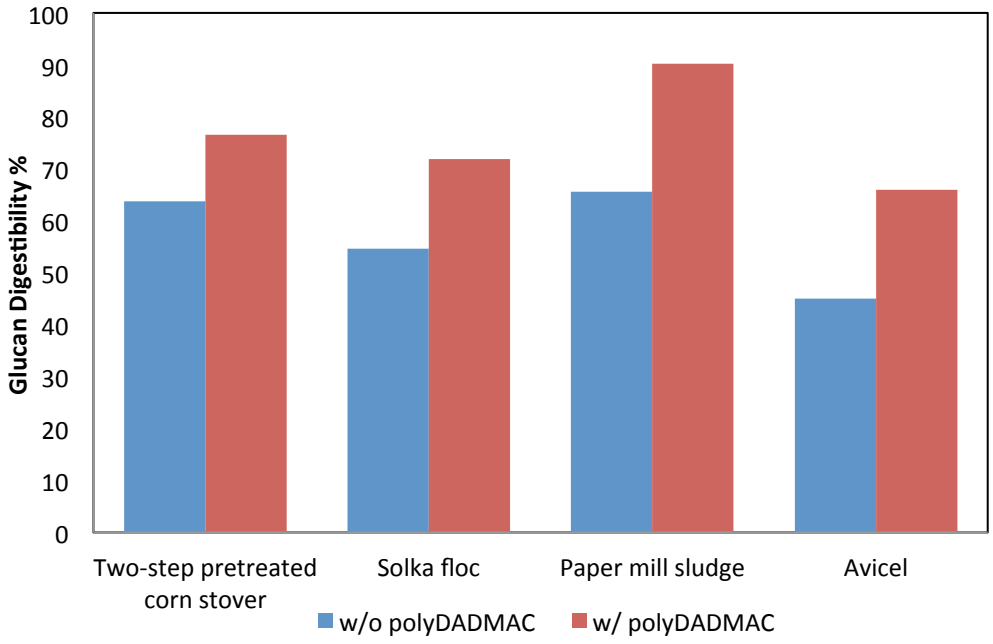
Figure III-8 Enzymatic hydrolysis of two-step pretreated corn stover with and without addition of PolyDADMAC

III.3.7 Discussion

Supplementary results from enzymatic hydrolysis of pretreated solids at 5% solid loadings, applying an enzyme dosage of 7.5 FPU/g glucan, are summarized in Fig. 10 and 11, corresponding to the pretreatment at 160 °C for 1 hour and 60 °C for 24 hours, respectively. In enzymatic hydrolysis of two-step pretreated corn stover at 160 °C, about 12.9% and 15% increases of glucan and xylan yields were achieved by impregnating SAA treated corn stover with polyDADMAC solution. Comparing sugar digestibilities from enzymatic hydrolysis at 2 and 5% solid loadings, the levels of increase of sugar yield from enzymatic hydrolysis at 5% solid loadings were relatively lower than that at 2% solid loading. For example, in enzymatic hydrolysis at 5% regenerated Avicel, the increase of glucan digestibility (20.9%) was 10% less than that at 2% solid loading (31%, as shown in Fig. 7). This was in accordance with the solid effects that decreased sugar yield was usually achieved in enzymatic hydrolysis at increasing solid loading even with a fixed enzyme-to-substrate ratio, likely due to the inhibition of enzyme adsorption or end-product (Kristensen et al., 2009; Wang et al., 2011). Therefore, the increase of sugar digestibility at 5% solid loading is still of great significance.

Moreover, in terms of enhancement of enzymatic hydrolysis, the effect of polyDADMAC was more significant on lignin-free biomass than lignocellulosic biomass. The level of increase of glucan digestibility in enzymatic hydrolysis of 5% solka floc was 17.3%, compared to the 12.75% increase for the two-step pretreated corn stover. This suggests that lignin might hinder the interaction of polyDADMAC to cellulosic fibers by recalling the results that higher level of increase of sugar digestibility was achieved when

applying polyDADMAC to the SAA pretreatment at higher temperature, which have a higher delignification. The other evidence would be that due to the complex formation between polyDADMAC and lignin, more residue lignin was left in the SAA pretreatment with polyDADMAC addition than that SAA pretreatment.



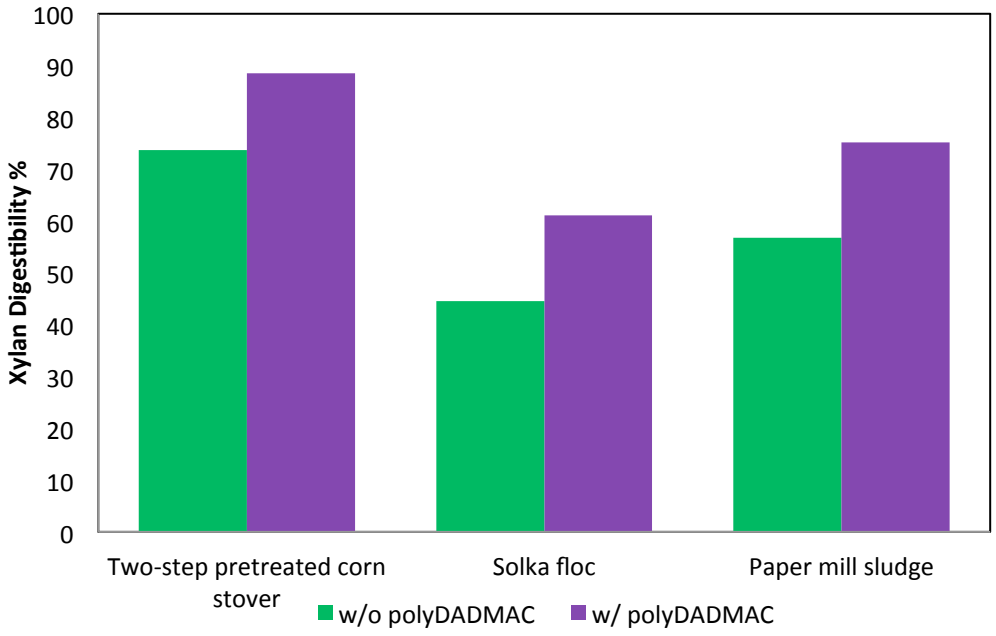


Figure III-9 120-hour yield from enzymatic hydrolysis at 5% solid loading (160 °C 1hour)

Finally, the effect of polyDADMAC on cellulosic biomass is independent on pretreatment temperature. It indicates that it could be used as an easily-handled and effective additive for post-pretreatment of lignocellulose or even additive in enzymatic hydrolysis, as reported by (Reye et al., 2011b) although a different cationic polymer.

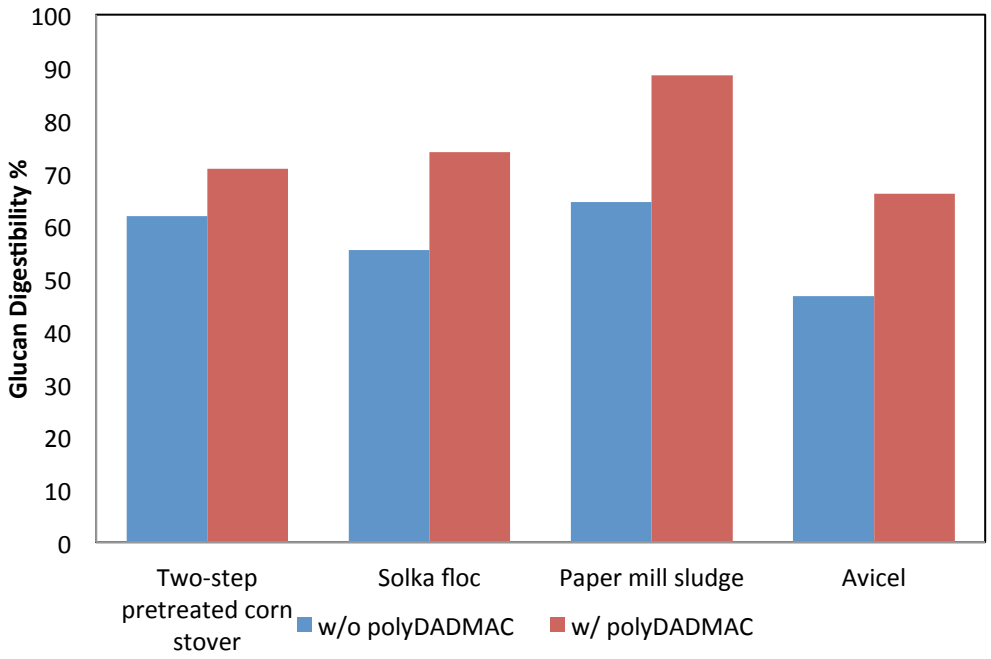
PolyDADMAC was found to have similar chemical structure and functional groups to many species of ionic liquid, such as, pyrrolidinium, piperidinium, etc. Ionic liquid pretreatment has been reported to deconstruct cellulose by reducing its degree of polymerization or crystallinity (Brandt et al., 2013; Singh et al., 2013). The anions of ionic liquid, preferably with strong polarity, such as chloride, acetate, etc., are able to break down the inter-and-intra molecular hydrogen bonds of cellulose.

Cellulosic fibers are formed by holding together millions of linearly chained polysaccharides of D-glucose through extensive hydrogen bonds between hydroxyl groups from one chain and oxygen atoms from the neighbor chain. The anions of ionic liquid with strong polarity readily attack and break down the hydrogen bonds between different chains (Zhang et al., 2005). Of course, the deconstruction of cellulose depends on the type of ionic liquid, biomass and pretreatment conditions as well. PolyDADMAC links together large quantities of strong polar chloride anions and DADMAC cations, possessing remarkable properties of ionic liquid especially with strong capability to partially deconstruct the cellulose even used in minute dosage as shown in this study.

III.4 Conclusion

In considering the minute amounts of polyDADMAC applied in this study, it is an economically effective chemical for improving the efficiency of enzymatic hydrolysis or

reducing the enzyme dosage for a specific conversion. Although it is effective to be used jointly as alkaline pretreatment additive, it is more effective to be applied individually as post-pretreatment reagent due to the hindrance of lignin. The permeation of polyDADMAC into cellulosic fibers allows the disruption of inter-and-intra hydrogen bonds between cellulose, enlarging its surface area, which hence facilitates the enzyme access to fibers.



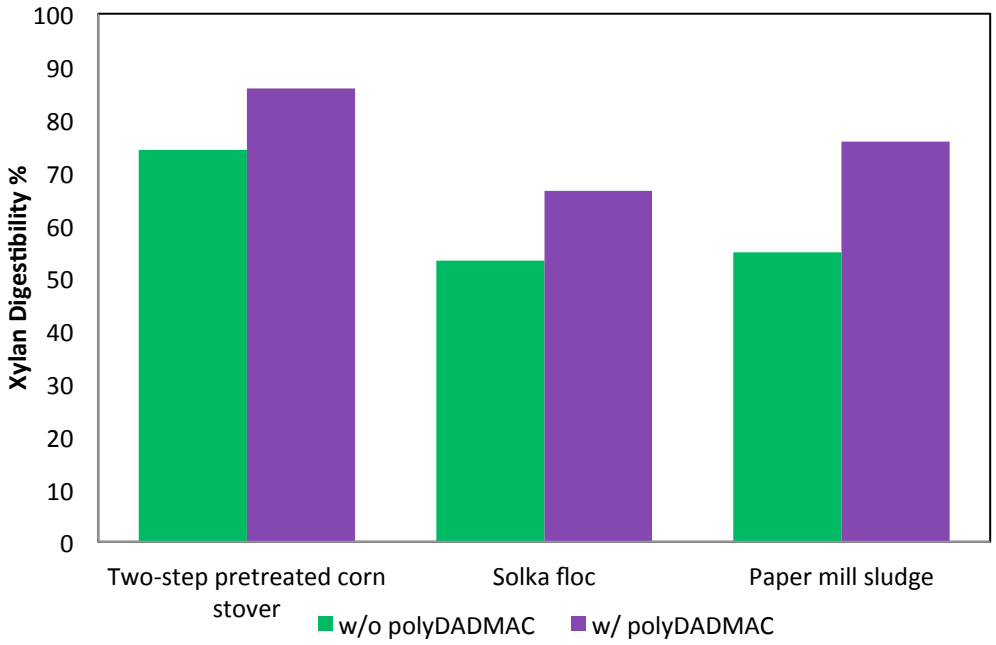


Figure III-10 120-hour yield from enzymatic hydrolysis at 5% solid loading (60 °C 24 hours)

Acknowledgements

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IV. Effect of Tween 80 on separate hydrolysis and fermentation and simultaneous saccharification and fermentation of alkali-pretreated switchgrass into butanol

Abstract

In this study, switchgrass was investigated as a feedstock for production of acetone, butanol and ethanol by ABE fermentation. For this purpose, switchgrass was first subjected to alkaline pretreatment, which is known to break down its recalcitrance and to increase enzyme accessibility. Both simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) were applied to alkali-pretreated switchgrass with Clostridium acetobutylicum (ATCC-824). In order to assess the overall bioconversion efficiency with switchgrass as feedstock, control tests with Avicel and pure sugars as substrate were also carried out. The results from SSF of Avicel indicated that enzyme loading played a key role in the bioconversion yields. SSF of alkali-pretreated switchgrass could be as efficient as SSF of Avicel and addition of Tween 80 was found to enhance the bioconversion yield quite significantly. However, attempts at SSF were strongly limited by solid loading with 6 wt. % as maximum level. With an enzyme loading of 15 FPU/g glucan, SSF of 6% well-pretreated switchgrass produced 12.2 g/L of total solvents, the level of which was far from the inhibition threshold for this specific culture. Therefore, SHF was further applied and achieved comparable levels of solvents as in ABE fermentation with glucose as substrate. We also proposed that utilizing butylene as extractant to separate fermentation products from broth. The details of pretreatment conditions, fermentation profiles as well as the prospect of butylene as extractant are discussed. Keywords: Butanol, switchgrass, fermentation, pretreatment

IV.1 Introduction

Butanol is an advanced biofuel, which could be used directly as drop-in biofuel or upgraded into high-molecular-weight jet fuel or biodiesel (Anbarasan et al., 2012; Durre, 2008; Tashiro et al., 2013). Additionally, owing to its versatile physical properties, butanol has been widely used as superb solvent, extractant and platform for chemical synthesis (Durre, 2008). In 2014, n-butanol has a global market of 3 million MT ($5 billion) and the market is expected to expand at 120,000 MT per year (Green, 2011). In addition to the traditional pathway from petrochemical synthesis for butanol production, microbial fermentation renders an alternative approach, i.e., bio-based butanol. One of the key challenges for commercial development of this bioprocess is to resort to renewable and abundant feedstock, instead of food-based sources.

Switchgrass is a perennial herbaceous biomass with high carbohydrate content (50-65%). Its adaptability to grow under infertile conditions makes switchgrass abundantly available across North American (Kim et al., 2011; Tao et al., 2011). It has been identified by the National Renewable Energy Laboratory (NREL) as one of the dedicated energy crops suitable as raw materials for bioenergy production (Kim et al., 2011; Shi et al., 2011; Tao et al., 2011). As a superior biofuel over ethanol, butanol has attractive features (higher energy density, lower anti-knock index, etc.) and thus, can be blended into gasoline or diesel at any ratio (Harvey & Meylemans, 2011).

Previous studies on bioconversion of switchgrass into butanol indicated that detoxification of hydrolysate was necessarily required, which would otherwise led to severe inhibition on both the culture growth and solvent production (Liu et al., 2015; Qureshi et al., 2010b). The formation of lignocellulose degradation products during

pretreatment contributes to the majority of the toxic compounds, typically including aliphatic acids (formic acid and acetic acid), sugar-degraded furan derivatives (furfural, HMF) and lignin-degraded phenolic compounds (ferulic acid, ρ-coumaric acid, etc.) (Alves Silva et al., 2013). The soluble lignin content and lignin-degraded phenolic compounds have been identified as the most toxic inhibitors to the solventogenic *Clostridia* species (Ezeji & Blaschek, 2008b; Lee et al., 2015; Liu et al., 2015; Wang & Chen, 2011). (Liu et al., 2015) reported fermentation of un-detoxified switchgrass hydrolysate resulted to weak performance in that only 1 g/L of butanol was produced. However, upon detoxification of the hydrolysate with activated carbon, the soluble lignin content was greatly reduced and the final butanol titer was increased to 11g/L (Liu et al., 2015). Similarly, (Qureshi et al., 2010b) reported that a two-fold dilution of switchgrass hydrolysate with the addition of DI water significantly improved the final ABE titer from 1.48 g/L for the undetoxified hydrolyzate to 14.61 g/L.

Overliming (Qureshi et al., 2010b; Sun & Liu, 2012) and adsorption with either activated carbon or ion-exchange resin (Kudahettige-Nilsson et al., 2015; Liu et al., 2015) are traditional detoxification methods. Major concerns associated with these methods are that they would not only bring up additional cost to the overall bioprocess but also lead to the loss of considerable portion of fermentable sugars (Alves Silva et al., 2013; Lee et al., 2015). Advanced methods with an emphasis on *in-situ* detoxification effect have been recently developed with the use of reducing agent (Alriksson et al., 2011; Alves Silva et al., 2013) or non-ionic surfactant (Dhamole et al., 2013; Lee et al., 2015). The surfactant-based detoxification was previously reported to improve the fermentability of corn stover hydrolyzate for ethanol production (Dhamole et al., 2013) and rice straw hydrolyzate for

butyric acid production (Lee et al., 2015). Addition of minute amount of Tween 80, a non-ionic surfactant, in rice straw hydrolysate significantly increased the butyric acid production from 0.1 g/L for the undetoxified hydrolysate to 8.7 g/L (Lee et al., 2015).

In the present study, alkaline pretreatment is chosen as the pretreatment method among various leading technologies in that it disrupts the recalcitrance of switchgrass by partial delignification and preserves most of the structural carbohydrates (Kim et al., 2011; Tao et al., 2011). In terms of the bioconversion strategies for biobutanol production, both simultaneous saccharification and fermentation (SSF) (Guan et al., 2016; Qureshi et al., 2008b; Shah et al., 1991a) and separate hydrolysis and fermentation (SHF) (Gao et al., 2014; Liu et al., 2015; Qureshi et al., 2010b) have been proven as feasible approaches. Although the two strategies have different process configurations, both of them were previously reported to produce ABE solvent at the level comparable to that of pure sugar control (Guan et al., 2016; Sun & Liu, 2012). The bioconversion efficiency of SSF and SHF for biobutanol production was, yet, rarely compared. The objective of this study seeks to assess the effectiveness of the surfactant-based detoxification in fermentative bio-butanol production from alkali-pretreated switchgrass. The bioconversion features of SSF and SHF are also compared in conjunction with the overall bioconversion yield and productivity.

IV.2 Materials and method

IV.2.1. Feedstock, Enzyme and Microorganism

Switchgrass was provided by Ceres Inc. (Thousand Oaks, CA). Cellulase Cellic® C-Tec 2 (Batch No. VCNI0001) was gifted from Novozymes, North America (Franklinton, NC). The protein content and specific activity for C-Tec2 are 255 mg

protein/ml and 119 FPU/mL. Clostridium *acetobutylicum* ATCC-824™ (Lot NO. 58727357) was purchased from American Type Culture Collection (ATCC). Sodium hydroxide, Tween 80, model inhibitors (furfural, HMF, ferulic acid, *p*-coumaric, vanillic aid, 4-hydroxybenzoic acid) are purchased from Sigma-Aldrich (St. Louis, MO.).

IV.2.2. Feedstock pretreatment

Switchgrass was grounded and the sample with particle size between 20-60 meshes was collected for subsequent purpose. The sample was pretreated by soaking in sodium hydroxide solution in stainless steel reactor (1.375 "ID×6"L) under the following conditions: NaOH concentration (2 wt.%), liquid-to-solid ratio of 9:1, 60 °C, 24 hrs. After pretreatment, the solids were washed with water till the filtrate pH reached above 6.0. The solids were then partially dewatered until moisture content reduced to below 50% before further processing. The solid composition of the sample before and after pretreatment was determined according to the NREL standard analytical procedure (NREL/TP-510-42618).

IV.2.3. Enzymatic hydrolysis of alkali-pretreated switchgrass

Enzymatic hydrolysis (2%(w/v) solid loading and 10 FPU/g-glucan) was performed to estimate the digestibility of pretreated switchgrass. The experiment was carried out in batch mode with 50 mL working volume over 72 h periods. Sodium citrate (0.05 M, pH 4.8) was used as buffer maintaining optimal pH environment for robust enzyme activity during enzymatic hydrolysis. And sodium azide (0.01% (w/v)) was added as antibiotics to prevent microbial contamination.

IV.2.4. Culture Maintenance and Inoculum Preparation

The stocks of C. *acetobutylicum* ATCC 824 was maintained as spores at -20 °C in Difco™ Thioglycollate medium (Sigma-Aldrich) supplemented with 20 %(v/v) of

glycerol. The spores were revived under anaerobic conditions (N_2) by inoculating one loop of the spore suspension in the Difco^{TM} Elliker Broth (Sigma-Aldrich) and incubated for 24 h at 36 °C. The second generation of revived culture (OD_{600}=1.3) was used as the seed culture for fermentation.

IV.2.5. ABE fermentation with pure sugar as feed and toxicity test

ABE fermentation with glucose/xylose as substrate was carried out in 125 mL serum bottle with a working volume of 50mL. P2 medium was chosen as the fermentation medium for inorganic minerals supply. The formula of P2 medium is as followings (g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; NaCl, 0.01; ammonium acetate, 2.2; yeast extract, 1.0. After sugar addition, the medium was filter-sterilized through a 0.45-μm-syringe filter (VWR) and then decanted into a sterile serum bottle. Upon seed-culture inoculation (5% (v/v)), the bottle was flushed with nitrogen gas for 5 min and crimp-sealed with rubber stopper. To maintain the anaerobic condition, the bottle was kept sealed throughout the fermentation course and aliquots of samples were collected with 1.0 mL syringe at 12-h interval. Fermentation was performed at 36 °C and 150 rpm.

The toxicity test of model inhibitor was performed in the glucose-based medium. A number of typical toxic compounds were tested, including furfural, hydroxymethylfurfural (HMF), *p*-coumaric, ferulic acid, vanillic acid and 4-hydroxybenzoic acid. Briefly, the individual inhibitor was added into the fermentation medium at various concentrations (0.25-2 g/L). The cell growth (OD600) was measured after 36 h cultivation. To investigate the detoxification effect of Tween80, the test with and without addition of Tween80 (0.2 wt.%) were run in parallel.

IV.2.6. Simultaneous saccharification and fermentation (SSF) of alkali-pretreated switchgrass

SSF test was performed accordingly by replacing the substrate of sugar with alkali-pretreated switchgrass. Upon loaded with pretreated solids and P2 medium, the serum bottle was autoclaved at 121 °C for 15 min. After sterilization, cellulase enzyme (CTec-2) was added at an enzyme loading of 15 FPU/g-solid and the actively growing seed-culture was inoculated at 5% (w/v). Alkali-pretreated switchgrass was hydrolyzed into glucose and xylose during SSF, serving as the carbon source for fermentation. SSF of alkali-pretreated solid was tested with various solid loadings (4, 5, 6 and 7% (w/v)). Due to the insoluble properties of lignocellulose solid, the upper-limit solid loading was constrained to 7%, resulting to limited level of free water available in the broth. The overall bioconversion yield of SSF was calculated on the weight basis as the amount of ABE formation divided by the total sugars in the feed of pretreated solids.

IV.2.7. Separate hydrolysis and fermentation (SHF) of alkali-pretreated switchgrass

SHF was performed with the solid loading of 5 and 7% following two separate steps, i.e., enzymatic hydrolysis and fermentation. In contrast to the method described for enzymatic hydrolysis in section 2.3, P2 medium (pH 6.7) was used as the buffer and no antibiotic was added for the hydrolyzate production. The effect of Tween 80 on enzymatic hydrolysis was assessed by running parallel tests with and without Tween 80 addition. After 72 h enzymatic hydrolysis, the residual solid was separated from the liquid portion via filtration and the liquor was collected as hydrolyzate for subsequent fermentation. The hydrolyzate was then sterilized by passing through a 0.45-μm filter and fermentation was performed in a similar manner as that of pure sugar. The solvent yield

of hydrolyzate fermentation was calculated as the amount of ABE formation divided by the total sugars in the hydrolyzate and the overall bioconversion yield of SHF was calculated as the amount of ABE formation divided by the total sugars in the feed of pretreated solids.

IV.2.8. Analysis

The optical density of *C. acetobutylicum* ATCC 824 in the inoculum was measured by a UV-vis spectrophotometer () at wavelength of 600 nm. The enzymatic digestibility of alkali-pretreated switchgrass was calculated from the released glucose content on 120 h basis, as a percentage of the theoretical sugars available in the substrates. Released glucose/xylose concentration during enzymatic hydrolysis was analyzed with HPLC equiped with Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA). The fermentation product (i.e., acetone, butanol, ethanol, acetic acid and butyric acid) in this study was analyzed by HPLC equipped with Refractive Index detector (Shodex, Japan) and Aminex HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA).

IV.3 Results and Discussion

IV.3.1 Chemical composition and enzymatic digestibility

The chemical compositions of switchgrass before and after pretreatment were analyzed and the results are presented in Table IV.1. Untreated switchgrass solid was analyzed to contain 59.8% of structural carbohydrates with glucan (36.9%) and xylan (19.2%) as the leading components, 20.0% of lignin and 13.04% of unquantified extractives. Upon subjecting to the NaOH pretreatment, the lignin content was reduced to

10.4%, corresponding to a 69.4% of delignification. As a result of significant delignification, the glucan/xylan content increased to 58.1/21.8%. The pretreatment partially solubilize the structural carbohydrates, leading to a 1.8/6.4% loss in glucan/xylan content.

Table IV-1 Chemical composition and enzymatic digestibility of switchgrass

Component (%)	Untreated	Pretreated[a]
Glucan	35.9±0.85	58.1±0.41
Xylan	19.2±0.61	21.8±0.23
Galactan	1.4±0.09	0.6±0.03
Arabinan	3.3±0.14	2.2±0.18
K-lignin	19.96±0.34	10.4±0.15
Ash	4.10	6.20
Acetyl	3.10	0.14
Mass Closure	86.96	99.44
Solid Recovery%	-	58.70
Delignification%	-	69.40
Glucan Digestibility[b]%	11.8±0.84	75.3±1.15
Xylan Digestibility[b]%	9.4±0.72	61.2±0.84

a. Pretreatment condition: NaOH (2 wt.%), solid/liquid ratio: 1/10, 60 ºC for 24 h

b. Enzymatic hydrolysis was performed at 2% solid loading (w/v) under an enzyme loading of 10 FPU/g-glucan. And the enzymatic digestibility was based on 72 h.

In the enzymatic digestibility test (Table IV.1), enzymatic hydrolysis of untreated solids achieved only 11.8/9.4% of glucan/xylan digestibility over 72 h. However, for the alkali-pretreated solid, the 72-h glucan/xylan digestibility substantially increased to 75.3/61.2%. The fact that alkaline pretreatment was able to preserve the majority of glucan and xylan while to substantially enhance the enzymatic digestibility suggested an appropriate pretreatment method for biobutanol production since both the hexose and

pentose could be utilized as carbon source by the solventogenic *Clostridia* species (Guan et al., 2016).

IV.3.2 The inhibitory effect of pretreated solids on ABE fermentation

Initial attempts at SSF of alkali-pretreated switchgrass (5% solid loading) resulted to an inferior bioconversion performance in that only small amounts of ABE (3g/L) but significant level of acids (10.1 g/L) were produced **(Fig. IV.1)**. The fact that SSF progressed in presence of significant level of glucose, from 10 g/L at 24-hr point to 12 g/L at 120-hr point, indicated that enzyme hydrolysis was not likely to be a causing factor for the weak performance.

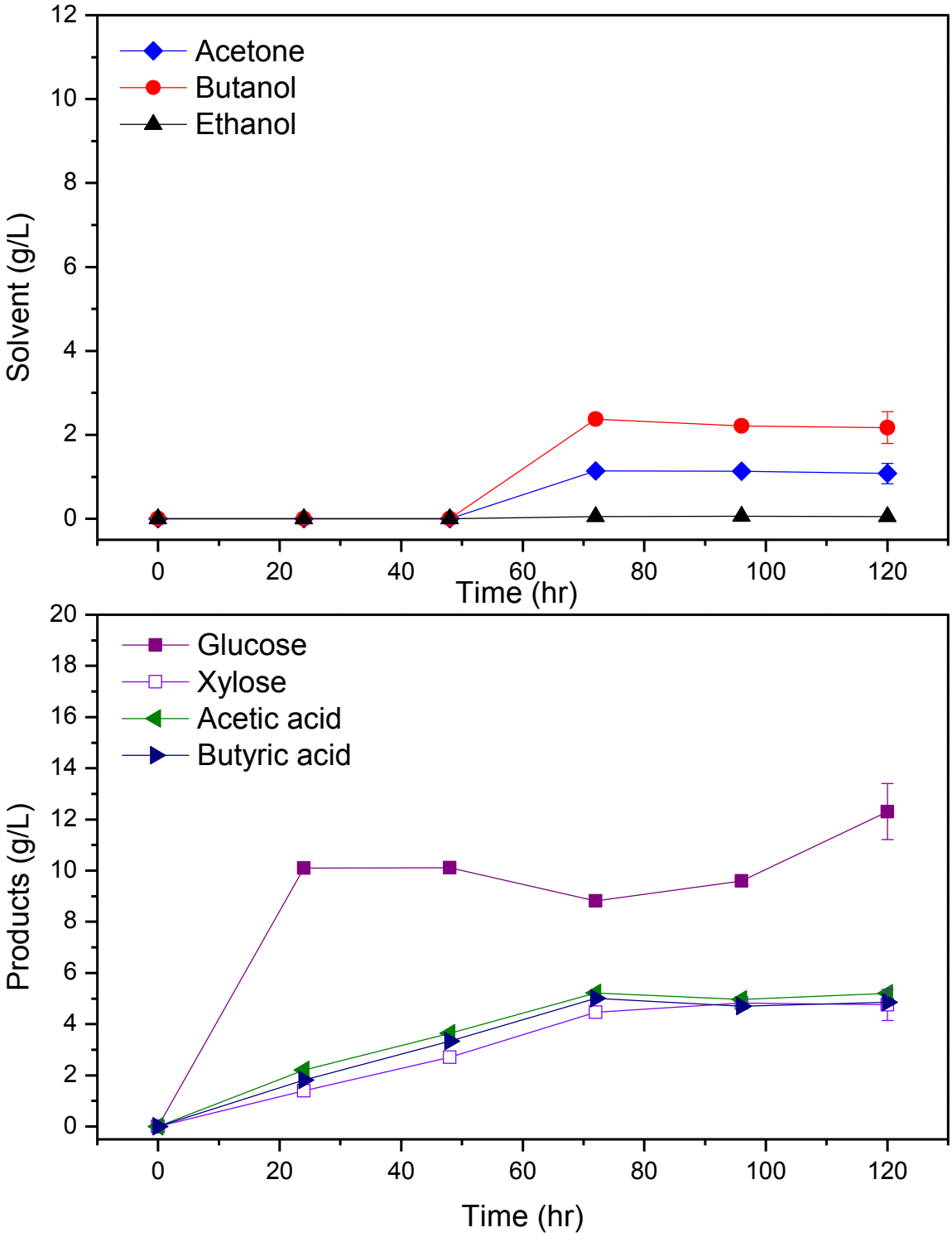

Figure IV-1 The inhibitory effects of alkali-pretreated switchgrass on SSF

The inhibitory effect of pretreated solids was further investigated in ABE fermentation with pure sugar as feed **(Table 2)**. In the test with glucose as substrate (50 g/L), a total of 15.4 g/L of ABE solvent with 9.1 g/L of butanol was produced from 49.2 g/L of glucose over 96-h period, giving a fermentation yield of 0.31 (g-solvent/g-glucose) and productivity of 0.16 g/L/h. However, when the fermentation was performed in the presence of 5% pretreated solids, the solvent production was decreased to a great extent such that only 8.2 g/L of solvent was produced, leaving 15.7 g/L of glucose unutilized at the end of fermentation. In the case of xylose-based medium, addition of alkali-pretreated solid inhibited the fermentation in a similar manner as the acids (9.4 g/L of acetic acid and 6.1 g/L of butyric acid) became the primary fermentation products and the fermentation terminated with substantial xylose (19.1 g/L) available. At the end of fermentation, a total of 4.5 g/L of ABE solvent was produced, which was substantially lower than that of xylose control (12.6 g/L).

As the sole inhibitory component in the pretreated solids, the residual lignin content appeared to be the source of inhibition on the microbial fermentation. The toxicity test of model inhibitors on the cell growth of *Clostridium acetobutylicum* ATCC824 demonstrated that a total of four lignin-degraded phenolic compounds among the tested compounds had potent inhibition on the cell growth (**Fig 2**). The inhibitory concentration (IC_{50}) resulting to a 50% inhibition on the cell growth was measured to be in the range of 0.5-1 g/L for *p*-coumaric, vanillic acid and 4-hydroxybenzoic acid and of 1-1.5 g/L for ferulic acid. The results for *p*-coumaric acid and ferulic acid are in consistent with previous report (Ezeji & Blaschek, 2008b). Vanillic acid and 4-hydroxybenzoic acid, as commonly lignin-derived phenolic compounds in the

lignocellulose pretreatment, were also found to be rather toxic to the solventogenic *Clostridia* although they might not lead to a complete inhibition.

Table IV-2 ABE fermentation with pure sugar as feed in presence of pretreated solids

	Glucose-based medium			Xylose-based medium		
Tween 80[a]	w/o	w/o	w/	w/o	w/o	w/
Solid Loading	-	5%	5%	-	5%	5%
Acetone (g/L)	5.40	2.69	4.40	3.84	1.90	3.82
Butanol (g/L)	9.10	5.30	8.10	7.59	2.50	6.80
ABE (g/L)	15.40	8.20	13.30	12.60	4.50	11.29
Yield (g/g)[b]	0.31	0.16	0.27	0.25	0.09	0.23
Productivity ($g/L^{-1}h^{-1}$)	0.16	0.09	0.14	0.13	0.05	0.12
Acetic acid (g/L)	1.32	7.70	2.93	1.92	9.42	1.98
Butyric acid (g/L)	0.95	4.80	1.68	1.14	6.10	1.31
Rsugar[c]	0.84	15.70	2.80	4.60	19.10	5.39

a. "w/o" indicates the fermentation was performed without Tween 80 addition; "w/" indicates the fermentation was performed with Tween 80 addition at a dosage of 1% (v/v).

b. The solvent yield was calculated based on the sugar initially present in the fermentation medium.

c. "Rsugar" indicates the residual sugar present in the medium at the end of fermentation

Particularly notable is that all of these inhibitors are hydrophobic compounds with 4-hydroxybenzoic acid having the highest water-solubility (5 g/L) at room temperature, followed by vanillic acid (1.5 g/L), ferulic acid (0.78 g/L) and *p*-coumaric (sparingly soluble). A number of studies have shown direct contact of these compounds with the *Clostridia* cell would damage the functionality of cell membrane (hydrophobicity, redox balance, etc.) and thus, disrupt the microbial metabolism (Ezeji & Blaschek, 2008b; Lee et al., 2015; Liu et al., 2015; Ujor et al., 2014). Consequently, acid crash or premature cessation of the fermentation typically occurred (Liu et al., 2015; Ujor et al., 2014), which is in good agreement with the results in the present study.

It was believed that the hydrophobic sites of surface-active agent possess a higher affinity to these compounds and thus, weakens its interaction to cell membrane, serving as a detoxification agent (Dhamole et al., 2013; Lee et al., 2015). The toxicity test (**Fig. 2**) indicated that as a result of surfactant addition, the inhibition effect of these compounds (other than 4-hydroxybenzoic acid) was greatly reduced and the cell density in the fermentation broth was substantially increased. One of possible reason behind the ineffectiveness of Tween 80 on the 4-hydroxybenzoic acid-induced inhibition might be due to its relatively higher water solubility, resulting to a different inhibition mechanism. Accordingly, as indicated in **Table 2**, addition of Tween 80 in ABE fermentation in presence of pretreated solids greatly enhanced the solvent production to the level near that of control test in either glucose- or xylose-based medium.

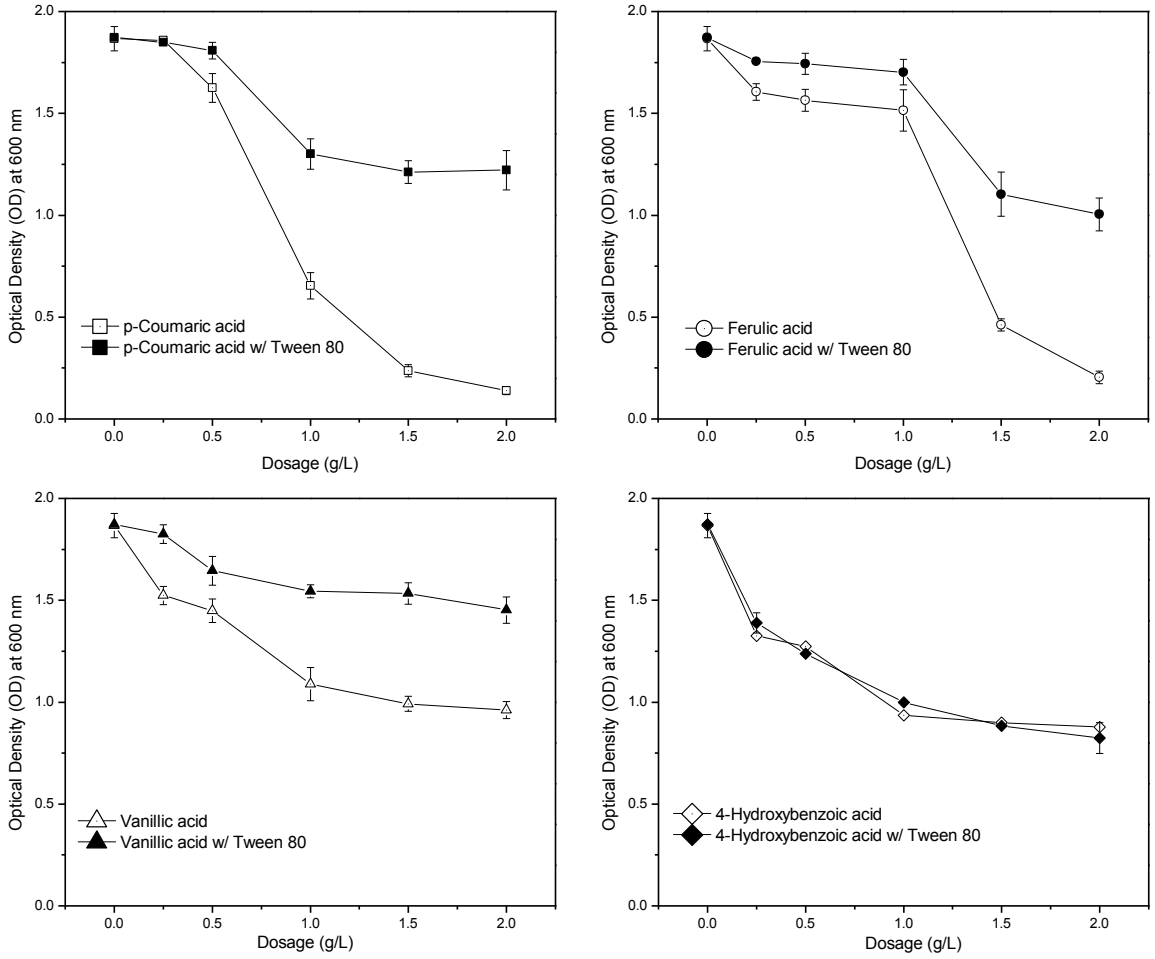

Figure IV-2 Effects of model inhibitors on the cell growth of C. acetobutylicum ATCC 824 and the detoxification effect of Twwen 80

IV.3.3 Simultaneous saccharification and fermentation (SSF) of alkali-pretreated switchgrass

With the addition of non-ionic surfactant Tween 80, SSF of alkali-pretreated switchgrass under the solid loading of 5% and an enzyme loading of 15 FPU/g-solid exhibited a typical biphasic pattern of ABE fermentation (**Fig. 3**). At the acidogenic phase, acetic acid and butyric acid were primarily produced, reaching to 3.7 g/L and 4.2 g/L at 36-h point. Glucose was continuously released from enzymatic hydrolysis and

accumulated to 10.4 g/L. In solventogenic phase, with rapid sugar consumption and acid re-assimilation, the ABE solvent was produced at significant rate. At the end of fermentation, a total of 12.3 g/L ABE solvent was produced, including 4.2 g/L of acetone, 7.8 g/L of butanol and 0.33 g/L of ethanol. The overall bioconversion yield and productivity reached to 0.28 (g-solvent/g-sugar) and 0.10 g $L^{-1}h^{-1}$.

It has been clarified in previous study that enzymatic hydrolysis is a rate-limiting step in SSF for biobutanol production and sufficient enzymatic hydrolysis is required to ensure proper saccharification rate and bioconversion yield (Guan et al., 2016). Therefore, a fairly high enzyme loading (15 FPU/g-solid) was applied in the present SSF study. To investigate the effects of solid loading on the solvent production, SSF was performed applying various solid loadings. SSF of alkali-pretreated switchgrass under the solid loadings of 4, 5, 6 and 7% produced a total of 10.0, 12.3, 12.4 and 9.3 g/L of solvent, corresponding to an overall bioconversion yield of 0.28, 0.28, 0.23 and 0.15 (g-solvent/g-sugar) (**Fig. 4**). The solid loading at 5% (w/v) appeared to be the optimal level to be applied in SSF achieving highest solvent production while giving the bioconversion yield near that of sugar control. Beyond that solid loading, the bioconversion yield had sharply decreased. Particular notable is that the upper limit of solid loading SSF can be employed is restricted at 7% (w/v), at which point the fermentation broth exhibited extremely high viscosity due to limited level of free-water. The lack of fluidity at 7% solid loading is often documented as "solid effects", severely impeding enzymatic hydrolysis, which might account for the sharp decrease in solvent production (Jorgensen et al., 2007; Kristensen et al., 2007).

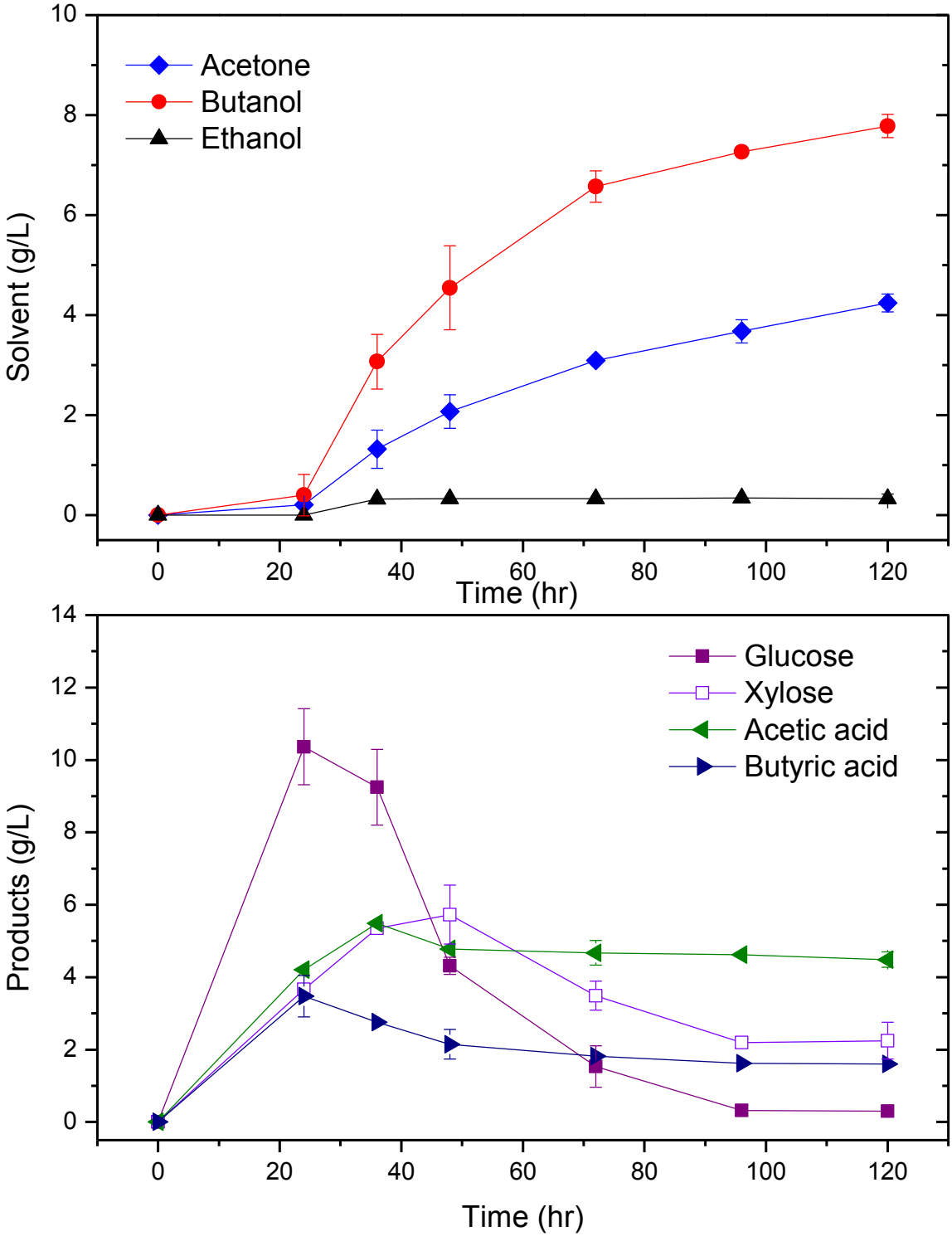

Figure IV-3 Simultaneous saccharification and fermentation (SSF) of alkali-pretreated switchgrass with the addition of Tween 80

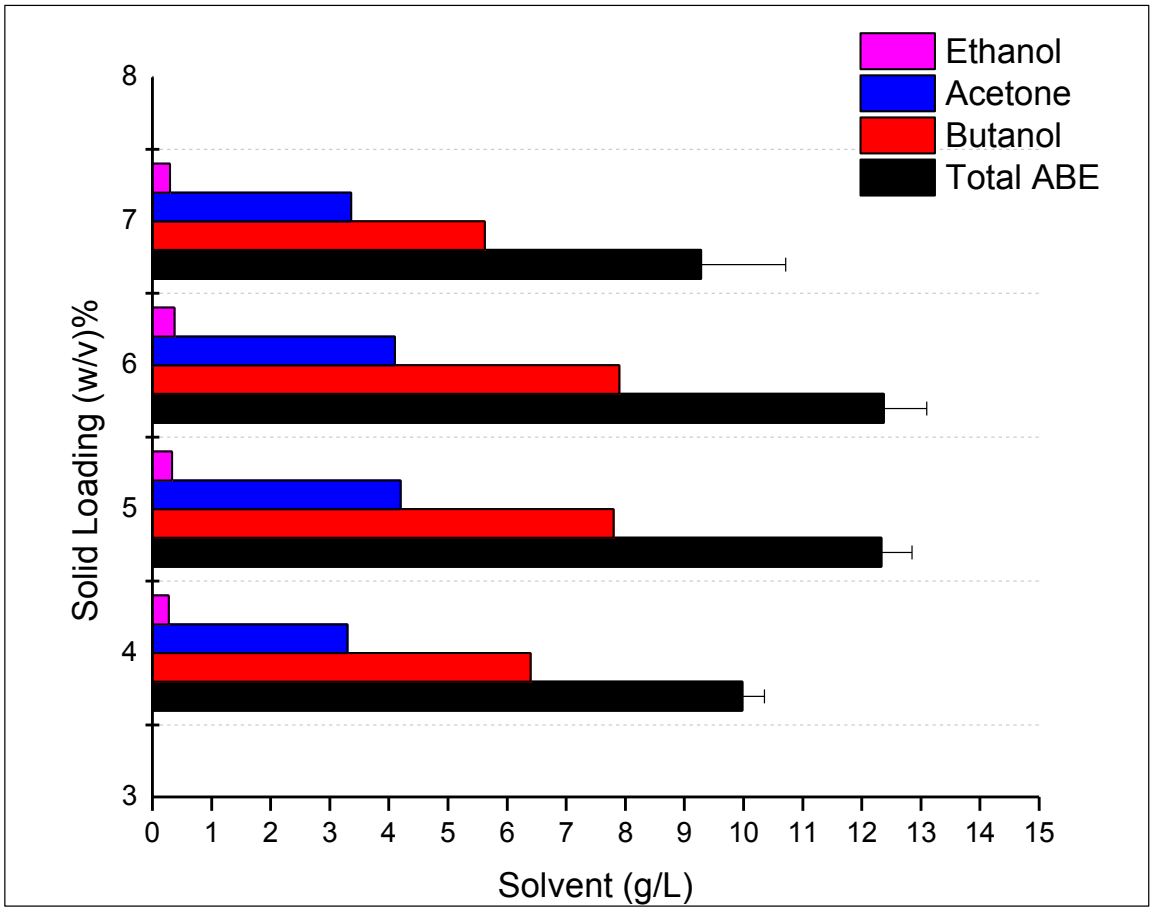

Figure IV-4 Simultaneous saccharification and fermentation (SSF) of alkali-pretreated switchgrass under various solid loadings

IV.3.4 Separate hydrolysis and fermentation of alkali-pretreated switchgrass

Although SSF offers several advantages by integrating enzymatic hydrolysis and fermentation into one step (Guan et al., 2016), the solid loading is proven to be a huge process constraint. As a counterpart bioconversion strategy, SHF was performed targeting higher solid loading for biobutanol production from alkali-pretreated switchgrass. The hydrolysate of alkali-pretreated switchgrass was achieved as described in section 2.7 (**Table 3**). After 72-h enzymatic hydrolysis with 5% solid loading, 22.2 g/L of glucose

and 7.5 g/L of xylose were released in the hydrolysate, corresponding to 68.7% of glucan digestibility and 60.2% of xylan digestibility. Addition of Tween 80 in enzymatic hydrolysis resulted in 11.5% increase in glucan digestibility and 16.3% increase in xylan digestibility. In this case, the total reducing sugar reached to 35.4 g/L, including 25.9 g/L of glucose and 9.5 g/L of xylose. The boosting effect of non-ionic surfactant on enzymatic hydrolysis is in consistent with previous reports (Borjesson et al., 2007; Kristensen et al., 2007). And the prevalent explanation for this effect is that the surfactant covers up the residual lignin surface via hydrophobic interaction and thus reduces the non-productive binding of enzyme on lignin (Borjesson et al., 2007; Kristensen et al., 2007). It is of great interest to know that surfactant serves not only as detoxification agent for biobutanol production but also as enzyme activity enhancers that increasing the saccharification yield.

Table IV-3 Effect of Tween 80 on enzymatic hydrolysis of alkali-pretreated switchgrass

Solid Loading	5%		7%	
Tween 80	w/o	w/	w/o	w/
Enzyme loading	15 FPU/g-glucan		15 FPU/g-glucan	
Glucan Digestibility%[a]	68.70	80.20	64.34	78.18
Xylan Digestibility%[a]	60.18	76.43	58.16	73.87
Glucose (g/L)	22.20	25.86	29.05	35.29
Xylose (g/L)	7.50	9.50	10.12	12.85

The hydrolysate from enzymatic hydrolysis of alkali-pretreated switchgrass (5% solid loading) with Tween 80 addition was further subjected to ABE fermentation. Time-course fermentation profile (**Fig. 5**) indicates the hydrolysate (glucose, 25.9 g/L; xylose, 9.5 g/L) could be directly fermented into ABE production without the need of extra detoxification. Within the acid production phase, the fermentation progressed with rapid

sugar consumption. Although both glucose and xylose are consumed by the culture for solvent production, glucose appeared to be the preferred choice for the *Clostridium acetobutylicum* ATCC 824 exhibiting a greater consumption rate. In the solventogenic phase, the sugar level declined to near zero toward the end (72 h). Fermentation of the hydrolysate produced a total of 9.9 g/L of solvent (butanol, 6.1 g/L; acetone, 3.4 g/L; and ethanol, 0.4 g/L), giving a bioconversion yield of 0.28 (g-solvent/g-sugar in the hydrolysate) and a productivity of 0.14 g/L/h. However, the overall bioconversion yield based on charged sugar in the pretreated solid was equivalent to 0.22 (g-solvent/g-sugar in the pretreated solids), which was less than that achieved in SSF (0.28 g-solvent/g-sugar). Additionally, although fermentation of hydrolysate took shorter time to complete (72 h in SHF V.S. 120 h in SSF), the overall productivity of SHF (0.07 g/L/h) in consideration of the 72-h enzymatic hydrolysis was also lower than that of SSF (0.10 g/L/h).

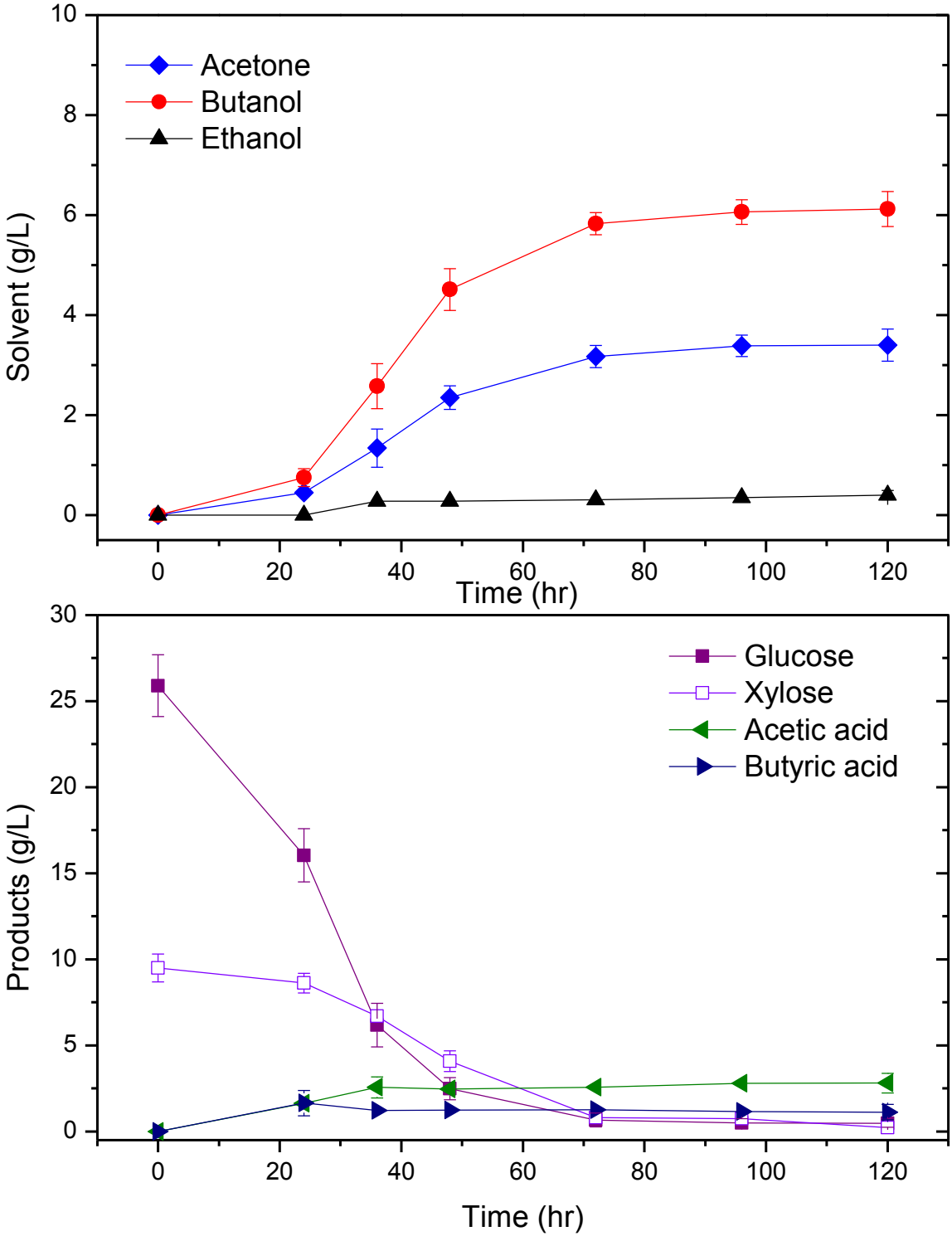

Figure IV-5 Separate hydrolysis and fermentation of alkali-pretreated switchgrass with 5% solid loading

The hydrolyzate obtained in the case of 7% solid loading contained a total of 48.2 g/L of reducing sugars, including 35.3 g/L of glucose and 12.9 g/L of xylose. After 72h fermentation, a total of 14.3 g/L of ABE solvent was produced including 5.1 g/L of acetone, 8.5 g/L of butanol and 0.7 g/L of ethanol (**Fig. 6**). The solvent yield based on the initial sugar present in the hydrolyzate reached to 0.29 (g-solvent/g-sugar in hydrolyzate), which was equivalent to an overall bioconversion yield of 0.23 (g-solvent/g-sugar in pretreated solid). Interestingly, in this case with 7% solid loading, the overall bioconversion yield of SHF was seen a significant improvement over that of SSF (0.15 g-solvent/g-sugar).

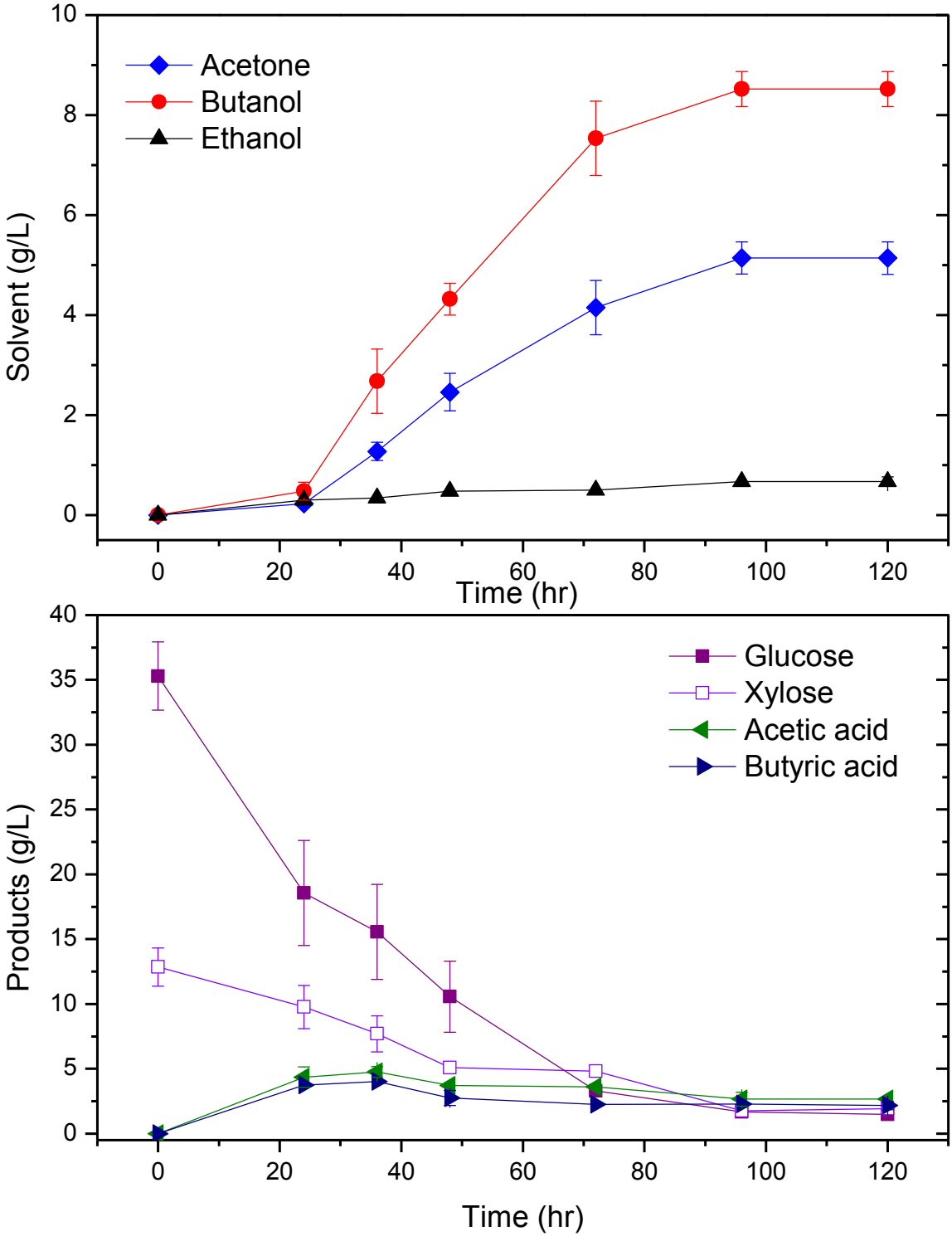

Figure IV-6 Separate hydrolysis and fermentation of alkali-pretreated switchgrass with 7% solid loading

IV.3.5 Discussion

Unlike dilute acid pretreatment, one of the major advantages of using alkaline pretreatment is that the hydrolyzate of alkali-pretreated solids contains fewer amounts of inhibitors (acetic acid, furan derivatives and phenolic compounds). Even through the alkaline pretreatment considerably removed 69.4% of lignin in the present study, the alkali-pretreated solid was still found to be inhibitory to the culture on SSF. (Zhang et al., 2014) reported enzymatic hydrolysis of NaOH-pretreated corn stover released low-molecular-weight phenols in the hydrolyzate and the concentration of phenolic compounds linearly increased with solid loading (Zhang et al., 2014). Major source of these phenolic compounds comes from the surface lignin re-precipitated on the surface of cellulose and hemicellulose during pretreatment (Alvira et al., 2013; Zhang et al., 2014). The phenolic compounds have been well-documented as the most toxic inhibitors impeding fermentative biobutanol production from lignocellulosic feedstocks (Ezeji & Blaschek, 2008b; Lee et al., 2015).

Various types of poly(ethylene oxide)-based non-ionic surfactants were particularly effective in coping with the phenol-induced microbial inhibition (Dhamole et al., 2015; Dhamole et al., 2013; Dhamole et al., 2012; Lee et al., 2015). In one study, addition of surfactant L62D or L62LF in simulated hydrolysate from dilute-acid treated corn stover precipitated out above 80% of phenolic compounds after separating the surfactant phase from aqueous phase via cloud point separation (Dhamole et al., 2013). (Dhamole et al., 2012) also employed the use of non-ionic surfactant to reduce the end product inhibition (butanol, also slightly soluble in water) on ABE fermentation, which resulted to a 25% improvement on solvent production. The toxicity test revealed that

addition of Tween 80 could greatly alleviate the inhibitory effect of phenolic compounds on the cell growth. (Lee et al., 2015) reported similar detoxification effects of Tween 80 on fermentation of the hydrolysate of dilute-acid treated rice straw for butyric acid production. Apart from the detoxification effect, non-ionic surfactant also acted as biocompatible activity enhancers for cellulase enzyme, bringing up significant improvement on the saccharification yield. These effects collectively led to substantial improvement on biobutanol production from alkali-pretreated switchgrass as presented in this study.

A comparison of ABE production via SSF or SHF from various studies was summarized in **Table 4**. SSF has been identified as a superior bioconversion strategy over SHF from a process integration standpoint. Also, SSF might give higher bioconversion yield than SHF as presented in the present study. However, when dealing with lignocellulosic feedstocks, the solid loading in SSF projects a huge process constraint, limiting the final solvent concentration, which in turn projects a technical challenge for the downstream solvent recovery (Jorgensen et al., 2007). In a relevant study, SSF of steam-explosion treated wood chips was limited at 5% solid loading and required sufficient enzyme loading to produce 11-13 g/L of ABE whereas when it comes to acrons, a starchy materials, SSF could process up to 8% solid loading, producing 16-21 g/L of ABE solvent (Sasaki et al., 2014). SHF might be a better choice when it comes to process higher solid loading and achieve concentrated final solvent concentration albeit with a slightly low overall productivity. In the present study, the fermentation yield (0.29 g-solvent/g-sugar in hydrolyzate) was found to be comparable to that from fermentation of activated-carbon detoxified hydrolyzate of hydrothermolysis-pretreated switchgrass (0.30

g-ABE/g-sugar consumed) (Liu et al., 2015), but significantly lower than that (Gao et al., 2014) had reported (0.39 g-ABE/g-sugar in the hydrolyzate). In their study, (Gao et al., 2014) utilized a different type of solvent-producing strain (*C. saccharobutylicum* DSM 13864) with higher inhibition tolerance, which might lead to the difference in the bioconversion yield. In most of referenced cases, regardless of the pretreatment type, sufficient enzyme loading is required to achieve concentrated sugar syrup and detoxification are also required to improve the fermentability of switchgrass hydrolyzate, which makes Tween 80 an attractive additives in biobutanol production

Table IV-4 Comparison of ABE production from various studies

Feedstocks	Solid Loading	Pretreatment	Detoxification	Fermentation	Culture	ABE (g/L)	ABE Yield (g/g sugar)	Reference
witchgrass	5%	Alkali	Tween 80	SSF	*C. acetobutylicum* ATCC 824	12.3	0.28	Present study
Wood chips	5%	Steam	Extraction[a]	SSF	*C. acetobutylicum* NBRC 13948	13.4	NA	(Sasaki et al., 201
Acorns	5%	None	None	SSF	*C. acetobutylicum* NBRC 13948	16.7	NA	(Sasaki et al., 201
Paper sludge	7%	None	None	SSF	*C. acetobutylicum* ATCC 824	17.1	0.24	(Guan et al., 201
Wheat straw	NA	Dilute acid	None	SSF SSF+GS[e]	*C. beijerinckii* P260	11.9 21.4	0.27 0.31	(Qureshi et al., 2008b)
witchgrass	7%	Alkali	Tween 80	SHF	*C. acetobutylicum* ATCC 824	14.3	0.29	Present study
Acorns	5%	None	None	SSF	*C. acetobutylicum* NBRC 13948	16.7	NA	(Sasaki et al., 201
Wood chips	5%	Steam	Extraction[a]	SHF	*C. acetobutylicum* NBRC 13948	15.3	NA	(Sasaki et al., 201
witchgrass	7%	Dilute acid	NA Dilution[b]	SHF	*C. beijerinckii* P260	1.5 14.6	NA 0.39	(Qureshi et al., 2010b)
witchgrass	10%	Hydrothermal	None pH adjustment Adsorption[c] Adsorption[d]	SHF	*C. acetobutylicum* ATCC 824	1.0 5.6 14.5 16.8	0.12 0.16 0.30 0.30	(Liu et al., 2015)
witchgrass	10%	Alkali	None	SHF	*C. saccharobutylicum* DSM 13864	22.7	0.40	(Gao et al., 2014

a. Extraction: Two stage extraction (water+methanol extraction), removed low-molecular-weight lignin from the steam-exploded solids.

IV.4 Conclusion

The present study demonstrated the non-ionic surfactant was an effective additive coping with phenolic compounds-induced inhibition on the fermentative butr production. Addition of Tween 80 in either SSF or SHF substantially enhanced solvent production from alkali-pretreated switchgrass, giving a bioconversion yield r that of pure sugar control. In terms of bioconversion strategy, although SSF achie higher bioconversion yield and productivity, SHF is capable of process higher s loading, attaining a more concentrated final solvent concentration.

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V. Acetone-butanol-ethanol (ABE) production from fermentation of hot-water extracted hemicellulose hydrolysate of pulping woods

Abstract

In Kraft pulping process, hemicellulose portion of wood is usually discharged as waste stream into the black liquor, representing a highly underutilized sugar source. In this study, the hemicellulose portion is investigated as feedstock for production of acetone, butanol and ethanol by ABE fermentation with Clostridium *acetobutylicum.* For this purpose, hot-water treatment is applied to Poplar (hardwood) and Southern pine (softwood) to extract the hemicellulose portion prior to the pulping process without damaging the cellulose quality. Control test on ABE fermentation with pure sugar as substrate indicated that both glucose and xylose could be utilized as carbon source for solvent production with comparable bioconversion efficiency. The culture could consume up to 57 g/L of glucose or xylose, producing ABE solvent at the levels of 17-18 g/L with a total solvent yield of 30-31% (g-solvent/g-sugar). It is shown that during the extraction process, various toxic compounds for the culture are generated, including furfural, HMF and lignin-degraded phenolic compounds. Therefore, the hemicellulose hydrolysate is required to be detoxified prior to fermentation. The effectiveness of different detoxification methods, i.e., overliming, ion exchange and activated carbon adsorption, is assessed. The details of hot-water extraction conditions, performance of detoxification as well as the fermentation profiles are discussed.

Keywords: Acetone-butanol-ethanol (ABE), hemicellulose hydrolysate, Clostridium *acetobutylicum*, pretreatment

V.1 Introduction

Pulp and paper mills project a great opportunity for integrated forest biorefinery to produce, in addition to pulp and fiber products, value-added biochemicals with the established technology and infrastructure (Amidon & Liu, 2009; Helmerius et al., 2010). In a traditional Kraft pulping process, woodchips are fractionated into major product of pulp and a number of byproducts ended up as a complex mixture in the black liquor, including extractive-derived tall oil, hemicellulose-degraded oligomeric and monomeric sugars, and lignin-degraded phenolic compounds (Helmerius et al., 2010; Huang & Ragauskas, 2013; Huang et al., 2010; Yoon et al., 2008). For the need of pulping chemical (NaOH and Na2S) regeneration, black liquor is concentrated; tall oil is recovered as a profitable byproduct, whereas hemicellulose and lignin fraction is incinerated in the recovery boiler for energy and power generation (Helmerius et al., 2010). In consideration of the heating value of hemicellulose (13.6 MJ/kg) is relatively low in comparison with that of lignin (27.0 MJ/kg), the idea of utilizing hemicellulose portion of pulping wood as an underutilized sugar source for value-added biochemical production has been proposed to diversify the product portfolio and generate extra revenue for the pulp mills (Helmerius et al., 2010; Huang & Ragauskas, 2013; Yoon et al., 2008; Yoon & van Heiningen, 2010).

Biobutanol is one of such high-value products with versatile industrial applications and expanding market demand (Duerre, 2008; Green, 2011). Fermentative production of butanol has typically been performed with solvent-producing *Clostridium* species with acetone, butanol and ethanol (ABE) as major fermentation products. One of

the attractive features about ABE fermentation is that the wild-type *Clostridium* culture is capable of catabolizing both hexose (e.g., mannose, the leading component in softwood hemicellulose) and pentose (e.g., xylose, the dominant component in hardwood hemicellulose) for solvent production (Ezeji & Blaschek, 2008b; Guan et al., 2016). The fact that papermaking industry generally combines hardwood and softwood pulp together to achieve a desired paper property (e.g., strength, brightness, or so) makes both types of hemicellulose abundantly available yet underutilized in pulp mills.

Given the complex nature of black liquor, it is rather challenging to recover the dissolved hemicellulose from black liquor. (Kudahettige-Nilsson et al., 2015) investigated hemicellulose recovery from birch Kraft black liquor via acid precipitation and demonstrated the difficulty of utilizing the recovered xylan as feedstock for ABE fermentation. In their study, aside from high level of microbial inhibitors, fermentation of well-detoxified xylan hydrolysate resulted to a rather lower bioconversion yield (0.12 g-solvent/g-sugar) than that of xylose control (0.34 g-solvent/g-sugar) (Kudahettige-Nilsson et al., 2015). One possible reason for this inferior fermentation is that considerable portion of solubilized polysaccharides were degraded into hydroxyl carboxylic acids under the alkaline condition in the black liquor (Helmerius et al., 2010; Sjostrom, 1991).

Implementation of a hemicellulose extraction (also named as pre-extraction or pre-hydrolysis) step to recover the hemicellulose sugar prior to the chemical pulping process has been a more common and practical approach (Helmerius et al., 2010; Huang & Ragauskas, 2013). Existing technologies for hemicellulose extraction have been specifically developed for the production of the dissolving pulp (>90% cellulose) by

applying hot-water or dilute solution of acid or alkali (Hamaguchi et al., 2013; Huang & Ragauskas, 2013; Huang et al., 2010). A key criterion about hemicellulose extraction is not to cause measurable degradation in the subsequent pulp quality and yield. As a more frequent practice, hot-water pre-extraction typically imposes slight damage on the subsequent pulp yield and property in comparison with dilute acid treatment while at the same time, it could generate more concentrated hemicellulose pre-hydrolysate with less amounts of lignin-degraded and inorganic microbial inhibitors than dilute alkaline extraction (Hamaguchi et al., 2013; Helmerius et al., 2010; Huang & Ragauskas, 2013).

(Helmerius et al., 2010) reported the use of enzymatically-hydrolyzed xylose-rich prehydrolysate from hot-water treatment of silver birch (hardwood) as sugar source for fermentative production of succinic acid with genetically modified culture of *Escherichia coli AFP 184*. Similarly, (Kang et al., 2012) reported bioconversion of the hemicellulose prehydrolysate from hot-water treatment of southern pine (softwood) into bioethanol with the yeast culture of *Saccharomyces cerevisiae* ATCC 200062. Detoxification of the hemicellulose prehydrolysate with either overliming or charcoal adsorption, however, was commonly required in their study (Helmerius et al., 2010; Kang et al., 2012). It is well-documented that hot-water treatment generates various types of potential toxic compounds for the microbial fermentation (e.g., aliphatic acids, sugar-degraded furan derivatives and lignin-degraded phenolic compounds) (Ezeji & Blaschek, 2008b; Liu et al., 2015; Sun & Liu, 2012; Villarreal et al., 2006). (Sun & Liu, 2012) previously investigated the utilization of dilute acid-hydrolyzed prehydrolysate as feedstock for ABE production. In their study, a combined detoxification of nano-filtration and overliming

was applied to ensure a solvent production of 11 g/L of ABE, which would otherwise be inhibited to the level of 0.8 g/L (Sun & Liu, 2012).

The present study seeks to investigate the use of hemicellulose prehydrolysate following hot-water treatment of two types of pulping wood: hybrid poplar (hardwood) and softwood pine (softwood) as feedstock for biobutanol production. The chemical compositions of the prehyrolysate (i.e., carbohydrates and degradation products) from hybrid poplar and southern pine were comparatively assessed, followed by a toxicity test of the model degradation compounds. Detoxification treatment (overliming and adsorption) was applied to improve the fermentability of the prehydrolysate. The effectiveness of detoxification was discussed in conjunction with the fermentation test.

V.2 Materials and methods

V.2.1 Pulping woods, enzyme and microorganism

Debarked hybrid poplar was collected from a forest products laboratory at Auburn University (Auburn, AL) and southern pine was provided from Rock-Tenn Co (Demopolis, AL). The two raw materials were grounded and screened as woodchips with an average particle size of 0.4×0.4×0.2 in. The woodchips was then stored at 4 ºC. Cellulase (C-Tec 2, Lot No. VCNI0001) with protein content and specific activity of 255 mg-protein/mL and 119 FPU/mL was supplied form Novozymes, North America (Franklinton, NC). Multifact xylanase with protein content of 42 mg/mL and Multifact pectinase with protein content of 82 mg/mL was gifted from Genencor (Paulo Alto, CA). The microorganism (*Clostridium acetobutylicum* ATCC 824) for the ABE fermentation was purchased from American Type Culture Collection.

V.2.2 Hemicellulose extraction with hot-water treatment

Hemicellulose extraction was performed with hot-water treatment in a 4 L Parr reactor (Parr Instrument Co., Moline, IL) under the following conditions: liquid-to-solid ratio of 5, 170 ºC, and 1 h. The conditions of hot-water treatment was referenced from a previous study and corresponded to a H-factor of 925, an indicator for the severity of hot-water treatment, under which the resulting pulp quality and yield were not subjected to sizable degradation (Kang et al., 2012). The Parr reactor was equipped with a temperature controller and a motor-driven stirring bar. Prior to the hot-water treatment, 500 g (oven-dried weight) of woodchip was pre-soaked overnight at room temperature in 2.5 L DI water. Upon the completion of hot-water treatment, the slurry was separated via vacuum filtration and the liquid portion was collected as pre-hydrolysate. The dilute prehydrolysate was then concentrated via evaporation with Rotavapor (Büchi RE-121, Switzerland). The pre-hydrolysate and the treated solids were analyzed.

V.2.3 Culture maintenance and seed-culture preparation

The spore suspension of *C. acetobutylicum* was maintained at -20 ºC on Elliker Broth (BD Difco™) with the addition of glycerol (20 %(v/v)). The method for culture maintenance and inoculum preparation has been detailed in previous study (Guan et al., 2016).

V.2.4 Detoxification of concentrated hemicellulose pre-hydrolysate

Detoxification with overliming or charcoal adsorption was applied to improve the fermentability of hemicellulose prehydrolysate. The pH of concentrated poplar pre-hydrolysate/southern pine prehydrolysate was 3.3/3.4. Overliming was performed to adjust the pH of the prehydrolysate near 10 with the addition of calcium oxide (CaO) and incubated at 60 °C, 150 rpm. Upon 6-h incubation, the pH of prehydrolysate was decreased to approximately 6.7. The lime-treated prehydrolysate was then centrifuged to remove the precipitate.

Batch-mode adsorption was performed as a detoxification method for concentrated pre-hydrolysate. Granular activated charcoal with particle size of 20-40 mesh was used as adsorbents. Before detoxification treatment, charcoal was rinsed with DI water on a filter paper to remove the impurities and oven-dried overnight at 45 °C. The adsorbents were loaded in the pre-hydrolysate at 5% (w/v). The mixture was then incubated at 60 °C and 150 rpm for 6h to reach the adsorption equilibrium. The detoxified pre-hydrolysate was recovered via centrifugation. Following adsorption, the pH of the detoxified pre-hydrolysate was adjusted to 6.5-7 with the addition of CaO. Microfiltration (0.45-μm filter) was then applied to remove the suspended particles from overliming/adsorption-treated pre-hydrolysate. The chemical composition of detoxified pre-hydrolysate was analyzed.

V.2.5 ABE fermentation with pure sugar as feed and toxicity test

The fermentation tests in the present study were carried out anaerobically at 36 °C at 150 rpm in 125 mL serum bottle with a working volume of 50mL. P2 medium was

chosen as the fermentation medium. Detailed procedure for ABE fermentation with pure sugar as substrate has been described in previous study (Guan et al., 2016). The toxicity test was performed with the addition of various model potential inhibitors in the fermentation medium with pure sugar as feed. A number of typical toxic compounds were tested, including furfural, hydroxymethylfurfural (HMF), *p*-coumaric, ferulic acid, vanillic acid and 4-hydroxybenzoic acid. Briefly, the individual inhibitor was added in the range of 0.25-2 g/L. The cell growth (OD600) was measured after 36 h inoculation.

The formula of P2 medium is as followings (g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; NaCl, 0.01; ammonium acetate, 2.2; yeast extract, 1.0.

V.2.6 Simultaneous saccharification and fermentation of hemicellulose pre-hydrolysate

When it came to utilize the hemicellulose pre-hydrolysate as feedstock, simultaneous saccharification and fermentation (SSF) was used as the bioconversion strategy. The organic and mineral nutrients of P2 medium were directly supplemented to the pre-hydrolysate. The pre-hydrolysate was then filter-sterilized by passing through a 0.45-μm syringe filter (VWR) and then decanted into a sterile serum bottle. The enzyme was then added after sterilization. For the xylan-rich poplar pre-hydrolysate, a combination of cellulase (5 mg-protein/g-xylan) and xylanase (25 mg-protein/g-xylan) was loaded, whereas an enzyme cocktail of cellulase (5 mg-protein/g-xylan), xylanase (10 mg-protein/g-xylan) and pectinase (10 mg-protein/g-mannan) was applied when it came to the pre-hydrolysate of southern pine with mannan as the leading component. The headspace of the serum bottle was flushed with nitrogen gas to develop the anaerobic

condition. The seed-culture was then aseptically inoculated at 6%(v/v). The bottle was kept sealed to maintain the anaerobic condition (N_2). Aliquots of samples were collected with 1.0 mL syringe at 12-h interval. (CaCO3)

V.2.7 Analytical methods

The solid composition of woodchips before and after pretreatment was analyzed according to NREL standard analytical procedure (NREL/TP-510-42618). The chemical composition of hemicellulose pre-hydrolysate (hot-water extracted, concentrated and detoxified) was also analyzed via secondary hydrolysis as described in the protocol (NREL/TP-510-42623). The pre-hydrolysate was characterized as the carbohydrate content (oligosaccharide and monomeric sugar) and degradation products (acetic acid, furfural, HMF and soluble lignin content). The oligosaccharide in the pre-hydrolysate was calculated by subtracting the monomeric sugar content in the pre-hydrolysate from the total monomeric sugar content after secondary hydrolysis. The soluble lignin content in the hydrolysate was measured by a UV-vis spectrophotometer (BioTek Instruments, VT) and reported as the optical density (OD) at wavelength of 290 nm.

Sugar analysis was quantified with HPLC equipped with Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA). The fermentation products (i.e., acetone, butanol, ethanol, acetic acid and butyric acid) in this study were analyzed by HPLC equipped with Refractive Index detector (Shodex, Japan) and Aminex HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA). The solvent yield was calculated on the weight basis as the amount of ABE production divided by the total sugar in the pre-hydrolysate.

V.3 Results and Discussion

V.3.1 Hemicellulose extraction with hot-water treatment

Composition analysis of the woodchips gave clear indication about the degree of hemicellulose removal and overall weight loss of woodchips after hot-water treatment (Table 1). Major components of untreated hybrid poplar consisted of 42.2% of glucan, 18.7% of xylan and 27.5% of lignin. The hot-water treatment at H-factor of 925 resulted in a total of 15.7% of weight loss, primarily coming from the removal of xylan (8% loss) and extraneous extractives (acetyl, protein, etc.). The treated poplar sample, however, exhibited no significant decrease in other components, particularly the glucan content (1% loss), in comparison with the untreated sample.

Table V-1 Chemical composition of hybrid poplar (hardwood) and southern pine (softwood)

Component (%)	Hybrid Poplar		Southern Pine	
	Untreated	Treated*	Untreated	Treated*
Glucan	42.2	41.2	40.6	39.4
Xylan	18.7	10.7	6.8	4.5
Galactan	0.8	0.5	2.8	1.4
Arabinan	0.5	0.3	1.1	0.7
Mannan	2.4	1.9	9.6	5.3
Lignin	27.5	25.8	32.8	30.7
ash	0.6	0.3	0.5	0.3
Acetyl	5.5	3.0	2.5	1.7
Solid Recovery	-	84.3	-	86.4

Note. * The solid composition of hot-water treated sample is calculated on the basis of untreated raw sample.

Untreated southern pine was analyzed to primarily contain 40.6% of glucan, 6.8% of xylan, 9.6% of mannan and 32.8% of lignin. Hemicellulose portion represents the most distinct difference in chemical composition between hardwood and softwood. The backbone of hardwood hemicellulose comprises dominantly acetylated 4-O-methyl glucuronoxylan whereas the leading building-block of softwood hemicellulose is glucomannan, followed by glucuronoxylan (Helmerius et al., 2010; Huang & Ragauskas, 2013). In this case, 4.3% of mannan and 2.3% of xylan were removed following the hot-water treatment, which, together with water-soluble extractives, collectively led to 13.6% of overall weight loss. The hot-water treatment had caused slight loss (1.2%) in cellulose content, indicating the process is highly selective for hemicellulose portion for both the hardwood and softwood. (Huang & Ragauskas, 2013) previously demonstrated the hemicellulose pre-extraction did not typically affect the degree of polymerization (DP) of the cellulose content. The resulting pulp quality and yield could be experimentally determined, which were not included in the present study. In facts, various studies have validated the potential impacts of hot-water extraction on the resulting pulp property and yield could be largely offset by optimization of pre-extraction process or adjusting the downstream pulping conditions, e.g., cooking time, pulping chemical dosage (Huang & Ragauskas, 2013; Yoon et al., 2010; Yoon et al., 2008).

The chemical composition of the hemicellulose prehydrolysate is presented in Table 2. Hot-water treatment of hybrid poplar released a total of 22.2 g/L of carbohydrates with xylose (16.8 g/L) as the dominant component in the prehydrolysate. Particularly notable is that the majority of the carbohydrates were oligosaccharides (17.9 g/L) and only a small portion in the prehydrolysate was present in the form of the

monomeric sugars (4.3 g/L). When it came to the southern pine-derived hemicellulose prehydrolysate, a total of 20.5 g/L of carbohydrates with 14.7 g/L of oligosaccharides was acquired after hot-water treatment. In this case , mannose (8.4 g/L) became the leading component, followed by xylose (4.3 g/L) and galactose (3.3 g/L). The hot-water treatment (also called self-hydrolysis) works by cleaving off the acetyl groups in hemicellulose backbone, simultaneously releasing the polysaccharides and acetic acid in the aqueous solution (Hamaguchi et al., 2013; Helmerius et al., 2010). Consequently, the increased acidity further hydrolyzes the polysaccacharides into oligomers and monomers. The ratio of oligomer to monomer was known to be dependent on the acidity of the aqueous phase (Helmerius et al., 2010; Jun et al., 2012). Dilute sulfuric acid-assisted hot-water extraction was known to generate monomer-dominant pre-hydrolysate but resulted to a great reduction in the pulping yield (Amidon & Liu, 2009; Helmerius et al., 2010; Jun et al., 2012; Liu et al., 2015; Sun & Liu, 2012).

Table V-2 Chemical composition of hot-water extracted hemicellulose prehydrolyzate

Component (g/L)	Hybrid Poplar (Hardwood)			Southern Pine (Softwood)		
	Monomer	Oligomer	Total	Monomer	Oligomer	Total
Glucose	0.3	1.2	1.4	0.4	1.6	2.0
Xylose	2.4	14.3	16.8	1.1	3.0	4.1
Galactose	0.6	0.9	1.5	0.7	2.1	2.8
Arabinose	0.5	0.0	0.5	1.5	0.3	1.7
Mannose	0.5	1.5	2.0	0.6	8.2	8.7
Total	4.3	17.9	22.2	4.3	15.0	19.3
Acetic acid	2.0	-	4.8	0.7	-	1.6
HMF	0.0	-	0.0	0.1	-	0.2
Furfural	0.4	-	1.3	0.1	-	0.4
Soluble lignin	3.1	-	-	3.6	-	-

Note:

a. Monomer represents the concentration of monomeric sugars and oligomer represents the concentration of oligosaccharides.

b. The value reported is UV-Vis absorbance (10x dilution) at wavelength of 290 nm, which linearly correlates with the concentration of soluble lignin content.

The hemicellulose prehydrolysate was concentrated to increase the carbohydrates content and thus, the final fermentation product titer, which would otherwise raise the technical challenge for product recovery (Guan et al., 2016). Accordingly, the total carbohydrate in the concentrated poplar/southern pine prehydrolysate was increased to 49.1/53.1 g/L (Table 3).

Table V-3 Effects of detoxification on chemical composition of concentrated prehydrolysate

Components (g/L)	Hybrid poplar prehydrolysate			Southern pine prehydrolysate		
	Concentrated	Overliming	Charcoal	Concentrated	Overliming	Charco
Glucose	3.8	3.5	2.3	5.4	5.3	4.9
Xylose	36.1	34.5	33.5	12.6	10.9	11.8
Galactose	3.7	2.8	2.8	7.5	7.5	6.5
Arabinose	1.1	0.7	1.3	2.5	2.0	2.4
Mannose	4.5	3.8	3.5	24.3	22.6	20.9
Total	49.1	45.4	43.3	52.4	48.3	46.6
pH	3.3	6.7	3.7	3.4	6.1	3.6
Acetic acid	9.5	10.2	5.4	3.8	3.6	2.1
HMF	1.0	ND	0.1	1.4	ND	0.3
Furfural	1.8	ND	0.6	1.3	ND	0.7
Soluble lignin	3.1	2.9	0.3	3.6	3.4	0.4

a. ND indicates non-detectable.

b. The value reported as UV-Vis absorbance (10x dilution) at wavelength of 290 nm, which linearly correlates with the concentration of soluble lignin content.

V.3.2 Detoxification of concentrated hemicellulose prehydrolysate

Albeit hot-water treatment generated pre-hydrolysate rich in carbohydrates content, which could be further hydrolyzed into fermentable sugars, the formation of various degradation products, including acetic acid, furfural, HMF and phenolic compounds, were known as potential inhibitors for the subsequent microbial fermentation (Table 2). Sugar-degraded furfural or HMF at the present level (less than 2 g/L) in the concentrated prehydrolysate was reported to be toxic to the culture of yeast or E. *coli* in bio-ethanol fermentation but not to the solvent-producing C. *acetobutylicum* in ABE fermentation (Ezeji & Blaschek, 2008b; Sun & Liu, 2012).

In poplar prehydrolysate, acetic acid was present at the level of 2.0 g/L, which was, however, substantially increased to 4.8 g/L after secondary hydrolysis. This indicated the dissolved oligosaccharides carried considerable portion of acetyl group, which was not released as acetic acid until the oligomer was completely hydrolyzed. The release of acetic acid in SSF might potentially disrupt buffering effect of fermentation medium, which has been verified as a critical limiting factor for the ABE fermentation (Yang et al., 2013a). The overall acetic acid content in the poplar prehydrolysate (4.8 g/L) was substantially higher than that in the southern pine prehydrolysate (1.6 g/L), which was in agreement with the common notion that the majority of acetyl group linked to the glucuronoxylan backbone of hemicellulose (Amidon & Liu, 2009; Liu et al., 2012).

Additionally, during the hot-water treatment, lignin-degraded compounds partially precipitated as high-molecular-weight insoluble particles while the other parts dissolved as soluble phenolic compounds in the pre-hydrolysate (Kang et al., 2012; Sun

& Liu, 2012). Various model phenolic compounds (*p*-coumaric, ferulic acid, vanillic acid and 4-hydroxybenzoic acid) were identified as rather toxic compounds for the solventogenic *Clostridia* culture, as presented in the toxicity test (Fig. 1). These compounds started to inhibit the cell growth when their concentrations reached to 0.25-0.5g/L. Ferulic acid and *p*-coumaric acid was found to lead to near complete inhibition on cell growth when their concentration reached to 1.5 g/L, whereas vanillic acid and 4-hydroxybenzoic acid at the level of 1 g/L resulted to a maximum 50% reduction in the cell growth. The results for *p*-coumaric acid and ferulic acid were in generally consistent with previous report (Ezeji & Blaschek, 2008b). It should be noted that *p*-coumaric acid and ferulic acid are sparingly water-soluble while vanillic acid and 4-hydroxybenzoic acid are slightly water-soluble. The inhibitory mechanism of these phenolic compounds had been proposed by disrupting the function of *Clostridium* cell membrane via hydrophobic interaction (Ezeji & Blaschek, 2008b; Liu et al., 2015; Sun & Liu, 2012).

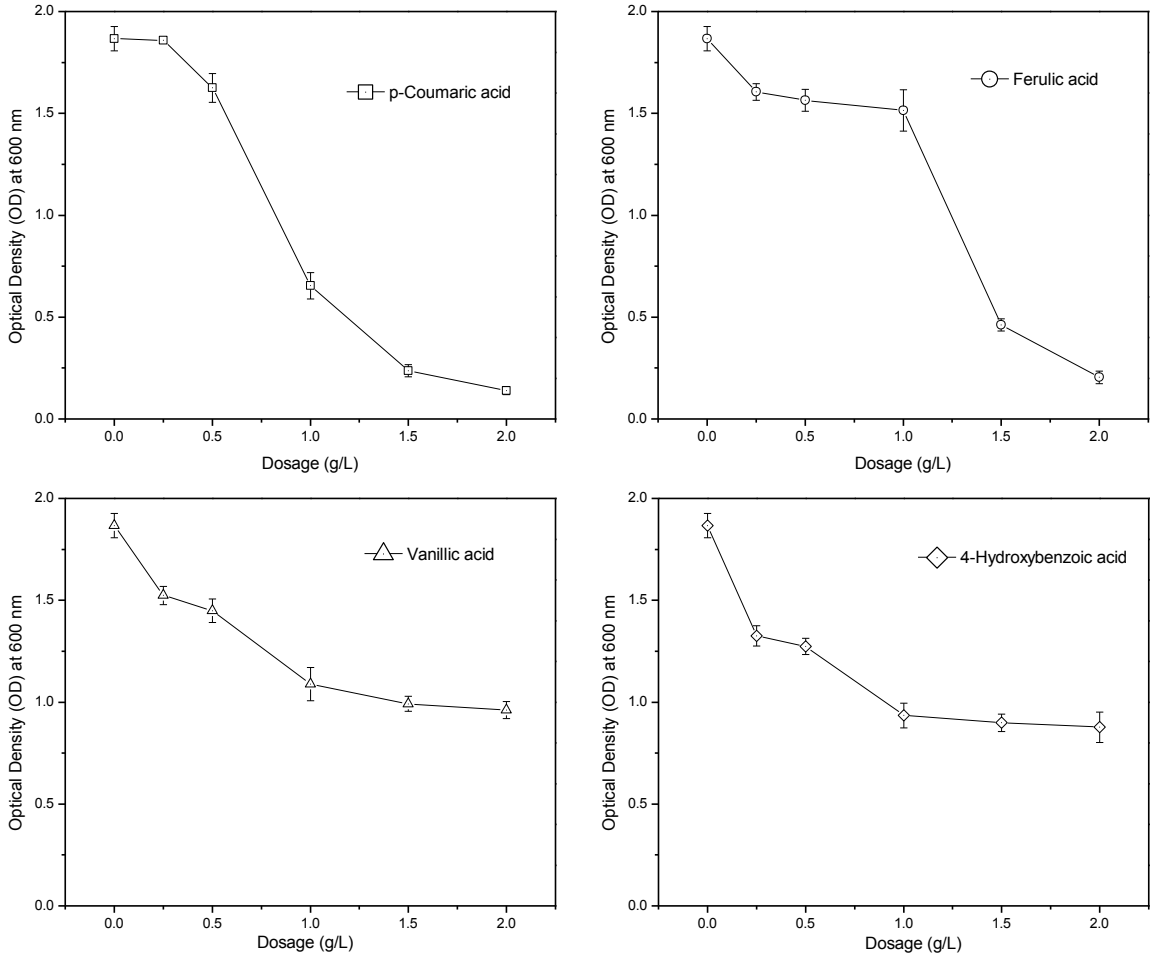

Figure V-1 Effects of model phenolic inhibitors on ABE fermentation

SSF of the untreated pre-hydrolysate had not resulted to any cell growth and solvent production. Detoxification with overliming and activated charcoal adsorption were therefore applied to improve the fermentability of the concentrated pre-hydrolysate. The effect of detoxification on the chemical composition of concentrated pre-hydrolysate was presented in Table 3. A common yet unfavorable feature was that both overliming and charcoal adsorption had caused the loss of carbohydrates. Composition analysis revealed that total sugar content (49.1 g/L) in concentrated poplar pre-hydrolysate was notably decreased to 45.4 g/L/43.3 g/L following the overliming/charcoal adsorption

treatment (Table 3). Similarly, overliming/activated charcoal treatment had decreased the total sugar content in concentrated southern pine pre-hydrolsate from 52.4 g/L to 48.3 g/L/46.6 g/L. It is known that activated charcoal detoxifies the pre-hydrolysate by physical adsorption whereas overliming works by precipitation of the toxic compounds (Liu et al., 2015; Sun & Liu, 2012; Wang & Chen, 2011). It was primarily the oligomeric sugars rather than the monomer that contributed to the carbohydrates loss (results were not shown), probably due to their higher affinity to lime and activated carbon.

On the other hand, overliming treatment had increased the pH of pre-hydrolysate from 3.3 to 6.7, which are suitable for ABE fermentation. But overliming was found to be ineffective in the removal of acetic acid and soluble lignin content. In contrast to the overliming treatment, activated charcoal adsorption had resulted to substantial removal of soluble lignin content (more than 90%) as evidenced by a great reduction of the UV-Vis absorbance (Table 3). Additionally, the adsorption had considerably removed the unbound acetic acid in the poplar pre-hydrolysate and reduced the total acetic acid concentration to 5.4 g/L, from 9.5 g/L for the un-detoxified pre-hydrolysate. But no dramatic change in the pH of pre-hydrolysate was observed. Therefore, the pH of the pre-hydrolysate was adjusted to 6.5-7.0 with the addition of CaO prior to fermentation. Detoxification with overliming or adsorption affected the chemical composition of southern pine pre-hydrolysate in a similar manner (Table 3). The effectiveness of activated charcoal treatment on removal of phenolic compounds was in agreement with previous report (Kudahettige-Nilsson et al., 2015; Liu et al., 2015; Villarreal et al., 2006; Wang & Chen, 2011).

V.3.3 Simultaneous saccharification and fermentation of overliming detoxified hemicellulose pre-hydrolysate

ABE fermentation with mixed sugar as feed was performed as control test (Table 4). As the poplar pre-hydrolysate control, fermentation of synthetic sugar medium (xylose, 35.4 g/L; glucose, 5.0 g/L; mannose, 4.7 g/L; galactose, 5.3 g/L) produced 13.6 g/L of ABE solvent with 8.5 g/L of butanol over 96-h period, giving a fermentation yield of 0.27 (g-solvent/g-sugar) and productivity of 0.14 g/L/h (Table 4). In comparison, a total of 15.3 g/L of ABE solvent with 9.3 g/L of butanol was produced from a mixed sugar control of concentrated pine pre-hydrolysate (glucose, 5.1 g/L xylose, 14.7 g/L; galactose, 5.2 g/L; mannose, 25.1 g/L). The fermentation yield and productivity reached to 0.31 (g-ABE/g-solvent) and 0.16 g/L/h.

Table V-4 ABE fermentation of mixed sugars and detoxified prehydrolysate

	Concentrated poplar-derived prehydrolysate					Concentrated southern-pine prehydrolysate				
Products	Poplar Control	Untreated	CaO	Carbon	Carbon+CaCO3	Pine control	Untreated	CaO	Carbon	Carbon+CaCO3
Sugar (g/L)	50.3	49.1	45.4	43.3	43.3	50.1	52.4	48.3	46.6	46.6
Acetone (g/L)	4.1	-	0.6	2.1	3.5	5.1	-	1.7	4.2	4
Butanol (g/L)	8.5	-	1.1	5.2	6.8	9.3	-	3.7	8.3	7.9
ABE (g/L)	13.6	-	1.8	7.6	10.8	15.3	-	5.7	13.2	12.6
Yield (g/g-sugar)	0.27	-	0.04	0.18	0.25	0.31	-	0.12	0.28	0.27
roductivity (g/L/h)	0.14	-	0.02	0.08	0.11	0.16	-	0.06	0.14	0.13
Acetic acid (g/L)	2.2	5.1	10.4	7.6	6.2	1.9	2.1	6.9	4.6	5.3
Butyric acid (g/L)	0.8	-	6.6	3.9	1.8	1.0	-	5.4	1.6	2.1
Rsugar (g/L)	0.8	40.8	10.4	4.6	0.9	1.2	45.5	8.1	1.1	0.6

Note

a. Synthetic medium consisted of a total of 50.2 g/L sugars, including xylose (35.4 g/L), glucose (5.0 g/L), mannose (4.7 g/L) and galactose (5.3 g/L).

b. Synthetic medium consisted of a total of 46.6 g/L sugars, including

Table V-5 Comparison of ABE production from various studies

Feedstocks	Pretreatment	Detoxification	Culture	ABE (g/L)	ABE Yield (g/g sugar)	Reference
oplar pre-hydrolysate	Hot-water	Activated charcoal	*C. acetobutylicum* ATCC 824	10.8	0.25	This study
?ine pre-hydrolysate				13.2	0.28	
ed wood pre-hydrolysate	Green liquor	Overliming	C. beijerinckii CC 101	5.8	0.28	(Lu et al., 2013)
		Activated charcoal		9.0	0.28	
		Ion-exchange resin		11.4	0.39	
Maple pre-hydrolysate	Hot-water	Nanofiltration+overliming	*C. acetobutylicum* ATCC 824	11.0	0.28	(Sun & Liu, 2012)
Xylan in black liquor	Kraft pulping	Activated charcoal	*C. acetobutylicum* ATCC 824	2.8	0.12	(Kudahettige-Nilsson et al., 2015)
dwood pre-hydrolysate	Hot-water	Flocculation	C. acetobutylicum ATCC 824	6.4	0.17	(Mechmech et al., 2015)
		Nanofiltration/flocculation		4.3	NA	
Acorns hydrolysate	Steam-explosion	Two-stage extraction	*C. acetobutylicum* NBRC 13948	15.3	NA	(Sasaki et al., 2014)
Switchgrass	Hydrothermo	None	*C. acetobutylicum* ATCC 824	1.0	0.12	(Liu et al., 2015)
		pH adjustment (NaOH)		5.6	0.16	
		NaOH+$CaCO_3$		7.9	0.	
		Adsorption		16.8	0.30	
Corn stover	Steam-explosion	Activated charcoal	*C. acetobutylicum* ATCC 824	12.4	0.30	(Wang & Chen, 2011)

Simultaneous saccharification and fermentation of detoxified pre-hydrolysate was performed as described in *section 2.6*. Attempt at SSF of overliming-treated poplar pre-hydrolysate was unsuccessful in that the acid (11.9 g/L), instead of the solvent (1.8 g/L), was primarily produced (so-called acid crash) with significant level of unused residual sugars (10.8 g/L) (Table 4). Similarly, for overliming-detoxified southern pine pre-hydrolysate, although SSF resulted a solvent production of 5.7 g/L, the acids (acetic, 4.8 g/L; butyric, 5.4 g/L) were the primary SSF products rather than the solvent. The solvent yield in SSF of overliming-detoxified pre-hydrolysate is far less than that in the mixed sugar control. It is known that ABE fermentation typically involves a bi-phasic metabolic pattern, in which the acid was firstly produced (acidogenic phase) and then re-assimilated for solvent production (solventogenic phase). Researchers have identified two possible reasons for the acid crash. Firstly, the buffering effect (pH) of fermentation medium was disrupted due to the accumulation of undissociated acid, disabling the acid re-assimilation and solvent production for the *Clostridium* culture (Cho et al., 2009; Liu et al., 2015; Ujor et al., 2014; Yang et al., 2013a). Additionally, the presence of soluble lignin content was also found to interfere with the transition from acidogenesis to solventogenesis, which substantially reduced the solvent production (Baral & Shah, 2014; Cho et al., 2009; Mechmech et al., 2015; Wang & Chen, 2011).

In contrast to the results in present study, (Sun & Liu, 2012) previously reported detoxification of hemicellulose hydrolysate with overliming had resulted a ten-fold increase in the solvent production from 0.8 g/L to 11 g/L. In their study, prior to overliming treatment, a nanofiltration was performed and had significantly removed the acetic acid and phenolic compounds from the hydrolysate, which might lead to the

different results. Given the fact that overliming treatment had not notably removed the acetic acid and soluble lignin content from the pre-hydrolysate, these compounds was believed to collectively lead to the low fermentation yield.

V.3.4 Simultaneous saccharification and fermentation of activated charcoal-detoxified hemicellulose pre-hydrolysate

Unlike overliming treatment, activated charcoal treatment had greatly removed the phenolic compounds and partially removed the acetic acid from the hemicellulose pre-hydrolysate. SSF of charcoal-treated poplar pre-hydrolysate achieved a total solvent production of 7.6 g/L with 5.2 g/L of butanol (Table 4). The solvent yield (0.18 g-solvent/g-sugar) was substantially higher than that of overliming-treated but still lower than that of mixed sugar control. At the end of fermentation, significant level of the acetic acid (7.6 g/L) and butyric acid (3.9 g/L) was present in the medium.

Supplementation of calcium carbonate in ABE fermentation has been reported to stabilize the pH of fermentation medium and thus, improve the solvent production (Liu et al., 2015; Yang et al., 2013a). In the present study, SSF of charcoal-detoxified pre-hydrolysate with the addition of $CaCO_3$ exhibited a typical pattern of ABE fermentation (Fig. 2). Within the first 24 h (acidogenic phase), acetic acid and butyric acid were concurrently produced, reaching to 7.6 g/L and 4.5 g/L at 24h. At the same time, monomeric xylose, released from enzymatic hydrolysis, accumulated to 13.4 g/L, corresponding to a 38% xylan digestibility. Afterwards, with gradual consumption of the sugars, acidogenic phase transitioned into solventogenic phase and ABE solvent was produced at significant rate. After 96-h fermentation, SSF produced a total of 10.8 g/L ABE solvent (acetone, 3.5 g/L; butanol, 6.8 g/L; ethanol, 0.5 g/L), leaving insignificant

level of residual sugars present in the fermentation medium. The solvent yield and productivity of SSF reached to 0.28 (g-solvent/g-sugar) and 0.13 g $L^{-1}h^{-1}$, which was comparable to that of mixed sugar control.

In the same way, activated charcoal detoxification had enabled a substantial improvement on the solvent production. In this case, SSF of activated charcoal detoxified does not require the addition of $CaCO_3$, probably due to its low acetic acid content in the pre-hydrolysate. The time-course profile of SSF exhibited a similar biphasic pattern (Fig. 3). In the acidogenic phase, monomeric mannose and xylose accumulated to 8.6 g/L and 6.1 g/L. In the solventogenic phase, although both mannose and xylose were utilized by the culture for solvent production, mannose appeared to be the preferred choice as evidenced by greater consumption rate than the xylose. Similar results about the sugar preference had been previously reported by (Ezeji & Blaschek, 2008b). In the end, SSF produced a total of 13.2 g/L ABE solvent (butanol, 8.3 g/L; acetone, 4.2 g/L), giving a solvent yield and productivity of 0.28 (g-solvent/g-sugar) and 0.14 g $L^{-1}h^{-1}$ (Table 4).

As a well-established detoxification method, activated charcoal treatment had been widely applied to improve the fermentability of lignocellulosic hydrolysates, including steam-treated corn stover (Wang & Chen, 2011), hydrothermo-treated switchgrass (Liu et al., 2015), dilute acid-treated eucalyptus (Villarreal et al., 2006). In the present study, activated carbon treatment substantially removed the phenolic compounds from both types of hemicellulose pre-hydrolysate. Consequently, SSF of charcoal-detoxified pre-hydrolysate achieved a final solvent titer and yield comparable to that of mixed sugar control.

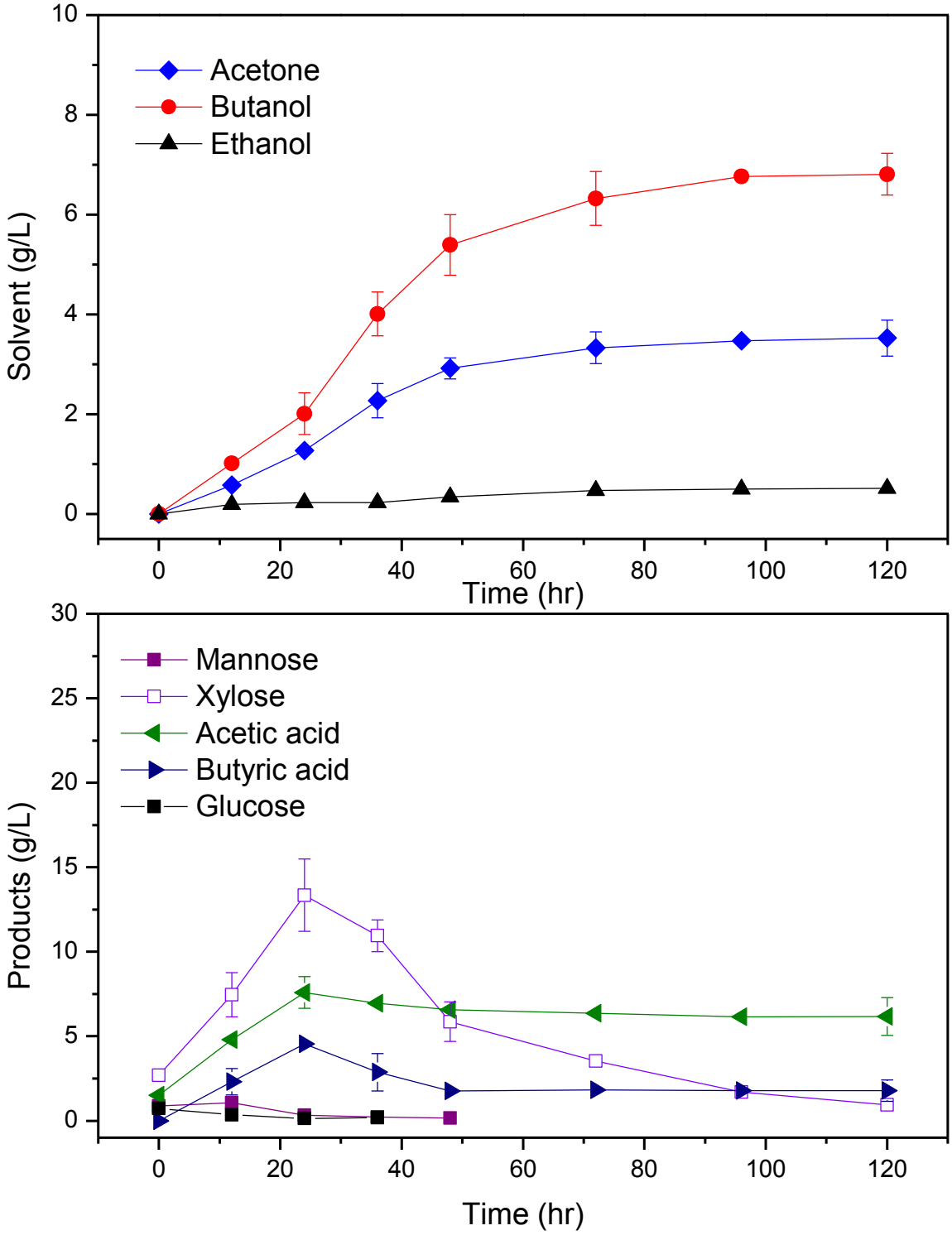

Figure V-2 Simultaneous saccharification and fermentation of activated carbon detoxified hybrid poplar pre-hydrolysate

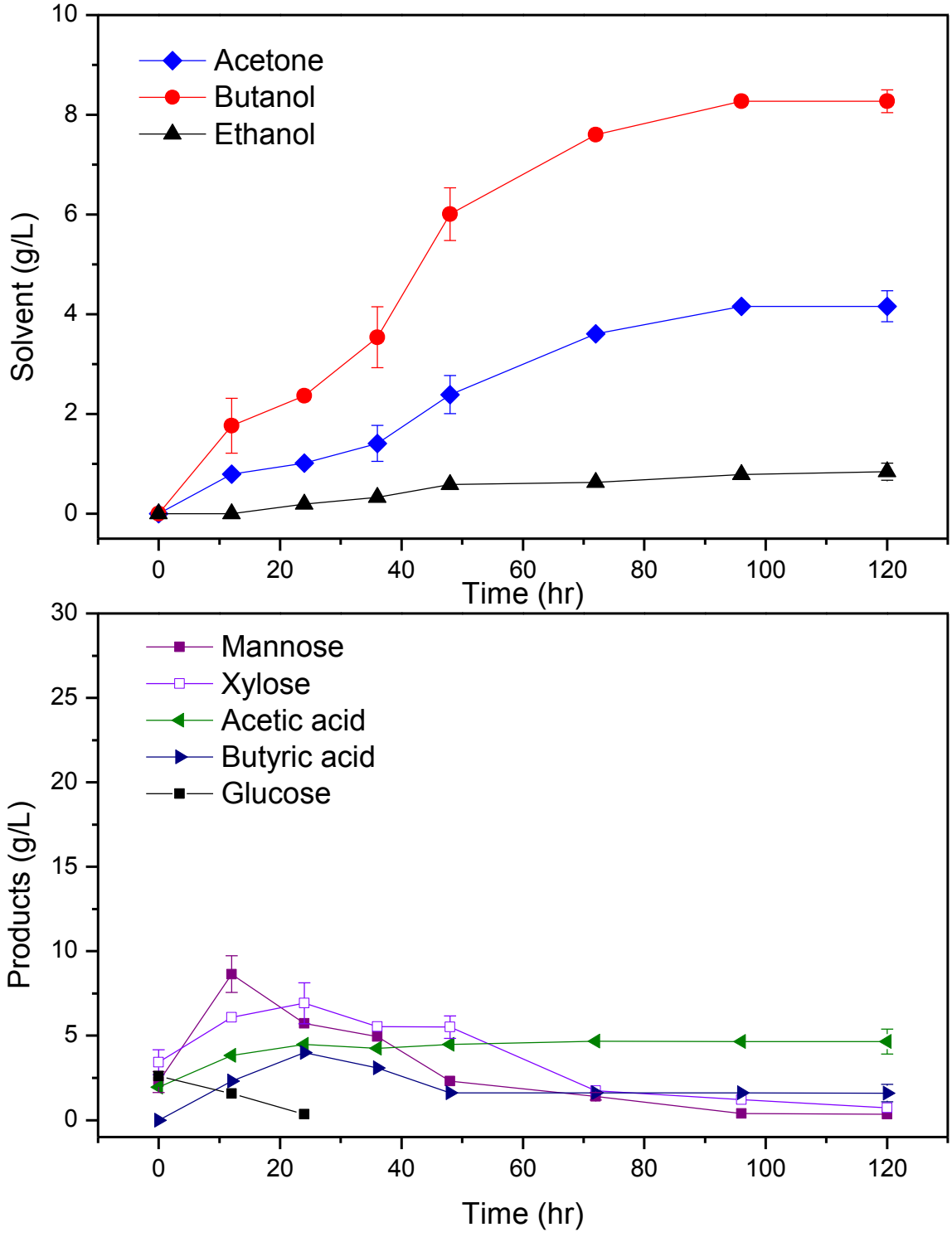

Figure V-3 Simultaneous saccharification and fermentation of activated carbon detoxified southern pine pre-hydrolysate

V.3.5 Discussion

On the premise of not impairing the pulp quality and yield, integration of hemicellulose pre-extraction to the traditional chemical pulping process has been proposed for the forest-based biorefinery (Amidon & Liu, 2009; Hamaguchi et al., 2013; Huang & Ragauskas, 2013; Kang et al., 2012). Hot-water extraction (typically, 150-210 ºC for 30-120 min) selectively removes hemicellulose from the wood while retaining the cellulose portion in the solids. The resulting pre-hydrolysate was primarily composed of xylan-oligosaccharides (hardwood) or mannan-and-xylan oligosaccharides (softwood), which can be enzymatically hydrolyzed into fermentable sugars.

The present study investigated the use of the hemicellulose pre-hydrolysate from hot-water treatment of two types of pulping wood as liquid sugar feedstocks for biobutanol production. A comparison of ABE production in the present study from relevant studies was summarized in Table 5. Due to the presence of toxic compounds (phenolic compounds) in the pre-hydrolysate, SSF of untreated pre-hydrolysate suffered from severe inhibition. Activated charcoal treatment was therefore applied and significantly improved the fermentability of the pre-hydrolysate. SSF of the adsorption-detoxified poplar/pine pre-hydrolysate had produced a total of 10.8 g/L/13.2 g/L of ABE solvent, resulting solvent yield comparable to that of mixed sugar control. Relevant study have reported the use of green liquor extracted hardwood hydrolysate (xylose, 42.7 g/L; glucose, 20.8 g/L) as feedstock for butanol production and compared the effects of various detoxification treatments (overliming, charcoal adsorption and ion-exchange resin adsorption) on the batch fermentation (Lu et al., 2013). In their study, fermentation of untreated pre-hydrolysate result a solvent production of 6.7 g/L whereas a total of

5.8/9.0/11.4 g/L of ABE solvent was produced following the overliming/activated charcoal/ion-exchange resin treatment (Lu et al., 2013). Additionally, (Liu et al., 2015) applied overliming/activated charcoal treatment to the hydrolysate of hydrothermo-pretreated switchgrass and confirmed that activated charcoal adsorption is more effective in coping with phenolic compounds-induced inhibition. Activated charcoal could be thermally or chemically regenerated after detoxification (Villarreal et al., 2006). However, the treatment conditions (e.g., charcoal loading and treatment time) should be further optimized to enable a great reduction of toxic compounds but minimize the carbohydrate loss.

V. 4 Conclusion

The present study demonstrated technical feasibility of utilizing hot-water extracted pre-hydrolysate as feedstock for ABE production. The hemicellulose pre-hydrolysate from both the hardwood poplar and softwood pine could be bio-converted into bio-butanol. Detoxification of the pre-hydrolysate was however required for the reduction of phenolic compounds. Activated charcoal treatment substantially improved the solvent production of 10.8/13.2 g/L for the poplar/pine-derived pre-hydrolysate, corresponding to a solvent yield of 0.25/0.28 g-solvent/g-sugar, which was near to that of mixed sugar control.

Acknowledgement

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VI. Acetone-butanol-ethanol production from Kraft paper mill sludge by simultaneous saccharification and fermentation

Abstract

Paper mill sludge (PS), a solid waste from pulp and paper industry, was investigated as a feedstock for acetone-butanol-ethanol (ABE) production by simultaneous saccharification and fermentation (SSF). ABE fermentation of paper sludge by *Clostridium acetobutylicum* requires partial removal of ash in PS to enhance its enzymatic digestibility. Enzymatic hydrolysis was found to be the rate-limiting step in the ABE production by SSF. A total of 16.4-18.0 g/L of ABE solvents were produced in the SSF of de-ashed PS with solid loading of 6.3-7.4% and enzyme loading of 10-15 FPU/g-glucan, and the final solvent yield reached 0.27 g/g sugars. These levels of solvent in SSF are comparable to those of glucose and cellulose controls, approaching to the upper limits. No pretreatment and pH control are needed in ABE fermentation of paper mill sludge, which makes it an attractive feedstock for production of butanol.

Keywords: paper mill sludge, *Clostridium acetobutylicum,* acetone-butanol-ethanol (ABE), simultaneous saccharification and fermentation (SSF)

VI.1 Introduction

Butanol is an advanced biofuel and a versatile platform chemical usable for synthesis of various industrial chemicals (Durre, 2008; Tashiro et al., 2013). Fermentative production of butanol has failed to compete with the chemical synthesis route from propylene since 1960s (Durre, 2008; Jones & Woods, 1986). Recently, bio-based butanol has been proposed as the next generation biofuel due to its properties superior to ethanol (higher energy density, less corrosiveness, and low vapor pressure) (Anbarasan et al., 2012; Durre, 2008; Tashiro et al., 2013).

The bioprocess for butanol production, however, is challenged by low solvent titer, high cost of feedstock and high energy consumption for solvent recovery. Strong end-product inhibition is known to be the major cause for the low solvent titer and low productivity, which in turn raises the cost of product recovery (Anbarasan et al., 2012; Tashiro et al., 2013). High feedstock cost has been cited as the primary reason for cessation of commercial bioprocess in 1960s (Jones & Woods, 1986). Conventional feedstocks for ABE fermentation are food-based sugars, starch and molasses. Although those substrates have good fermentability, their cost is too high to make it a viable bioprocess for butanol production via fermentation. Lignocellulosic biomass has therefore drawn attention as a feedstock for ABE production (Ezeji & Blaschek, 2008b; Lu et al., 2012; Qureshi et al., 2010a; Qureshi et al., 2010b).

Paper mill sludge, a waste material generated from pulp and paper plants, is one of such with a great potential as it has a number of attractive features as a raw material. Being a waste material, it carries zero or, in some cases, negative cost (elimination of disposal cost). Sludge from common pulping process (Kraft pulping) has low lignin

content. Therefore it does not require pretreatment prior to enzymatic hydrolysis. Pretreatment along with the detoxification of the post-pretreatment effluent is one of the major cost factors in bioconversion of lignocellulosic materials, especially in the case of ABE production from lignocellulose (Ezeji et al., 2007; Palmqvist & Hahn-Hagerdal, 2000; Qureshi et al., 2010a; Sun & Liu, 2012). Furthermore, the short fibers found in most Kraft mill sludges are readily hydrolyzed by enzymes into fermentable sugars (Lark et al., 1997; Marques et al., 2008). A major challenge for sludge as a feedstock is coping with high ash content originated from filler materials (clay, TiO_2, and $CaCO_3$), which severely impede the enzymatic hydrolysis (Lynd et al., 2001; Nikolov et al., 2000). Ash removal is therefore necessary for efficient bioconversion.

Simultaneous saccharification and fermentation (SSF) has been suggested to be more efficient bioconversion strategy than separate hydrolysis and fermentation (SHF) in ethanol production (Alkasrawi et al., 2003; Kadar et al., 2004). The advantages of the SSF over SHF include low equipment cost and alleviation of product inhibition of glucose and cellobiose on cellulase enzymes. Reduction of equipment and operation is achieved by performing enzymatic hydrolysis and fermentation concurrently in a single process. More importantly, SSF achieves high product yield and productivity keeping glucose level, consequently end-product inhibition on enzymatic hydrolysis low, since the sugar is simultaneously consumed by the microbes (Alkasrawi et al., 2003; Linde et al., 2008). SSF has been investigated to produce butanol from corn stover by *Clostridium beijerinckii* P260, however overliming detoxification of dilute acid pretreated substrates and hydrolysates was required (Qureshi et al., 2014). Shah et al. (1991) identified the effect pretreatment on converting hardwood substrates to butanol in a SSF process, they

revealed that both glucose and xylose could be utilized simultaneously by *C. acetobutylicum* ATCC 824 (Shah et al., 1991b). Calcium carbonate ($CaCO_3$) has been found to increase butanol yield significantly in a SSF process by enhancing the buffer capacities and the activities of NAD(P)H-dependent butyraldehyde and butanol dehydrogenases (Li et al., 2015). Since paper mill sludge in this study is a pretreated material from Kraft pulping, which contains mainly glucan, xylan and ash ($CaCO_3$), we hypothesize that both glucan and xylan in paper mill sludge can be directly converted into butanol in a SSF process.

Paper mill sludge has been evaluated as a feedstock for ethanol and lactic acid production, and results have been positive (Budhavaram & Fan, 2009b; Kang et al., 2011; Lark et al., 1997; Marques et al., 2008). It is, however, yet to be studied for butanol production. The objective of this study is to investigate the technical feasibility of bioconversion of paper mill sludge into acetone-butanol-ethanol (ABE) through simultaneous saccharification and fermentation (SSF). The main technical issues of our interest are to assess the effects of enzyme loading and solid loading on the SSF and to see how the results from the sludge compared with those of other biomass feedstocks.

VI.2 Materials and method

VI.2.1 Feedstock, enzyme and microorganism

Recycled Kraft paper mill sludge, collected from Boise Cascade (Jackson, AL), was used as feedstock for ABE production. Cellulase Cellic® C-Tec 2 (Batch No. VCNI0001) was a kind gift from Novozymes, North America (Franklinton, NC). The protein content and specific activity for C-Tec2 were 255 mg protein/mL and 119

FPU/mL. *Clostridium acetobutylicum* ATCC-824™ (Lot NO. 58727357) was purchased from American Type Culture Collection (ATCC). Avicel PH-101 was purchased from Sigma-Aldrich (St. Louis, Mo.). Switchgrass was provided by Ceres Inc. (Thousand Oaks, CA). Switchgrass was pretreated by soaking in 2 % (w/w) sodium hydroxide solution at 60 °C for 24 h. The liquid-to-solid ratio in switchgrass pretreatment was 9:1. After pretreatment, the solids were washed with water until the pH reached 6.0 before subjecting to further analysis. The chemical compositions of paper mill sludge and switchgrass were determined according to the NREL standard procedure (NREL/TP-510-42618). The solid composition of NaOH pretreated switchgrass was analyzed to contain 54.1% of glucan, 23.8% of xylan, 10.4% of lignin and 6.2% of ash.

VI.2.2 Ash removal of paper mill sludge

The ash in paper mill sludge was partially removed as previously described (Kang et al., 2011). Briefly, the sludge was first suspended in DI water at 3% (w/w) consistency and blended with bench-top stirrer (IKA® RW16, Germany) for 30 min. It was then dewatered by filtering through a 100-mesh screen. The dewatered sludge was dried at 45 °C until the moisture content reached below 10% for further processing and sample analysis. Multiple de-ashing cycles was applied to reduce the ash content of sludge to a level (6.1% ash) that can achieve acceptable saccharification rate. PS2 indicates the paper sludge de-ashed by two cycles of washing. PS3, PS4, etc. are defined accordingly.

VI.2.3 Culture maintenance and inoculum preparation

The stock of *C. acetobutylicum* was maintained as spores at -20 °C in Elliker broth (BD Difco™) supplemented with 20 % (v/v) of glycerol. The spores were revived

by inoculating one loop of the spore suspension in the Reinforced Clostridial HiVeg™ Broth (HiMedia Laboratories, India) and anaerobically incubated for 24 h at 36 °C. The inoculum was prepared by transferring 1 mL of actively growing culture into 50 mL of P2 medium supplemented with 10 g/L of glucose in a 100 mL screw-capped Pyrex bottle and anaerobically incubated for 24 h. The P2 medium formula was as follows (g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; NaCl, 0.01; ammonium acetate, 2.2; yeast extract, 1.0.

VI.2.4 Enzymatic hydrolysis of paper mill sludge

Enzymatic hydrolysis was performed to estimate the digestibility of de-ashed paper mill sludge. The experiment was carried out in batch mode using 125 ml Erlenmeyer flasks for 120 h under 50 °C and 150 rpm. Sodium citrate (0.05 M, pH 4.8) was used as buffer and 0.01% (w/v) of sodium azide was used as biocide to prevent microbial contamination. To assess the effect of ash content on enzymatic hydrolysis, the de-ashed paper mill sludge (4% glucan) was hydrolyzed with cellulases under an enzyme loading of 10 FPU/g glucan.

VI.2.5 ABE fermentation with glucose and xylose as substrates

ABE fermentation with glucose (44.8 g/L) and xylose (16.1 g/l) as substrates was carried out in 125 mL serum bottle with a working volume of 50 mL. P2 medium was used as inorganic mineral fermentation nutrients. The initial pH of the fermentation broth was adjusted to 6.7 with calcium carbonate (5.0 g/L). The fermentation bottle was flushed with nitrogen gas for 5 min and crimp-sealed with rubber stopper. The bottles were loaded with sugars and fermentation medium, autoclaved at 121 °C for 15 min,

inoculated with the actively growing seed-culture at 5 % (v/v). Fermentation was carried out at 36 °C, 150 rpm, and under strict anaerobic condition. Aliquots of samples were taken with12-h interval.

VI.2.6 Simultaneous saccharification and fermentation (SSF) of de-ashed paper mill sludge, Avicel and alkali-pretreated switchgrass

SSF of paper mill sludge was performed at 36 °C and 150 rpm in a 125 mL serum bottle containing 50 mL of P2 medium with 3.8-7.4% of PS7. Anaerobic condition was developed by sparging nitrogen gas into the headspace of the bottle. The sealed bottles were autoclaved at 121 °C for 15 min. Cellulase enzyme (C-Tec2) was added after sterilization. Different solid loading of PS7 (3.8, 5.0, 6.3 and 7.4% w/v) was compared in SSF processes, which are equivalent to 36.6, 48.6, 60.6, 72.7 g/L sugars (glucose and xylose) given the carbohydrates were completely hydrolyzed. For each solid loading, three enzyme loadings were applied: 5, 10 and 15 FPU/g-glucan. For the solid loadings of 3.8% and 5.0%, *C. acetobutylicum* culture was inoculated at the same time as enzyme addition. For the solid loadings of 6.3% and 7.4%, there was insufficient fluidity to perform fermentation due to limited level of free water available in the beginning, the culture was inoculated after 12 h enzymatic hydrolysis, at which point the substrate was partially liquefied. Aliquots of samples were taken at 24, 48, 72, 96, and 120 h. SSF of Avicel and alkali-pretreated switchgrass were conducted under the same conditions.

VI.2.7 HPLC analysis

The enzymatic digestibility of de-ashed paper mill sludge was calculated from the released glucose content, as a percentage of the theoretical sugars available in the

substrates. The released glucose and xylose content during enzymatic hydrolysis were quantitated by HPLC with Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA). The fermentation product in this study was analyzed by HPLC equipped with refractive index detector (Shodex, Japan) and Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). The solvent yield was calculated on weight basis as the amount of ABE formation divided by the total sugars in the feed.

VI.2.8 SEM image analysis

The surface morphology of de-ashed sludge sample was examined with scanning electron microscopy (Carl Zesis, Model EVO-50, Thornwood, NY). For SEM examination, the samples were sputter-coated with gold using EMS 550X Sputter Coating Device (Electron Microscopy Sciences, Hatfield, PA). The SEM images were taken with1.0 and 3.0 kX magnifications under 20.0 kV beam.

VI.3 Results and discussion

VI.3.1 Chemical composition and enzymatic digestibility of de-ashed paper mill sludge

The chemical composition and enzymatic digestibility of unwashed and de-ashed paper mill sludges were determined (Table 1). Glucan (51.8%) and xylan (12.6%) were the two major organic components in the unwashed paper mill sludge. The ash content was as high as 32.7%. Ash in paper mill sludge appeared to inhibit enzymatic hydrolysis significantly (Table 1). After each cycle of water washing, the ash content in paper mill sludge was reduced gradually to 25.1%, 20.5% 12.3% and 6.1% in PS2, PS4, PS6 and PS7, respectively. Correspondingly, the glucan digestibility increased significantly from

0 in unwashed PS to 0.2%, 6.4%, 65.4% and 74.1% in PS2, PS4, PS6 and PS7, respectively. There is a strong inverse association (r^2=0.85) between ash content and glucan and xylan digestibility in paper mill sludge. SEM images (Fig.1) revealed clean and smooth surface of the washed sludge, contrasting with the rough surface with small particles attached on the surface of unwashed sludge. However, the de-ashing also led to considerable loss of short fibers. For example, about 24-25% of glucan was washed away in PS6 and PS7. The results indicated that de-ashing by water washing is critically needed for improving the glucan digestibility of paper mill sludge, but minimizing short fiber loss should be considered as well.

Table VI-1 Chemical composition and enzymatic digestibility of paper mill sludge

Component (%)	Unwashed	PS2[a]	PS4[a]	PS6[a]	PS7[a]
Glucan	51.8	55.5	59.4	66.4	71.8
Xylan	12.6	13.2	13.6	14.8	15.2
Mannan	1.5	1.3	1.5	1.1	1.7
Ash	32.7	25.1	20.5	12.3	6.1
Mass Closure	98.6	95.1	94.9	94.5	94.8
Solid Recovery (%)	-	85.3	68.1	59.1	54..0
Glucan Loss (%)	-	8.5	22.0	24.3	25.0
Xylan Loss (%)	-	11.1	27.0	31.0	34.9
Ash Removal (%)	-	34.6	57.5	77.7	90.0
Solid Loading[b] (%)	8.1	7.5	7.0	6.3	5.7
pH	7.4	7.4	6.4	5.5	5.3
Glucan Digestibility[c] (%)	0	0.2	6.4	65.4	74.1
Xylan Digestibility[c] (%)	0	0	9.2	71.7	94.3

a. In each de-ashing cycle, the paper mill sludge slurry was blended for 30 min at 3% (w/w) consistency and de-ashed sludge was collected by filtrating the slurry with a 100 mesh sieve. For the reference, PS4 was the de-ashed sample after 4-times of washing.

b. The enzymatic hydrolysis was evaluated for a given glucan loading of 4 % (w/v) and an enzyme loading of 10 FPU/g-glucan.

c. Enzymatic digestibility was based on 120 h.

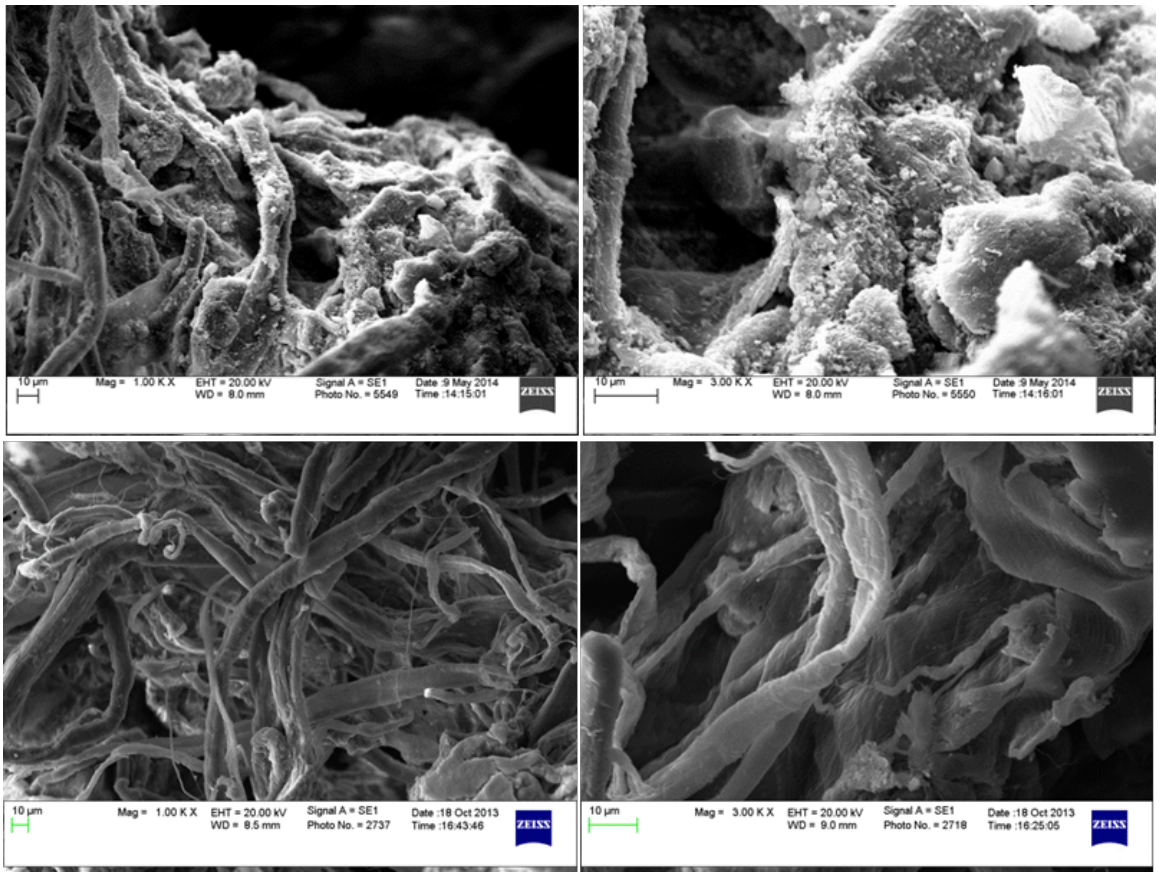

Figure VI-1 SEM images of pulp mill sludge before and after washing. The top two represent the untreated sludge sample pictured at 1000 X and 3000 X magnifications; The bottom two represent the de-ashed sludge (7 times washed) pictured at 1000 X and 3000 X magnifications.

Ash in the sludge is composed of inorganic salts, calcium carbonate being the predominant component. The presence of calcium carbonate can dramatically increase the pH of paper mill sludge suspension in water or buffer. The buffer pH changed from 4.8 with unwashed PS to 7.4, 7.4, 6.4, 5.5 and 5.3 with PS2, PS4, PS6 and PS7, respectively. The change of pH in solution has a significant impact on cellulase activity.

Wang et al. (2005) previously showed the specific activity of endoglucanase III decreased more than 90% at pH 7.0 (Wang et al., 2005). This negative effect of pH increase on enzyme was amplified by the high temperature in the enzymatic hydrolysis. Kang et al. (2010) observed enzymatic hydrolysis at higher temperature (50 °C) resulted in much lower glucan digestibility of primary paper mill sludge as comparing to the hydrolysis at 37 °C (Kang et al., 2010).

VI.3.2 Simultaneous saccharification and fermentation of Avicel, alkali-pretreated switchgrass and de-ashed paper mill sludge

SSF of Avicel, alkali-pretreated switchgrass and de-ashed paper mill sludge were compared and fermentation of mixed sugars (glucose and xylose) was used as a control. Mixed sugars fermentation showed both glucose and xylose were consumed and final solvent concentration reached 17.3 g/L (Fig. 2) and butanol was 10.4 g/L. At the end of fermentation (120), small amount of glucose (3.3 g/L) and xylose (2.1 g/L) were not utilized. ABE fermentation of mixed sugars gave the yield of 0.31(g/g sugars).

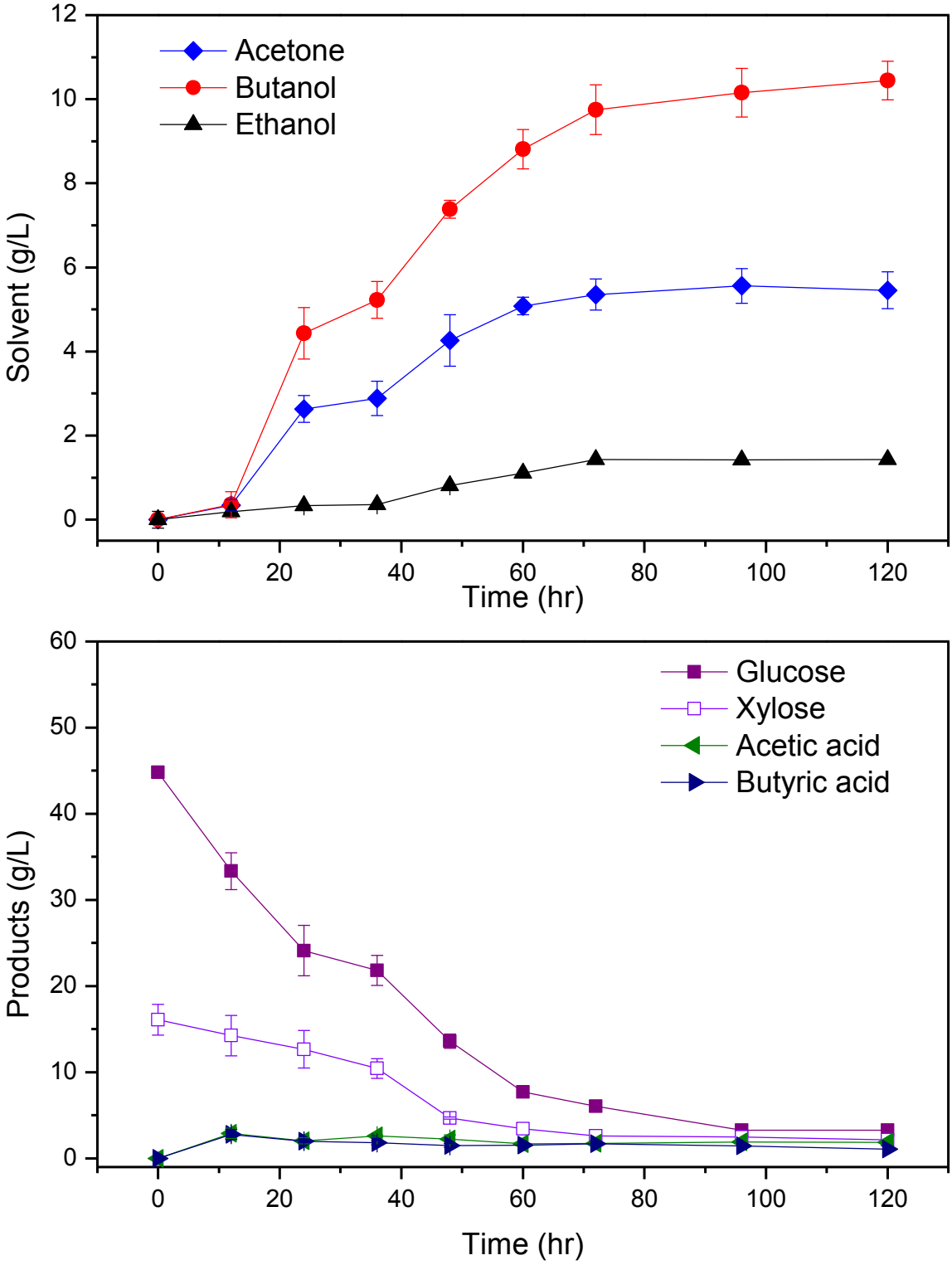

Figure VI-2 ABE fermentation of mixed sugar (44.8 g/L of glucose and 16.1 g/L of xylose).

SSF of Avicel was performed with a solid loading of 5.8% (w/v) and enzyme loading of 20 FPU/g-glucan (Fig. 2a). During the initial 24 h, glucose was quickly released to 12.0 g/L, and acetic and butyric acids were accumulated to 2.9 and 3.2 g/L, respectively. After that, glucose concentration decreased and more acids were re-assimilated to butanol, acetone and ethanol. Acetic and butyric acids were stabilized at 1.9 and 1.2 g/L after 72 h. At the end of SSF (120 h), ABE total solvents reached 16.8 g/L with 9.5 g/L of butanol. The ABE solvent yield was 0.28 (g/g glucose) and the productivity was 0.14 g/L/h.

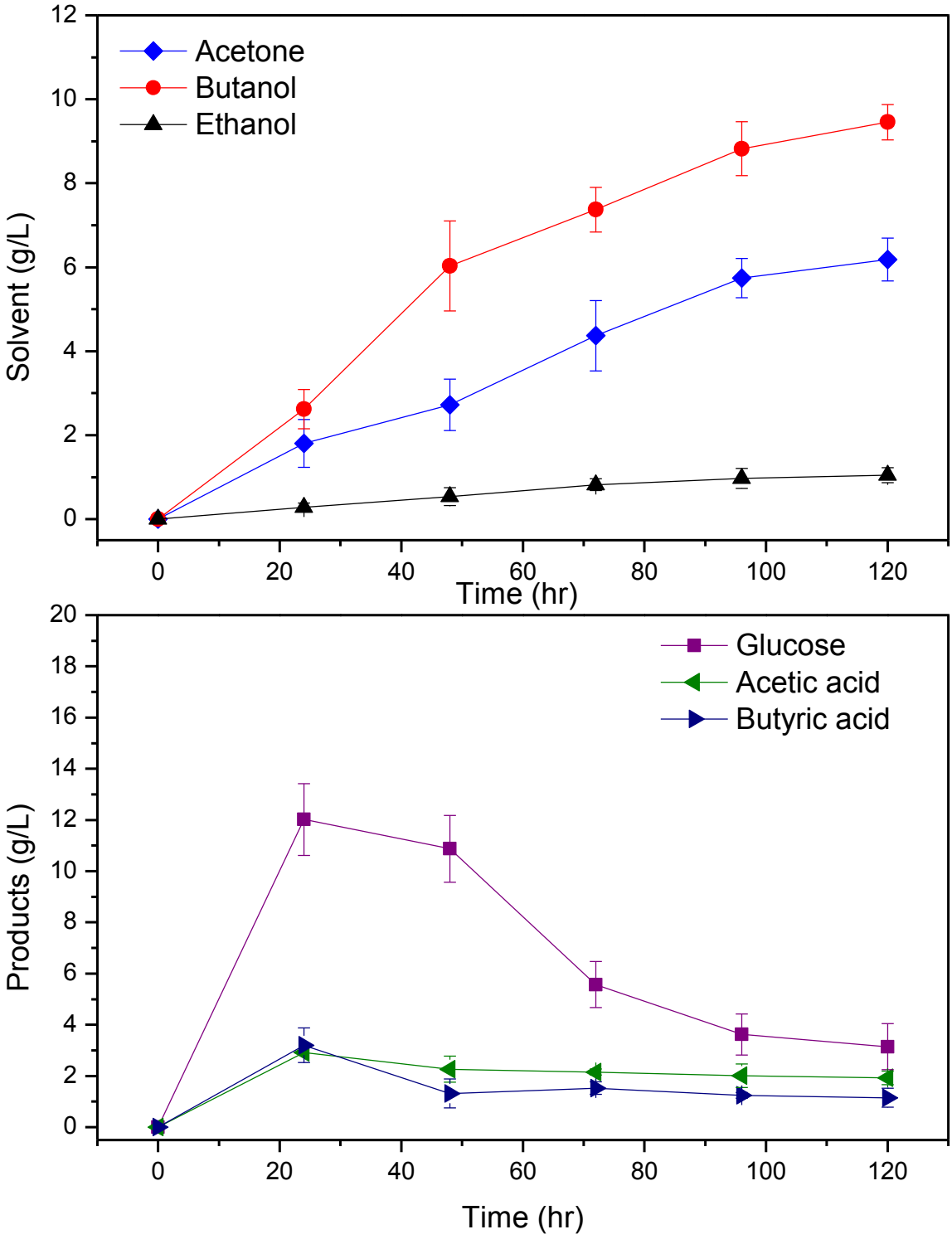

Figure VI-3 Simultaneous saccharification and fermentation of Avicel

SSF of alkali-pretreated switchgrass was performed with a solid loading of 5% and enzyme loading of 15 FPU/g-glucan (Fig. 4). In the first 24 h, glucose (10 g/L) and xylose (1.0 g//L) was released, small amount of acetic and butyric acids (2 g/L) was produced. No solvents were produced until 48 h, but the solvents production quickly stopped at 72 h. The final total solvents was very low (3 g/L). More than 9 g/L of glucose was present in the solution from 24 to 120 h. This indicated microbes could not actively consume sugars in the SSF of Alkali-pretreated switchgrass, and enzyme hydrolysis was not a rate-limiting step in SSF. It was most likely that residual lignin (10.4%) and other degradation compounds in alkali-pretreated switchgrass inhibited the microbial fermentation step. Similar results have been reported on converting dilute acid pretreated corn stover to butanol in a SSF process (Qureshi et al., 2014), they found the pretreated corn stover still contained toxic chemicals that inhibited cell growth and fermentation activity. A further detoxification of pretreated solid was required to improve the ABE yield.

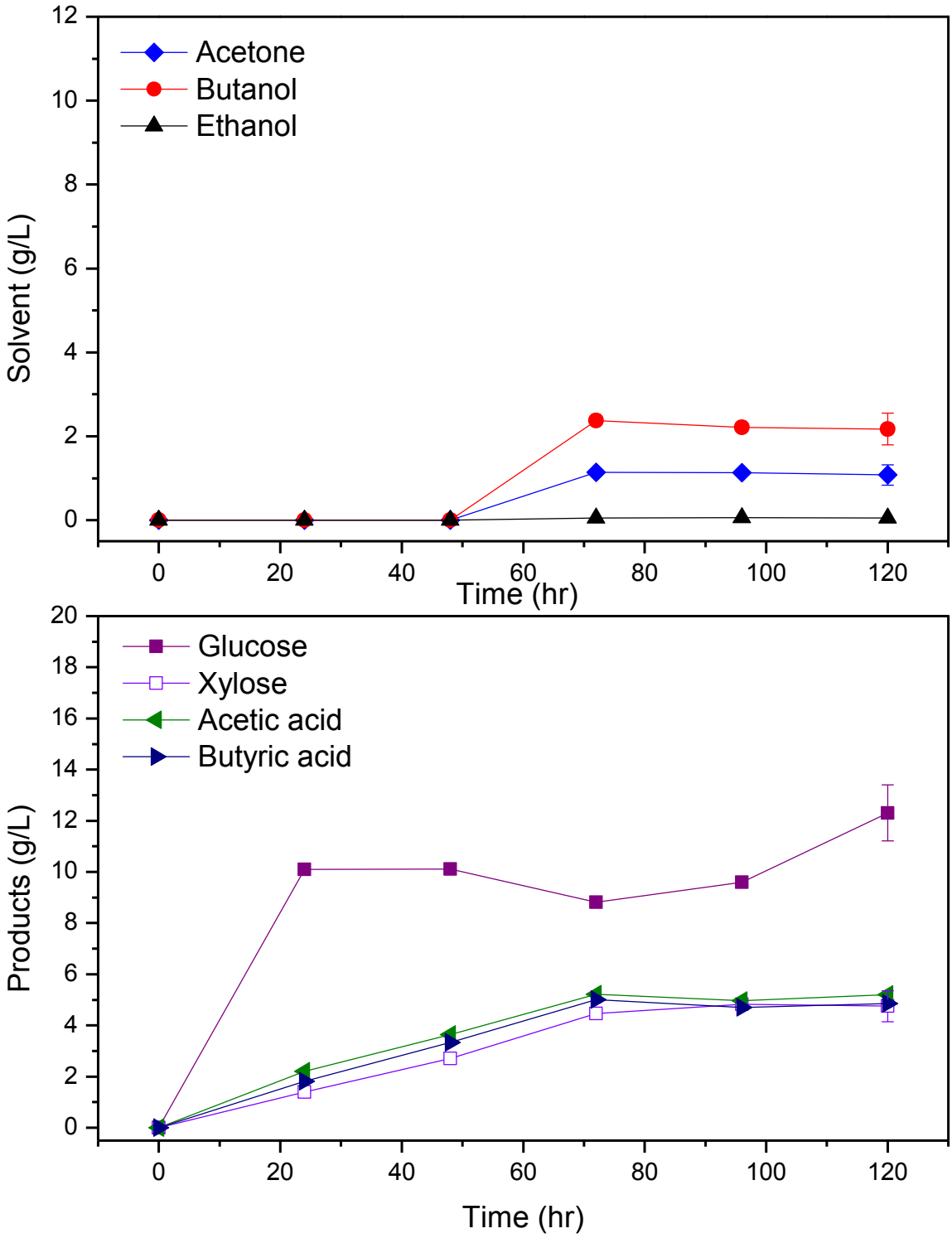

Figure VI-4 Simultaneous saccharification and fermentation of alkali-pretreated switchgrass

In contrast to alkali-pretreated switchgrass, SSF of de-ashed paper mill sludge (7.4% solid loading and 10 FPU) showed very good ABE solvents yield (Fig. 5). Specifically in the first 24 h, glucose and xylose reached 10 and 6 g/L, respectively. Both acetic and butyric acids reached 4.0 g/L, respectively. After 24 h, the glucose concentration continued to increase and reached at 16.1 g/L at 48 h, then decreased gradually to 5.0 g/L at 120 h as SSF progressed. The xylose concentration was kept at 6 g/L from 24 to 120 h. Butanol was steadily produced from 24 to 120 h and reached 9.7 g/L at the end of fermentation. The total ABE solvent concentration was 17.1 g/L, the solvent yield was 0.24 (g/g sugars) and the productivity was 0.20 g/L/h. As comparing to the SSF of Avicel, the total acids concentration (5.7 g/l) was significantly higher than that in SSF of de-ashed sludge. Most likely, it is due to the buffering effect of ash in paper mill sludge. The culture tends to produce more acids to maintain the medium pH near its optimum for solvent production. Most of the solvent-producing cultures used for ABE production has shown the tendency to produce high level of acids when it comes to the fermentation of the hydrolysates of lignocellulosic substrate (Qureshi et al., 2010b; Sun & Liu, 2012).

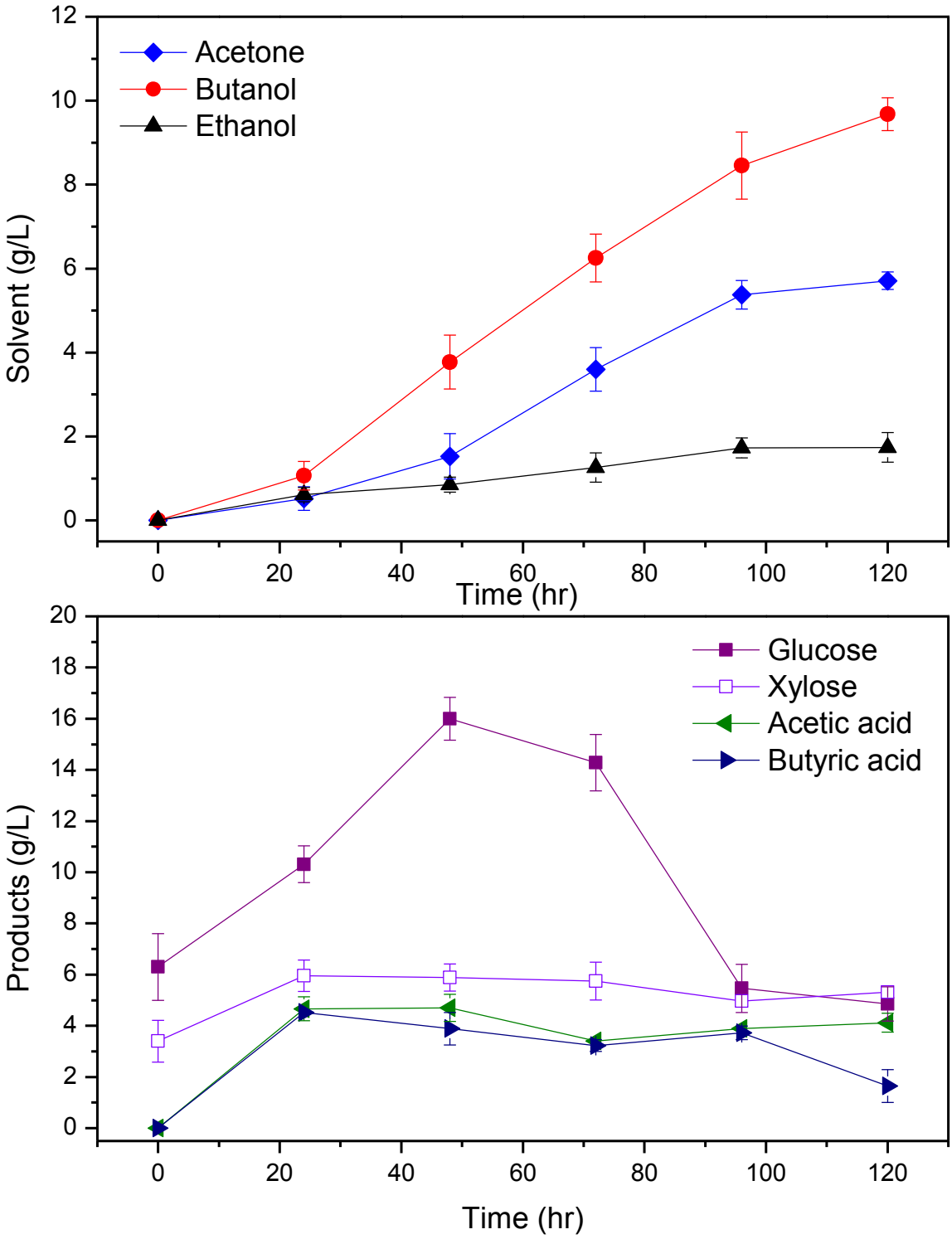

Figure VI-5 Simultaneous saccharification and fermentation (SSF) of PS7 under the solid loading of 7.4% and enzyme loading of 10 FPU/g-glucan).

VI.3.3 Effect of enzyme loading on SSF of de-ashed paper mill sludge

To examine the effect of enzyme loading on ABE production, SSF of PS7 was performed (3.8% solid loading) with different enzyme loading (5, 10, and 15 FPU/g-glucan) (Fig. VI-6a-c). Under 5 FPU, the glucose and xylose were released in a small amount (1 g/L) in the solution from 24 to 96 h (Fig. VI-6a). At 120 h, the glucose surged to 5.2 g/L. However, the low glucose concentration did not appear to inhibit the butanol production. Butanol increased from 0.3 g/L at 24 h to 5.3 g/L at 120 h. At the end of fermentation, the total solvent reached 7.4 g/L, the solvent yield was 0.20 g/g sugars and the productivity was 0.062 g/L/h.

When the enzyme dosage was increased to 10 FPU, about 2.3 and 0.40 g/L of glucose and xylose were released in the solution at 24 h. After that, both glucose and xylose concentration were kept less than 2 g/L until 120 h. The butyric acid first increased to 4.7 g/L at 24 h, and decreased gradually to 2.2 g/L at 120 h. The acetic acid increased to 4.5 g/L and was kept at the same level until 120 h. The butanol concentration increased linearly and reached 6.8 g/L at 120 h. The total solvent concentration reached 10.6 g/L, the solvent yield was 0.29 g/g sugars and the productivity was 0.088 g/L/h. As comparing to the SSF process at 5 FPU, both solvent yield and the productivity increased by 45%. This indicated enzymatic hydrolysis was a rate-limiting step in SSF process. It should be noted that xylose was also consumed in the SSF process; this agreed well with previous reports on *C. acetobutylicum* being able to ferment xylose to butanol as well (Ezeji & Blaschek, 2008b; Gao et al., 2014; Ounine et al., 1985).

When the enzyme loading was increased 15 FPU, there was no further improvement on the total solvent concentration. In the first 24 h, 2.8 and 1.8 g/L of

glucose and xylose were released in the solution. The glucose concentration continued to increase and reached 6.2 g/L at 48 h. After that, glucose decreased gradually and reached 0.1 g/L at 120 h. Xylose decreased quickly to less than 0.5 g/L and was kept at the same level until 120 h. The total solvent concentration reached 10.6 g/L, the solvent yield was 0.29 g/g sugars and the productivity was 0.088 g/L/h, which were similar to those in 10 FPU. This indicated that 10 FPU was sufficient in converting of paper mill sludge to butanol at 3.8% of solid loading.

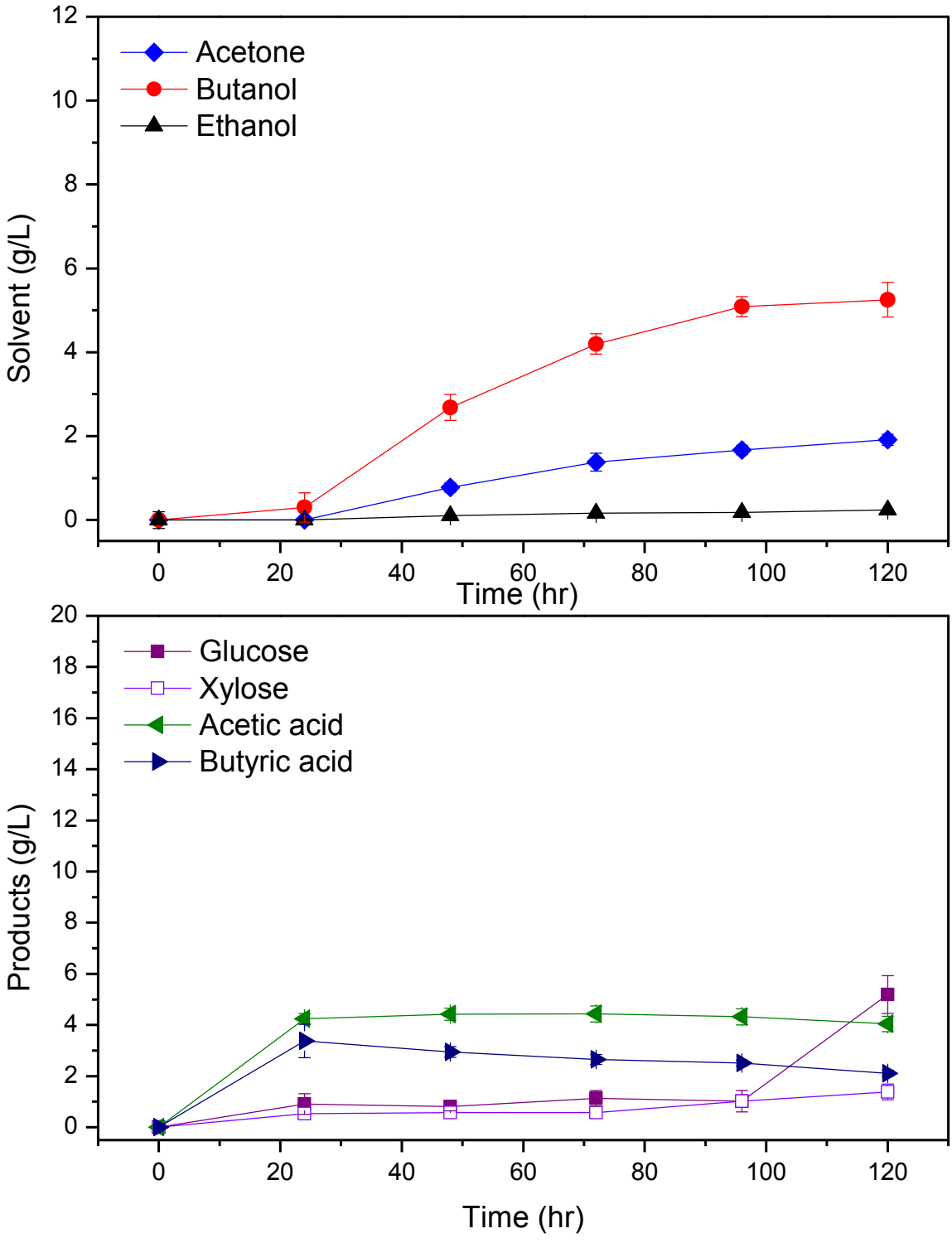
Acetone
Butanol
Ethanol
Solvent (g/L)
Time (hr)
Glucose
Xylose
Acetic acid
Butyric acid
Products (g/L)
Time (hr)

Fig. VI-6a.

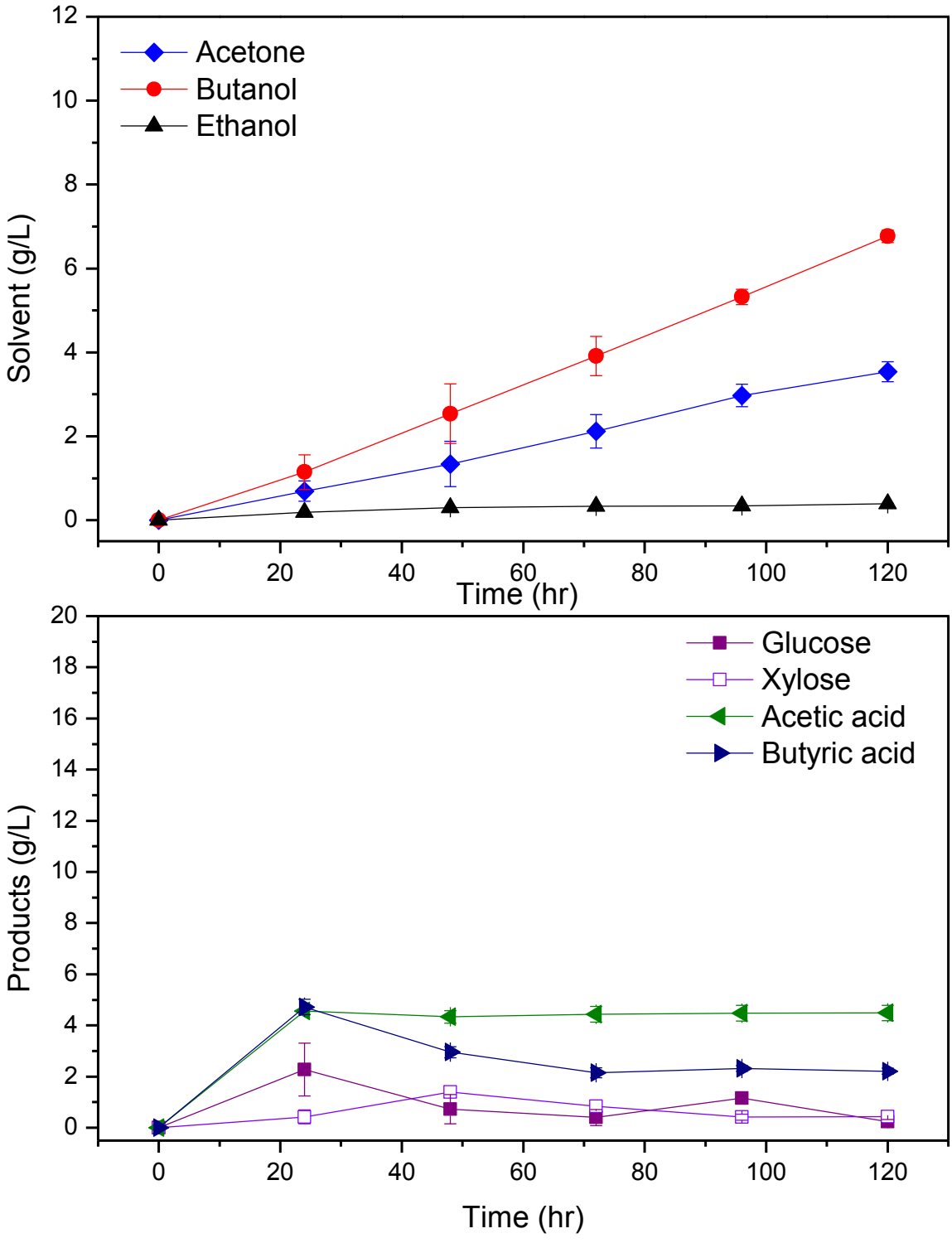
Acetone
Butanol
Ethanol
Solvent (g/L)
Time (hr)
Glucose
Xylose
Acetic acid
Butyric acid
Products (g/L)
Time (hr)

Fig. VI-6b.

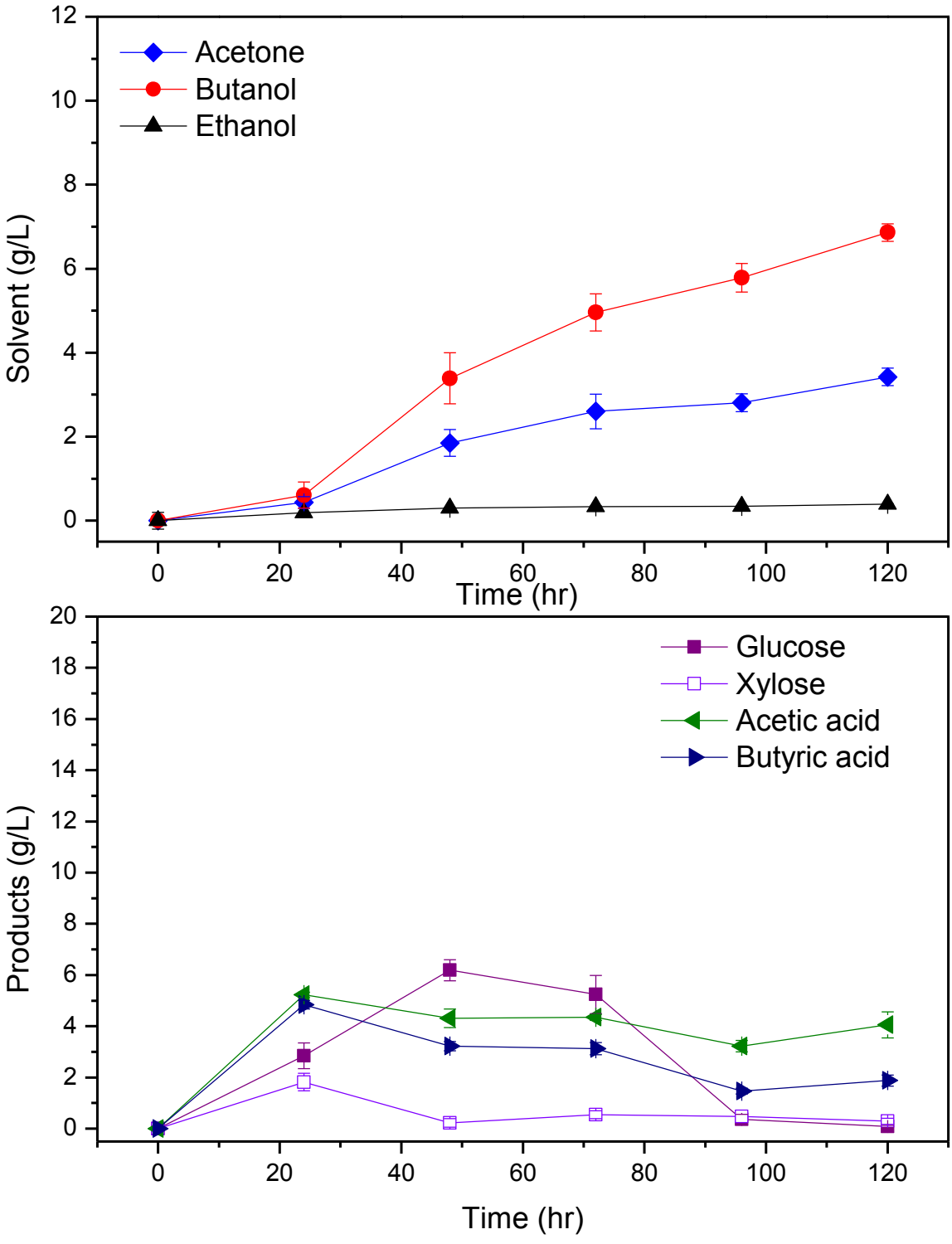

Figure VI-6c Effect of enzyme loading on simultaneous saccharification and fermentation (SSF) of PS7 under 3.8% of solid loading (a: 5 FPU/g-glucan, b: 10 FPU/g-glucan, c: 15 FPU/g-glucan).

VI.3.4 Effect of solid loading on the SSF of de-ashed paper mill sludge

To assess the effect of solid loading on ABE production, SSF of PS7 was performed under 15 FPU and different solid loading (5.0, 6.3 and 7.4%). At 6.3% solid loading and above, the slurry exhibited extremely high viscosity, which limited the nutrients transportation. Hence, a pre-hydrolysis was performed to liquefy the slurry before culture inoculation.

When the 5.0 % solid loading was used in SSF, glucose (8.2 g/L) and xylose (3.7 g/L) were quickly released in the solution at 24 h. After that, glucose further increased to 9.5 g/L at 48 h, and then decreased gradually to 0.4 g/L at 120 h. Xylose concentration were kept around 3.5 g/L until 120 h. The butyric acid first increased to 3.3 g/L at 24 h, and decreased gradually to 1.6 g/L at 120 h. The acetic acid increased to 3.7 g/L at 24 h and was kept at 3.0-3.7 until 120h. The butanol production was initiated early and reached 2.2 g/L at 24 h. At the end of fermentation, the total solvent concentration reached 14.5g/L, the solvent yield was 0.30 g/g sugars and the productivity was 0.12 g/L/h. When the solid loading increased to 6.3%, about 12.7 g/L of glucose and 5.3 g/L of xylose were released in the solution at 24 h. Glucose continued to increase and reached 15.4 g/l at 48 h. After that, glucose decreased quickly to 1.5 g/L from 48 to 120 h. Xylose decreased gradually to 2.3 g/L at 120 h. Butyric acid reached 3.7 g/L at 24 h, then decreased slowly to 1.8 at 120 h. Acetic acid reached 3.7 g/L at 24 h and was not changed until 120 h. At the end of fermentation, the total solvent concentration reached 16.3 g/L, the solvent yield was 0.27 g/g sugars and the solvent productivity was 0.14 g/L/h.

When the solid loading increased to 7.4, about 14.5 g/L of glucose and 6.7 g/L of xylose were released in the solution at 24 h. Glucose continued to increase and reached

18.0 g/L at 48 h. After that, glucose decreased quickly from 17.0 to 1.5 g/L during the period of 72-120 h. Xylose decreased slowly from 6.7 to 4.3 g/L at 120 h. Butyric acid reached 4.1 g/L at 24 h, then decreased to 1.8 at 120 h. Acetic acid reached 4.1 g/L at 24 h and was not changed until 120 h. At the end of fermentation, the total solvent concentration reached 18.0 g/L, the solvent yield was 0.275g/g sugars and the solvent productivity was 0.15 g/L/h. Comparing the solvent yield at different solid loading, higher solid loading resulted in lower solvent yield. It was most likely due to the decreasing hydrolysis yield at higher solid loading, which has been documented as the "solids effect" (Jorgensen et al., 2007; Kristensen et al., 2009).

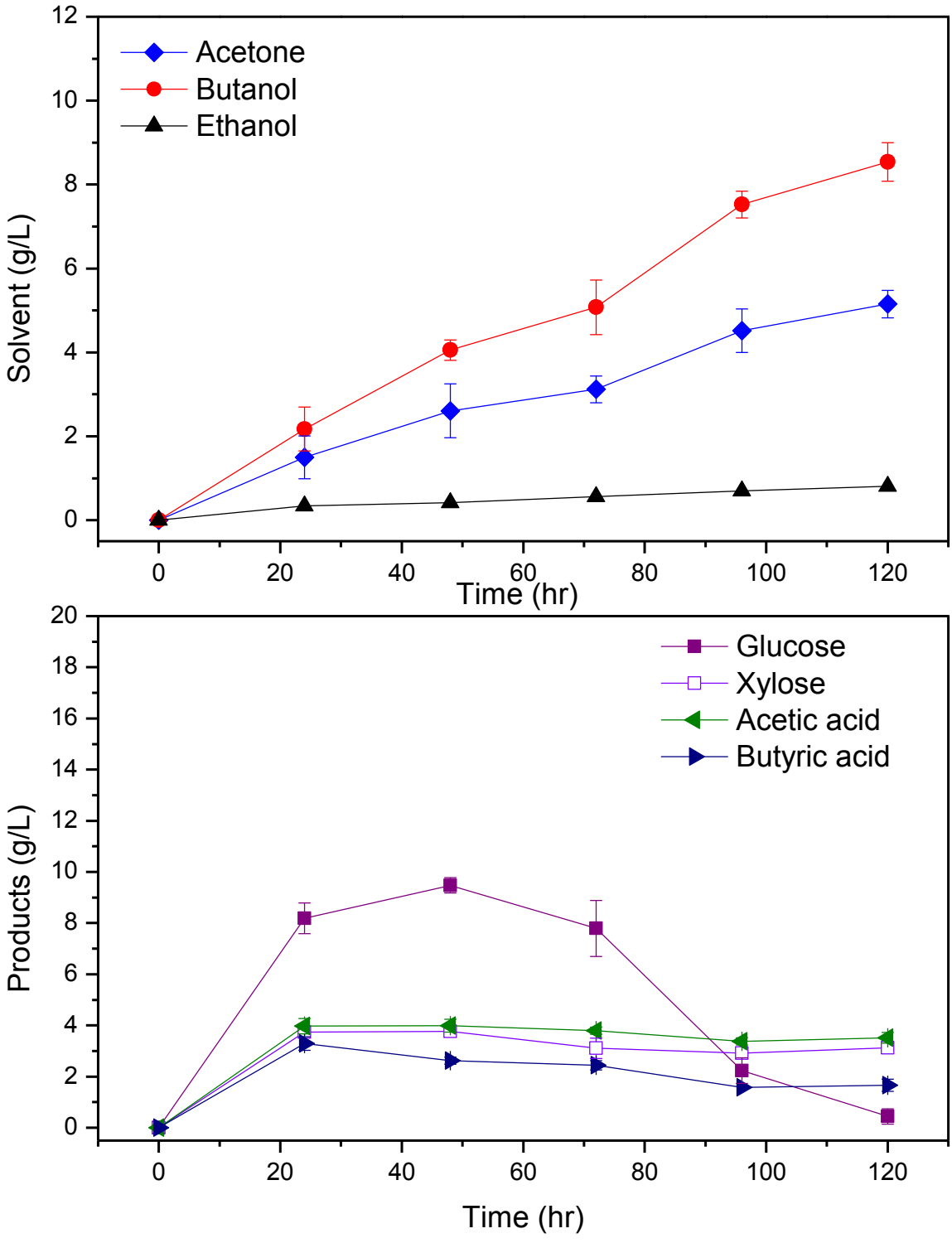
Acetone
Butanol
Ethanol
Solvent (g/L)
Time (hr)
Glucose
Xylose
Acetic acid
Butyric acid
Products (g/L)
Time (hr)

Fig. VI-7a.

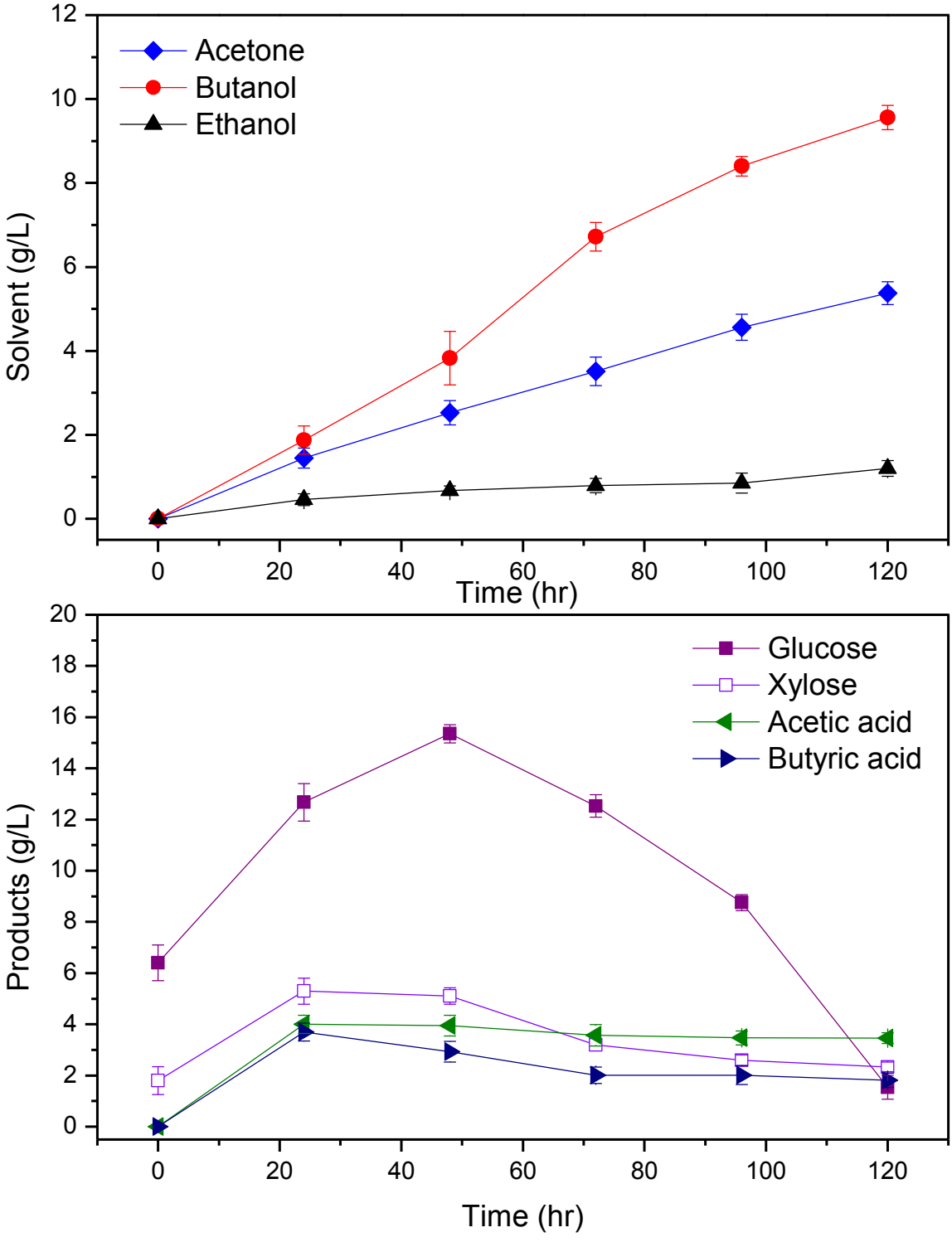
Acetone
Butanol
Ethanol
Solvent (g/L)
Time (hr)
Glucose
Xylose
Acetic acid
Butyric acid
Products (g/L)
Time (hr)

Fig. VI-7b.

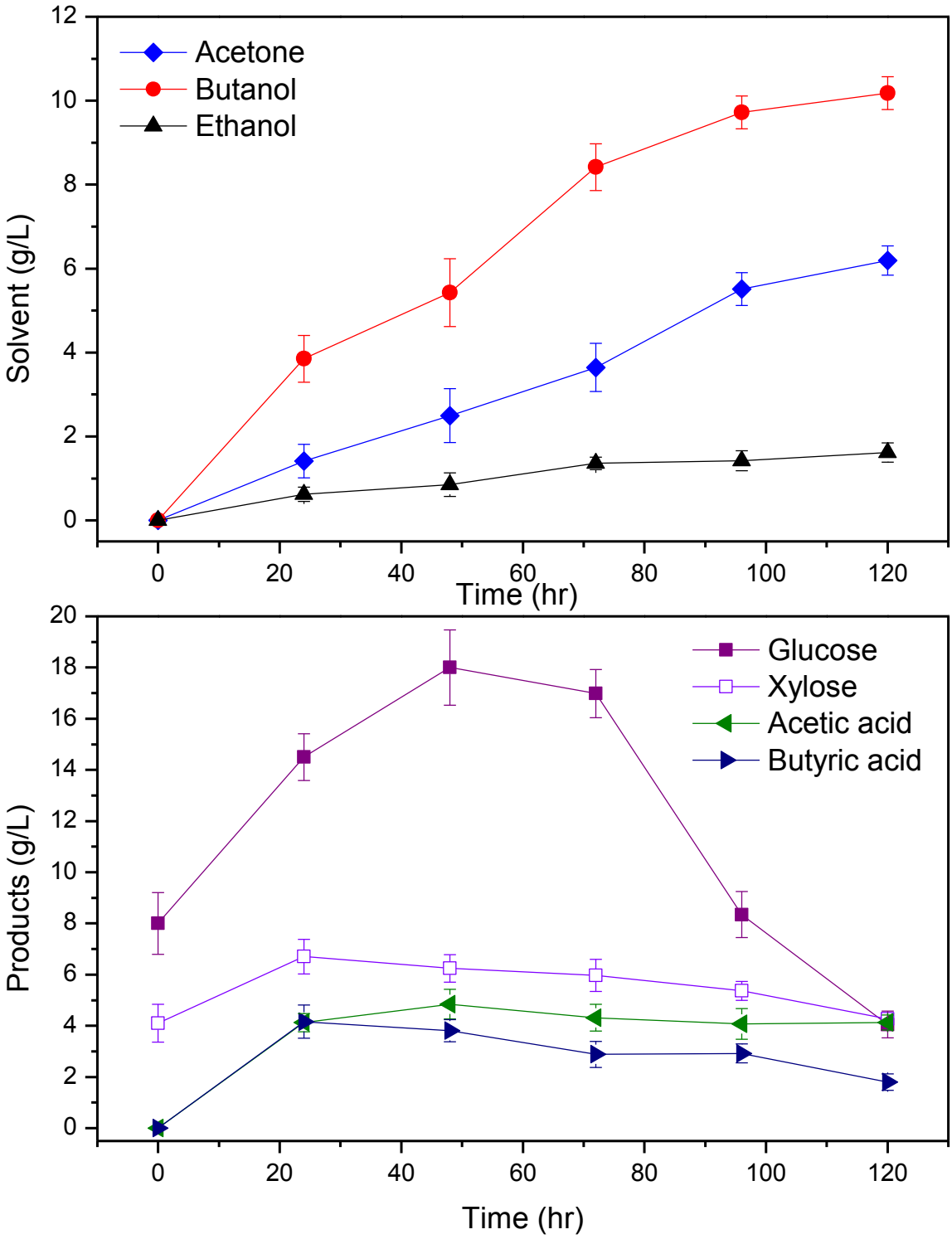

Figure VI-7 Effect of solid loading on simultaneous saccharification and fermentation (SSF) of PS7 under different solid loadings applying enzyme loading of 15 FPU/g-glucan (a: 5.0% solid loading, b: 6.3% solid loading, c: 7.4% of solid loading).

VI.3.5 Discussion

Paper mill sludge was utilized directly as feedstock for butanol production via simultaneous saccharification and fermentation. Effects of solid loading and enzyme loading on SSF of PS7 were compared (Table 2). Under a solid loading of 6.3-7.4% and enzyme loading of 10-15 FPU/g-glucan, a total of 16.4-18.0 g/L of ABE solvent was produced, which is comparable to that of the control test (pure cellulose). In comparing ABE production from different substrates in SSF processes, the final ABE solvent concentrations from paper sludge were 5-34% higher than that from dilute acid pretreated wheat straw (Table 3). The ABE yields from paper sludge were similar to that from dilute acid pretreated wheat straw. In comparing ABE production in SSF and SHF processes, the ABE yields in SHF process appeared relatively higher than those in SSF process, but the ABE final concentrations were similar. It should be noted that different enzyme loading and different *Clostridium* strains will affect the final ABE concentration and ABE yields was well. Two major advantages of paper mill sludge as feedstock for butanol production are that no pretreatment is needed and no pH control is required as ABE fermentation from sugars. Yang et al. previously found that supplementation of calcium carbonate in ABE fermentation from glucose significantly improved the solvent yield by stabilizing the pH of fermentation broth (Yang et al., 2013b). Remarkably, significant amount of $CaCO_3$ already exists in paper mill sludge and can be used as a buffering reagent in fermentation process. Previously, Budhavaram et al. investigated lactic acid production from paper sludge with *Bacillus coagulan* in a SSF process and found high lactic acid yield (>80%) were achieved without pH control because of the

buffering effect of $CaCO_3$ (Budhavaram & Fan, 2009a). However, excessive $CaoCO_3$ inhibited enzymatic hydrolysis. Margues et al. showed paper sludge needs to be neutralized before enzymatic hydrolysis (2008). It be noted that calcium carbonate also showed inhibition on glucose utilization in ethanol production from paper sludge by *zymomonas mobilis* (Zhang & Lynd, 2010), they found calcium carbonate and high temperature were responsible for the accumulation of glucose in high solid loading of paper sludge SSF process. This indicated utilization of paper sludge should not only consider the benefits of buffering effect of $CaCO_3$ in fermentation, but also take into account its inhibitory effect on enzymatic hydrolysis and glucose utilization. In addition, attempts to convert alkali-pretreated switchgrass to butanol in a SSF process have not been successful, residual lignin or other toxic compounds on the pretreated substrates potentially inhibit the cell growth and fermentation activity.

Table VI-2 Effects of solid loading and enzyme loading on SSF of paper mill sludge

Solid loading	**Enzyme Loading**	**Residual sugars (g/L)**		**Acids (g/L)**		**Solvents(g/L)**				**Solvent Yield[a] (g/g)**
(w/v)%	(FPU/g-glucan)	Glucose	Xylose	Acetic	Butyric	Acetone	Butanol	Ethanol	Total	
3.8	5	5.2	1.4	4.1	2.1	1.9	5.3	0.2	7.4	0.20
	10	0.3	0.4	4.9	2.2	3.5	6.8	0.4	10.6	0.29
	15	0.1	0.3	4.1	1.9	3.4	6.9	0.4	10.6	0.29
5	5	8	3	4.5	2.7	3.5	5.8	0.4	9.7	0.20
	10	6	3.2	4.2	2.2	4.1	7.7	0.7	12.6	0.26
	15	0.5	3.1	3.5	1.6	5.2	8.5	0.8	14.5	0.30
6.3	5	7.2	3.8	4.2	1.9	4.6	7.7	1.0	13.3	0.22
	10	3.2	3.3	4.0	1.9	5.6	9.1	1.1	15.7	0.26
	15	1.5	2.3	3.5	1.8	5.4	9.6	1.4	16.3	0.27
7.4	5	8.9	4.1	4.1	1.4	5.8	8.7	1.2	15.8	0.22
	10	4.9	5.3	4.1	1.6	5.7	9.7	1.7	17.1	0.24
	15	4.1	4.3	4.1	1.8	6.2	10.2	1.6	18.0	0.25

[a] the solvent yield was calculated from the total solvents over the theoretical amounts of sugars (glucose and xylose) in the PS7.

Table VI-3 ABE production from different feedstocks in SSF and SHF processes

Feedstock	Pretreatment	Detoxification	Fermentation	Culture	ABE (g/L)	ABE Yield (g/g sugars)	References
Paper sludge	None	NA	SSF	*C. acetobutylicum* ATCC 824	12.6-18.0	0.24-0.30	This study
Wheat straw	Dilute acid	NA	SSF SSF+GS[d]	*C. beijerinckii* P260	11.9 21.4	0.27 0.31	(Qureshi et al., 2008a)
DDGS[a]	Dilute acid Liquid hot water AFEX	Overliming NA NA	SHF	*C. acetobutylicum* ATCC 824	12.1 11.4 9.0	0.31 0.31 0.32	(Ezeji & Blaschek, 2008a)
Switchgrass	Dilute acid	NA Dilution[c]	SHF	*C. beijerinckii* P260	1.5 14.6	NA 0.39	Qureshi et al., 2010b
Maple[b]	Liquid hot-water	Overliming	SHF	*C. acetobutylicum* ATCC 824	11.0	0.28	Sun & Liu, 2012
Switchgrass	NaOH	NA	SHF	*C. saccharobutylicum* DSM 13864	22.7	0.40	Gao et al., 2014

a. DDGS: Dried distillers' grains and solubles; b. Maple: maple hemicellulose hydrolysate; c. Dilution: the hydrolysate was diluted 2 times with DI water and then was supplemented with extra pure sugar; SSF+GS: SSF was performed together with gas stripping.

VI.4Conclusion

Kraft paper mill sludge has great potential as a feedstock for ABE production. It does not require chemical pretreatment or detoxification, but only needs to be partially de-ashed to improve the enzymatic hydrolyzability. Remarkably, the buffering effect of $CaCO_3$ in paper mill sludge can be effectively used in the SSF process to produce butanol, because pH control is a critical issue in butanol fermentation by *C. acetobutylicum*. The results showed butanol production from de-ashed paper mill sludge could be carried out under the solid loading of 6.3-7.4 wt% and enzyme loading of 10-15 FPU. Higher solid loading resulted in lower solvent yield, which indicated enzymatic hydrolysis still limited the SSF process.

Acknowledgement

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VII. Future work

In the future work, three primary tasks are proposed to continue focusing on the bioprocess for butanol production from lignocellulosic feedstock with *C. acetobutylicum*. The first task focuses on bioconversion of alkali-pretreated switchgrass into ABE solvents. The primary objective of this task is to investigate the technical feasibility of bioconversion of alkali-pretreated switchgrass into ABE though SSF. From previous experience with pulp mill sludge, SSF could not process concentrated solid loading toward an efficient productivity. Therefore, both SSF and SHF are applied and the yields are compared. The second task aims to integrate the *in situ* product recovery (ISPR) technique to the ABE fermentation process. The primary objective of the integrated process is to alleviate end-product inhibition and to improve the fermentation process productivity (Nielsen and Prather, 2009; Qureshi and Blaschek, 2001). The third task investigates the feasibility of bioconversion of hot-water extracted hemicellulose from woody biomass into ABE solvents. The hemicellulose portion of woody biomass represents a highly underutilized sugar source in the Kraft pulping process, which is often discharged as waste stream into the black liquor (Kudahettige-Nilssona et al., 2015; Shi et al., 2015).

VII.1 *In situ* extractive removal of ABE solvents with liquid butylene as extractant

Integration of in situ product recovery (ISPR) technique into the ABE fermentaiton

As demonstrated in the previous chapters, bio-butanol production from lignocellulose still faces the challenge of low low butanol titers, typically in the range of 10-13 g/L,

resulted from the strong end-products inhibition, which in turn causing dramatic energy input for products recovery (Jones and Woods, 1986; Nielsen and Prather. 2009). Integration of *in situ* product recovery into ABE fermentation process has been well-documented often as a straightforward technique to alleviate end-product inhibition and thus to increase solvent titres (Nielsen and Prather. 2009; Ezeji et al., 2003). With these techniques, it is feasible to achieve high solvents titers by processing more concentrated substrate, even by the fed-batch mode. For example, it has been reported that, with integration of gas stripping into the batch fermentation, Clostridium *beijerinckii* BA101 was able to continuously consume up to 161.7 g/L of glucose and produce as much as 75.9 g/L of total solvents, compared to the control and non-integrated batch fermentation where only 59.2 g/L of glucose was fermented producing 17.7 g/L of total solvents (Ezeji et al., 2003).

Extractive fermentation with liquid butylene as extractant

The objective of this task aims to integrate the ABE fermentation process with an *in situ* extractive removal of fermentation products with liquid butylene as extractant. The extraction performance of butylene to butanol had been demonstrated in industrial process for the synthesis of butanol from butylene under supercritical conditions (Jessop and Leitner, 1999). To the best of my knowledge, this is the first time that liquid butylene is introduced as an extractant for the solvents recovery of the ABE fermentation process. Utilization of liquid butylene as extractant for *in situ* solvents recovery offers several advantages. First, extractant regeneration and solvents purification can be easily performed through evaporation due to the low volatility of butylene at normal conditions (BP: -6.5°C) and completely immiscibility with aqueous. Second, butylene, as an

intermediate chemical when upgrading butanol into hydrocarbons, affords the opportunity to further integrate this process to the subsequent chemical catalysis, as indicated in Fig.VII-1.

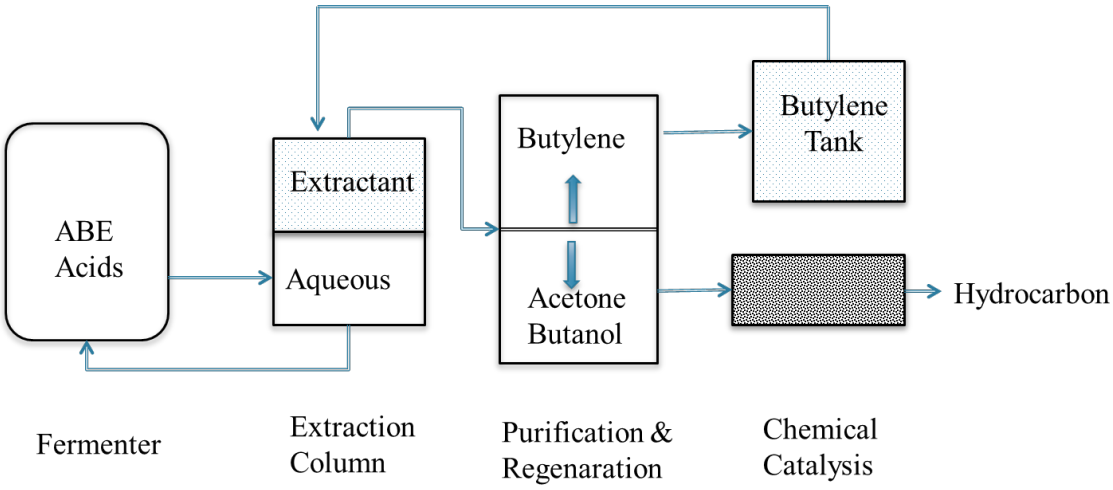


Fig. VII-1 Schematic diagram of integrated fermentation process and chemical catalysis

Preliminary Results

The distribution coefficients of broth-related products are tested under various mass fractions of extractant-to-aqueous and under various products concentrations and are summarized in Table 4. The results indicated that the products separated from the broth are mostly pure butanol and acetone. Both the acetone and butanol have severe inhibitory effects on the fermentation and can be converted to higher molecular hydrocarbons. The extraction performance is stable over the entire range of products concentration in the fermentation broth, as indicated in Fig. 2A for butanol and 2B for acetone.

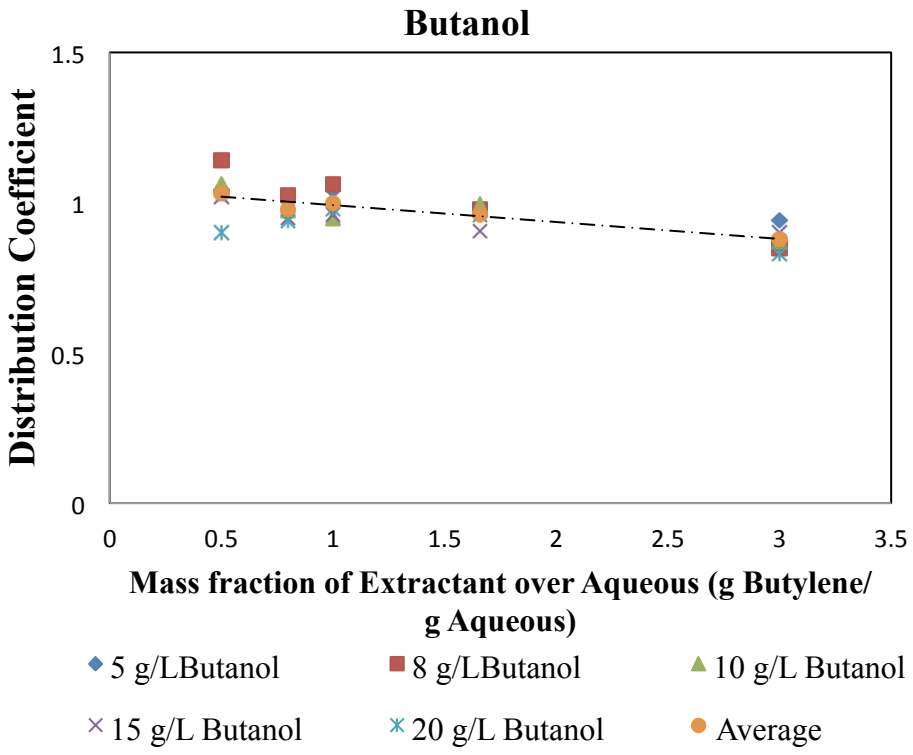
Table VII-1. Summarization of distribution coefficient of broth-related products [a]

Distribution Coefficient (wt.% solute in extractant/ wt.% of solute in aqueous						
Glucose	Xylose	Acetic acid	Butyric acid	Aetone	Butanol	Ethanol
-[b]	-	2.3×10^{-4}	0.12	0.5	1.03	0.05

Note:

a. Distribution coefficients are measured at 25 °C and 30 Psi

b. – indicates the distribution coefficient is negligible



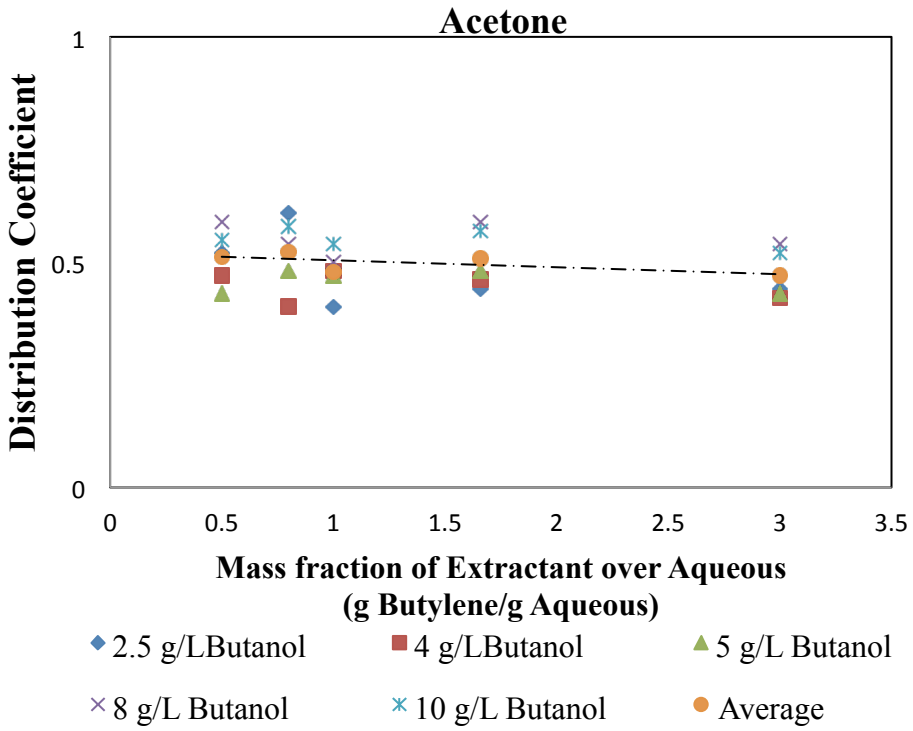


Fig. VII-2. Distribution coefficient of butanol and acetone in the aqueous-butylene system.

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