KEY FEATURES OF THERMOCHEMICAL PRETREATMENT INDUCED LIGNIN STRUCTURE TO SACCHARIFICATION AND FERMENTATION

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Abstract

This dissertation introduced and investigated an innovative biorefinery platform to convert urban waste derived lignocellulosic biomass into bioethanol and reactive lignin. Woody biomass derived from single or mixed species were investigated separately for softwood, hardwood, and then universally identical woods, respectively. Softwood contains large amount of easily fermentable hexoses (mannose plus glucose) and G-lignin, while hardwood is with more xylose and S-lignin; thereby the two types of woody biomass may be more feasible in different biorefinery approaches for valorization based on a comprehensive review on current processes (Chapter 2). Softwoods were subjected to sulfite pretreatment followed by the "whole slurry" saccharification and fermentation (SSF) process, which aims to utilize all the dissolved sugars (in pretreatment liquor) plus enzyme hydrolyzed sugars in fermentation. New insights on the duel effects of inhibiting/improving mechanisms of pretreatment derived lignosulfonates were studied by surface tension analysis, enzyme activities, and fitting of linearized kinetic models (Chapter 3). It was discovered that pretreatment liquor could increase the inhibiting effects to fermentation at high temperature, and an extremely high ethanol titer (82.1 g/L) were yielded by simply optimizing the operation temperature. Without the need of detoxification, the resulting ethanol titer is approaching the theoretical yield and is currently among the highest in softwood conversion (Chapter 4). Energy balance calculation was conducted to evaluate the trade between energy lose from temperature control and benefited higher energy yield from product titer. The net energy yield of the new process was 2,410 MJ per ton oven-dried wood, which is approximately 730-1,690 MJ higher than that of the other biorefinery processes. The water input before reclamation was 3.65 tons per ton dried wood, which is 25.8-51.2%

lower than most of the other processes (Chapter 5). Finally for hardwood, a new reactive lignin with great solvent solubility and preserved β-O-4 linkages was obtained from eucalypts after a modified organosolv pretreatment using 1,4-butanndiol (1,4-BDO). 2D HSQC NMR analysis showed that this 1,4-BDO lignin contain relative higher amount of β-O-4 linkages, indicating a higher integrity than ethanol pretreated lignin. This result agreed with ^{13}P NMR analysis and suggested that alcohols can quench the benzyl carbocation intermediate and formed ether linkage at the α position of the lignin. Although dioxane structure was not observed in the NMR spectra, solubility tests revealed that grafting aliphatic hydroxyl groups on 1,4-BDO lignin can increase its dissolution. This phenomenon was demonstrated for four other diols with similar structures; and more than 90% cellulose conversion was obtained from all the diol pretreated eucalyptus coupling with enzymatic hydrolysis at only 7.5 FPU/g glucan. Diol pretreatment offers an attractive reaction pathway to coincide with the trading among lignin fractionation, lignin structural integrity, and cellulose hydrolysis.

Publications arising from the thesis

1. Dong, C., Wang, Y., Zhang, H., Leu, S.-Y. 2018. Feasibility of high-concentration cellulosic bioethanol production from undetoxified whole Monterey pine slurry. *Bioresource technology*, **250**, 102-109.

2. Dong, C., Wang, Y., Chan, K., Akanksha, B., Leu, S. 2018. Temperature profiling to maximize energy yield with reduced water input in a lignocellulosic ethanol biorefinery. *Applied Energy*, **214**, 63-72.

3. Dong, C., Wang, Y., Leu, S. Toward complete utilization of lignocellulosic biomass in biorefinery: pretreatmens and applications. *Bioresource technology*. (To be submited)

4. Dong, C., Liu, H., Peng, Y., Leu, S. Insight into the Maximized Beneficial Effects of Lignosulfonate in the Whole Slurry Enzymatic Saccharification through Surface Activity Analysis. *Cellulose*. (To be submitted)

5. Dong, C., Meng, X., Yeung, C., Leu, S. Diol pretreatment to fractionate a reactive lignin in lignocellulosic biomass biorefinery. *Green chemistry*. (To be submited)

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Table of contents

List of abbreviations

DA	Dilute acid
SSF	Simultaneous saccharification and fermentation
LS	Lignosulfonate
RED	Relative energy difference
DES	Deep eutectic solvents
THF	Tetrahydrofuran
GVL	γ-Valerolactone
MW	Molecular weights
AlOH	Aliphatic hydroxyl
PhOH	Phenolic hydroxyl
CCR	Carbon catabolic repressio
SP	Sulfite pretreatment
U	Unwashed
W	Washed
H	Hydrolysate
HPLC	High-performance liquid chromatography
GPC	Gel permeation chromatography
FPU	Filter paper activity
NREL	National renewable energy laboratory
SED	Substrate enzymatic digestibility
CAC	Critical aggregation concentrations
CMC	Critical micelle concentration
CFU	Colony-forming units
NMR	Nuclear magnetic resonance
EtOH	Ethanol
MeOH	Methanol
2-ProOH	2-Propanol
2-BuOH	2-Butanol
EG	Ethylene glycol
1,3-PG	1,3-propanediol
1,3-BDO	1,3-butanediol
1,4-BDO	1,4-butanediol

Chapter 1 Introduction

Developing new generation biorefinery to convert lignocellulosic biomass into green fuels and valuable chemicals is an emerging strategy to progressively replace the conventional fossil fuel refinery. Woody biomass is amongst the most cost-effective feedstock for biorefinery due to its higher density and lower collection/transportation costs (Zhu & Pan, 2010; Zhu & Zhuang, 2012b). This type of feedstock is sustainably accessible in large quantities in various regions of the world. In Hong Kong, approximately 90,000-120,000 tonnes of wood product waste have been disposed of the landfills every year (HKEPD, 2012). The majority of the wood product waste (not including waste paper and paper products) were generated from municipal services as well as construction and demolition activities (Poon et al., 2001; Tam & Tam, 2006). The wood product waste has not been used by the existing recycle system and has been directly disposed of in the landfills. It occupies large amount of space and shortens the service life of landfill sites. There is an urgent need of new strategies to be developed or adopted for solid waste reduction and management. If the lignocellulosic biomass can be properly treated, the woody product waste can become a useful feedstock for production of new generation fuels and valuable chemicals.

Thermochemical pretreatment is the first and a crucial unit process in a biorefinery. This process aims to reduce the recalcitrance of lignocellulosic biomass against chemical and biological degradation. Wood cell is structurally and chemically more robust than most of the other biomass, and hence lots of research efforts have been made to dissociate this type of feedstock. Pretreatment methods such as alkaline(Chen et al., 2015b) (Wu et al., 2011), dilute acid (DA) (Liu & Bao,

2017; Zhang et al., 2011), and steam explosion (López-Linares et al., 2015) have achieved varied levels of success, while sulfite and organosolv pretreatments are shown to be particularly effective to improve the digestibility of high recalcitrant biomass. Sulfite pretreatment at low pH can dissolve most of hemicellulose and convert lignin into lignosulfonate, resulting in more than 90% (w/w) cellulose conversion within 24 h of enzymatic hydrolysis (at an enzyme loading of 15 FPU/g glucan). Lignosulfonate can act as a surfactant and further enhance the enzymatic hydrolysis, which make the whole slurry (solid plus the liquid fraction) simultaneous saccharification and fermentation (SSF) process feasible. Washed organosolv pretreated substrate showed similar level of cellulose conversion as the sulfite pretreated substrate, and the sulfur-free lignin product is with high potential of valorization. The two pretreatment approaches were investigated and evaluated in significant details in this dissertation.

A key challenge of using woody waste for biofuels is of the inconsistent quality of the feedstock. The commonly discussed forestry residues have been collected from signal forest. The plant species contributed to the feedstock are simple and with the chemical composition roughly predictable. However, the woody biomass derived from urban waste is a mixture of decomposed wood products originated from different sources and is composed of a wide verity of different species. In Hong Kong, more than 99% of the woody materials and related products were imported materials originated from external sources, *e.g.*, China, U.S., Southeast Asia, and Europe (HKCSD, 2012a; HKCSD, 2012b). The chemical compositions of different wood species vary significantly. For example, lignin contents of softwoods are 26-32 %, hardwoods are 20-25%, and bamboos are 15-30% (Sjöström, 1993).

Higher lignin contents increases the recalcitrant of the biomass to enzymatic hydrolysis (Nakagame et al., 2010). Removing only hemicelluloses by DA is sometimes sufficient enough for treating biomass with low lignin contents (Leu & Zhu, 2012), but lignin removal, at least partially, is essential for treating softwoods. The biorefinery technology should be modified to manage the inconsistency of feedstock as increased pretreatment severity also results in irreversible impacts such as generation of toxic compounds and aggregated lignin degradation/condensation. Softwood species has high hexosan content and hence the related substrate is favorable for ethanol production using commercially available yeast (*Saccharomyces cerevisiae*) that can only consumes hexoses but not pentoses. Thus, sulfite pretreatment was selected to treat softwood species as most of its dissolved hemicelluloses can be fermented with the hydrolysate by whole slurry SSF. Hardwood contains more xylose which are less preferred by yeast but useful to produce valuable chemicals after chemical processing, so organosolv pretreatment was applied to produce less condensed lignin.

In Chapter 3, a series of enzymatic saccharification experiments were designed and conducted to elucidate the enhancing and inhibitory mechanisms of lignin by-products to whole slurry SSF process. Lignosulfonate (LS) in the sulfite pretreated slurry could act as surfactant and benefit cellulose enzymatic saccharification, while inhibiting effects were still observed in some conditions. The enzymatic activities and reaction kinetics of the hydrolysis process under a well-controlled LS-enzyme complex were investigated. The enhancing/inhibiting duel effects were studied by examining the specific binding affinity of enzyme to substrate and product formation, using linearized models and enzyme activity

measurements. Strategies to overcome the inhibitory effects of the LS for whole slurry process were provided to validate the proposed mechanism.

With basic understanding of the surfactant enhanced enzymatic saccharification, a bioethanol production process was established in Chapter 4 by using undetoxified whole slurry of Monterey pine. While sulfite pretreatment enabled the whole pretreated slurry (solid plus the liquid fraction) to be directly applicable in SSF, less than 50 g/L ethanol had been achieved in previous studies, which cannot compare with the performance of lignin-free substrates even though higher fermentable sugars was obtained in the hydrolysate. SSF were optimized to facilitate the performance of commercial cellulase and *S. cerevisiae*. The pretreatment whole slurry was prehydrolyzed and then fermented with various doses of concentrated pretreatment spent liquor at different temperatures. A modified SSF was successfully built up to achieve a high ethanol titer 82 g/L which has never been achieved previously with softwood biomass.

In Chapter 5, different fed-batch approaches were applied to maximize the rates and yield of hydrolysis of the sulfite pretreated Monterey pine feedstock. Water-soluble contents in the pretreatment spent liquor were fractionated and purified to study the possible components inhibiting the metabolisms of the yeast. Numerical analysis using both experimental and literature data were conducted to calculate the energy and water footprints in the process, which were then compared with the other recent biorefinery techniques. Complete utilization of the whole slurry with reduced heat stress indeed showed outstanding energy production and water saving potential.

For hardwood, chapter 6 aims to minimize lignin condensation in organosolv pretreatment with diols, which act as nucleophile to react with the C_α benzylic carbocation. Hildebrand solubility theory suggested that diols have smaller relative energy difference (RED) with lignin than other alcohols, implying a greater lignin dissolution capability of this solvent. Meanwhile, diols have one more hydroxyl group, which was hypothesized to form stable acetals to prevent lignin condensation in comparison with monohydric alcohols. By varying the pretreatment solvents, substrates with unique properties were formed during organosolv pretreatment and their susceptibility to enzymatic hydrolysis was evaluated. The structural changes on lignin were observed by analyzing the lignin properties including molecular weight, structural information, and hydroxyl group contents using NMR technology.

Chapter 2 Literature review: toward complete utilization of lignocellulosic biomass in biorefinery

2.1 Introduction

Lignocellulosic biomass is a valuable resource of sustainable energy and chemicals (Robledo‐Abad et al., 2017; Wang et al., 2018). Bioenergy is renewable and its utilization shall progressively substitute the conventional refinery upon depletion of the fossil fuels or in order to mitigate global warming. In addition, biomass provides the only source of renewable polymers and pharmaceuticals and hence development of biorefinery has been related to benefit the regional economy and environmental management (Nikodinoska et al., 2017). Non-food based biomass derived from municipal and agricultural wastes are sustainable feedstock of biorefinery, which can be collected, processed, and utilized without scarifying food security (Alexander et al., 2015). Bioconversion of biomassBiorefinery includes a series of unit processes, *i.e*., pretreatment, saccharification, fermentation, and separation (Valdivia et al., 2016). Pretreatment is the primary process affecting the rest of the downstream processes and hence is the major focus in our study. Pretreatment has been developed to reduce the biomass recalcitrance, *i.e*., the capability to protect plant cell wall from microbial and enzymatic deconstruction, and thereby benefit the subsequent saccharification and fermentation without significant inputs of energy or chemicals. This chapter introduces some basic concept of lignocellulosic structure and the state-of-the art pretreatment techniques toward whole biomass utilization in biorefinery.

2.2 Lignocellulosic Feedstock

Cellulose, hemicellulose and lignin are the main building block polymers which build up the cell wall of lignocellulose. The minor components include pectin, inorganic compounds and extractives. The following review summarizes the basic chemistry of lignocellulosic biomass.

2.2.1 The molecular of cell wall

Cellulose is the most abundant biopolymer on earth. It constitutes approximately 40% of the wood and can be synthesized in plants, algae, and bacteria. Cellulose is a liner polysaccharide consists of D-glucose units linked together by β-(1-4)-glycosidic bonds (Fig. 2.1). The degree of polymerization (DP) of native cellulose varies from 500 to 14,000 glucose units, and wood fibers cellulose showed a very low in polydispersity (Goring & Timell, 1962). In living plant, an elementary fibril is formed by approximately 36 individual cellulose chains through hydrogen bonds and Van der Waals force. The elementary fibrils are packed into larger microfibrils with 5-50 nm in diameter and several micrometers in length. These microfibrils include the amorphous and crystalline regions with significant alignments (Neralla, 2012). The amorphous domains are regularly distributed along the microfibrils, while in the crystalline regions cellulose chains are closely packed together by a strong and highly intricate intra- and intermolecular hydrogen-bond network (Fig. 2.1). Compared with crystalline domains, the amorphous regions of cellulose microfibrils are more susceptible to be attacked. They can be hydrolyzed selectively when the lignocellulosic biomass are subjected to mechanical, chemical and enzymatic treatment (Beck-Candanedo et al., 2005; Bondeson et al., 2006;

Filson et al., 2009).

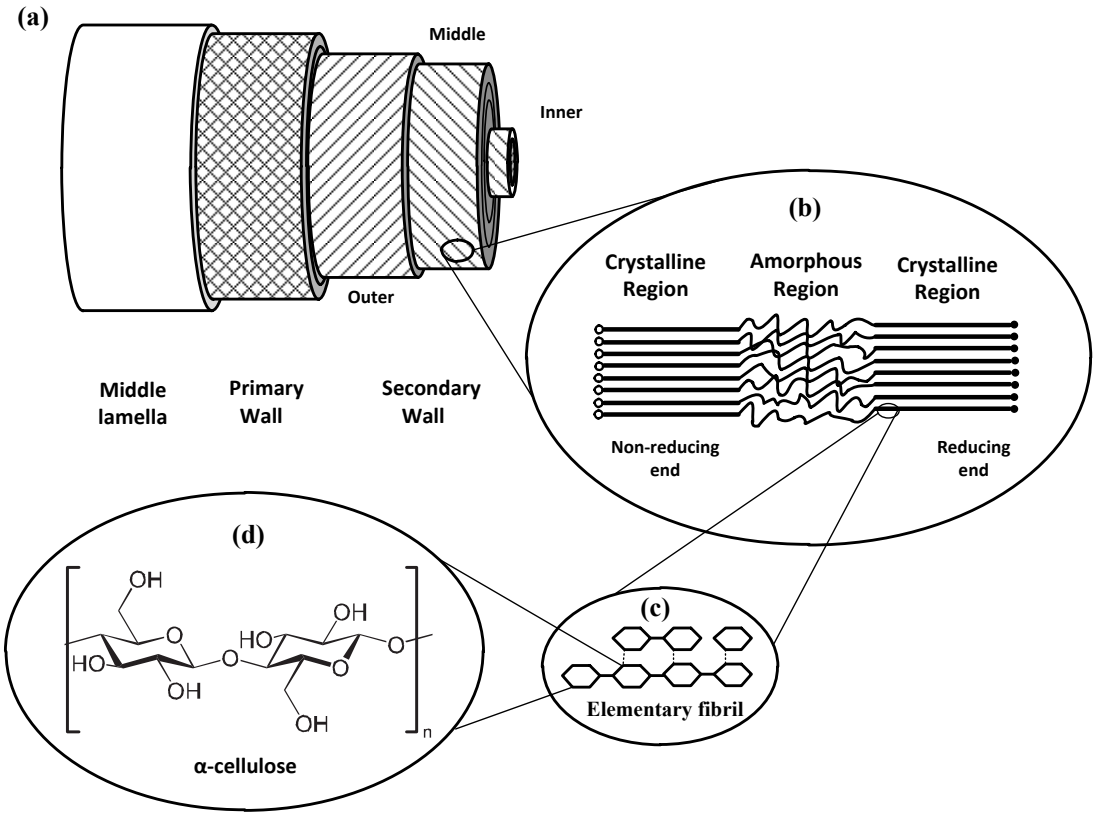


Fig. 2.1 Scheme of cellulose in plant cell (a) plant cell wall; (b) cellulose molecular chains within the crystalline region of cellulose microfibril; (c) elementary fibril and (d) two α-cellulose. Note: the dimension of the cell wall is not in scale, the direction of microfibrils alignment is not based on real image. This diagram was re-plotted based on Anderson and Kerr (1938) and Gordon and Hsieh (2006).

In living plants, the crystalline structure is denoted as Cellulose I, which is a mixture of two crystalline forms I_α and I_β (Vanderhart & Atalla, 1984). According to CP/MAS ^{13}C-NMR spectra, Cellulose I_α was converted to cellulose I_β during kraft pulping but not during the sulfite pulping (Hult et al., 2002). Igarashi, et al (Igarashi et al., 2006) compared the enzyme kinetics of Cel7A towards the two crystalline forms and found that maximum adsorption of Cel7A on Cellulose I_β is higher than that from Cellulose I_α, but the cellobiose production is lower for the former than the later. The specific enzyme activity on Cellulose I_α may be higher than on Cellulose I_β.

Furthermore, when Cellulose I_α was converted to Cellulose I_β (by pretreatment), the specific enzyme activity was decreased and became similar as Cellulose I_β at the same surface density.

Hemicelluloses are group of heterogeneous polysaccharides built up of several different monomers such as mannose, arabinose, xylose, galactose and glucose. Hemicellulose contributes to 20-30% of the wood cell (Fengel & Wegener, 1984). The existence of hemicelluloses strengthen the cell wall by creating interaction with cellulose and lignin (Himmel, 2008). The DP for hemicellulose is much lower than cellulose, *i.e.*, 100 and 200 (Fengel & Wegener, 1984). The major hemicelluloses in hardwoods is glucuronoxylan, and in softwoodsare galactoglucomannans and arabinoglucuronoxylan (David & Nobuo, 2000; Fengel & Wegener, 1984). Glucuronoxylan has the backbone of β-(1→4)-linked xylopyranose units. At approximately every 10^{th} xylose unit of xylan the xylose unit is substituted with a α-(1→2)-linked 4-*o*-methylglucuronic acid residue, and most of the hydroxyl groups at C2 and C3 of the xylose units are substituted with acetyl acid residues. Galactoglucomannans is a liner or slightly branched chain composed of β-(1→4)-linked D-mannopyranose and D-glucopyranose units. Some galactoglucomannans with low galactose content (galactose to glucose to mannose ratio is 0.1-0.2:1:3-4) were referred to glucomannan; and the others are with higher galactose to glucose to mannose ratios of 1:1:3. On an average of one group per 3-4 hexose units, the hydroxyl groups at position C2 and C3 in the backbone units are partly substituted by O-acetyl groups. Another type of xylan named arabinoglucuronoxylan has a backbone of β-(1→4)-linked xylopyranose units. Compared with the glucomannan xylans are more resistance during the Kraft

cooking (Kibblewhite & Brookes, 1976), therefore residual xylan both occurs in softwood and hardwood Kraft pulps.

Lignins are three dimensional amorphous polymers which are biosynthesized from the polymerization of three monolignols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 2.3) (Chakar & Ragauskas, 2004). The lignin precursors in hardwoods are coniferyl alcohol and sinapyl alcohol. In softwoods, *p*-coumaryl alcohol is the main precursor of lignin. Lignins constitute approximately 30% of the dry weight of wood which limit the presentation of water into the wood cells and make wood very compact (Himmel, 2008).

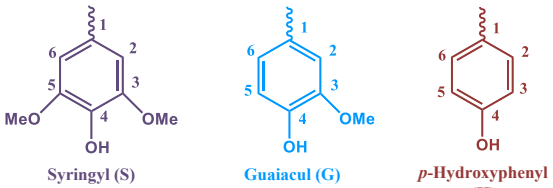


Fig. 2.3 The three building blocks of lignin (Chakar & Ragauskas, 2004).

The exact structure of protolignin remains virtually unknown due to their complex and irregular structure and high difficulty of isolating native lignin. Improvements in methods for identifying degradation products, many of the structure features of lignin have been elucidated by spectroscopic methods. Example linkages were depicted in Fig. 2.4 and Table 2.1 summarized the abundance of common linkages (Ede & Kilpelainen, 1995; Karhunen et al., 1995a; Karhunen et al., 1995b; Lewis & Yamamoto, 1990; Sjostrom, 1981). The β-aryl (β-O-4) and α-aryl (α-O-4) ether linkages are the representative chemical linkages in plant cell wall, accounting at least 43-50% and 6-8% (in number) in softwood, respectively (Li et al., 2015).

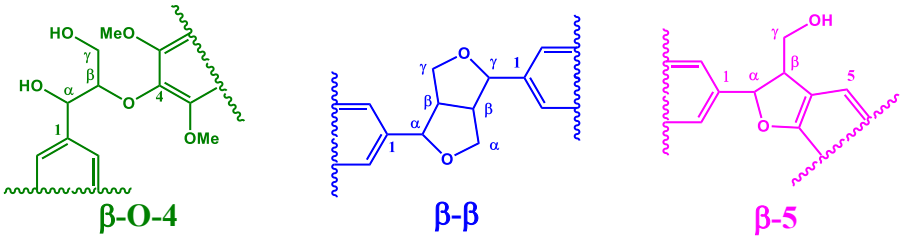


Fig. 2.4 Example linkages between phenylpropane units in lignin.

Table 2.1 Proportions of different types of linkages connecting the monolignols in softwood lignin (Ede & Kilpelainen, 1995; Karhunen et al., 1995a; Karhunen et al., 1995b; Lewis & Yamamoto, 1990; Sjostrom, 1981)

Linkage type	Dimer structure	Approximate percentage
β-O-4	Phenylpropane β-aryl ether	45-50
α-O-4	Phenylpropane α-aryl ether	6-8
β-5	Phenylcoumaran	9-12
5-5	Biphenyl and dibenzodioxocin	18-25
4-O-5	Diaryl ether	4-8
β-1	1,2-Diaryl propane	7-10
β-β	β-β-Linked structures	3

The chemical bonds between lignin and carbohydrates were first proposed by Erdmann in 1866, which was used to explain the difficulty in separating lignin from woods (Erdmann, 1866). The hypothesis is evidenced by Smith degradation, reduction, methylation analysis, chromatography, NMR, spectroscopy and electron microscopy (Eriksson et al., 1980; Koshijima & (Author), 2010; Yuan et al., 2011; Yuan et al., 2012). Watanabe , et al (Koshijima & (Author), 2010) reviewed the proposed lignin-carbohydrate bonds recently: (1) the α-hydroxyl group of a lignin unite was etherified with a hydroxyl group or a carboxylic acid group of one carbohydrate. (2) An aliphatic or aromatic hydroxyl group of lignin was glycosylated by the reducing end group of carbohydrates. (3) Two hydroxyl groups of carbohydrate were linked to lignin by an acetal linkage.

2.3 Pretreatment

Pretreatment is the first and the most crucial step in biorefinery. Representative mechanisms of pretreatment include breaking down the structural linkages between hemicelluloses and lignin, disrupting hydrogen bonds in crystalline cellulose, and altering and redistributing structural components. Table 2.2 summarized the performances of a few recently published pretreatment strategies. All processes start with thermochemical pretreatment of biomass followed by solid/liquid separation, enzymatic hydrolysis (or saccharification), fermentation, and then separation. In general, pretreatment severity increase with the biomass recalcitrant and/or lignin contents of which ranked (from low to high) for herbal samples, agricultural residues, hardwood, and then softwood.

Pretreatment related biorefinery processes are roughly classified into three main groups (Fig. 2.5), *i.e.*, (I) lignin fractionation; (II) whole slurry simultaneous saccharification and fermentation (SSF); and (III) one-pot biorefinery. The key differences of the three major biorefinery options are on the applied reactions and conditions for lignin removal and utilization. Among the three biorefinery processes, Type I strategy focuses on lignin dissociation; Type II aims to achieve the highest product yield without lignin dissociation; and Type III attempts to dissociate the whole biomass. In Type I and Type III strategies, the dissolved lignins are harvested after precipitation (or regeneration) through dosing the lignin-rich spent liquor in anti-solvents. In Type II strategy the dissociated cell wall components are applied directly in the SSF process without separating the spent liquid from the substrate (Dong et al., 2018b).

Table 2.2 Summary of recently developed thermochemical decomposition processes for lignocellulosic biorefinery

Type	Pretreatment	Feedstock	Pretreatment condition				Chemical composition			Reference
			Chemical	T (°C)	t (min)	L/S ratio	OL[a]	RL[b]	OC[c]	Cellulose	
I	AFEX	Rice Straw	Ammonia	140	50	1.3	19.8	15.8	34.4	-	(Harun et al., 2013)
	Ammonia	Corn stover	NH_3	120	30	18	23	10.7	38	94.7	(da Costa Sousa et al., 2016)
	Alkaline	Corn stover	NaOH	80	2	12	14.9	13.0	36.4	98	(Chen et al., 2015b)
	Alkaline	Bagasse	NaOH	50	30	20	15.4	-	39	95	(Wu et al., 2011)
	Organosolv	Lodgepole pine	H_2SO_4/EtOH[d]	170	60	7	27.8	14.6	50.1	78.6	(Pan et al., 2007)
	Co-solvent	Corn stover	H_2SO_4/THF[e]	150	25	7	15	8.8	36	97.2	(Nguyen et al., 2015)
	γ-valerolactone	Hardwood	H_2SO_4/GVL[f]	120	60	7	38	14	38	97.3	(Shuai et al., 2016b)
	Acid hydrotrope	Poplar	pTsOH[g]	80	20	10	23.4	3.7	45.7	81.9	(Chen et al., 2017)
II	Steam explosion	Rapeseed straw	-	215	7.5	1	17.8	47.8	31.6	93.8	(López-Linares et al., 2015)
	CO_2 explosion	Rice husk	CO_2, ethanol	80	10	-	26	3.5	40	-	(Serna et al., 2016)
	Dilute acid	Corn stover	H_2SO_4	160	-	10	19.3	-	37.5	81.6	(Avci et al., 2013)
	Dilute acid	Rice straw	H_2SO_4	162	10	0.5	13.4	28.8	37	95.7	(Kapoor et al., 2017)
	Dry Acid	Corn stover	H_2SO_4	175	5	0.5	16.1	-	35.4	-	(Liu & Bao, 2017; Zhang et al., 2011)
	SPORL	Aspen	H_2SO_4/$NaHSO_3$	180	30	3	23	28.5	45.9	86.4	(Wang et al., 2009)
	pH-SPORL	Douglas-fir	H_2SO_4/$NaHSO_3$	165	75	3	29.3	32.4	41	99.1	(Cheng et al., 2015a)
III	Ionic liquid	Miscanthus giganteus	[TEA][HSO_4]	120	240	10	22.4	6.4	47.3	96.8	(Bian et al., 2017)

[a]OL = Original lignin (wt.%); [b]RL = residual lignin (wt.%); [c]OC = original cellulose (wt.%); [d]EtOH = ethanol; [e]THF = tetrahydrofuran; [f]GVL = γ-valerolactone; [g]p-toluenesulfonic acid.

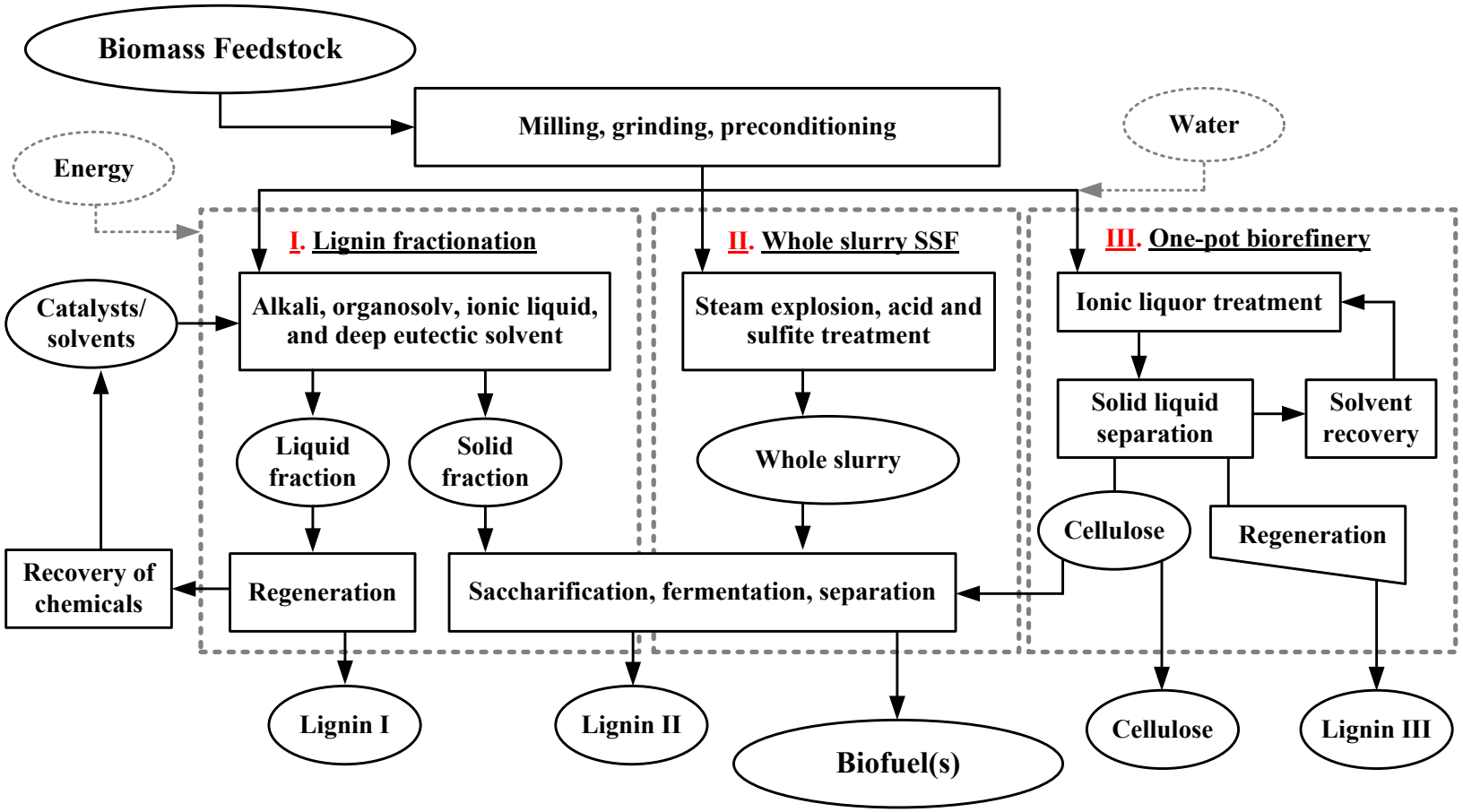


Fig. 2.5 Three biorefinery concepts for complete utilization of lignocellulosic biomass in bioenergy system.

The structure change of lignocellulose cell wall in the three types of biorefinery processes was presented in Fig. 2.6. The conceptual structure of lignocellulosic biomass was sketched based upon the representative references (Hsu et al., 1980; Mosier et al., 2005). Plant cell walls are composed of three components, *i.e.*, cellulosic fiber bundles (thin black lines), hemicellulose (thicker green lines), and lignin (thick red layers). Cellulosic fibers are aligned under either amorphous or crystal structures (shaded area). Hemicelluloses link randomly between cellulose and lignin, which covers outside the plant cell wall and protect it from biodegradation. Cellulosic fibers are not hydrolysable by simple reactions, but its crystallinity can be modified during the thermochemical process. With more complex physical and chemical features lignin is more difficult to dissociate than hemicelluloses.

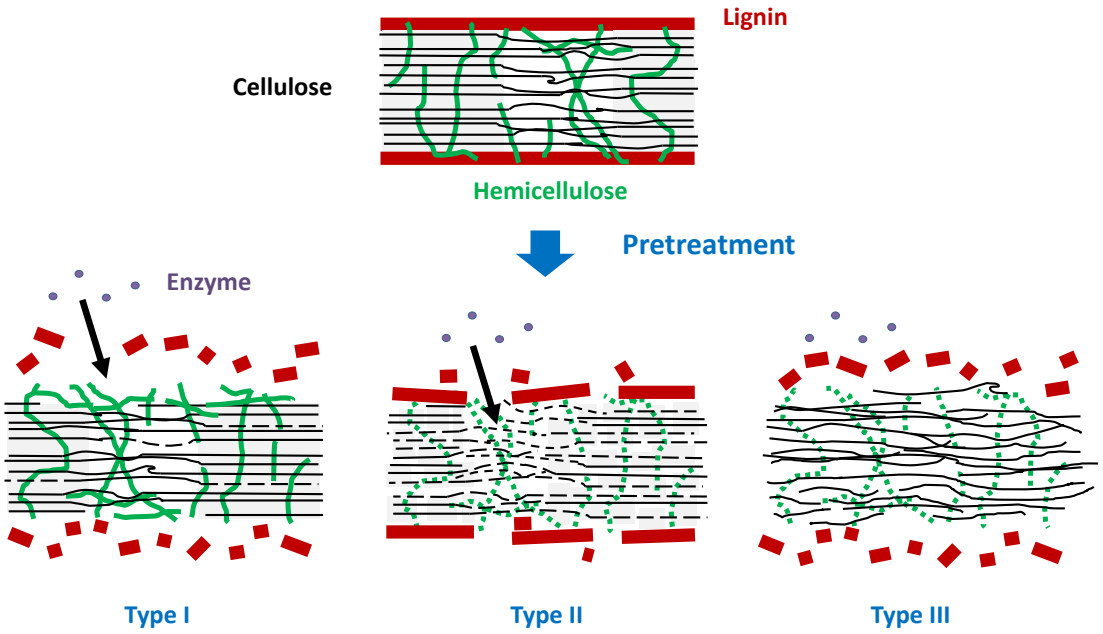


Fig. 2.6 Types of plant cell wall decompositions in the three classified biorefinery processes.

Type I biorefinery aims to dissociate the structural lignin and keep it in the spent liquor. Majority of hemicelluloses shall remain in the fiber bundles so the structural sugars can be recovered after enzymatic hydrolysis. Solid-liquid separation

is mandatory in this process so the dissociated lignin can be harvested for functional application and the remaining solvents/catalysts would not affect bioconversion. Enzymatic hydrolysis may be difficult if too much chemical reagents or lignin are remaining in the substrate (Turner et al., 2003). Type II biorefinery focuses on complete hemicelluloses removal using acid or other catalysts. Lignin is removed only partially by physical or chemical modifications. This process allows enzyme to access the cellulose through the inner surface for hydrolysis and therefore result in high substrate digestibility after enzymatic hydrolysis. Finally, Type III biorefinery is developed to decompose both lignin and hemicelluloses for functional applications. Cellulose shall become easily hydrolysable after swelling or regeneration with or without solid-liquid separation and/or washing. Based on those mechanisms different type of biorefinery concept can be selected for specific types of biomass or functions (*e.g.*, waste treatment, bioenergy harvesting, or production of functional chemicals).

2.3.1 Lignin Fractionation

Dissociation of lignin is the primary goal of Type I biorefinery. This process aims to produce polysaccharide-enriched substrates without damaging carbohydrates (Kim et al., 2016). After the thermochemical process the pretreated substrates are rich in polysaccharides and can be used as carbon sources for fermentation after enzymatic hydrolysis. Conventional wisdom believes that complete lignin dissociation is mandatory in any biorefinery as the residual lignin can interfere with the accessibility of the substrate to cellulase, but some other processes showed that complete removal of lignin may not be necessary if the surface characteristics of lignin can be properly modified (Leu & Zhu, 2013) (more detailed discussion of this approach has been provided in Chapter 3). In Type I biorefinery the pretreatment

spent liquor contains large amount of chemical reagents, solvents, and dissolved components and therefore the residual liquor need to be separated and rinsed away from the substrate before saccharification. The dissolved lignin in the spent liquor can be recovered through precipitation induced by anti-solvent(s). The recovered lignin can be used as biofuel or functional carriers/precursors in chemical processes (Upton & Kasko, 2015). Lignin fractionation in Type I biorefinery can be achieved by alkaline (Kim et al., 2016), organosolv (Zhao et al., 2017), ionic liquid (IL) (George et al., 2015), and/or deep eutectic solvents (DES) processes (van Osch et al., 2017) as summarized in the following sections:

2.3.1.1 Alkaline pretreatment

Alkaline pretreatment is a widely applied technique for delignification in pulp and paper industries (Heitner et al., 2016). The Kraft pulping process applies strong alkaline and Na_2S to achieve more than 90% lignin removal. Without causing significant damages to the polysaccharides this process aim to produce high quality fibers for papermaking (Gierer, 1980). Alkaline reagents such as metal-hydroxides, aqueous ammonia and ammonia hydroxide are often used in this process (Kim et al., 2016), while soda pretreatment is the most classic and widely applied approach for lignin dissociation. The advantages of the soda processes are of the high production rates, high substrate yields and possibility to treat different types of lignocellulosic feedstock (Heitner et al., 2016).

Based on the design concept of Type I biorefinery, Chen et al. (Chen et al., 2016) recently published an innovative Deacetylation and Mechanical Refining (DMR) process to effectively withdraw fermentable sugars from corn stover. Biomass was refined with 0.1 M NaOH solution and heated up to 80 °C for 2h. This

process (followed with the SSF processes) resulted in extremely high sugar and ethanol titers of 230 g/L and 10 vol.%, respectively in the fermentation broth. Da Costa Sousa et al. (da Costa Sousa et al., 2016) further proposed an alkaline based Extractive Ammonia (EA) process to dissociate lignin from corn stover. Liquid ammonia was charged into a high pressure vessel at a solvent to biomass ratio of 6:1 and heated to 120°C for 30 mins. The EA process successfully converted native (crystalline) cellulose into amorphous cellulose and has showed much higher recovery rate (45%) of high quality lignin in comparison with the conventional ammonia gas process. Alkaline pretreatment is a cost-effective process but the only issue is that the used reagents are not easily recyclable, making it less environmentally friendly than other processes.

2.3.1.2 Organosolv pretreatment

Organosolv pretreatment is an alternative approach to facilitate the product values of lignin (Zhao et al., 2017). Many organic solvents such as ethanol (Salapa et al., 2017), butanol (Lancefield et al., 2017), acetone (Smit & Huijgen, 2017), organic acids (Chen et al., 2015a) have been used in this process for lignin extraction (Zhao et al., 2017). Ethanol-water solution has been used to decompose lignin in conventional organosolv processes as its low solvent viscosity could favor solvent penetration into the cell wall (Pan et al., 2007). High purity lignin, cellulose, and hemicelluloses can be fractionated out of the biomass with almost no structure changes after organosolv pretreatment. Nguyen and Wyman et al. (Nguyen et al., 2015) introduced Co-Solvent-Enhanced Lignocellulosic Fractionation (CELF) process for high sugar yield by reduced enzyme consumption. Tetrahydrofuran (THF) miscible with aqueous dilute acid (DA) was employed to treated corn stover at

150 °C for 25 min. The process effectively removed more than 70% lignin and recovered 95% of glucose (theoretical yield) after enzymatic hydrolysis at low enzyme doses of 2 mg cellulase per g glucan.

Shuai and Luterbacher et al. (Shuai et al., 2016b) reported a mild pretreatment process using γ-Valerolactone (GVL) for hardwood conversion. The pretreatment process was carried out at 120 °C for 60 min. The process effectively removed up to 80% lignin and with 96-99% cellulose retaining in the substrates. Enzyme digestibility of the substrate was three times higher than those obtained by other organic solvents. Zhu et al., (Bian et al., 2017) developed an innovative low temperature (80°C) biorefinery process using *p*-toluenesulfonic acid (*p*-TsOH) to dissociate up to 85% birch wood lignin in 20 mins. This amount of lignin dissociation was not achievable under similar temperature or cooking time using conventional processes. In line with the outstanding treatment efficiencies, many studies have been carried out to improve the recyclability of used solvents (Alonso et al., 2017; Nguyen et al., 2015). Optimal conditions and recyclability of the organic solvents are functions of many process-specific parameters which will be covered in greater details in Section 2.6.13.

2.3.1.3 Ionic liquids and deep eutectic solvents

ILs and DESs are green solvents with great potential for delignification in biorefinery. ILs have been considered a possible option to decompose lignocellulosic biomass at low temperature (*i.e.*, 90-110°C) (Domínguez de María, 2014). However, conventional ILs such as imidazolium, pyridinium, and pyrrolidium salts are costly and less environmentally-friendly (Kumar et al., 2015). Many efforts have been made to evaluate the impacts of the IL related biorefinery and to improve the

applicability of ILs for biomass conversion. A discharge permit was recently granted by a DOE funded Advanced Biofuels Demonstration Unit for 28.8% [C_2mim][OAc] IL due to its low fish toxicity at the concentration (Sathitsuksanoh et al., 2014). George and Hallett et al., (George et al., 2015), aiming to produce low-cost solvents, synthesized a number of ILs containing $[HSO4]^-$ anion for biomass pretreatment. It was found that triethylammonium hydrogen sulfate IL achieved 75% efficiency (on cellulose digestibility) in comparison with the benchmark IL [C2C1im][OAc] when treating switchgrass. The cost of some ILs can be gradually reduced to a similar level of conventional pretreatment reagents. Socha et al., (Socha et al., 2014a) synthesized a series of tertiary amine-based ILs by using chemicals derived from lignin and hemicellulose. The new solvents resulted in almost complete digestion of switchgrass with 90-95% glucose and 70-75% xylose, respectively. The performance is comparable from conventional [C2mim][OAc] IL. A "closed-loop" concept was proposed by the authors for lignocellulosic biorefinery for future development.

DESs are a type of IL formed by mixing a Lewis or Brønsted acids with bases (Dai et al., 2013; Gunny et al., 2015). Representative DES are quaternary ammonium salts (*e.g.*, choline chloride, ChCl) mixing with hydrogen bond donors (*e.g.*, urea, lactic acid, or glycerol) (Paiva et al., 2014). DESs have been quickly developed in recent years for its low cost, high capability of selective dissolution of plant biomass, and low impacts to ecological systems (Zhang et al., 2012). Francisco et al. (Francisco et al., 2012) fabricated several DESs based on betaine, choline, and amino acids coupled with lactic or malic acid for dissolving large amount (12-15 wt.%) of lignin. Significant enrichment of cellulose was obtained after treating corncob residues with DESs at 115-150°C (Procentese et al., 2015). The subsequent enzymatic hydrolysis was enhanced in reaction rate and sugar yield, resulting in

recoveries of glucose and xylose as high as 86% and 63%, respectively (Procentese et al., 2015). In another study, rice straw was treated with a [ChCl][Lactic Acid] DES to achieve 90% lignin removal. The yield of dissolved lignin reached 68 mg/g biomass with 90% purity, but the substrate is much less digestible than that from alkali or DA pretreatment (Kumar et al., 2015). This is possibly due to the non-productive adsorption of lignin to cellulase as discussed in Section 4.2 of this paper.

2.3.2 Whole Slurry SSF

Whole slurry bioconversion is a recently developed strategy to maximize the energy yield and minimize water consumption in a biorefinery (Liu et al., 2016a). This process is often carried out after certain types of pretreatment. The pretreated substrates can be hydrolyzed and fermented together with the pretreatment spent liquor without detoxification (Cheng et al., 2015b). Complete utilization of polysaccharides can be achieved as significant amount of hexoses and/or pentoses can be recovered from the pretreatment liquor. Pretreatment liquor also contains many inhibitors, such as furfural, 5-(hydroxymethyl)furfural (HMF), organic acids, and phenolic compounds (Jönsson & Martín, 2016). Those inhibitors may be originated from the chemical reagents, solvents, reaction by-products, or derived from the biomass. They can be harmful to enzyme cellulase and/or fermentation cultures, hence hinder the reaction kinetics and/or product yield.

Steam explosion, DA and sulfite pretreatment are feasible approaches for whole slurry bioconversion. Those processes are designed to reduce the recalcitrant of lignocellulosic biomass without generating the inhibitors. Meanwhile, dissolved lignin after those processes shall not precipitate onto the substrates when the spent

liquor was diluted or after pH adjustment (Lan et al., 2013b). The pretreated slurry can be used directly in SSF without washing and hence the dissolved sugars in the pretreatment spent liquor can be used by the microorganisms (Dong et al., 2018b). The following sections summarize the basics of each pretreatment strategies.

2.3.2.1 Steam explosion

Steam explosion is among the most widely applied pretreatment processes for lignocellulosic biomass. This process applied the air blasts force created after an instant change of vapor pressure from a high temperature digester (180-200°C) into atmosphere pressure (Sun et al., 2014). The structure of lignocellulosic biomass can be loosen due to significant shearing forces and heat, resulting in physical separation of fibers, hemicellulose removal, melting and partial depolymerisation of lignin (Zhu et al., 2015). Steam explosion pretreatment has been applied in a few pilot-scale studies to convert rice straw at high sugar yield. Sharma et al. (Sharma et al., 2015) compared the performances of steam explosion pretreatment process with and without mixing dilute H_2SO_4 (0.5% w/w) in the process and reported an increased yield (81.8% vs. 77.1%), lower oligomer (3.3 g/L vs. 13.6 g/L), at reduced temperature (180°C vs. 200°C) in comparison with water explosion. The main concerns of steam explosion process are of its high operation temperature, which often result in high concentration of growth inhibitors and energy inputs. Ammonia fiber expansion (AFEX) (Harun et al., 2013) and CO_2 explosion (Serna et al., 2016) were developed to reduce the operation temperatures. The two pretreatment media are both with low boiling points and therefore the explosion effectives can be easily achieved at 100-140°C and 80 °C for AFEX and CO_2 explosion processes, respectively. Both processes resulted in reasonable lignin

removal and sugar yield (76% and 55% for AFEX and CO_2 treatment, respectively). Further optimization of the two gases based processes, however, are still in progress to reduce the operation costs for more economical application.

2.3.2.2 Dilute acid pretreatment

Dilute acid pretreatment is another commonly applied technique in a biorefinery. Jung et al. (Jung et al., 2015a) applied 0.2% (w/v) dilute malic acid to treat rice straw at 190°C for 20 mins. The pretreatment whole slurry was used in SSF processes without solid-liquid separation. A reasonable ethanol yield of 62.8 % was achieved by fermenting the broth with 20% solid contents. Ethanol titer achieved 11.2g/L in this process, which is not particularly high in comparison with washed substrates. Pretreatment by-products in the spent liquor showed significant inhibiting effects to the fermentation microorganisms. Castro et al., (Castro et al., 2014) utilized dilute phosphoric acid steam to decompose *Eucalyptus benthamii* chips at 180-200°C (5-15 mins). The ethanol yields of the processes were 275-304 L per tonne dried wood biomass.

Mclntosh et al. (McIntosh et al., 2016) compared a DA and a DA-Steam Explosion process to convert whole *Eucalyptus grandis* trees into bioethanol. The experiments were demonstrated in a lab- and a pilot- scale reactor, respectively. Substrate prepared after the pilot-scale reactor (150L, 180°C/15 min/2.4 wt.% H_2SO_4 followed by steam explosion) showed higher digestibility (71.8%) than one prepared in lab system (2L, 190°C/5 min/4.8 wt.% H_2SO_4). The unwashed substrates were then charged into 2L and 300L fermentation systems for pre-saccharification simultaneous saccharification fermentation (PSSF) process. The ethanol yields of the two processes were 82.5 and 113 kg/ton biomass.

Kapoor et al., (Kapoor et al., 2017) also performed a pilot-scale experiment using a screw feeder horizontal to convert rice straw into bioethanol. The DA process (0.35 wt.% H_2SO_4, 162 °C and 10 min) resulted in a total 77% sugar recovery when the pretreated substrate was hydrolyzed at 20-25 wt.% solid loading and 50 FPU/g-residue enzyme loads. Finally, Liu et al., (Liu & Bao, 2017) developed a dry acid pretreatment process to treat a wet corn stover at an extremely high solid content (50%). The biomass was presoaked in 2 wt.% H_2SO_4 before pretreatment, which was carried out at 175 °C for 5 mins. The whole slurry was then sent directly to SSF after pH adjustment. Because minimum amount of water was applied in the pretreatment process the solid content of the fermentation broth can be controlled at very high level (30%), resulting an extremely high ethanol titer (10.8 v/v%) after the SSF.

2.3.2.3 Sulfite pretreatment

DA and steam explosion processes have shown their potentials in whole slurry SSF. With addition of pretreatment liquor more sugars were provided to fermentation organisms. However, the product yields and titers still cannot achieve the levels as one provided by washed substrates (Dong et al., 2018a). Residual lignin and pretreatment by-products (inhibitors) create strong impacts to hydrolysis and fermentation (Jönsson et al., 2013b). Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) pretreatment was developed to reduce this impact for high efficiency whole slurry SSF. The key design concept of SPORL was to modify the hydrophobicity of lignin with complete hemicellulose removal (Wang et al., 2009). Enzymatic efficiency and tendency of re-condensation can be improved and hence SSF became feasible even without complete delignification. Lan et al. (Lan et

al., 2013a) converted SPORL pretreated lodgepole pine biomass and achieved 47.4 g/L ethanol titer at a solid content of 19.5% without detoxification. In our further study presented in Chapter 4, we confirmed and overcame the heat stresses associated with the phenolic based inhibitors in this process (Dong et al., 2018a). Extremely high ethanol yield and titer was obtained by reducing the fermentation temperature from 35°C to 28°C for treating softwood. In this case, fermentation rate was a more sensitive parameter than hydrolysis when converting sulfite pretreated substrate in the whole slurry SSF process.

2.3.3 One-Pot Biorefinery

As described in the previous section that many ILs, *i.e.*, 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc] or [Emim][Ac]) can effectively dissociate lignin and hemicelluloses (Socha et al., 2014b; Sun et al., 2009), as well as reduce the crystallinity of cellulose (Cheng et al., 2012). In Type I biorefinery the dissociated lignin and hemicelluloses can be withdrawn from the cellulose-rich substrate (Viell et al., 2016). The de-crystallized substrate can be regenerated after washing and is easily hydrolysable by cellulase. The only concern of IL pretreatment processes is the high complexity of the multi-scale processes and the negative impacts of IL to enzyme activity. To overcome this problem Shi et al. (Shi et al., 2013) developed an innovative one-pot strategy to simultaneously extract the fermentable sugars, recover lignin, and recycle the IL in one-pot. A thermostable IL tolerant enzyme cocktail (JTherm) was used to hydrolyze the IL pretreated substrate. The authors successfully harvested 81.2% glucose and 87.4% xylose in 72 h in an IL solution under 10% solid loading. Furthermore, Xu et al. (Xu et al., 2015) discovered that cellulase obtained from *Trichoderma aureoviride* showed high

stability in the [Emim][DP] IL. Grewal et al. (Grewal et al., 2017) immobilized *Trichoderma reesei* cellulase to improve their stable in [EMIM][Ac].The immobilized cellulase was used to hydrolyze IL pretreated sugarcane bagasse and wheat straw for at least two cycles.

2.4 Pretreatment induced biomass recalcitrant factors

Biomatrix opening as well as lignin and/or hemicelluloses removal represents the most basic pretreatment mechanisms to increase the digestibility of biomass, but the performances can be related many factors (Chen et al., 2015a), *i.e*., the structure of cell wall, cellulose crystallinity, DP, cellulose grafted functional groups and lignin structure. This section reviewed the related factor in greater details.

2.4.1 Cellulose related factors

Pretreatment modifies the structure of lignocellulosic biomass and cellulose, which makes cellulose more accessible to enzymes. Factors affecting cellulose bioconversion are complicated due to existence of various components derived from the biomass during pretreatment. The following sections review the major pretreatment induced changes of cellulose-related features on enzymatic hydrolysis.

2.4.1.1 Crystalline

Cellulosic fibrils are parallel cellulose chains linked together in a highly ordered structure through hydrogen bonds (Neralla, 2012). Crystalline cellulose is compact and highly resistant to chemical and biological hydrolysis. As the minimum pore size on crystalline cellulose is less than 2 nm and the size of enzyme cellulase is 3-18 nm, cellulase can only digest the cellulose crystal from its surfaces [10]. Meanwhile, as

crystalline cellulose is highly hydrophobic, it can irreversibly bind with cellulase which lowers the amount of active enzyme in the hydrolysates. The amorphous cellulose obtained 10-fold hydrolysis rate as that of Cellulose I due to the 4-fold higher gross specific surface area.

2.4.1.2 Degrees of polymerization

The DP of cellulose represents the number of glucose in the molecule. Based on catalytic preferences of cellulase exoglucanases (Karimi & Taherzadeh, 2016), it appears that the greater the number of reducing (non-reducing) ends, the greater its cellulose accessibility. The lower the DP, the more reducing and non-reducing ends are available, and more cellulases would be expected to be able to work at one time while exposing more sites for endoglucanases attack. Hallac et al (2011) found that cellulose DP and fiber length have little effect on cellulase adsorption and the results of enzymatic hydrolysis of organosolv pretreated softwood shows indicated that synergistic effects of enzymatic hydrolysis could not be neglected during saccharification (Hallac & Ragauskas, 2011; Pan, 2012).

2.4.1.3 Grafted functional groups on cellulose

Grafting of functional groups on cellulose surface is an unexpected result of organosolv pretreatment processes. Pan et al. (Pan et al., 2006b; Pan & Sano, 2005) reported that hydrophobic acetate can be grafted on cellulose during acetic acid pretreatment. The acetyl groups can intercept with the binding of the catalytic domain of cellulases to cellulose. Substrate digestibility of an acetic acid-pretreated wood substrate was as low as 10% even when 60% of lignin was removed from the cell wall. Dilute alkali treatment at 50 °C removed the acetyl groups and hence

improved the cellulose conversion to 60%. Zhao et al. (Zhao & Liu, 2012) reported a similar pattern when treating sugarcane bagasse with formic acid. Cellulose formylation occurred with the pretreatment processes to achieve 80% lignin removal. Removal of the formyl groups using dilute alkali increased the cellulose digestibility by approximately 40%. Li et al. (Shuai et al., 2016b) discovered that GVL as a carboxylate ester also grafted on the cellulose surface through transesterification. After incubating with a dilute alkali solution the digestibility of the GVL-treated substrate increased significantly to approximately 98%. Since glucose yields of original GVL-treated substrate are over 60%, grafting is likely less severe when comparing with what occurs during acetic acid pretreatment.

2.4.2 Lignin related factors

Lignin (residues or suspended) is the most important factors limiting the rate and extent of enzymatic hydrolysis of pretreated substrate/hydrolysates (Li & Zheng, 2017). Besides providing a physical barrier to cover the cellulosic fibrils, lignin also causes recalcitrance through chemical interference by irreversibly binding cellulase enzymes and preventing further enzymatic activity on cellulose (Liu et al., 2016a).

2.4.2.1 Physicochemical properties of lignin

Lignins are amorphous, hydrophobic biopolymers composed of many phenolic monolignols. The potentials to cleave the two basic ether linkages are process-specific and has been presented in Table 2.6 (Sjostrom, 2013). Alkaline pretreatment can cleave α-O-4 linkage in phenolic structure but not the non-phenolic structures; organosolv pretreatment under acidic environment can cleave both α-O-4

and β-O-4 linkage; and sulfite treatment process can only cleave α-O-4 ether linkage but not β-O-4 linkage.

Table 2.3 Effectiveness of bond cleavage through various reagents

Pretreatment*	α-O-4 linkage		β-O-4 linkage	
	phenolic structures	non-phenolic structures	phenolic structures	non-phenolic structures
Alkali (I)	Yes	No	Yes	Yes
Acid + organosolv (I)	Yes	Yes	Yes	Yes
Acid + sulfite (II)	Yes	Yes	No	No

*Symbols in the parentheses represent the type of biorefinery approach

The chemical reactions in cleaving the two representative ether linkages under different types of biorefinery processes were illustrated in Fig. 2.3(a) and 2.3(b). The presented model lignin structures include two G-lignins linked at the α-O-4 position. The monolignol at the bottom right contains hydroxyl group linking to its phenolic structure at the 4th position while its α- and β- positions both linked with other lignins. The monolignol at the upper left linked with other lignins at the 4th and β-positions, but its α- position linked only with hydroxyl group. The concept diagram demonstrated the major functions and limitations of applied reagents and/or catalysts to cell wall decomposition during thermochemical processes.

Cleavage of the α-O-4 and β-O-4 ether linkage linkages under alkaline pretreatment (Type I biorefinery) was shown in Fig. 2.7(a). Cleavage of lignin at elevated pH can initiate most rapidly at the hydroxyl group at the 4th position of the phenolic structure, but this reaction can carried out only very slowly when the 4th position had been linked with another lignin. During pretreatment, more hydroxyl structure can be created to continue the cleavage of the linkages. Alkali can cleave some hydrolysable linkages in lignin and polysaccharides, which result in a

reduction in DP and crystallinity of the substrate (Kim et al., 2016). For Type II biorefinery which carry out at low pH, bond cleavage can start randomly at either the α- or β- positions disregarding whether there is a hydroxyl group bonding with the aromatic ring at the 4th carbon. In addition, acid based biorefinery processes can effectively cleave glucosidic linkages in hemicelluloses and amorphous cellulose, which further improve the enzyme accessibility and promote enzymatic hydrolysis (Leu & Zhu, 2013).

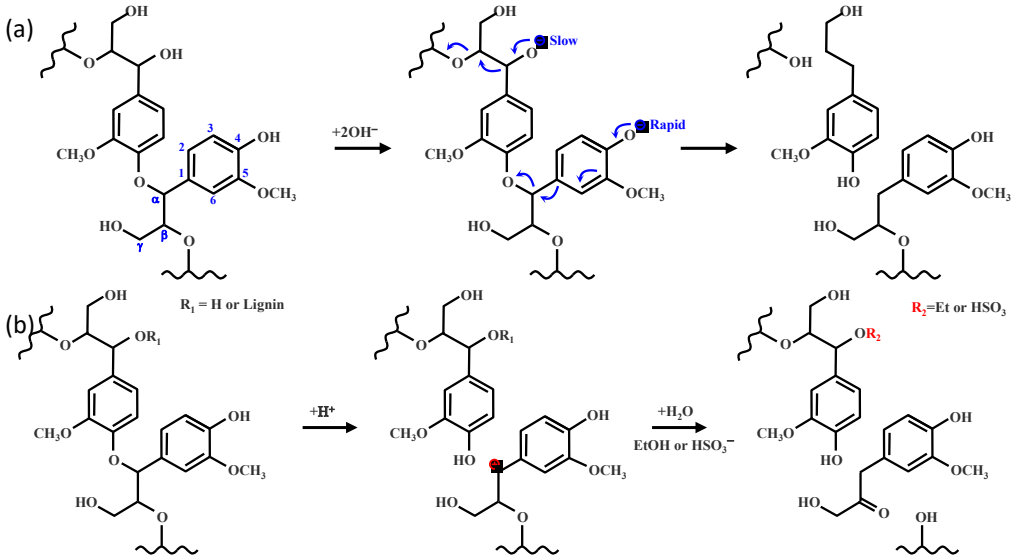


Fig. 2.7 (a) Cleavage of α-O-4 and β-O-4 bonds in alkaline pretreatment process; (b)cleavage of α-O-4 and β-O-4 bonds in dilute acid, organosolv, and sulfite pretreatment (SPORL) processes.

Lignins differed in molecular weights (MW), aliphatic hydroxyl (AlOH), phenolic hydroxyl (PhOH), and hydrophobicity after pretreatment. During pretreatment, lignin was depolymerized and formed in different sizes. Meanwhile, depolymerization of lignin caused by the cleavage of α-O-4 and β-O-4 ether linkages formed new phenolic hydroxyls (Yang & Pan, 2016). Thus, MW was negatively correlated with phenolic hydroxyl content. Even though hydroxyl group are hydrophilic, the quantity of the hydroxyl groups was not sufficient to affect or

change the hydrophobic nature of lignin. It has been proved that hydrophobicity of lignin was dominated by hydrophobic aromatic backbone of lignin that was related to the degree of condensation, which was affected by the severity of pretreatment (*e.g.*, chemical doses, cooking temperature and time). With different regents, the original functional groups were replaced by aliphatic hydroxyl, carboxyl or sulfonic group. Sulfonic group can reduce the hydrophobicity more effectively than other groups because sulfonic acid is a stronger acid (pKa=-3).

Pretreatment induced alternation of some lignins may cause inhibition to enzymatic saccharification, *e.g.*, the well-known non-productive bonds of lignin to enzyme. The non-covalent bonds governing between cellulase and lignin include hydrophobic interactions, electrostatic interactions, and hydrogen bonding. Hydrophobic interactions have been proposed as a major feature in the non-productive binding of cellulase to lignin. Direct measurement of adhesion forces between Kraft lignin and cellulase by Atomic Force Microscopy (AFM) showed a strong attractive force during the engaging of a hydrophobic functionalized AFM tip and the silicon wafer immobilized *T. reesei* cellulase. Molecular interactions between cellulase and hydrophobic tips were 13% and 43% higher than those with tips carrying COOH and OH groups, respectively. Adsorption kinetic analysis of lignin showed that both affinity constants and binding strengths are positively correlated with the hydrophobicity of lignin (Yang & Pan, 2016). DelRio et al. (2011) pointed out that introduction of hydrophilic groups (*e.g.*, carboxyl and sulfonic groups) can enhance the electrostatic repulsion between the lignins and the enzyme and thereby reduce the enzyme adsorption on the modified lignins (Del Rio et al., 2011). In addition, phenolic hydroxyl group of lignin also plays a key role in lignin-induced enzyme inhibition, and therefore hydroxypropylation of lignin to

block the phenolic hydroxyl groups significantly reduced the lignin inhibition (adsorption parameters of lignin did not change substantially). In other words, phenolic hydroxyl groups were effective to reduce enzyme activity but were less relevant to enzyme adsorption on lignin.

2.4.2.2 Surface activity of lignin

The presence of aromatic backbone and large amount of polar functional groups makes the lignin macromolecule diphilic. This structure, in turn, increases the surface activity of lignin and its derivatives and may influence enzymatic saccharification and production of secondary products when remaining or being introduced into the hydrolysates. In order to clarify the impacts of changed lignin characteristics and surface properties to enzymatic hydrolysis a comprehensive study was conducted and reported in Chapter 3.

2.4.3 Inhibitors released during pretreatment

During thermochemical pretreatment, in order to achieve a good enzymatic digestibility of cellulose, the operational conditions are tuned to remove hemicelluloses and/or lignin from the lignocellulosic matrix. Achieving high degrees of solubilization of hemicelluloses and/or lignin unavoidably leads to degradation of the solubilized fragments. The amount and nature of the formed degradation products, many of which are inhibitory to inhibit enzymatic hydrolysis as well as microbial fermentation steps, is directly related to the pretreatment method and conditions.

Under the acidic conditions typical for processes, such as steam explosion, acid pretreatment, sulfite pretreatment and organosolv pretreatment, the pentoses and

uronic acids resulting from hydrolysis of the hemicelluloses undergo dehydration with formation of 2-furaldehyde, hereafter referred to as furfural, while the hexoses are dehydrated to 5-hydroxymethyl-2-furaldehyde, hereafter referred to as HMF. HMF and furfural are unstable in the dehydrative medium, and can be subjected to further degradation to levulinic acid, formic acid, and to condensation reactions with formation of resins. Acetic acid was also formed as a result of the hydrolysis of the acetyl groups of hemicelluloses.

Under alkaline conditions the carbohydrates are better preserved than at low pH, while peeling reactions lead to endwise degradation of polysaccharides with formation of carboxylic acids (Jönsson & Martín, 2016). Acetic acid, formed by saponification of the acetyl groups, is another typical inhibiting product of alkaline pretreatments. Further, phenolic and aromatic compounds are formed from lignin and biomass extractives regardless of whether an acid or alkaline catalyst is added to the reaction. The large number and the diversity of the aromatic compounds found in different lignocellulose hydrolysates make identification and quantification of separate compounds complicated. Folin-Ciocalteu method is the most convenient approach to analyze the total phenolic contents in lignocellulose hydrolysates, while it should be avoided in experiments with redox reagents (such as reduced sulfur compunds including dithionite, dithiothreitol, and sulfite) (Jönsson et al., 2013b).

To overcome the inhibitory effects of pretreatment products in the pretreatment hydrolysate, a few techniques have been developed to remove the toxic chemical residues in the hydrolysates, including physical (evaporation and membrane separation), chemical (over-liming, activated charcoal treatment, ion exchange, neutralization and organic solvent extraction), and biological (treatment with laccase or peroxidase) techniques. However, these additional detoxification steps increase

the overall costs due not only to the capital and chemical costs but also to the loss of sugars. Novel methods for detoxification without the need for separate process steps are needed to be designed and developed for industrial implementation.

2.5 Valorization of building block chemicals

Complete utilization of the whole plant cell components is the primary target of the new generation biorefinery. While the first generation biofuel has been available industrially and continuously optimized recent research on bioenergy and biorefinery have broadened its focus to the other side of biomass spectrum, *e.g.*, from rapidly degradable sugars/starch (food crops), cellulosic fibers (mainly for paper making) and then to lignin (such as lignin monomers and value-added products). New values of the building block components have been discovered with various new challenges toward large scale applications as summarized in the following section.

2.5.1 Utilization of Pentoses and Hexoses in Fermentation System

Production of biofuels and bio-based chemicals to replace fossil products requires generation of revenue from each fraction of the feedstock. Lignocellulosic biorefinery will be more economically feasible if all the carbohydrates in the biomass can be harvested and utilized in the downstream processes. Complete consumption of carbohydrates relies on proper selection of fermentation strains and operation processes to consume the produced carbon.

In Type II biorefinery, sugar stream prepared after enzymatic hydrolysis may consist of up to more than 30% pentose (C5) over the total sugars (Ha et al., 2011). Those C5 sugar is generated in form of xylose existing in hardwood and herbal species. By using special or engineered strains a few co-fermentation technology has been

successfully applied to simultaneously utilize glucose and xylose in many cases, with conversion yields close to 95% (Wang et al., 2015). Among the natural pentose-fermenting yeasts, *Scheffersomyces stipitis* is able to provide high ethanol yields from pentoses. This strain is among the most promising yeasts for pentose fermentation due to its low nutrient requirements, high resistance to contaminants (Nakanishi et al., 2017; Yaguchi et al., 2018). It has been used as a fermenting microorganism for lignocellulosic hydrolysates from olive tree pruning biomass, sunflower seed hull, red oak wood, or sugar cane bagasse (Ma et al., 2017; Ruiz et al., 2017). Recombinant microorganisms such as *Escherichia coli* have been developed to generate strains very tolerant to acetic acid and can achieve high ethanol productions (Pedraza et al., 2016; Sandoval & Papoutsakis, 2016). It has been successfully applied to ferment different lignocellulosic residues for bioethanol, including sugarcane bagasse, *Eucalyptus*, sweet sorghum bagasse, or corn stover (Gupta & Verma, 2015).

Furthermore, xylose is a less favorable sugar to be utilized by fermentation species than glucose. Glucose induced carbon catabolic repression (CCR) is a frequently found problem which limited the utilization of C5 sugars. While capable to utilize xylose and glucose fermentation strains tend to prefer glucose than xylose as carbon source, resulting significant amount of xylose wasted after fermentation under the CCR condition (Wang et al., 2014). Pretreatment specific co-fermentation strategies of utilizing cellobiose with xylose have been demonstrated to overcome the CCR problem for production of ethanol (Ha et al., 2011; Tomás‐Pejó & Olsson, 2015), butanol (Noguchi et al., 2013) and lactic acid (Wang et al., 2014). The bioconversion process can be performed separately (separate hydrolysis and

fermentation, SHF); in combine with enzymatic hydrolysis (simultaneous saccharification and fermentation, SSF); as well as combine enzyme production with enzymatic hydrolysis (consolidated bioprocessing, CBP) (Loaces et al., 2017). Several fermentation modes have been developed to mitigate the solid content limitation and to increase the productivity of the overall process, such as semi-continuous (Brethauer & Studer, 2014), repeated batch (Sanda et al., 2011), continuous fermentation with (Ma et al., 2017) or without cell recycling (Andersen et al., 2015). In addition, numerous efforts have been made to optimize fermentation conditions such as pH or temperature to ease the non-isothermal conditions for saccharification and hydrolysis (Dong et al., 2018a; Narayanan et al., 2016).

2.5.2 Cellulosic Materials

Dissociation and regeneration of cellulose may be directly conducted in Type III biorefinery or a joint process in Type I biorefinery (for lignin removal such as Kraft pulp) followed by Type III process (for cellulose dissociation). Cellulose dissociated into ILs or other solution can be regenerated to form into many different value-added products, such as fibers, films, aerogels and scaffolds. Some recently published cellulose-based materials developed over the last few years were demonstrated in Table 2.4. Kosan et al. (Kosan et al., 2008) used five kinds of ILs to dissociate cellulose and preparing regenerated cellulose fibres. The regenerated fibres all showed superior tensile than Lyceoll fiber. Li et al. (Li et al., 2014) investigated the effect of wet spinning and draw rations on the structures and mechanical properties of the cellulose fibers regenerated from [Emim][Ac]. The maximum tenacity of the regenerated cellulose fibers reached as high as 2.73 cN/dtex. Kargl et al. (Kargl et al., 2015) prepared thin film by spin coating from

[Emim][Ac]/DMSO mixtures. The film can be used for packaging, biomedical materials, and other functional purposes. Turner et al. (Turner et al., 2005) prepared surface functionalized cellulose composite films using an 1-butyl-3-methylimidazolium chloride ([C4mim][Cl]) IL. This film can be used as sensing devices to detect various compounds including polyphenols, aromatic amines, and aminophenols and as solid support materials for enzyme-catalyzed transformations. Cellulose-starch composite gel was obtained by keeping the homogeneous mixture of cellulose (10wt.%) and starch (5wt.%) in [Bmim][Cl] IL for several days at room temperature (Kadokawa et al., 2009). Oshima et al. (Oshima et al., 2014) produced celuulose aerogel from [Bmim][Cl] IL. Iminodiacetic acid groups were successfully introduced to the aerogel for immobilized metal affinity adsorption of proteins, indicating its potential function as polymer supports with high protein adsorption capacities. Zhou et al. (Zhou et al., 2014b) demonstrated a facile method to prepare polyaniline (PANI)-decorated cellulose aerogel via a dissolve/regeneration route using [Bmim][Cl] IL. PANIcellulose aerogel exhibited excellent photocatalytic activity towards the degradation of methylene blue. Kimura (Kimura et al., 2014) et al dissolved cellulose in [Emim][Ac] and [Dema][Fa] with a water content of 18 wt.% and then irradiated with γ-rays under aerated condition to produce new cellulose gels. The electronic conductivity of the cellulose was found to be 3.0 mS cm^{-1}, which has great potential for applications in optoelectronic and medical devices. Singh et al. (Singh et al., 2013) prepared scaffolds from cellulose and silk blends in [Emim][Ac]. The scaffolds can direct stem cell chondrogenic fate, which can be used for in vitro tissue engineering or stimulating endogenous stem cells to initiate cartilage repair.

Table 2.4 Cellulose products derived from IL pretreated/regenerated cellulose

Ionic liquor	Process	Product	Application	Ref.
[Bmim][Cl], [Emim][Cl], [Bmim][Ac] and [Emim][Ac]	Dry-wet spinning process	Fiber	Textile, Filtration	(Kosan et al., 2008)
[Emim][Ac]	Wet spinning process	Fiber	Textile, Filtration	(Li et al., 2014)
[C4mim][Cl]	Spin coating	Film	Sensing devices	(Turner et al., 2005)
[Emim][Ac]/DMSO	Spin coating	Film	Packaging, optical products	(Kargl et al., 2015)
[Bmim][Cl]	Long time aging	Aerogel	Not specified	(Kadokawa et al., 2009)
[Bmim][Cl]	Heating-cooling process	Aerogel	Protein adsorption	(Oshima et al., 2014)
[Bmim][Cl]	Heating-cooling process	Aerogel	Photocatalysis	(Zhou et al., 2014b)
[Emim][Ac] and [Dema][Fa]	Irradiation crosslinking	Aerogel	Optoelectronic and medical devices	(Kimura et al., 2014)
[Emim][Ac]	Heating-cooling process	Scaffolds	Tissue engineering	(Singh et al., 2013)

2.5.3 Functional lignin

Lignin is the second largest carbonaceous component in the plant call wall (20-35 wt.%). It is also the only renewable source of aromatic compound in nature environment. The structure, purity, and properties of lignins isolated from biorefinery processes largely depend on the feedstock and the delignification process. Alkali lignin can be harvested from the precipitate of acidified pretreatment liquor (pH<2) (Mingliu et al., 2013). Lignin generated from organosolv pretreatment process contained high purity of phenolic structures and trace amounts of carbohydrates (Pan et al., 2005a). This sulfur-free component is a suitable precursor of many chemical processes (Pan, 2012).

2.5.3.1 Production of lignin monomers

Lignin has been used to produce lignin monomers after direct hydrogenolysis. However, the product yields of the hydrogenolysis processes were largely dependent on the quality of lignin. The yield of conversion for solid untreated biomass was 45-55 mol.% while the conversion of organosolv pretreated lignin decreased significantly to 5-20 mol.% (Shuai et al., 2016a; Xu et al., 2014). The reduction of yield is mostly due to lignin condensation during the pretreatment process (Fig. 2.8(a)). The results of 2D HSQC NMR spectra suggested that lignin structure obtained after GVL pretreatment was similar to native lignin, which could be attributed to the low acid level and extraction temperatures achievable using GVL solvent (Luterbacher et al., 2015). The theoretical yields of monomer were still below 70% from GVL pretreated corn stover and below 40% for maple wood, indicating that lignin condensation still occurred during the thermochemical process. Luo et al. (Luo & Abu-Omar, 2018) compared three organosolv (*i.e.*, methanol,

acetone, and acetic acid) for extraction of native lignin from wild-type and genetically modified poplars. Methanol extracted lignin gave the best yield of >60% of aromatic products (*i.e.*, guaiacol, isoeugenol, and 4-propenyl syringol). NMR spectroscopy demonstrated that methanol served as a nucleophile reacted with the Cα benzylic carbocation formed during the organosolv pretreatment while minimizing lignin condensation during the pretreatment process (Fig. 2.8(b)). Shuai et al. (Shuai et al., 2016a) added formaldehyde during biomass pretreatment produces a soluble lignin fraction that can be converted to guaiacyl and syringyl monomers at near theoretical yields during subsequent hydrogenolysis (47 mole % of Klason lignin for beech and 78 mole % for a high-syringyl transgenic poplar, Fig. 2.8(c)). These yields were three to seven times those obtained without formaldehyde, which prevented lignin condensation by forming 1, 3-dioxane structures with lignin side-chain hydroxyl groups.

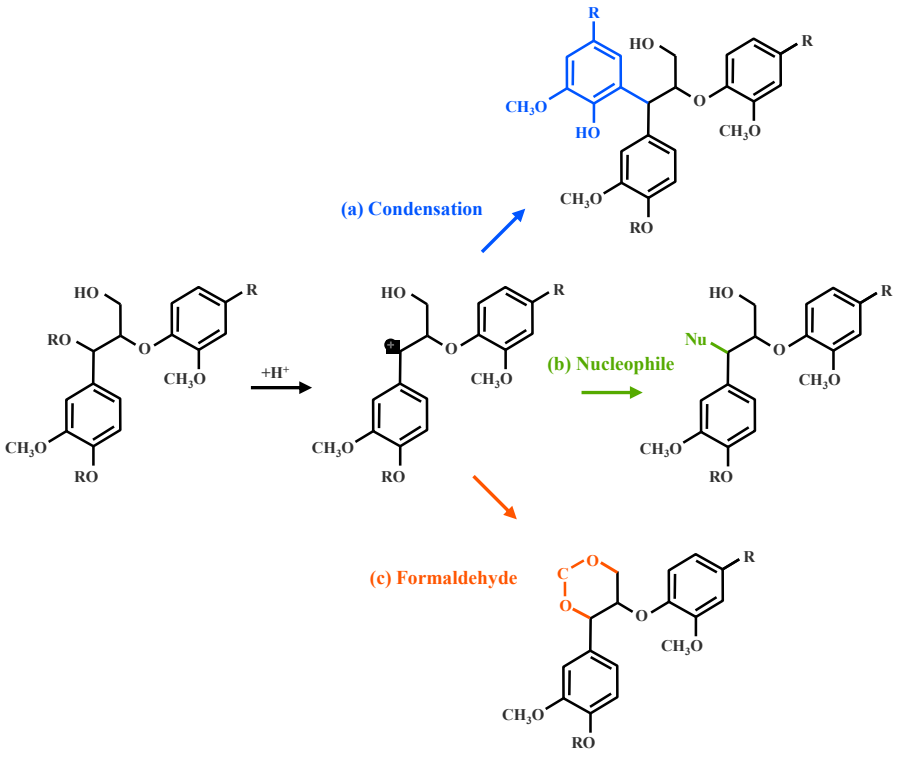


Fig. 2.8 Condensation or stabilization of dissociated lignin.

2.5.3.2 Lignin based resins

Converting lignin into monomers is an energy intensive process, and hence lignin is currently more valuable serving as a fuel than chemical precursor. The heating value of pure lignin is 26.7 GJ/ton, which is economically competitive or even better than coal (20.9 GJ/ton) (Archambault‐Léger et al., 2015). Direct synthesis of solvent dissociated lignin for new materials without converting it into monomer could be economical and environmentally-attractive for lignin valorization. Xu and Ferdosian (Xu & Ferdosian, 2017) reviewed the applicability of lignin-based phenol-formaldehyde (LPF) resins using lignosulfonate, kraft lignin, organosolv lignin, biorefinery residues, and two other lignins. The authors suggested that biorefinery derived lignin may substitute the utilization of phenol by 30-50 wt.% without chemical modification. Low molecular weight depolymerized organosolv lignin after water-ethanol treatment in hydrogen environment were with more reactive sites and resulted in an high substitution rate of 75 wt.% of the LPF resins. Lignin can also be hydroxymethylated (methylolated) or phenolated to reduce water adsorption and increase the impact strengths of the final products (Jiang et al., 2018). In a recent publication, Li and Sattely (Li et al., 2018) synthesized a formaldehyde-free adhesive using kraft lignin and a few non-toxic crosslinkers (*e.g.*, ethylene glycol diglycidyl ether) and evaluated the properties of the corresponding plywood products. The new resins and products showed outstanding characteristics in mechanical properties to meet the related standard and were comparable to the typical formaldehyde based products.

2.5.3.3 Lignin carbon fiber

In addition to green resin production, lignin is a potential feedstock for carbon fiber production. Lignin is a biopolymer with outstanding characteristics such as high carbon content, low cost, great renewability, and high availability in large scale (Strassberger et al., 2014). Application of lignin for carbon fiber production was first reported in the 1960s (Bajpai, 2017). The related techniques and industries have not been widely applied commercially, which is mostly related to the growth of petroleum economy over time. Manufacturing process of the lignin carbon fibers include spinning, stabilization, carbonization, and applications. In recent studies lignin after chemical modification (*e.g.*, butyrated) was mixed with polyacrylonitrile (PAN) and then electrospun into ultrafine carbon fibers (Ding et al., 2016). Li and Yuan et al., (Li et al., 2017) investigated the properties of lignin carbon fibers generated after fractionated lignin and suggested that higher molecular weight, less hydroxyl groups, and more linear structures can improve the quality of lignin derived carbon fibers. For more details, Fang, Sun et al., (Fang et al., 2017) provided a comprehensive review on the essential criteria to manufacture lignin-based carbon fibers.

2.6 Sustainability concerns of biorefinery

Bioconversion is an environmentally attractive option to valorize biomass derived from organic wastes. Biorefinery in comparing with direct incineration provides can better preserve the valuable components in the plant cells for functional utilization in the future. With large amount of new findings it is foreseeable that the related techniques could be commercialized in a very near future. The related environment and technical concerns, however, has not been categorized or widely disclosed in most of the literatures, which most likely owes to lack of information of

different processes. Meanwhile, biorefinery is a multi-staged process including many different processes in a broad range of technical discipline, *e.g.*, plant science, thermochemical systems, and biological process. Selection of techniques for different applications needs to consider the region- and technique-specific limitations, such as industrial background, supportive experts, and infrastructures. This chapter summarizes the environmental and technical criteria of three basic biorefinery concepts toward sustainable development of biomass valorization,

2.6.1 Phases of reagents

Environmental issues of biorefinery system may be referred to direct contamination or in-direct impacts due to excessive waste of resources. Direct environmental impacts such as effluent toxicity to fish or emissions of volatile organic compounds (VOCs) can be directly monitored by instruments and the acceptable values have been well-regulated by the stakeholders. Indirect environmental measures such as reactor profiles, risks of operation, and water footprints are more relevant to process design/control conditions and are more difficult to regulate. Elevated operation pressure induced by the applied chemicals at the desirable temperature is a critical parameter affecting the reactor profile, operation difficulty, and capital costs of the biorefinery. Fig. 2.9 shows the phase diagrams of typical solvents used in the pretreatment processes (curves) and the representative ranges of operation temperatures (symbols) based upon published works. The optimal operation pressures of different pretreatment processes ranged dramatically from approximately 10 kPa (GVL) to nearly 10,000 kPa (ammonia) at 120°C. The vapor pressure of the reagent solvent increases nearly exponentially with increased operation temperature in the confined space. Typical autoclaves operating

are designed to hold 100-110 kPa, which is approximately equivalent to the pressure of water vapor operating at 100-121°C, respectively. Other low-boiling point solvents such as THF or ethanol require special high-profile reactor to hold at least 1-1.2 MPa pressure at temperature between 150-210°C. This requirement limits the applicability under various conditions or region.

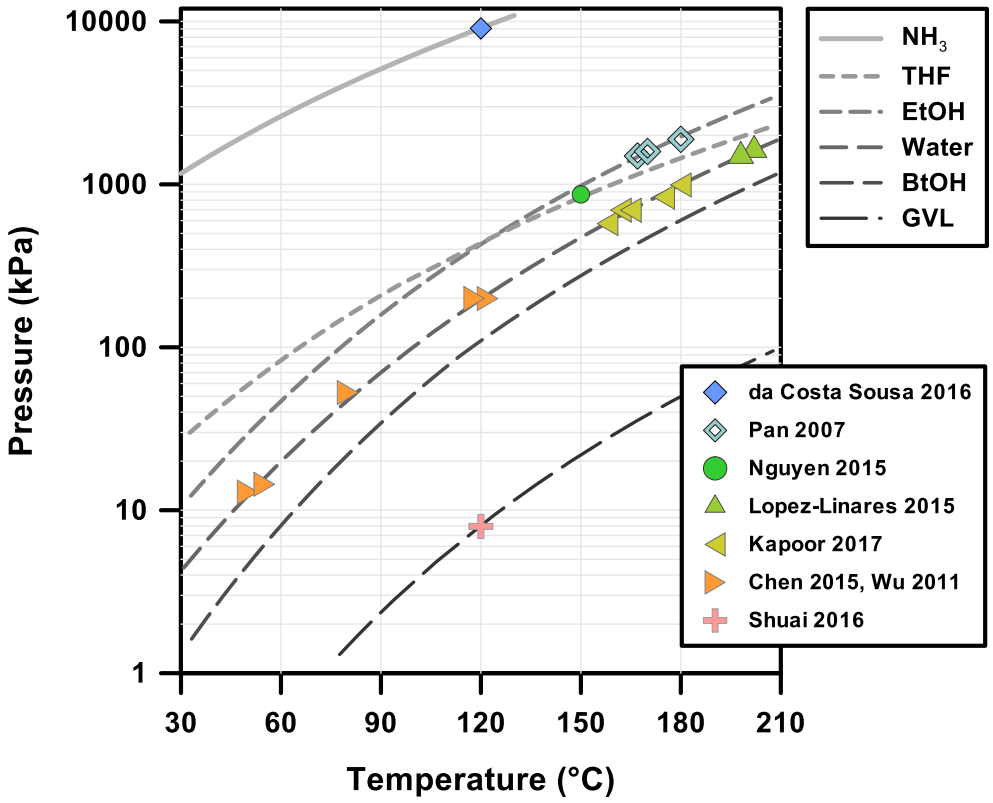


Fig. 2.9 Temperature-pressure relationships (curves) of different solvents for biomass pretreatment. Symbols represent the practical ranges of pretreatment temperatures used in published studies. Note: the pressures were presented in log scale.

2.6.2 Energy-water nexus

The effectiveness of plant cell wall dissociation is roughly related to the liquid/solid (L/S) ratios during the thermochemical pretreatment process. The average L/S ratios of representative pretreatment processes were demonstrated in Fig. 2.10. The liquid portion is contributed by water and co-solvents (*e.g.*, the organosolv

processes) and the error bars showed the standard deviations of different processes. With increased cooking temperatures the L/S ratios were gradually reduced for withdrawing similar amounts of cellulose (80%) from the feedstock in the studies. Meanwhile, the residue lignin contents also decreased with increasing temperature (results not showed), which may be due to the reduced severity of chemical reagents/co-solvent(s) used in the processes. The broadest ranges of L/S ratios and cooking temperature were observed for the alkaline pretreatment. Organosolv processes cover a wide temperature ranges but not the L/S ratio. Dilute acid and SPORL pretreatment can be conducted at different temperature with wide variety while water vapor based steam explosion can be only conducted at high temperature (>200°C).

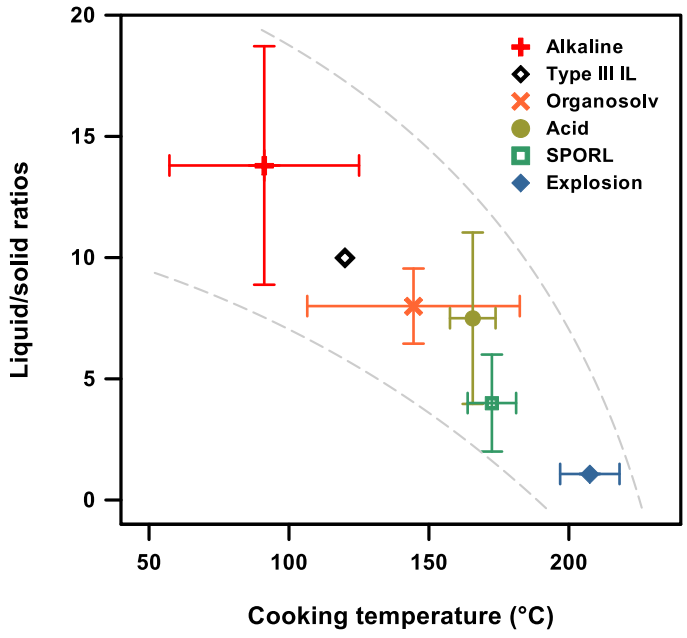


Fig. 2.10 Liquid/solid ratios of published pretreatment processes as a function of treatment temperature. Water (with co-solvent) consumption reduced with increased temperature.

Biorefinery energy is directly related to the amount of liquids (solvent + water) used in the pretreatment and SSF processes (Dong et al., 2018a). The categorized

water consumptions for different biorefinery systems were presented in Fig. 2.11, which includes the cooking, washing, and fermentation waters. When more water was used in the pretreatment and fermentation processes more energy was needed in the cooking and distillation processes (more detailed calculation of the water-energy nexus please referred to Dong et al., (Dong et al., 2018a)). Alkaline pretreatment processes used larger amount of water (12.8 w/w for cooking, and 9.7 w/w for washing/fermentation water) than other processes, which may be due to its capability in delignification at lower cooking temperatures (Fig. 2.11). SPORL and one-pot IL biorefinery required less amount of water (3.7-4.0 w/w for cooking and 4.4-6.2 w/w for washing and fermentation water), but it use smaller amount of fermentation water. It should be also noted that the numbers reported here were just to reflect the current status of recently published works with promising performances. It does not represent the final capacities for full-scale applications. It showed the possible directions for future development while it should not be used for making any conclusive comparison. Meanwhile, all the washing processes were assumed to be accomplishable by using three times of rinsing waters for one unit mass of pretreated biomass (in dried weight) and the solid loads of effective SSF were all at 15 wt.% disregarding the small variations among different publications.

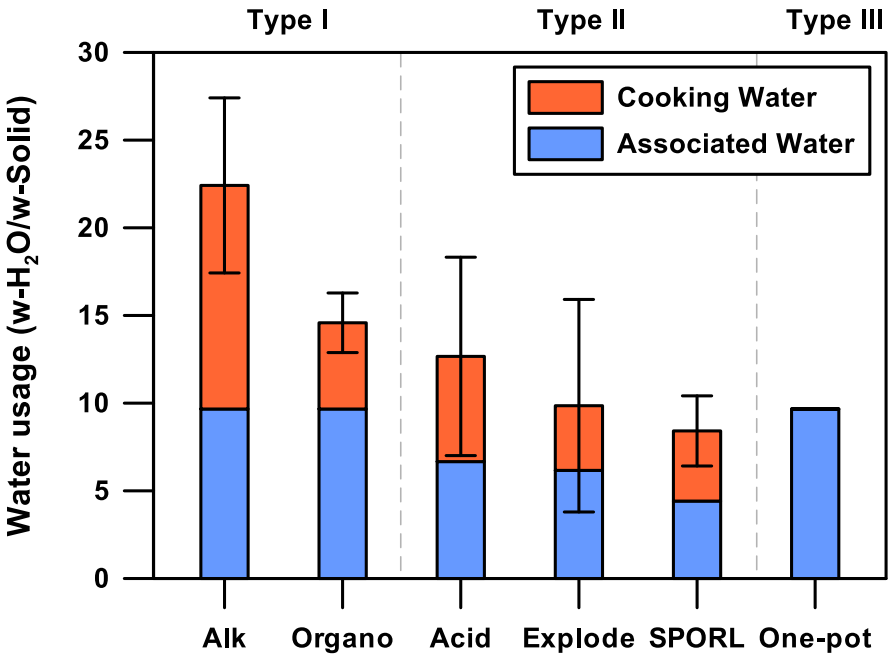


Fig. 2.11 Total water consumption of different biorefinery processes. The cooking water refers to the water used in the pretreatment processes. Associated water includes resining water and water used in the SFF processes. Error bars show the standard deviations of the data.

Table 2.5 Recyclability of reagents

Pretreatment	Chemical	Recovery process	Recovery Ratio	Energy	Capital	Ref.
Alkaline	NaOH	Kraft process	84.0%	Middle	Middle	(García et al., 2011)
	NH_3	Evaporation	99.6%	Low	Low	(da Costa Sousa et al., 2016)
Organosolv	Ethanol	Distillation	93.0%	Middle	Low	(García et al., 2011; Liu et al., 2018)
	Ethylene glycol	Distillation	70.0%	High	Low	(Alriols et al., 2009)
	GVL	CO_2 extraction	99.5%	Middle	High	(Shuai et al., 2016b)
	p-TSOH	Crystallization technology	NG	Low	Low	(Chen et al., 2017)
Ionic liquor	[C2mim][O2CH][a] and [C2mim][OAc][b]	Membrane separation	Concentrated from 5% to 50%	Low	High	(Lynam et al., 2016)
	[Dbne][Dep][c]	Evaporation	Reused at least 5 times	High	Low	(Liu et al., 2017b)
	[Bmim][PF6][d]	Liquid-liquid extraction	Product yield reduced from 93% to 83% in the 2nd cycle	Low	High	(Fukuyama et al., 2002)
	[Bmim][PF6][d]	Supercritical CO_2 extraction	>95% organic solutes were separated from an ionic liquid	Low	High	(Blanchard & Brennecke, 2001)
	[Emim][Ac]	Freeze crystallization	72.3%	Middle	Low	(Liu et al., 2018)
	[Emim][Dep]	Freeze crystallization	59.1%	Middle	Low	(Liu et al., 2018)

[a]1-ethyl-3-methylimidazolium formate; [b]1-ethyl-3-methylimidazolium acetate; [c]1-ethyl-1,5-diazabicyclo[4.3.0]-non-5-enium diethylphosphate; [d]1-n-butyl-3-methylimidazolium hexafluorophosphate 2.5.3. Recovery of chemicals

2.6.3 Recovery of chemicals

Many of the chemical reagents used in the biorefinery processes are costly and must be recycled to achieve economical and environmentally-sound operation. Lignin in the liquid fraction of alkaline-based pretreatments can be recovered after lignin precipitation induced by pH modification. This alkaline based spent liquid is therefore not appropriate to be used in the SSF for whole slurry conversion. Organosolv and IL also need to be washed away to prevent enzyme inhibition. More specifically, if the biomass has been completely dissolved in the ionic liquor, an anti-solvent must be used to regenerate the pretreated biomass before further processing. Water is often used as a rinsing agent and an anti-solvent in Type I and Type III biorefinery. Some representative recycling processes of chemicals were presented in Table 2.5.

Garcı´a et al., (García et al., 2011) recovered 84% NaOH from the spent liquor of an alkaline pretreatment process. The spent liquor was first concentrated to 60% solid content and then burned for energy without lignin regeneration. The incombustible solid residue contained large amount of Na_2CO_3 and was regenerated to $CaCO_3$ and NaOH by mixing with $Ca(OH)_2$. In an ammonia pretreatment process, NH_3 was evaporated directly from the pretreated slurry to recycle up to 99.6% NH_3 used in the biorefinery process (Type I) (da Costa Sousa et al., 2016). Ethanol is a favorable solvent in the biorefinery process as it is easily recoverable (García et al., 2011). Because ethanol is also the product of SSF process, recycle of the pretreatment solvents can be conducted by the distillation unit after mixing the resining water with the fermentation broth. This process allows recovery of 93% ethanol. Alriols et al., (Alriols et al., 2009) simulated an optimized design for

ethylene glycol (high boiling point)-water distillation. A liquid stream containing 82 wt.% ethylene glycol, furfural, acetic acid and 14 wt.% water was isolated and a residual stream composed by lignin, sugars and remaining ethylene glycol (18 wt.%) was obtained. This resulted in 70 and 80 wt.% reduction of ethylene glycol and water, respectively.

Shuai et al., (Shuai et al., 2016b) recovered over 99.5% of GVL from pretreatment slurries by using a modified liquid-CO_2 extraction process. Liquid CO_2 was continuously flowed through the slurry at high pressure to recover the low-boiling point solvent into a low-pressure vessel. This approach can completely separate the solvent from dissolved sugars, lignin and solid materials and may be applicable to other solvents with similar characteristics. Bian, Zhu et al., (Bian et al., 2017) recycled *p*-TsOH using a commercially proven crystallization technology by repeatedly cooling the concentrated spent acid solution to ambient temperature. As *p*-TsOH has relatively low water solubility than sugars it is possibly that the process can easily conducted in a whole slurry process. Furthermore, direct contact membrane distillation is a low temperature and ambient pressure process which can be applied to separate water from ILs, of which Lynam et al. (Lynam et al., 2016) increased the solvent recovery rate from 5 wt.% to 50 wt.% from the pretreatment liquor. Fukuyama et al., (Liu et al., 2017b) investigated the reusability of the ([Dbne][Dep]) from wool keratin liquor. The IL rich liquor was condensed by rotary evaporation at 120°C to increase the recyclability of IL by five times. Blanchard et al., (Blanchard & Brennecke, 2001) also used CO_2 to extract organic solutes from an [Bmim][PF6] IL and recovered up to 95% solutes. Liu et al., (Liu et al., 2018) developed a freeze crystallization/evaporation process for recycling ILs from a

cellulose spinning process. Compared to evaporation only for recycling of [Emim][Ac] and [Emim][Dep], the energy requirement for the new process was significantly reduced by approximately 25% in the new process. Recycle of chemicals is a complex process and its performance can considerably affect the operation costs of the biorefinery process. However, most of the processes are technically more feasible than pretreatment or fermentation and many instruments are readily available industrially.

2.7 Visions and future development

Applying biorefinery for waste valorization is a prospective way to mitigate climate changes without damaging food security. The three biorefinery strategies reviewed in this study all aim to maximize the benefits of bioconversion processes but limitations still existed to be resolved. Type I biorefinery is a relatively mature process (*e.g.*, Kraft pulp) that can produce high quality lignin (Lignin I in Fig. 2.5) and cellulose; but it is energy intensive and may not completely harvest the hemicelluloses for fermentation. Type II biorefinery can produce highest titer of fermentation products; but its lignin by-products (Lignin II) are with lower values and may create more negative impacts to the environment. Finally, Type III biorefinery is with high potential and, without the need of high profile digesters, very simple to operate; but the IL can be very costly and difficult to recycle. Meanwhile, water consumption of the related processes should not be overlooked as it is closely related to energy consumption, reagent recyclability, product yields, and environmental problems. The parameters provided in this review addressed the

difficulty of biorefinery and should be optimized with proper experiments before region-wide applications.

Chapter 3 Insight into the maximized beneficial effects of lignosulfonate in the whole slurry enzymatic saccharification through surface activity analysis

3.1 Introduction

Enzymatic saccharification of cellulose is the primary step in biorefinery processes for converting lignocellulosic biomass into biofuels and other chemicals (Bozell & Petersen, 2010). Cellulose in the lignocellulosic biomass is physically and chemically protected by the highly robust heterogeneous ultrastructure of lignin and hemicelluloses (Fengel & Wegener, 1983) that can effectively resist the biodegradation carried out through enzymatic saccharification (Sweeney & Xu, 2012). In a biomass biorefinery, substantial loading of enzyme is required to break down the lignocellulose into fermentable sugars. The high enzyme content significantly increases the cost of the process whereby enzymatic hydrolysis can account to approximately 27 to 40% the total cost of the overall bioconversion processes (Ferreira et al., 2009; Ma et al., 2008).

Introducing surfactants to the hydrolysis reaction is a promising technique to increase the rate and yield of the enzymatic hydrolysis, and consequently to reduce the enzyme loading and operating costs (Mackenzie & Francis, 2014; Okino et al., 2013; Yang et al., 2011). Surfactants can enhance both the enzyme solubility and enzyme activity during the hydrolysis, and hence increasing the efficiency of the hydrolysis reaction (Chen et al., 2006; Mahammad et al., 2010; Xiang et al., 2006). Due to their strong interaction with hydrophobic surfaces, surfactants can bind to the lignin residuals of the substrate to reduce the non-productive enzyme adsorption and

to increase the amount of available enzymes (Kumar & Wyman, 2009b; Yang et al., 2011). Surfactants can also protect enzymes from heat and agitation, and therefore assisting to maintain the enzyme activity in the hydrolysate (Mackenzie & Francis, 2014; Okino et al., 2013; Wang et al., 2006). One plausible mechanism to this protection effect is that the "reverse micelles" formed by the surfactants can reduce the detrimental effects of the ambient environment on the enzymes (Chen et al., 2006; Xiang et al., 2006). Meanwhile, surfactants may enhance the enzyme stability by reducing the surface tension of the substrate and facilitate enzyme adsorption and desorption (Mahammad et al., 2010; Park et al., 2002; Tilton et al., 1991).

LS are lignin-derived products which exist in sulfite pretreatment hydrolysates (Wang et al., 2013c). Despite the well-accepted concept that pretreatment hydrolysate exhibits an inhibitory role to enzymes, some studies have recently demonstrated the beneficial effects of LS on the cellulose saccharification. This finding has rendered the whole slurry fermentation process a one-pot reaction, where the pretreated substrate can be hydrolyzed and fermented with the pretreatment liquor to increase the yield and reduce the water consumption (Cheng et al., 2015c; Cheng et al., 2014a; Zhou et al., 2014a; Zhou et al., 2013e). The inhibitory or enhancing effect of the pretreatment liquor has been related to the properties and amount of the LS (Lou et al., 2014; Wang et al., 2013b; Zhou et al., 2013d). Wang et al. (2013b) found that the enzymatic digestibility of cellulose decreased rapidly when a small amount of LS was introduced in the hydrolysates, but the performance of the hydrolysis reaction improved when the dose of the LS surpassed a critical concentration. Other studies showed that LS with high molecular weight and low degree of sulfonation inhibited the saccharification process, while low molecular

weight LS with high degree of sulfonation enhanced the enzymatic hydrolysis (Lou et al., 2014; Zhou et al., 2013d). At high LS doses, the inhibition effect of the LS with low molecular weight and high sulfonation was also observed (Lou et al., 2014). Despite several sporadic hypotheses, to the best of our knowledge, no systematic study has been conducted on the effect of LS to enhance/inhibit the saccharification process. In order to properly design the LS-assisted saccharification system, a more solid understanding of the mechanism of the LS influence on the enzymatic hydrolysis may deem necessary (Lou et al., 2014; Wang et al., 2013b; Zhou et al., 2013d).

In this study, the enzymatic activities and reaction kinetics of the hydrolysis process under a well-controlled LS-enzyme complex have been investigated. The objective of this study is to elucidate the enhancing and inhibitory mechanisms of enzymatic saccharification by examining the specific binding affinity of enzyme to substrate and product formation, using the appropriate models as well as the enzyme activity. Strategies to overcome the inhibitory effects of the LS for whole slurry fermentation process have also been provided to validate the proposed mechanism.

3.2. Materials and methods

3.2.1 Materials

Monterey pinewood chips were purchased from Tianjin Ji Xing wood processing factory (Tianjin, China). Commercial cellulase enzymes Cellic Ctec2 were generously provided by Novozymes Investment Co. Ltd. (Beijing, China). Two types of LS with different characteristics were purchased. Both of them were derived from the sulfonation of Kraft lignin. The first type of LS (denoted as LS1) was

purchased from Xiya Chemical Industry Co. Ltd. (Shangdong, China). The other type of LS (denoted as LS2) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). The LS used in the present study was centrifuged to remove water insoluble. It was then separated and purified using dialysis membrane with a cutoff molecular weight of 500 Da. Table 3.1 lists the sulfur contents and molecular weights of both lignin samples.

Table 3.1 Molecular weight distribution and elemental sulfur content of the LS

Lignosulfonate	M_n**(Da)**	M_w**(Da)**	M_w/ M_n	**Sulfur (mmol/g)**
LS1	1265	1331	1.05	2.8
LS2	6592	31230	4.74	2.2

3.2.2 Sulfite pretreatment

The laboratory-scale sulfite pretreatment process was conducted in a 4-L rotary-type electric heating digester (KRK 2611, Tokyo, Japan) (Cheng et al., 2014a). In each batch, a total of 500 g wood chips were pretreated using the sulfite pretreatment method under three predesigned conditions, DA with 1.2% (v/w) sulfuric acid, SP10 with 10% (w/w) bisulfite and 1.2% (v/w) sulfuric acid and SP20 with 20% (w/w) bisulfite and 1.9% (v/w) sulfuric acid (Table 3.2). The temperature profile of the heating process was monitored using a thermocouple. The digester was heated to 165°C at a rate of 5˚C/min and was maintained at that temperature with a fluctuation of ±1°C for 75 min. The digester was then water-cooled to reach the room temperature before collecting the substrate and the liquor. The substrate and the spent liquor were separated by using a nylon cloth and weighed separately. The spent liquors from the pretreatment of the SP10 and SP20 were denoted as H-SP10

and H-SP20. Also, symbols U and W were used in Table 3.2 to designate the unwashed and washed substrates, respectively.

3.2.3 Chemical composition the substrates and liquors

The chemical compositions of the solid samples were measured according to the procedures established by National Renewable Energy Laboratory (Chum & Overend, 1992). Briefly, the solid samples were ground to pass through a 20-mesh sieve before a two-stage acid hydrolysis (72% H_2SO_4, 30 °C, 1 h dilution to 4%, 121 °C, 1 h). Concentrations of sugars and inhibitors in the hydrolysates were analyzed on a high-performance liquid chromatography (HPLC, Shimadzu Corp., Kyoto, Japan) equipped with a refractive index detector. The monosaccharides (glucose, xylose, arabinose, and galactose) and cellobiose in all the hydrolysates were analyzed by an Aminex HPX-87P column (Bio-Rad) at 85°C at an eluent (deionized water) flow rate of 0.6 ml/min. Acid insoluble lignin content was determined gravimetrically after hydrolysis.

Table 3.2 Pretreatment conditions and chemical compositions of monterey pine and the substrates by sulfite pretreatment method

Pretreatment Label	Pretreatment conditions	pH	Samples	Glucan	Xylan	Galactan	Mannan	Acid Insoluble Lignin	Yield
Monterey pine	N/A	N/A	N/A	41.8%	3.7%	4.6%	12.1%	25.2%	100%
DA	165 ºC, 75 min, 1.2% (v/w) H_2SO_4,	0.4	Washed solid substrate (w/w)	54.9%	ND	ND	ND	40.3%	43.2%
SP10W	165 ºC, 75 min, 1.2% (v/w) H_2SO_4, 10% (w/w) $NaHSO_3$	1.2	Washed solid substrate (w/w)	59.8%	0.2%	ND	ND	29.3%	57.5%
SP10U			Unwashed solid substrate (w/w)	47.9%	1.6%	1.3%	6.8%	25.1%	79.2%
H-SP10*			Pretreatment hydrolysate (g/L)	13.9	6.9	5.8	26.7	N/A	15.6%
SP20W	165 ºC, 75 min, 1.9% (v/w) H_2SO_4, 20% (w/w) $NaHSO_3$	1.2	Washed solid substrate (w/w)	79.1%	0.2%	ND	ND	10.7%	37.8%
SP20U			Unwashed solid substrate (W/W)	48.3%	1.6%	1.2%	6.8%	15.2%	76.8%
H-SP20*			Pretreatment hydrolysate (g/L)	13.2	6.7	5.8	26.8	N/A	16.7%

N/A: Not applicable; ND: Not detectable; * Carbohydrates in H-SP10 and H-SP20 are form of both monosugars and oligosugars

3.2.4 Sulfur content analysis and determination of lignin molecular weight

The sulfur content of LS was analyzed using the bomb-washing standard method of American Society for Testing and Materials (ASTM, E775-87). Gel permeation chromatography (GPC) coupled with ShodexOHpak SB-804 HQ and SB-806 HQ column were employed to determine the molecular weight of LS. A $NaNO_3$ aqueous solution of 0.10 mol/L was used as eluent at a flow rate of 1.0 mL/min. The eluent was determined by the means of Evaporative Light Scattering detector (ELSD, Wyatt, USA) and dextran was used as the criterion.

3.2.5 Surface activities of lignosulfonates

Surface activities of the lignosulfonates were examined by recording the alterations in the surface tension of the enzyme solutions with increasing the lignosulfonates concentration using a DCAT 21 tensiometer (DataPhysics, Filderstadt, Germany). The enzyme concentration was kept at the same amount as used in the hydrolysis experiment.

3.2.6 Hydrolysis experiment

Enzymatic hydrolysis was conducted in 150 mL flasks at 50°C with a total working volume of 50 mL. The pH level of the solution was adjusted to 5.5, as the nonproductive binding of cellulase to lignin on the substrate can be significantly reduced at this pH value (Lou et al., 2013). The Ctec2 loading was fixed at 5 FPU/g glucan. At regular intervals, 500 μL sample was taken and centrifuged at 7,000 rpm for 5 min. The glucose contents of the supernatants were analyzed by HPLC, as described in the preceding

sections. The substrate enzymatic digestibility (SED) of the solid samples was calculated by taking the mass fraction of the dissolved sugars over the total glucan of the substrates.

3.2.7 Enzyme activity assay

Filter paper activity (FPU) was measured according to according to the National Renewable Energy Laboratory (NREL) technical report, NREL/TP-510-42628. Relatively enzyme activity (*EA*, %) was calculated by the following equation:

$$EA = \frac{FPU_t}{FPU_{initial}} \times 100 \qquad (1)$$

where FPU_t is the enzymatic hydrolysis measured at time t.

3.2.8 Michaelis-Menten equation

Using the Langmuir isotherm model, it has been well-documented that lignosulfonates can reduce the nonproductive binding of enzyme to lignin (Kumar & Wyman, 2009a; Lou et al., 2013). However, due to the inability of this model to distinguish between the specific and nonspecific binding of enzyme to the substrate, the effect of lignosulfonate on the specific binding of enzymes has not been explored yet. Therefore, Michaelis-Menten model, which is based on the specific binding affinity of the enzyme to the substrate and can analyze the rate of the product formation, was used to study the enzyme kinetics (Bezerra et al., 2013). The initial rate of hydrolysis (v_0) can be expressed as:

$$v_0 = \frac{v_{max} \cdot S_0}{K_m + S_0} \quad (2)$$

where v_{max} is the maximum rate of hydrolysis, S_0 is the initial concentration of substrate, and K_m is the Michaelis constant physically representing the concentration of substrate when the hydrolysis rate reaches $v_{max}/2$. K_m is considered as an index of affinity between the substrate and enzyme.

3.3 Results and discussion

3.3.1 Effects of pretreatment severity on enzyme hydrolysis

In this study, sulfite pretreatment has been applied to break down the robust lignocellulose structure to enhance the enzyme accessibility to cellulose. The trends of the SED change in different lignosulfonate and acid concentrations have been presented in Fig. 3.1. When no bisulfite is available in the pretreatment process, the biomass-to-sugar conversion is very low (39%). As shown in Table 3.2, pretreating the feedstock with acid only could dissolve the major fraction of the hemicellulose from the lignocellulose, but no delignification could occur *(i.e.,* lignin was enriched from 25.20% to 40.26%), although lignin condensation is probable to take place. The physical blockage of the cellulose by the lignin in the lignocellulose and non-productive binding of the enzyme to the lignin can limit the enzyme hydrolysis and result in the low SED when no bisulfite is utilized in the pretreatment process (Alvira et al., 2010). As the concentration of the bisulfite increases up to 10%, the SED value drastically rises to

around 93%. Sulfite pretreatment method introduces sulfonic groups on the benzylic carbons of the lignin, partially dissolves the lignin and increases its hydrophilicity (Liu et al., 2010). After sulfonation, the hydrophobic interaction between the lignin residues and the enzymes is reduced (Wang et al. 2013a). Lignosulfonate present in the pretreated hydrolysate can also prevent the nonproductive binding of the cellulase to the lignin leading to the enhanced lignocellulose saccharification (Wang et al. 2013b). It was interesting to note that the SED of the whole slurry SP20 substrate was lower than the whole slurry SP10 (75% vs 93%, respectively) (Fig. 3.1). Cheng et al. (2014) reported that, at a fixed acid concentration of 1.2 % (w/w), 8-10% (w/w) bisulfite was the optimal dosage in the pretreatment of softwood chips (Cheng et al., 2014a). However, when the dosage of bisulfite is increased, the pH value in the pretreatment system also increases. pH rise results in a lower hemicellulose removal, decreases the mean pore size of the substrate and limits the accessibility and probability of the cellulose to be hydrolyzed (Cheng et al., 2014a). Therefore, it is practical to relate the changes in the SED to solely bisulfite concentration, as the hemicellulose concentration is not identical in different experimental conditions. In this work, in order to avoid any pH increase, as the bisulfite dosage of the pretreatment process is increased from 10% to 20% (w/w), more acid (1.2%-1.9%, w/w) is simultaneously introduced to the pretreatment process. According to Table 3.2, while insoluble lignin is reduced from 29.27% to 10.68%, almost all hemicellulose is removed and therefore the decrease in the SED cannot be attributed to the hemicellulose blockage. Rather, the enzyme saccharification of lignocellulose is inhibited by higher sulfur contents in SP10H and SP20H, whereas it was previously believed that higher sulfonation is always a favorable strategy (Lou et al., 2014; Zhou et

al., 2013d). This often-misinterpreted phenomenon will be dealt with in more detail in subsequent sections.

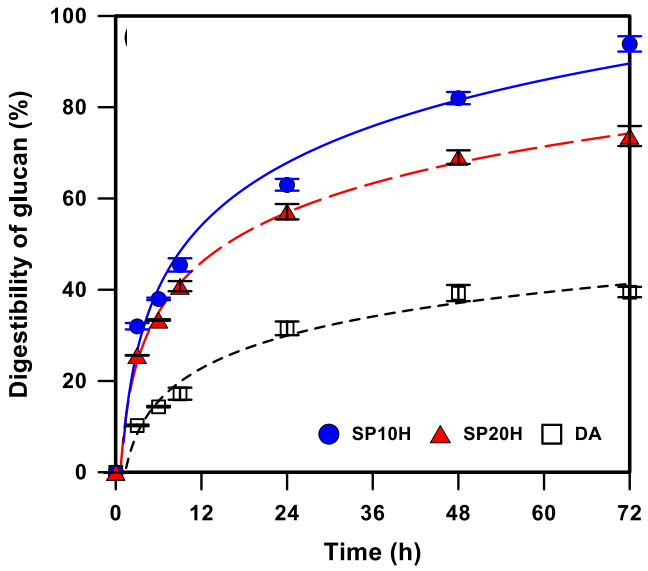


Fig. 3.1 Effects of sulfite loading on the enzymatic digestibility (SED) of sulfite pretreated substrates. Substrate content and acetate buffer concentration are 2% (w/v) and 0.1 M, respectively. H: whole slurry.

3.3.2 Surface activity of lignosulfonates

Surface activity is related to the efficiency of surfactants in increasing enzyme hydrolysis (Cheng et al., 2014b). Fig. 3.2 shows the surface tension of the enzyme solution as a function of the lignosulfonate concentration. For each lignosulfonate, two break points were obtained at the intersections of the regression lines. The intersection point T_1 (LS1: 1.6×10^{-2} g/L, LS2: 2.3 g/L) represents the critical aggregation concentrations (CAC) corresponding to the onset of micelle formation on the enzyme. The intersection point T_2 (LS1: 3.6 g/L) signifies that the enzymes are saturated with lignosulfonates. At low concentrations, lignosulfonates are adsorbed at the air-liquid interface of the solution surface and consequently the surface tension exhibits a

decreasing trend. When the air-liquid interface is crowded with lignosulfonate (T_1), the lignosulfonate binds to the enzyme and the surface tension of the solution is not significantly altered and thus, reaches a plateau (between T_1 and T_2) until a critical micelle concentration (CMC) is achieved. At excessive concentrations of the lignosulfonates, the enzymes are saturated with the lignosulfonate micelles, resulting in the adsorption of the lignosulfonates to the air-liquid interface. This poses further reduction in the surface tension of the solution when the lignosulfonate concentration exceeds the CMC. Since the value of the $T_{1,\,LS1}$ was smaller than the $T_{1,\,LS2}$ and $T_{2,\,LS2}$ is not achieved (*i.e.,* the enzyme was not saturated with the LS2 in the range of the tested concentrations), it can be concluded that LS1 has a higher surface activity than LS2. The lower molecular weight and higher degree of sulfonation for the LS1 compared with the LS2 accounts for the higher surface activity of the former lignosulfonate.

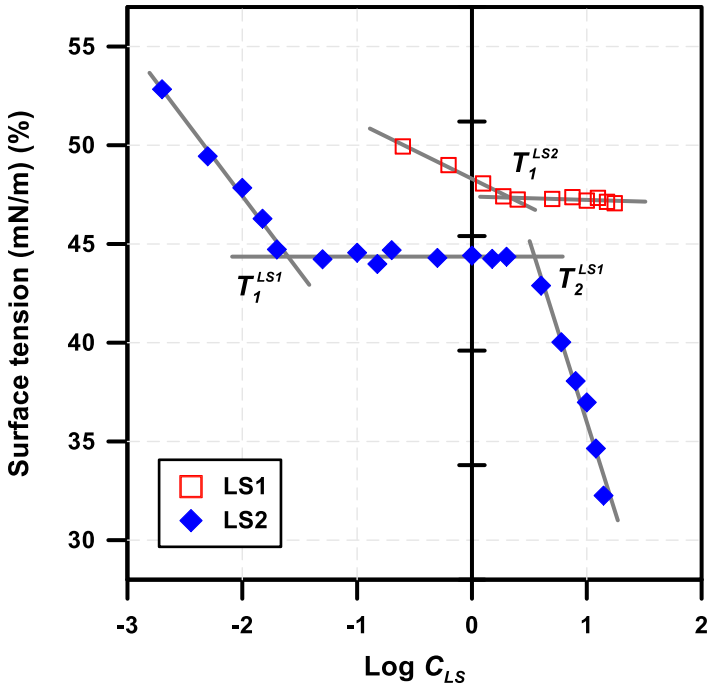


Fig. 3.2 Surface tension-concentration isotherm for two lignosulfonates. T_1: critical aggregation concentrations; T_2: critical micelle concentration.

3.3.3 Effect of lignosulfonate dosage on enzyme hydrolysis

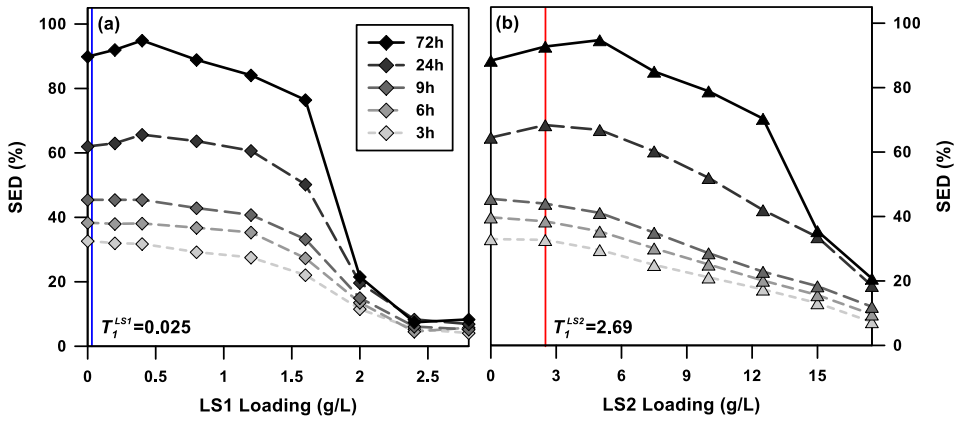


Fig. 3.3 Effects of LS1 (a) and LS2 (b) on the enzymatic digestibility of the SP10W substrate. Substrate content and acetate buffer concentration are 2% (w/v) and 0.1 M, respectively.

It is well-documented that the efficiency of the enzymatic saccharification is highly dependent on both the properties and dose of lignosulfonates (Cheng et al., 2014b; Lou et al., 2014; Zhou et al., 2013d). Nonetheless, some contradictory trends on the effect of lignosulfonates on the enzymatic saccharification have been reported. While low-molecular weight lignosulfonates with higher degree of sulfonation were shown to be more effective in enhancing the enzymatic saccharification (Lou et al., 2014; Zhou et al., 2013d), both enhancing and inhibitory effects were found when the dose of the introduced lignosulfonates varied in the hydrolysates (Lou et al., 2014). In order to elucidate the controversial phenomenon and maximize the LS beneficial effects, SP10W substrate was selected to perform the enzymatic hydrolysis with different dosage of LS. According to Fig. 3.3, it has been found that the enzymatic hydrolysis is not only a function of the lignosulfonate loading, but also the hydrolysis time. The effect of lignosulfonate loading on the SED can be categorized into three stages; Stage I (low LS

loading), Stage II (medium LS loading), and Stage III (high LS loading). At Stage I (<0.4 g/L for LS1 and <5 g/L for LS2), the introduction of the lignosulfonate has a negligible impact on the enzymatic saccharification of the substrate in the first 9 hours. When the hydrolysis time is extended to over 24 hours, an enhancement in the enzymatic saccharification is observed at low LS concentrations. At a hydrolysis time of 72 hours, the introduction of 0.4 g/L LS1 and 5 g/L LS2, increases the SED to over 95%. Introduction of the LS beyond the optimum loading amount (Stage II), however, has an adverse impact on the conversion of the lignocellulose to sugars. This trend is more intense at prolonged hydrolysis durations, where the SED decreases to 78% and 70% at LS1 and LS2 loadings of 1.6 and 13 g/L, respectively. Excessive LS loading, Stage III, results in a drastic reduction in the lignocellulose-to-sugar conversion.

Despite the similar trends observed for the SED changes with the two lignosulfonate, the range of the LS loading for each stage is, however, very different for the two types of the LS, where LS1 loadings window for various stages are almost 10 times lower than that of the LS2. For instance, the optimum LS1 and LS2 loadings to achieve the highest SED are 0.4 and 5 g/L, respectively. This implies the significance of the surface activity of the lignosulfonate in the conversion efficiency of the lignocellulose.

Lou et al. (2014) and Zhou et al. (2013d) showed that lignosulfonates with lower molecular weights and higher degree of sulfonation can have an enhancing effect on the SED, whereas higher molecular weight and less sulfonated lignosulfonates may lead to inhibiting effect. Nevertheless, this study verifies that lignosulfonates can exhibit either enhancing or inhibiting behavior depending on their dosage. As demonstrated in Fig. 3.2,

the LS1 with lower molecular weight and higher degree of sulfonation has higher surface activity. Therefore, it can bind to the enzymes ($T_{1, LS1}$), saturate it ($T_{2, LS1}$) and influence the enzyme hydrolysis more effectively at a lower dosage.

3.3.4 Mechanistic insight into the LS inhibition effect for enzymatic saccharification in the initial reaction period

Table 3.3 The initial rate (v) of enzyme hydrolysis at different conditions

Lignosulfonate (g/L)		Substrate content (w/v, %)			
		2%	**2.5%**	**3.3%**	**5%**
Control	0	1.54	1.81	2.06	2.62
	0.4	1.50	1.73	2.03	2.56
LS1	1.2	1.35	1.56	1.86	2.39
	2.8	0.10	0.13	0.20	0.70
	5	1.51	1.76	2.03	2.58
LS2	7.5	1.39	1.62	1.94	2.41
	17.5	0.14	0.20	0.27	0.61

v: g/Lh

Wang et al. (2013c) discussed that the formation of lignosulfonate-cellulase complexes can diminish the nonproductive binding of cellulase to lignin and therefore enhance the glucan conversion into sugars. However, it was not clarified whether the complexes could also influence the specific binding of enzyme and affect the enzyme activity. In order to shed light to the mechanistic impact of lignosulfonates on enzymes, a series of hydrolysis experiments under controlled conditions are carried out at different lignosulfonate dosages and substrate contents (Table 3.3). The initial hydrolysis rates (v_0) in the experimental conditions are calculated according to the method developed by Kim et al. (Kim et al., 1982). As summarized in Table 3.4, the initial hydrolysis rate increases

with the increasing initial substrate concentration and decreases when more lignosulfonate is added. Particularly, the initial hydrolysis rate became very small when the dosages of the LS1 and LS2 were set at 2.8 g/L and 17.5 g/L, respectively.

Table 3.4 The K_m and V_{max} calculated by Linewaeaver-Burk method

Lignosulfonate (g/L)		***K*m (w/v, %)**	***V*max (g/Lh)**	***R***
Control	0	4%	4.66	0.9960
LS1	0.4	4.3%	4.73	0.9973
	1.2	5%	4.75	0.9979
LS2	5	4.2%	4.70	0.9963
	7.5	4.8%	4.71	0.9981

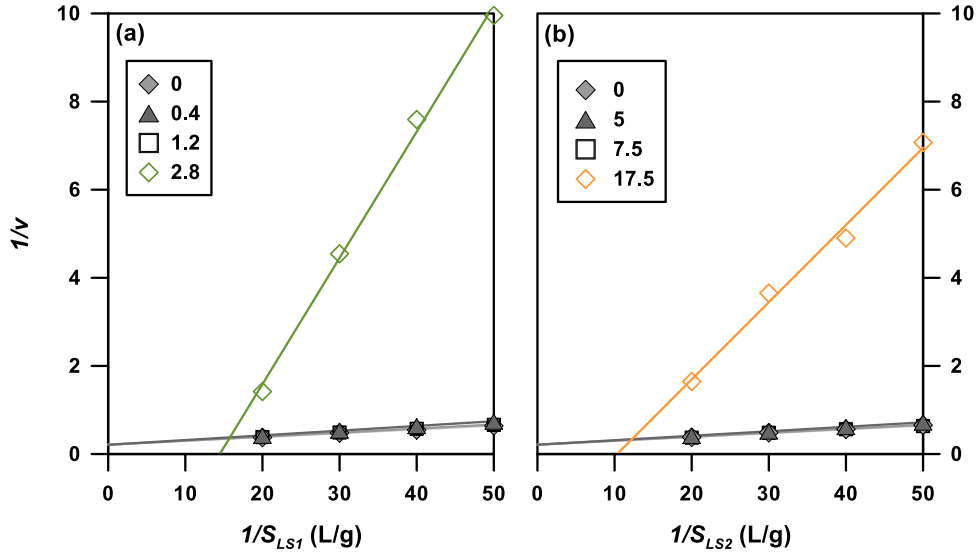


Fig. 3.4 Kinetic analysis of SP10W substrate enzyme hydrolysis in the presence of several concentrations of the inhibitor at different substrate concentrations. LS1: (a); LS2: (b). The legend represents the dosages (g/L) of LS. *v*: initial rate of enzymatic hydrolysis; [*S*]: substrate concentration (w/w, %).

In order to clarify the inhibition mechanism of the lignosulfonates, initial rates are incorporated in the Lineweaver-Burk equation and the kinetic parameters, v_{max} and K_m, are calculated (Fig. 3.4 and Table 3.4). Generally, in substrate saccharification processes, inhibitors can affect the reaction via three different routes: competitive, noncompetitive or uncompetitive (Yeh et al., 2010). In competitive inhibition, inhibitor is bound to the active site of enzyme and prevents the binding of enzyme to substrate, but the ability of enzyme-substrate to form a product is not affected. Hence, the maximum velocity (v_{max}) of the reaction does not change by the presence of the inhibitor, while the apparent affinity of the substrate to the specific binding site is decreased. On the other hand, non-competitive inhibition reduces the maximum velocity (v_{max}) without changing the specific binding affinity (K_m) of the enzyme to the substrate. Also, the Lineweaver–Burk plot for an uncompetitive inhibitor produces a line parallel to the original enzyme-substrate plot (Bezerra et al., 2013). When excessive amounts of the LS (2.8 g/L for LS1 and 17.5 g/L for LS2) are introduced into the reaction, negative v_{max} values are obtained implying the immediate deactivation of the enzymes upon the introduction of the LS into the hydrolysates. Meanwhile, as tabulated in Table 3.4, the half-reaction constant, K_m, increases from 4% (w/v) for the control sample with no lignosulfonate to 5% (w/v) when 1.2 g/L LS1 is introduced. Despite a considerable change in the K_m value, the rate of the cellulase-substrate complex conversion into glucose (v_{max}) does not change with the introduction of LS1. This suggests that competitive inhibition occurs between the inhibitor and the substrate in the enzyme hydrolysis, demonstrating that the LS and the substrate combine with the same enzyme site. When LS2 is introduced into the system, similar trend is observed, where the reaction rates do not alter, but the K_m

values changed significantly. This further verifies the hypothesis of competitive inhibition.

3.3.5 Effects of the lignosulfonate content on the enzyme activitie

Enzyme deactivation as a result of nonproductive binding of cellulase to lignin and other unfavorable components can be reduced by using surfactants for enhanced enzyme stability (Mackenzie & Francis, 2014; Okino et al., 2013; Ye et al., 2014). Lignosulfonates may play two distinct opposing roles in the enzymatic hydrolysis; they can be considered either as lignin-derived substances that may deactivate the cellulase or as surfactants that may stabilize the cellulase. In order to elucidate which role is more dominant in the hydrolysis reaction, it is necessary to determine the enzyme activity as lignosulfonates bind to cellulase nonproductively. According to Fig. 3.5, excessive loading of lignosulfonate completely deactivates the enzyme due to the fact that high loading of anionic surfactants can damage the structure of the enzymes. However, the enzyme stability was slightly enhanced with the introduction of low concentrations of the lignosulfonates. While the enzyme activities dropped to 48% and 18% of their initial activity after 24 h and 72 h incubation in the absence of lignosulfonate, enzyme activity loss is slightly enhanced to 58% and 25% as 1.6 g/L LS1 was used as surfactant (Fig. 5a). Similar trend is also observed as the concentration of LS2 increases (Fig. 5b). While the introduction of 15 g/L LS2 deactivated the enzyme after 72 h, 12.5 g/L LS2 leads to the enhancement of the enzyme activity to 57% and 26% after 24 h and 72 h, respectively. Several mechanisms account for the enzyme stabilization by employing an

appropriate amount of lignosulfonate. In the absence of lignosulfonate, enzymes can easily be exposed to the air-liquid interface which results in a significant enzyme deactivation (Kim et al., 1982). Nonetheless, when lignosulfonates are introduced, they occupy the surface of the solution and thus reduce the enzyme exposure to the air/liquid interface and hence, protect it from deactivation. In addition to the surface exposure of enzyme, the adsorption and desorption of enzymes to lignin can also lead to their activity loss (Ye et al., 2014). Lignosulfonates can block the nonspecific binding of cellulase by binding on lignin in the same manner as a nonionic surfactant (Wang et al., 2013b). Moreover, lignosulfonates can employ their ionic nature and mitigate the nonproductive binding of cellulase to lignin as a result of the electrostatic repulsion between the LS-cellulase complex and lignin (Wang et al., 2013c).

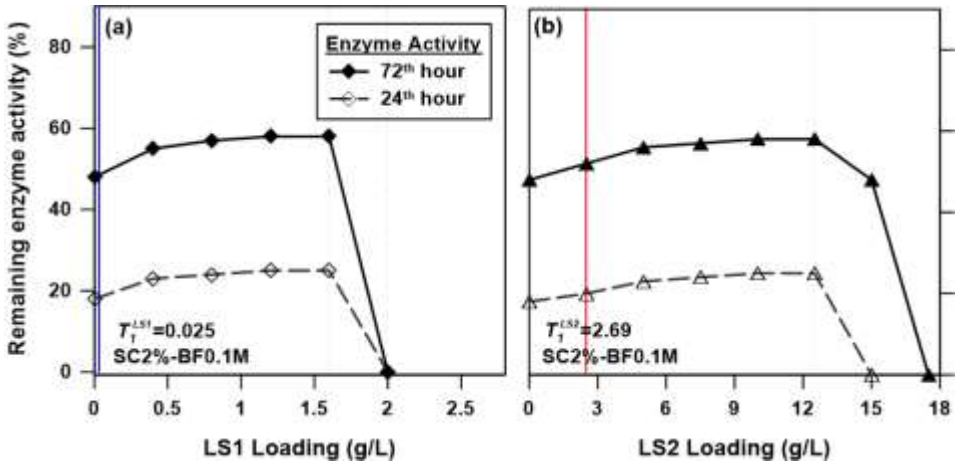


Fig. 3.5 Effects of LS1 (a) and LS2 (b) on enzymatic activity (column) and SED (line) at different LS loading. SC: substrate content and BF: acetate buffer concentration, respectively.

Notably, although the enzyme activity increases by increasing the LS loading up to

1.6 g/L LS1 and 12.5 g/L LS2, the trend is not identical to the lignocellulose-to-sugar conversion, where the SED decreases by increasing the LS content over the optimum loading (Fig. 3.5). Retention of the enzyme activity implies that the competitive inhibition is mainly responsible for the decrease of the SED in Stage II. In addition, if no inhibition occurs, the enzyme activity should theoretically follow a steadily increasing trend by increasing the LS loading. However, the rate of the increase in the enzyme activity is significantly reduced after the LS1 and LS2 loadings are increased over 0.4 g/L and 5.0 g/L, respectively, indicating a "relative" decrease in the enzyme activity compared with the theoretical one. The reason is that competitive inhibition also affects the enzyme activity results obtained by the filter paper method. This trend is in line with the already-discussed mechanism of competitive inhibition which is mainly responsible for the decrease of the SED at medium dosage of lignosulfonate (Stage II).

3.3.6 Effects of the substrate content and buffer concentration on the optimum content of lignosulfonate

In the previous sections, it was elucidated that competitive inhibition is the main reason for the reduction in the lignocellulose conversion. In order to overcome this challenge two approaches can be employed, increasing the substrate content (Bezerra et al., 2013) and buffer concentration. As the substrate content increases from 2% to 5%, the optimum dosage of LS1 increases from 0.4 g/L to 1.2 g/L and LS2 from 5 g/L to 7.5 g/L (Fig. 3.6). In addition, the SED_{max} of the SP10W substrate recovers from 27% to 79% at LS1 loading of 2.0 g/L which confirms that the deactivation of enzyme can also be prevented as the substrate content increases. This is related to the fact that the insoluble

lignin can adsorb lignosulfonate and thus can mediate the interaction between lignosulfonate and enzyme.

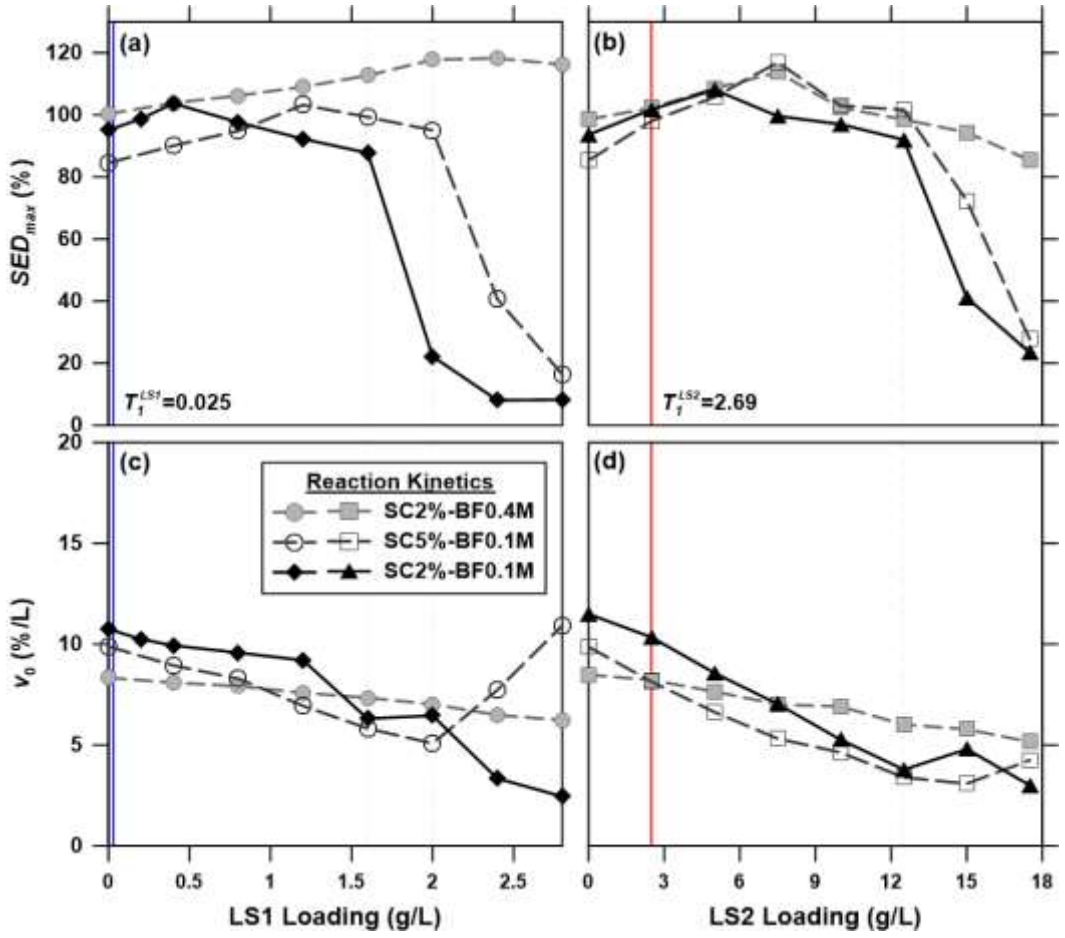


Fig. 3.6 Effects of LS1 (a, c) and LS2 (b, d) on enzymatic digestibility of SP10W substrate. SC: substrate content and BF: acetate buffer concentration, respectively.

The effect of buffer concentration on the enzyme hydrolysis is also investigated in this study. The optimal dosage of LS1 increases from 0.4 g/L to 2.0 g/L and LS2 from 5 g/L to 7.5 g/L when the concentration of buffer enhances from 0.1 M to 0.4 M (Fig. 3.6). Also, enzyme deactivation is not observed even at excessive LS loading in the case of

both types of lignosulfonate. Liu et al. (2010) discovered that metal compounds can deactivate the adsorption sites of lignin for enzymes to reduce or eliminate nonproductive enzyme adsorption. Consequently, sodium ions from the buffer solution can form complexes with the lignosulfonate and reduce the amount of the lignosulfonate binding to the enzyme. Thus, adding salt to the solution is an effective way to eliminate the inhibition of the lignosulfonate.

3.3.7. Effects of lignosulfonates on enzyme hydrolysis at their optimal dosage

A conceptual model was introduced to elucidate the contradictory inhibiting/enhancing effects of the lignosulfonate on the enzymatic hydrolysis of the sulfite pretreated lignocellulosic substrate at its optimal dosage (Fig. 3.7). The enzyme-lignosulfonate interactions can have two distinct effects: competitive inhibition and stabilization. Most cellulose-degrading enzymes have a two-domain structure that consists of a catalytic and a cellulose-binding domain connected by a linker region (Linder et al., 1996). Lignosulfonate can bind with the two domains randomly after the surface of the hydrolysate is crowded with lignosulfonate (Fig. 3.2). The parameters determined by Michaelis-Menten equation show that only competitive inhibition is exerted by the lignosulfonates at their optimal dosage. It is indicated that lignosulfonates that cover the binding domain can decrease the specific binding affinity of enzymes to cellulose. As a result, the initial rates of hydrolysis are decreased when the lignosulfonates are incorporated. The ability of the catalytic domain to convert the cellulose to glucose is not inhibited when the lignosulfonates bind to the domain unproductively. On the other hand, the negatively-charged lignosulfonate-enzyme

complex is more hydrophilic than the pure enzyme. Also, the sulfonated lignin residue from the sulfite pretreatment process is hydrophilic and negatively-charged. Thus, the decrease in the hydrophobic interaction between the LS−enzyme and sulfonated lignin as well as the electrostatic repulsion can reduce the nonproductive interaction. Furthermore, lignosulfonate adsorbed at the surface of air-liquid surface prevents the enzyme exposing to the air. Thus, enzyme stabilization is promoted and the final yield of the hydrolysis experiment is enhanced.

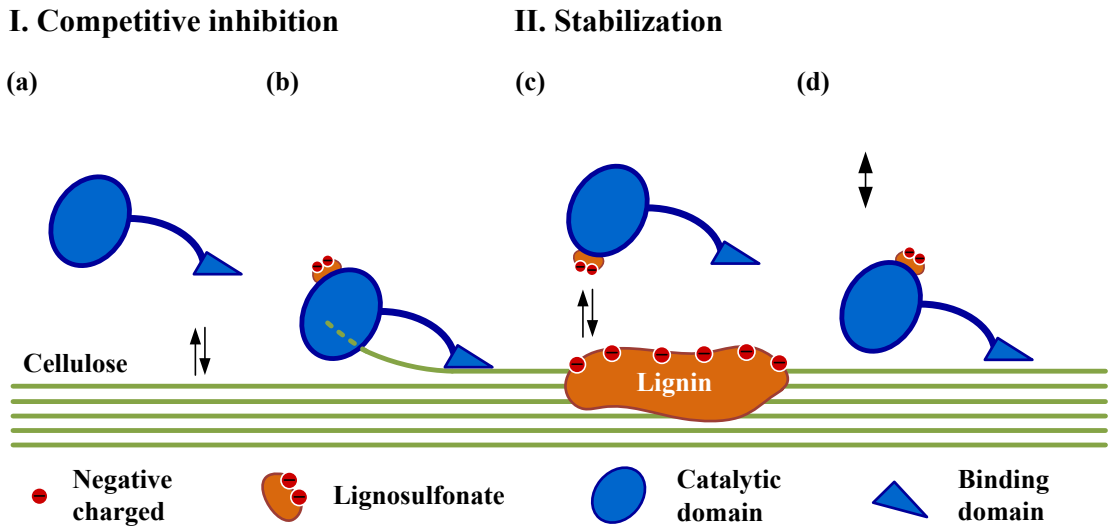


Fig. 3.7 Conceptual schematic of the effect of the LS on the enzyme hydrolysis at its optimal dosage. (a) Covering the specific binding site; (b) No inhibition of product formation; (c) Reduction in non-productive binding of enzyme to lignin; and (d) Preventing the exposure of enzyme to the air.

3.4. Conclusions

This chapter shed light into the contradictory information about the inhibitory /enhancing effect of lignosulfonates in enzymatic hydrolysis. Besides deactivation, LS with higher surface activity influences the enzyme hydrolysis more effectively through two opposing mechanisms. Competitive inhibition of enzyme by LS reduces the specific

binding affinity between enzyme and cellulose; hence inhibits the enzyme hydrolysis. The presence of LS results in enzyme stability and enhances the digestibility. Competitive inhibition was the dominating factor at excess LS loading. Increasing the solid substrate content and buffer concentration are proven to be promising in overcoming the inhibition effect of LS in the whole slurry hydrolysis process.

Chapter 4 Feasibility of high-concentration cellulosic bioethanol production from undetoxified whole Monterey pine slurry

4.1 Introduction

Cellulosic bioethanol is considered as a green, renewable alternative to fossil fuels, which attribute to mitigate global climate change and facilitate sustainable rural economic development (Bondesson et al., 2013; Koppram et al., 2014). Generally, cellulosic bioethanol production requires the following major steps: (1) pretreatment of cellulose-based biomass, (2) separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF), and (3) subsequent separation by distillation (Liu et al., 2014). Among these, distillation of ethanol from fermentation broth is an energy intensive step accounts for even more than 80% of total energy demand in the whole industrial process (Sassner et al., 2008). In order to reduce the distillation cost, a strategy of SSF at high substrate concentrations was developed, which means high sugar input and potentially high ethanol concentrations (Sassner et al., 2008). It has been calculated that the energy ratio (in/out) of distillation can be reduced by approximately 25% when ethanol concentration from SSF process increasing from 60 g/L to 80 g/L, although literature values for cellulosic ethanol concentrations have rarely exceeded 60 g/L (Chen et al., 2016). Therefore, high-concentration cellulosic bioethanol producing by SSF process is attractive but remains challenging because of the heterogeneous features of cellulose-based biomass (Koppram et al., 2014; Maurya et al., 2015).

Lignocellulose that consists mainly cellulose, hemicellulose and lignin in an intricate structure is the mainstream feedstock for cellulosic bioethanol production considering its great availability, sustainability, and low cost compared to pure sugars (Fengel & Wegener, 1983). Preferentially, bioconversion of cellulosic bioethanol needs lignocellulosic biomass to be of high cellulose content and inedible species (Koppram et al., 2014). Monterey pine, a fast growing woody biomass, is a good substrate candidate since its higher cellulose content up to 48% and compositional uniformity (Torr et al., 2012) (Koppram et al., 2014). Moreover, Monterey pine has been extensively cultivated as a plantation timber in various temperate parts of the world, especially in several marginal environments that are otherwise not suitable for edible crop cultivation (FOODS, 2003). Thus, Monterey pine should be an attractive feedstock for production of cellulosic bioethanol.

Enhancement of cellulosic bioethanol converted from woody biomass is always hindered by the presence of lignin, which forms a physical barrier to any enzyme (Leu & Zhu, 2013; Wi et al., 2015). Therefore, a pretreatment is necessarily performed prior to SSF to remove lignin from biomass cell walls. However, actually lignin cannot be completely removed during most of the pretreatment processes (Zhou et al., 2013a). The residual lignin increases the insoluble solid consistency of fermentation broth, which introduces operational difficulties (Koppram et al., 2014). On the other hand, the hydrophobic property of lignin may result to non-productive adsorption of cellulose which significantly lowers the efficiency of SSF (Liu et al., 2016a; Lou et al., 2013; Palonen et al., 2004).

Recently, series of high severity pretreatment have been conducted to reduce lignin content to less than 5% (w/w) for achieving high concentrations of fermented sugars and/or cellulosic bioethanol product (Cui et al., 2014; Elliston et al., 2013; Zhang et al., 2010). Zhang et al. (2010) pretreated corncobs with a two-step (acid plus alkali) process realized a substrate with 3% (w/w) lignin content and a final ethanol concentation of 84.7 g/L was observed (Zhang et al., 2010). Cui et al. (2014) reduced the lignin content in oil palm empty fruit bunch from 19% to 5% (w/w) by a Formiline (formic acid plus lime) process results to 83.6 g/L ethanol in the final fermentation broth (Cui et al., 2014). Elliston et al. (2013) generated 92 g/L ethanol using waste paper, which contains only 1% (w/w) lignin (Elliston et al., 2013). Nevertheless, extensive lignin removal from lignocellulose biomass requires excessive chemical and energy input in these pretreatment processes. Although Chen et al. (2016) converted pretreated corn stover with 13% (w/w) lignin to 86 g/L ethanol by co-fermenting hexose with pentose using engineered *rZymomonas* (Chen et al., 2016), most of lignocellulosic feedstock, especially the softwood specie consists higher lignin at 26-29%, which becomes a major obstacle for efficiently lignocellulose hydrolysis (Zhu & Pan, 2010).

Zhu et al. (2009) evaluated sulfite pretreatment to satisfy enzymatic cellulose conversion by sulfonating 34% (w/w) content of lignin in woody biomass to a hydrophilic type (i.e., lignosulfonate) (Zhu et al., 2009). As the result, more than 90% (w/w) cellulose conversion was achieved within 24 h of hydrolysis (Leu et al., 2013). Moreover, the dissolved lignosulfonate in pretreatment hydrolysate acted as a surfactant further enhanced the enzymatic hydrolysis enabling the whole pretreated slurry (solid plus the liquid fraction) to be directly used in SSF (Wang et al., 2013a; Zhou et al.,

2013c). However, only 48.9 g/L ethanol was observed by using *Saccharomyce cerevisiae* YRH400 in this study (Cheng et al., 2015a). To date, there are no reports on production of high concentration cellulosic bioethanol through SSF of high lignin content woody biomass. Thus, the aim of this present work was to establish an efficient cellulosic bioethanol production process by using undetoxified whole slurry of Monterey pine.

4.2. Materials and methods

4.2.1. Materials

Monterey pine wood chips and Commercial cellulase Cellic Ctec2 were prepared as described in Chapter 3 section 3.2.1. Thermae-tolerant alcoholic active dry yeast (*Saccharomyce cerevisiae*) was obtained from Angel Yeast Co. Ltd. (Hubei, China). All other chemicals used were of ACS reagent grade.

4.2.2 Sulfite pretreatment and mechanical size reduction

Pretreatment process was described in Chapter 3 section 3.2.2.

4.2.3 SSF experiments

SSF experiments were performed by loading the pretreated biomass (either the whole slurry or washed substrate) in 500-ml fermenters with 200-ml working volume. The spent liquor was added to a fixed amount of unwashed substrate, and the mixture was adjusted to pH 5.5-6.2 with the addition of solid calcium hydroxide. The enzyme loading used in SSF was 15 FPU/g substrate. The experiments were initiated by enzyme

addition and prehydrolysis for 24 h at 50°C and 200 rpm. The temperature was then reduced to 28°C or 35°C, and 5 g/L dry *S. cerevisiae* was inoculated into the hydrolysate. Additional nutrients, 1 g/L yeast extract and 2 g/L peptone were supplemented into the fermentation media.

4.2.4 Fed-batch SSF experiments

The fed-batch SSF experiments were started with an initial unwashed solids at 15% (w/w) loading with additional pretreatment spent liquor. Another 5% dry unwashed solids was added at 12 h, giving a final unwashed solid loading of 20%. The fermentation conditions, enzyme loadings, and nutrient concentrations were the same as in the batch experiments.

4.2.5 Fermentation

Fermentations using synthetic mediums (as described below) and pretreatment spent liquor were performed to evaluate the tolerance of *S. cerevisiae* to inhibitors. The fermentations were conducted in 500-ml fermenters with 200-ml working volume that incubated at 35°C for 24 h. The synthetic mediums had the same concentration of HMF, furfural and acetic acid as the pretreatment spent liquor and contained 200 g/L hexose, 1 g/L yeast extract and 2 g/L peptone. Pretreatment spent liquor was obtained after the filtration of the slurry. Since some of sugars present in the liquid fraction were oligomers, a mild acid post-hydrolysis (4% [w/w] H_2SO_4, 121°C and 60 min) was needed to make the monomeric sugars available to be utilized by *S. cerevisiae*. The pretreatment spent liquor was supplemented with glucose and nutrient to make it also

contain 200 g/L hexose, 1 g/L yeast extract and 2 g/L peptone. Control experiments were performed under the same conditions without inhibitors.

4.2.6 Pretreatment spent liquor concentration

To conduct high solid SSF using the whole slurry (solid plus liquor fraction) with up to 25% unwashed solids, the spent liquor was concentrated using a vacuum rotary evaporator. The evaporation temperature was set to approximately 52°C to avoid sugar degradation. The volumetric concentration ratios of 2.02 were determined based on the initial and final liquor volumes (Table 4.1).

Table 4.1 Chemical composition of concentrated pretreatment spent liquor

	Unconcentrated liquor	**Concentrated liquor**
Volume (mL)	1000	496
Solid content (g/L)[a]	132	256
Glucose (g/L)	11.4	22.2
Xylose (g/L)	6.9	13.4
Galactose (g/L)	4.9	9.1
Mannose (g/L)	20.1	38.2
Inhibitors		
HMF (g/L)	2.4	4.1
Furfural (g/L)	1.7	ND
Acetic acid (g/L)	3.5	5.2

[a]The number is calculated based on the oven dry weight.
ND: Not detectable

4.2.7 Measurement of viable cell density

Colony-forming units (CFU) were measured to estimate the viable cell density. A total of 100 μL fermentation slurry was sampled and diluted with sterile water. Then, 100 μL of each diluted sample was plated on a YPD agar medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone). The dilution rate for each sample was varied to

guarantee the number of colonies on a single plate between 20 and 200. The plates were then cultured at 28°C for 48 h. Single colonies formed on the plates were counted, and viable cell density was calculated accordingly.

4.2.8 Analysis of sugars, inhibitors and fermentation products

Sugars, inhibitors and fermentation products were analyzed based on the procedure described in Chapter 3 section 3.2.3.

4.3 Results and discussion

4.3.1 Compositional analysis of Monterey pine

Untreated Monterey pine chips were composed of 41.8% (w/w) glucan, 3.7% (w/w) xylan, 4.6% (w/w) galactan, 12.1% (w/w) mannan and 25.2% (w/w) acid insoluble lignin as shown in Table 4.2. This composition was comparable to that of pine used in other studies (Zhou et al., 2013e; Zhu et al., 2009). After pine chips were pretreated with sulfite, the total solids recovery was 94.8% (w/w) with 79.2% (w/w) in the unwashed solids fraction and 15.6% (w/w) in the spent liquor as soluble and suspended solids. As a result, recoveries of glucan, xylan and mannan were determined as 88.2% (w/w), 60.9% (w/w) and 42.2% (w/w), respectively. A total of 82.3% (w/w) of the glucan was retained in the insoluble solids of the pretreated Monterey pine, whereas almost all the recovered xylan and mannan was solubilized in the liquid fraction. Successful conversion of Monterey pine into cellulosic bioethanol will therefore require efficient co-utilization of insoluble solids as well as the soluble fraction derived from pretreatment process.

Table 4.2 Chemical composition of untreated and sulfite pretreated Monterey pine

Components of substrates	Untreated wood chips	Sulfite pretreated washed substrates	Sulfite pretreated unwashed substrates	Unconcentrated liquid (spent liquor)
Glucan (%)	41.8	59.8/2.3[a]	44.8/84.9[a]	11.4/3.3[b]
Xylan (%)	3.7	0.2/2.7[a]	1.8/38.5[a]	6.9/22.4[b]
Galactan (%)	4.6	ND	1.0/17.2[a]	4.9/12.8[b]
Mannan (%)	12.1	ND	3.4/22.3[a]	20.1/19.9[b]
Acid insoluble lignin (%)	25.2	29.2/66.7[a]	25.1/78.9[a]	NA
HMF (%)	NA	ND	0.4/NA	2.4/NA[b]
Fufural (%)	NA	ND	0.3/NA	1.7/NA[b]
Acetic acid (%)	NA	ND	0.6/NA	3.5/NA[b]
Total mass yield[c] (%)	100	57.5	79.2	15.6

[a]The first number is the percentage of component in the substrate, the second number is saccharide yield
[b]The first number is the concentration (g/L) of component in the spent liquor, the second number is saccharide yield
[c]The number is calculated based on the oven dry weight
NA: Not applicable; ND: Not detectable

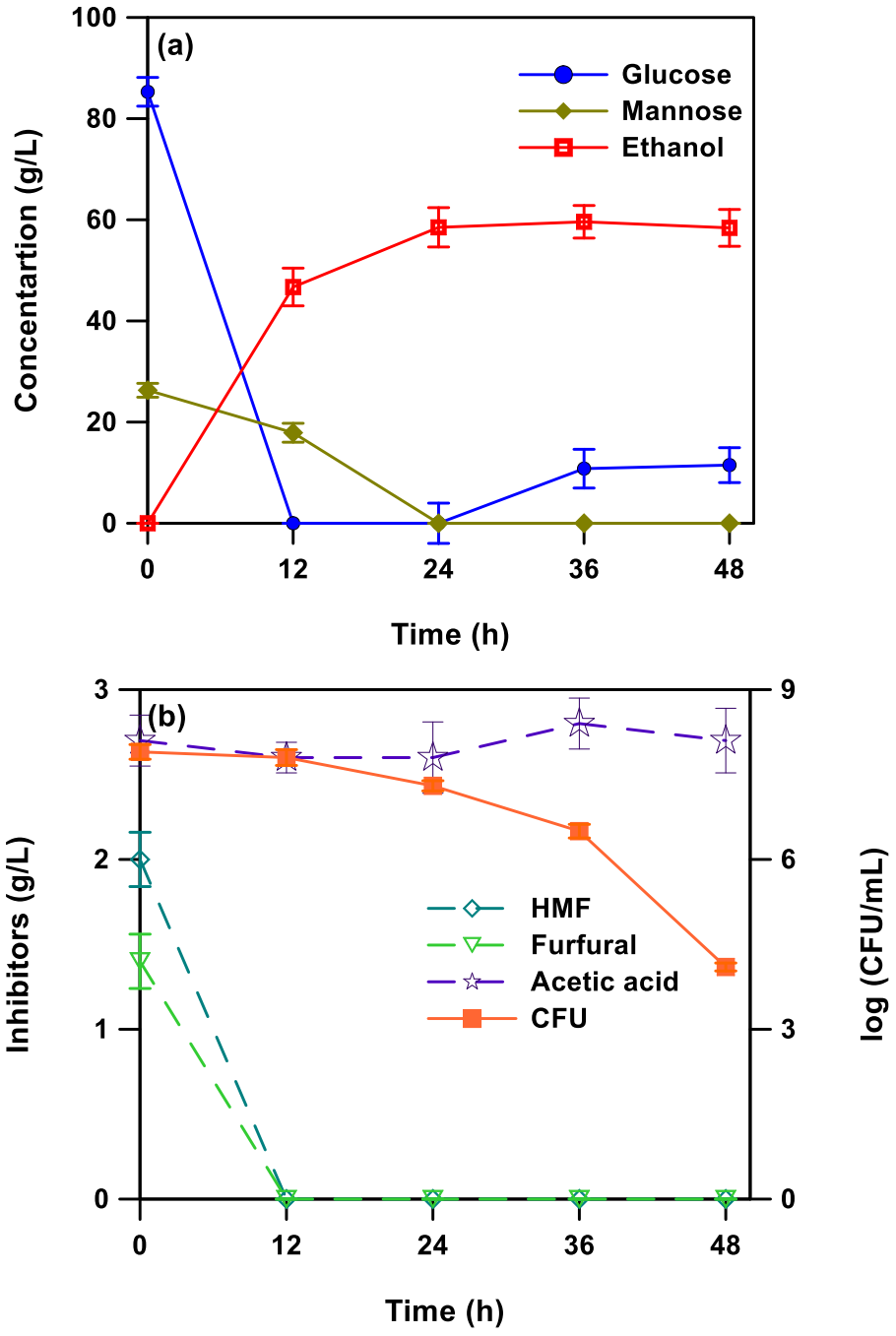


Fig. 4.1 Time course for SSF process using the whole slurry at 20% (w/w) unwashed solid loading: (a) sugars and ethanol; (b) inhibitors and cell viability. Symbols: closed circles, glucose concentration; closed rhombus, mannose concentration; opened squares, ethanol concentration; opened rhombus, HMF concentration; opened inverted triangles, furfural concentration; opened pentagrams, acetic acid concentrations; closed squares, CFU. Data points represent the mean values from three independent experiments.

4.3.2 Whole slurry SSF of sulfite pretreated Monterey pine

In this study, we initially conducted SSF of unwashed solids at 20% (w/w) loading with additional pretreatment spent liquor (Fig. 4.1). The slurry was prehydrolyzed for 24 h prior to *S. cerevisiae* inoculation when 85.3 g/L glucose and 26.3 g/L mannose were generated. All sugars in this mixture were completely consumed within 24 h, which indicated that *S. cerevisiae* fermented glucose and mannose as readily as it was released. Accordingly, 59.3 g/L ethanol was observed within 48 h and no further increase in the latter period of SSF. However, it is noticed that 11.2 g/L residual glucose was accumulated in the slurry, which indicated the cellulase was still activated whereas ethanol production had finished. The final ethanol concentration was lower than 84.7 g/L obtained by using washed pretreated corncob with *S. cerevisiae* (Zhang et al., 2010). The lower fermentability was primarily caused by the presence of toxic components, which inhibited the growth and fermentation activity of *S. cerevisiae*. Such a deleterious effect of pretreatment spent liquor was also reported by Gu et al. (Gu et al., 2014) and Jung et al. (Jung et al., 2015b).

It is well known that HMF, furfural and acetic acid are specifically fermentation inhibitors in the pretreatment spent liquor (Koopman et al., 2010). As shown in Fig. 4.1b, HMF and furfural were completely metabolized by *S. cerevisiae* in the SSF process within 12 h. Although *S. cerevisiae* can convert HMF to 2,5-bis-hydroxymethyfural (Taherzadeh et al., 2000) and furfural to furfuryl alcohol (Diaz De Villegas et al., 1992) under anaerobic conditions, the cell viability was continuously decreased after extinction of HMF and furfural. On the other hand, the elimination of acetic acid during SSF was limited, which indicated that acetic acid might be responsible for such poor

fermentability. More work should to be done to evaluate the inhibition of the pretreatment spent liquor during cellulosic bioethanol conversion.

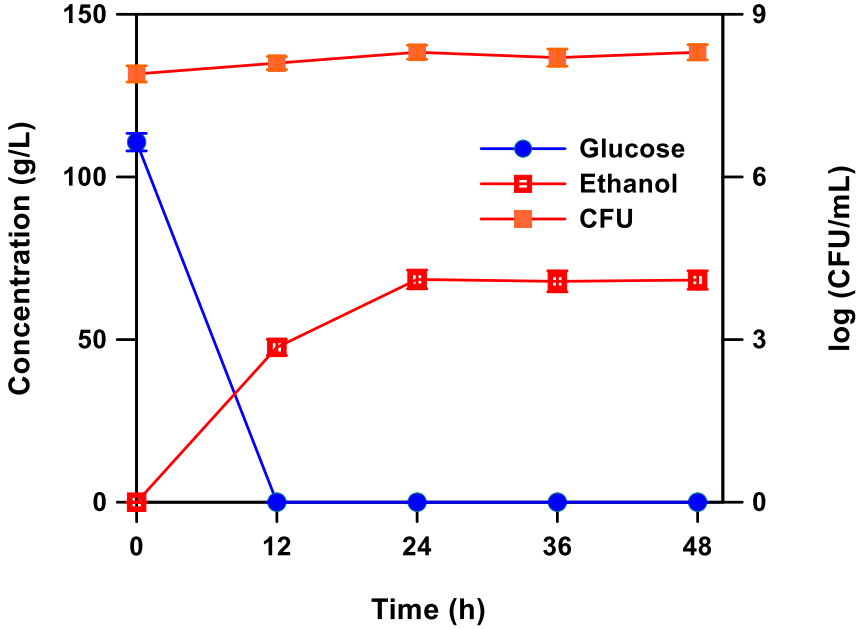


Fig. 4.2 Time course for SSF process using the washed substrate at 20% (w/w) solid loading. Symbols: closed circles, glucose concentration; opened squares, ethanol concentration; closed squares, CFU. Data points represent the mean values from three independent experiments.

4.3.3 Evaluation of the inhibiting effect on pretreated spent liquor

4.3.3.1 Effect of washing strategy on the SSF with sulfite pretreated Monterey pine

To evaluate the effect of present inhibitors in the liquid fraction of slurry, the pretreated Monterey pine was washed thoroughly with tap water. Washing the slurry removed soluble hemicellulose and lignin, resulting in high cellulose content in the substrate (Table 4.2). Prehydrolysis of washed substrate at 20% (w/w) solid loading released 110.7 g/L glucose over 24 h (Fig. 4.2). Glucose was exhausted within 24 h of inoculation, and its concentration remained low throughout the SSF process. Ethanol concentration reached 68.5 g/L at 24 h of SSF, and *S. cerevisiae* showed no reduction in

cell viability. These results reconfirmed the deleterious effect of pretreatment spent liquor on SSF. Although it has been reported that washed pretreated biomass gave higher ethanol concentrations (Alfani et al., 2000), a washing step in the ethanol production process would increase the production cost and cause a considerable loss of sugars, which cannot be applied in a large-scale process (Öhgren et al., 2007).

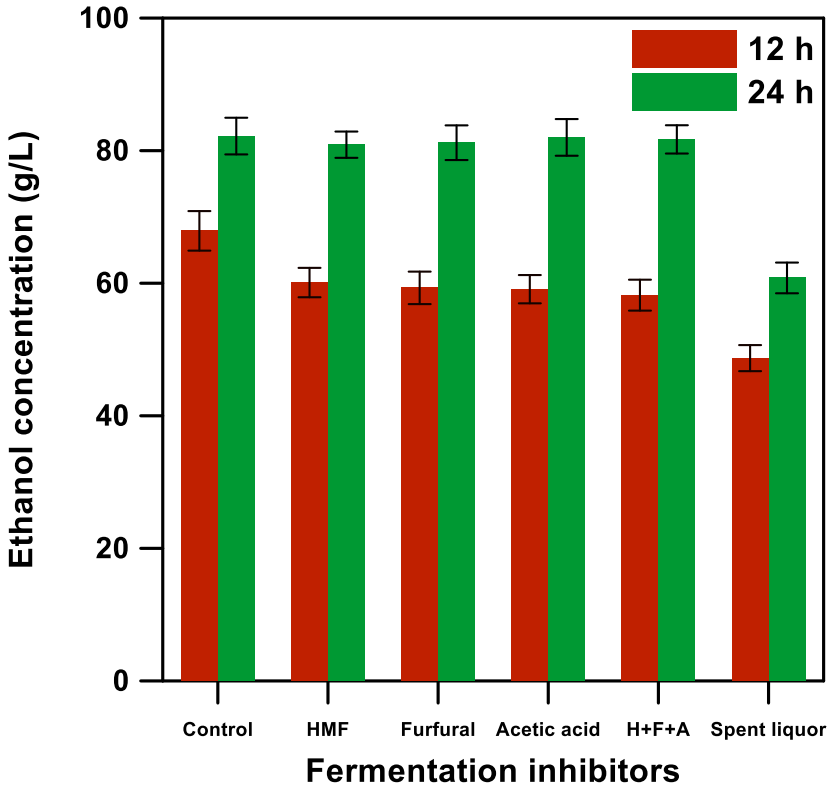


Fig. 4.3 Ethanol production formation by Saccharomyces cerevisiae during the fermentation of synthetic mediums and pretreatment spent liquor. H+F+A, HMF, Fufural and Acetic acid. Data points represent the mean values from three independent experiments.

4.3.3.2 Effect of inhibitors and the pretreatment spent liquor on fermentation

Fermentation with synthetic mediums and pretreatment spent liquor was performed to evaluate the *S. cerevisiae* tolerance to inhibitors. The results of ethanol production at 12 h and 24 h are shown in Fig. 4.3. The final ethanol production was not affected by the

presence of HMF, furfural and acetic acid at the concentration tested. However, the pretreatment spent liquor significantly affected the fermentation performance of *S. cerevisiae*, and only 60.2 g/L ethanol was produced during the 24 h fermentation. Therefore, inhibition could not be attributed solely to HMF, furfural and acetic acid. Other toxic components, such as phenolic compounds and aromatics, may be responsible for the relatively low fermentability (Oliva et al., 2003; Palmqvist & Hahn-Hägerdal, 2000). Types and concentrations of inhibitors are strongly depend on the feedstock and pretreatment methods, whereas large number and diversity of inhibitors found in different lignocellulose hydrolysates make identification and quantification of separate compounds complicated (Jönsson et al., 2013b). Furthermore, previous studies also indicated that fermentability of a real pretreatment spent liquor differs from fermentability of a synthetic medium with the same amount of the major hydrolysate inhibitors due to the importance of inhibitors that cannot be identified, which indicate synergistic effects between several inhibitory compounds (Jönsson & Martín, 2016).

4.3.3.3 Effect of temperatures on fermentation with pretreatment spent liquor

It was reported that the combination of higher temperature along with inhibitors poses a synergistic detrimental effect on fermentation process (Mutturi & Lidén, 2014). To investigate the effect of temperature on the performance of *S. cerevisiae*, the pretreatment spent liquor and YPD broth was fermented at 24°C, 28°C, 32°C, 35°C, 38°C, 40°C, 43°C and 50°C (Fig. 4.4), respectively. Fig. 4.4 showed that the ethanol concentration in the YPD broth at 24 h were still more than 80 g/L when the temperature

increased to 35°C. While the ethanol concentration in the pretreatment spent liquor at 24 h significantly decreased from 82.3 g/L to 62.6 g/L as the temperature increased from 28°C to 35°C, which indicated 28°C is the optimal temperature for *S. cerevisiae* tolerance to the present inhibitors.

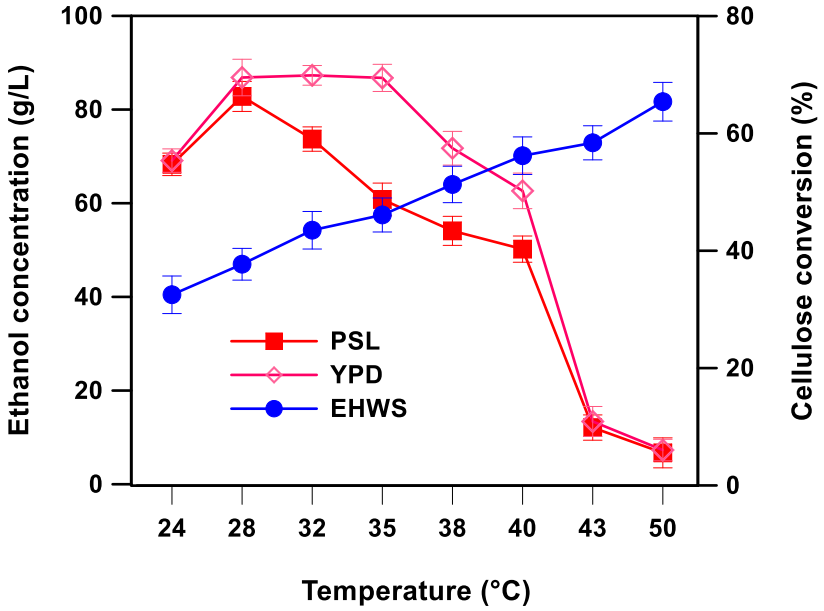


Fig. 4.4 Fermentation of pretreatment spent liquor (PSL) and YPD broth, and enzymatic hydrolysis of the whole slurry (EHWS) at different temperatures. Solid loading of the whole slurry is 20% (w/w). Ethanol concentration and cellulose conversion was detected at 24 h. Symbols: closed squares and triangles, ethanol concentration; closed circles, cellulose conversion. Data points represent the mean values from three independent experiments.

Compared to SHF, SSF is usually preferred in industry processes due to the low cost, reduced contamination risk, and lower sugar inhibitory effects (Liu et al., 2014; Lu et al., 2013). However, there are still several obstacles to efficient conversion of cellulosic bioethanol by SSF process, such as the optimum temperature discrepancies between saccharolytic enzymes and fermentation microbes (Taherzadeh & Karimi, 2007). The ideal ethanol producing temperature is approximately 28°C for most *S.*

cerevisiae species, whereas 50°C for hydrolysis by cellulases, so an appropriate temperature is necessary for the SSF process. It was found that cellulose conversion of the whole slurry at 24 h decreased from 65.4% to 32.5% when the hydrolysis temperature reduced from 50°C to 24°C (Fig. 4.4). Thus, 35°C or higher temperature was always used in the SSF process to strike a balance between the optimal temperature for the cellulosic enzymes and that for *S. cerevisiae* (Abdel-Banat et al., 2010). It has been reported that the thermal- and ethanol- tolerant *S. cerevisiae* used in this study performed better at 39°C in SSF of steam-exploded corn stover (Liu et al., 2014). However, the final ethanol production was less than 60 g/L as investigated. In this present study, *S. cerevisiae* performed better at 28°C when it was exposed to the high concentration ethanol (80 g/L) and inhibitors (Fig. 4.4). Thus, the whole Monterey pine slurry temperature at 28°C significantly improved ethanol concentration in SSF by *S. cerevisiae*.

4.3.4 Improvement of the whole slurry SSF by lowering temperature

Herein, we suggested a modified SSF process with prehydrolyzing the whole slurry at 50°C and then reducing the temperature to 28°C to perform the SSF process. The modified SSF with unwashed substrate at 20% solid loading with additional pretreatment spent liquor showed robust ethanol production of ethanol using *S. cerevisiae*, as shown in Fig. 4.5a. Ethanol concentration reached 65.6 g/L within 24 h of inoculation. Glucose accumulation was not observed in the SSF process. As observed in Fig. 4.5c, HMF and furfural were completely digested within 12 h, and *S. cerevisiae* showed no decrease in cell viability.

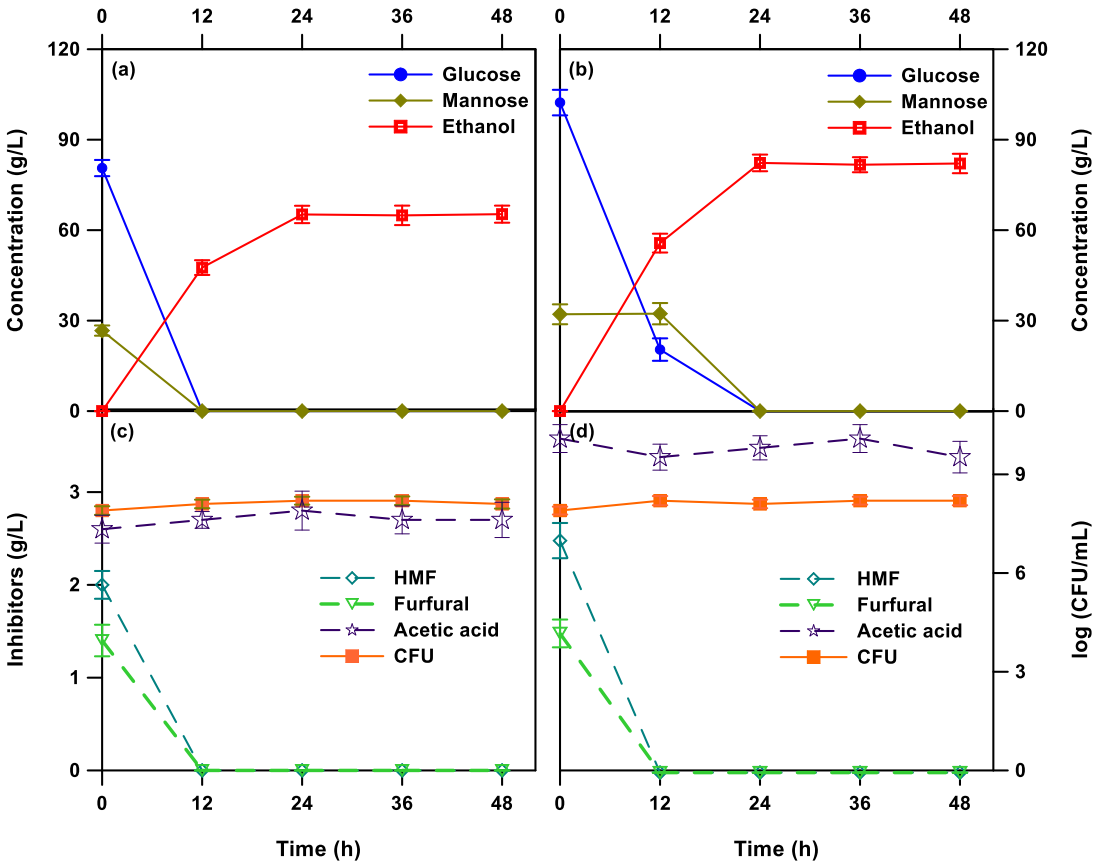


Fig. 4.5 Time course for SSF process using the whole slurry at 20% (w/w, a and c) and 25% (w/w, b and d) unwashed solid loading. Symbols: closed circles, glucose concentration; closed rhombus, mannose concentration; opened squares, ethanol concentration; opened rhombus, HMF concentration; opened inverted triangles, furfural concentration; opened pentagrams, acetic acid concentrations; closed squares, CFU. Data points represent the mean values from three independent experiments.

The whole slurry was further used for producing higher ethanol concentration by increasing solid loading to 25% (w/w) (Fig. 4.5b). The slurry was prehydrolyzed for 24 h and released 112.3 g/L glucose. As a result, 82.1 g/L ethanol concentration was observed after *S. cerevisiae* was inoculated for 24 h.

To achieve a high ethanol concentration, the biomass loading must be increased to a considerablely high level, which typically results in high concentrations of inhibitors in the SSF process (Jönsson et al., 2013a; Koppram et al., 2014). These inhibitors

significantly reduced the final ethanol production in the SSF process, which is one of the major barriers to the development of an economically viable process for cellulosic ethanol production (Jönsson et al., 2013a; Jönsson & Martín, 2016; Sivagurunathan et al., 2017). To overcome the issues related to the inhibitory compounds in the pretreatment spent liquor, some techniques on detoxifying the liquor by removing the toxic chemical residues have been reported, including physical (evaporation and membrane separation), chemical (over-liming, activated charcoal treatment, ion exchange, neutralization and organic solvent extraction), and biological (treatment with laccase or peroxidase) techniques (Jönsson et al., 2013b). However, these additional detoxification steps increase the overall costs due not only to the capital and chemical costs but also to the loss of sugars. For the whole slurry SSF process of the sulfite pretreated biomass, overliming and XAD-4 adsorption were used to detoxify the pretreatment liquor (Tian et al., 2010). The ethanol productivity in the first 4 h and overall fermentation efficiency was increased, while the final concentration of ethanol was not improved due to the sugar losses. Recently, “pH profiling” together with low temperature sulfite pretreatment was used to reduce inhibitor formation (Cheng et al., 2015a). Ethanol yield per ton of wood was increased by 27% in concentration, while ethanol concentration from one batch of fermentation was still 48.9 g/L. In the present work, the final ethanol concentration of the whole slurry SSF was successfully increased to 80 g/L by reducing the fermentation temperature to 28°C.

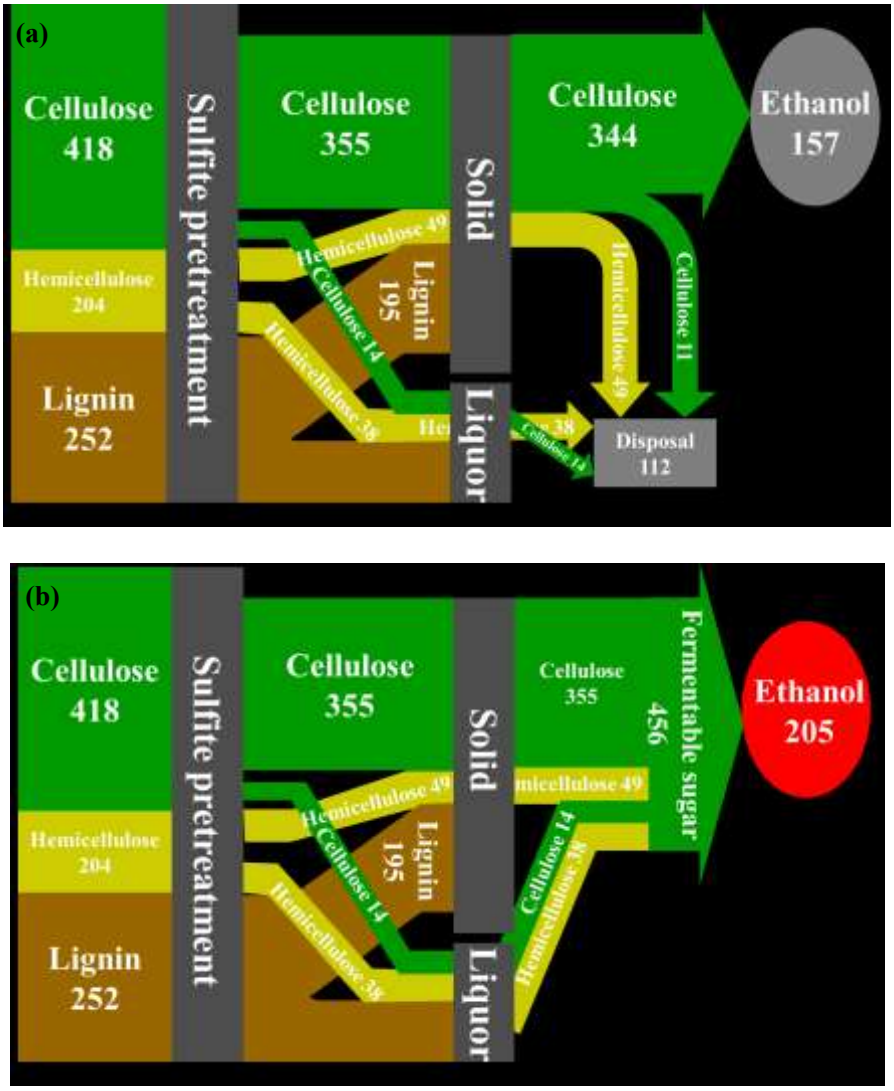


Fig. 4.6 Block diagram showing component mass balance (unit = kg). (a) washed substrate; (b) the whole slurry.

4.3.5. Mass balance

The overall mass balance of the whole slurry and washed substrate for ethanol production was conducted on the basis of the collection process. As shown in Fig. 4.6, a commercially viable ethanol concentration of 82 g/L was achieved with a yield of 205 kg/ton wood through the established biorefinery process. Generally, increasing ethanol concentration in fermentation broth is meaningful for decreasing energy consumption,

since distillation consumes a large part of energy for ethanol recovery. It has been found that energy consumption would be too intense, and regular distillation cannot economically separate ethanol if ethanol concentration in the fermentation broth is lower than 4% (w/w) (Huang & Zhang, 2011). 4% (w/w) of ethanol separation requires an energy input of 35% of its combustion energy. When ethanol concentration is 12% (w/w), distillation energy equals 12.6% of its combustion. In this work, the sulfite pretreated pine can obtain relatively high ethanol concentration (>8%, w/w), indicating that the energy ratio (in/out) of the distillation process can be reduced by approximately 30% compared to the previous work (Cheng et al., 2015a; Tian et al., 2010; Zhou et al., 2013c).

Table 4.3 shows the results with different conversion strategies to achieve high efficiency cellulosic bioethanol production. To obtain an ethanol concentration higher than 80 g/L, researchers performed high severity pretreatment to separate the lignin and break the structure of lignocellulose. In those processes, parts of cellulose and hemicellulose were also dissolved in the pretreatment liquor, whereas the pretreatment spent liquor was regularly washed out owing to its deleterious effect on SSF. The washing step led to only 115 kg/ton ethanol from corncob (Zhang et al., 2010) and 166 kg/ton from oil palm empty fruit bunches (Cui et al., 2014), respectively. It was reported that sulfite pretreatment was a potential solution to increase ethanol yield, and the solid sulfite pretreated substrate produced approximately 60% more ethanol than solid dilute acid substrate (Zhu et al., 2011). SSF with whole sulfite pretreated Lodgepole pine was further conducted at 20% total solid loading (18% unwashed solid loading with fixed amount of spent liquor) by Lan et al. (Lan et al., 2013a). Although they achieved high

ethanol yield at 225 kg/ton wood, maximum ethanol concentration of 47.4 g/L was observed. To the best of our knowledge, the present findings indicate the highest ethanol concentration of the whole slurry biorefinery process yet reported and exceed the technical and economic limits of industrial-scale ethanol distillation.

4.4. Conclusions

We first evaluate the feasibility to produce cellulosic bioethanol by using undetoxified whole Monterey pine slurry. It was found that the sulfite pretreatment spent liquor presented inhibitions on traditional SSF. By reducing operating temperature, tolerance of S. cerevisiae to inhibitors was enhanced at 28°C, which was future considered as a strategy to alleviate the inhibitions induced by the Monterey pine slurry. Ultimately, ethanol concentration was enhanced to 82.3 g/L by conducting SSF using hydrolysate up to 25% solid concentration. Consequently, an efficient system of cellulosic bioethanol production based on the SSF of whole Monterey pine slurry was successfully established.

Table 4.3 Comparison of lignin content and conversion strategy to achieve high efficiency ethanol production

Feedstock	Pretreatment	Acid insoluble lignin (%)		Detoxification strategy	Fermentation slurry	Solid loading (%)	$C_{ethanol}$ [a] (g/L)	$Y_{ethanol}$ [b] (kg/ton)	Ref.
		Untreated feedstock	Pretreated feedstock						
Corncob	Acid plus Alkali	18	3	Washing	Solid	39	85	115	Zhang et al., 2010
Oil Palm Empty Fruit Bunch	Formiline plus Deformylation	17	5	Washing	Solid	28	84	166	Cui et al., 2014
Waste paper	NA	1	NA	NA	Solid	25	92	NA	Elliston et al., 2013
Corn Stover	Deacetylation and Mechanical Refining	15	13	Washing	Solid	28	86	-	Chen et al., 2016
Lodgepole Pine	Sulfite pretreatment	29	23	Undetoxification	Whole slurry	18	48	225	Lan et al., 2013
Momentary Pine	Sulfite pretreatment	25	23	Undetoxification	Whole slurry	25	82	205	This work

[a]Ethanol concentration
[b]Ethanol yield
NA: Not applicable

Chapter 5 Temperature profiling to maximize energy yield with reduced water input in a lignocellulosic ethanol biorefinery

5.1 Introduction

Global climate change and increased energy demand have boosted tremendous efforts in developing innovative technologies to extract biofuel and chemicals from renewable sources. Cellulosic ethanol has been regarded as an attractive alternative to first-generation biofuels and fossil fuels (Achinas & Euverink, 2016; Debnath et al., 2017; Lynd et al., 2017). Woody biomass generated from the forest industry and municipal wastes have been considered the most promising feedstock for cellulosic ethanol production, which benefits the communities in the social, economic, and environmental perspectives (Cambero & Sowlati, 2016; Zhu & Zhuang, 2012a). Woody biomass, in comparison with other lignocellulosic biomass (*i.e.*, corn stover or rice straw), can be generated year-round and with specific physiochemical features such as high density, low moisture contents, and low inorganic contents, which benefit its logistics and transportation when serving as a feedstock for biofuel production (Zhu & Zhuang, 2012a).

The major economic barrier to a wider application of woody biomass in biorefineries is its well-known high recalcitrance to bioconversion. Woody biomass is a complex microstructure composed of cellulose, hemicelluloses, and lignin. This structure needs to be decomposed to enhance the accessibility of cellulase to cellulose before being converted into fermentable sugars and the related products (Leu & Zhu,

2013; Loow et al., 2015). The recent pretreatment techniques for woody biomass conversion, such as steam explosion (Bondesson et al., 2013), dilute acid (Avci et al., 2013), co-solvent pretreatment using tetrahydrofuran (THF) (Nguyen et al., 2015; Nguyen et al., 2016), γ-valerolactone (GVL) (Alonso et al., 2011), and sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) (Cheng et al., 2015a; Zhu et al., 2009), have been successfully developed to achieve remarkable sugar yields (>90% conversion of glucan to glucose) at reasonable enzyme loadings (*i.e.*, 5-15 filter paper unit, or FPU, per g glucan in biomass). These innovative pretreatment techniques have different mechanisms, which aim to mitigate (*i.e.*, steam explosion, dilute acid), modify (*i.e.*, sulfite treatment), and/or decompose the structural lignin and hemicellulose (*i.e.*, co-organosolv) in the plant cell wall for different applications. For example, sulfite pretreatment can enhance the sugar yield of the overall processes. Organosolv process aims to recover the value-added aromatic monomers. Acid catalysts are typically applied to remove hemicelluloses and has been applied in all the stated pretreatment processes (Liu et al., 2016a).

Following the pretreatment process, the simultaneous saccharification and fermentation (SSF) or prehydrolysis SSF (PSSF) processes have been applied to convert cellulose/hemicelluloses into fermentable sugars and to produce the fermentation products at the same time (Zabed et al., 2016). As increasing ethanol titer can significantly reduce the distillation energy, one major target of PSSF is to increase the solids loading of the substrate in the fermentation broth while reducing the impacts of growth-inhibiting factors on the fermentation microorganisms. Optimization of the PSSF process is complex as the operating temperatures differ significantly between

enzymatic hydrolysis (50°C) and fermentation (30-45°C) (Zabed et al., 2016; Zhou et al., 2013b). The pretreatment spent liquor remaining in the substrate or hydrolysate, furthermore, may contain significant amounts of pretreatment by-products, *i.e.*, 5-(Hydroxymethyl)furfural (HMF), furfural, and phenolic compounds, which may seriously affect the efficiency of either enzymatic hydrolysis, yeast fermentation, or both (Jönsson & Martín, 2016; Tian et al., 2011). Washing or detoxification has been applied to improve the digestibility and fermentability of the substrate, but both of those approaches result in significant losses of sugars and lowering the final ethanol yield. Energy, water, and chemical inputs may be increased due to the toxic impacts of pretreatment by-products or accumulation of lignin in the fermentation broth.

Zhu et al. (Zhu et al., 2010) recently introduced the "whole slurry" SSF process to directly apply sulfite pretreated Lodgepole pine chips with the pretreatment spent liquor to produce ethanol without detoxification. As sulfite pretreatment (SP) aimed to modify the surface of residual and dissolved lignin to satisfy enzymatic cellulose conversion without complete removal of lignin (Liu et al., 2016a), ethanol titer was expected to be significantly improved by completely utilizing the hexoses for fermentation. However, the maximum ethanol titer was still at 52.8 g/L (Lan et al., 2013a). The solutions needed to overcome the possible impacts of the components in the pretreatment spent liquor have not yet been identified.

This study aims to clarify three essential knowledge gaps toward the economical application of the softwood-to-bioethanol biorefinery processes for maximizing the energy yield of the pretreated substrate. Complete utilization of woody biomass is an economically and environmentally attractive targets for process development, but the

key mechanism to improve the ethanol titer to achieve similar levels as in other low-lignin-content biomass (Chen et al., 2016; Liu et al., 2017a) is still unclear. The energy consumption platform of different operating conditions, especially the relationships among the SSF process, ethanol titer, and distillation energy has not been well-developed. The water-and-energy nexus for the most recent bioconversion processes for woody biomass conversion has never been reported in previous research works.

In this study, proper configurations of the SSF processes were investigated through changing the operating conditions to facilitate the performances of the commercial cellulase and yeast. The whole slurry substrate was prehydrolyzed under different operating conditions (*i.e.*, prehydrolysis times, solid contents, and concentrations of the mixed spent liquor); and then different fed-batch approaches were applied to maximize the rates of hydrolysis. Water-soluble contents in the pretreatment spent liquor were fractionated and purified to study the possible components inhibiting the metabolisms of the yeast, while the tolerance of yeast to the inhibitors was investigated by changing the fermentation temperatures. Numerical analysis using both experimental and literature data on energy and water footprints were conducted and compared with the other recent biorefinery techniques. The findings of this study provide an opportunity to complete utilization of various feedstocks, such as energy crops, agriculture residues, and woody biomass.

5.2 Materials and methods

5.2.1 Setup of the biorefinery platform

The conceptual diagram of the proposed biorefinery process and the related energy/water footprints of each unit process are shown in Fig. 5.1. The woody biomass is hammer-milled before installing into the pretreatment vessel. The wood chips are cooked under designed pretreatment conditions (Cheng et al., 2015a) and then transferred to the solid-liquid separator. The solid fraction of the pretreated wood is disc-milled together with a portion of the pretreatment spent liquid, and the remainder of the spent liquor is concentrated by using an evaporator. The mixture of the milled substrate and concentrated spent liquor is then discharged into a fermenter for PSSF and then the fermentation broth is distilled to recover the ethanol. The major energy consuming units of the overall process include the hammer mill, the pretreatment vessel, the disc mill, the evaporator, and the distillation system; and the sole energy output is the purified ethanol. For water footprint analysis, the pretreatment and size reduction processes are the major water consumers while water can be harvested (potentially recyclable) from the evaporator and distillation tower. In this study, it has been assumed that the water is not recycled for any purpose. For treating one ton softwood chips the approximate sizes of the instruments are designed as follows: the pretreatment vessel and fermenter are with a working volume of 5,000 L each; the concentration unit is with maximum capacity of 3,000 L; and the distillation tower is with 27 cm diameter (*i.e.*, which is calculated by using a tower loading factor of 1.5 cm/sec [23] (Katzen et al., 1997)).

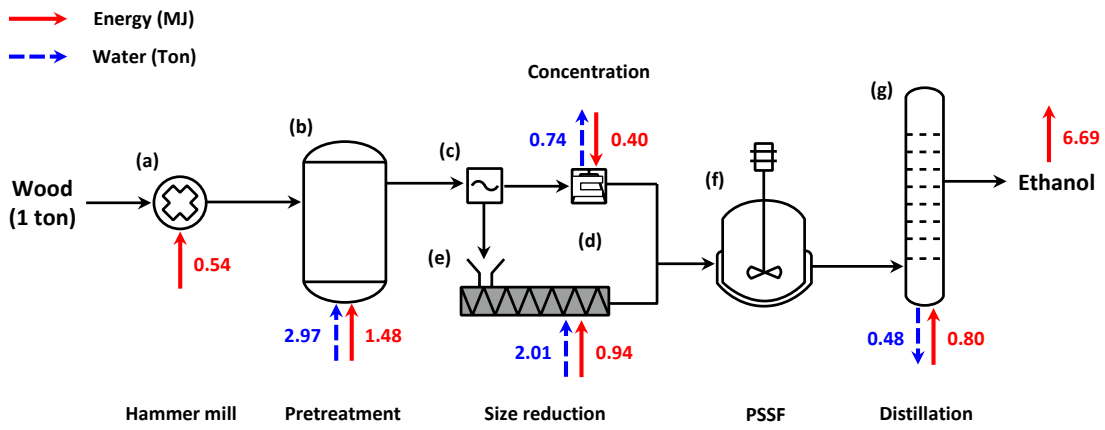


Fig. 5.1 Flow diagram of the water and energy balances of different unit processes in the proposed biorefinery for conversion of 1 ton softwood feedstock in ethanol.

5.2.2 Materials

Monterey pine wood chips and Commercial cellulase Cellic Ctec2 were prepared as described in Chapter 3 section 3.2.1. Thermae-tolerant alcoholic active dry yeast (*Saccharomyce cerevisiae*) was prepared as described in Chapter 4 section 4.2.1. All the other chemical reagents were of ACS reagent grade.

5.2.3 Pretreatment

Pretreatment process was described in Chapter 3 section 3.2.2.

Table 5.1 Experiment conditions of the SSF processes

Test Code[a]	Substrate	Time of Prehydrolysis (h)	Initial solid (%)	Fed substrate (%)	Fed time (h)	Temperature (°C)
W24P35	Washed substrate	24	20	5	12	35
H24P28	Whole slurry	24	20	5	12	28
W12F35	Whole slurry	12	20	5	12	35
H12F28	Washed substrate	12	20	5	12	28
W0F35	Whole slurry	0	20	5	12	35
H0F28	Washed substrate	0	20	5	12	28

[a] Test codes show four operation conditions for process optimization; the first symbol represent the substrate condition, where H = whole slurry and W = washed substrate; the following number 0, 12, 24 are the prehydrolysis time; P = substrate was fed at prehydrolysis time, F = substrate was fed at fermentation time, respectively; and the last number show the fermentation temperature.

5.2.4 PSSF experiments

The designed conditions of the PSSF experiments are presented in Table 5.1. The experiments were conducted by loading 100 mL of the mixture of the substrate and the other reagents in 250 mL Erlenmeyer flasks. The enzyme loadings, nutrient concentrations, and inoculation conditions of the yeast were fixed among all the experiments. In summary, the pH of the substrate was adjusted to pH 5.5-6.2 by calcium hydroxide and the cellulase load was fixed at 15 FPU/g-substrate during hydrolysis. The yeasts were grown in yeast extract peptone dextrose (YPD) medium (*i.e.*, mixtures of 20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone) at 35°C for 16 to 24 h before

mixing into the hydrolysate. The yeast was then dosed into the hydrolysate at a rate of 5 g/L.

Prehydrolysis was conducted in a shaking incubator at 50°C and 200 rpm for different periods of time. The activated yeast was added to the flasks after 36 h of prehydrolysis, and the operating temperature and shaking speed were reduced to 28-35°C and 130 rpm, respectively. The fed-batch experiments were started with an initial solids content of 20 wt% for the whole slurry substrate (Table 5.1). After a period of hydrolysis, an additional amount of sugar or substrate accounting for 5 wt% of the total solids content in the hydrolysate was mixed into the fermentation broth, giving a final solids content of 25 wt% for the whole slurry processes. To maintain the high solids contents, the fed-substrate and pretreatment liquors were concentrated by using a lab-scale evaporator. Triplicate experiments were conducted for each test and liquid samples of 1.5 mL were collected from the fermentation broth for further analyses at a sampling frequency of once every 12 hours.

5.2.5 Inhibition of fermentation

Fermentations using synthetic medium and pretreatment spent liquor were performed to evaluate the tolerance of *S. cerevisiae* to inhibitors. The synthetic medium had the same concentration of HMF, furfural and acetic acid as the pretreatment spent liquor; and also contained 200 g/L hexose, 1 g/L yeast extract and 2 g/L peptone. Pretreatment spent liquor was obtained after the filtration of the slurry. To evaluate the inhibition effects of the water-soluble components on the SSF process, the pretreatment spent liquor was purified by using 3 kDa MWCO Amicon® Ultra 15 Filters (Millipore,

MA, USA) at 4,000 ×g for 30 minutes at room temperature. The <3 kDa filtrate was collected (FS3), and > 3 kDa retentate (RL3) was rinsed away with an excessive amount of distilled water in the filter tube until the HMF and furfural were no longer detected. The pretreatment spent liquor was provided with glucose and nutrients to final concentrations of 200 g/L hexose, 1 g/L yeast extract and 2 g/L peptone. Control experiments were performed under the same conditions without inhibitors.

5.2.6 Measurement of viable cell density

Measurement of viable cell density was described in Chapter 4 section 4.2.7.

5.2.7 Analysis of sugars, inhibitors and fermentation products

Sugars, inhibitors and fermentation products were analyzed based on the procedure described in Chapter 3 section 3.2.3.

5.2.8 Energy and water balance

The energy consumption of the major operating processes, *i.e.*, heating of the pretreatment digester, cooling of the pretreated substrate for hydrolysis and fermentation, and distillation of the fermentation broth to separate the products and residues (Fig. 5.1), were calculated based on the enthalpy of the related components in the systems (Huang & Zhang, 2011; Zhu & Zhuang, 2012a), and the general form of the energy consumption is demonstrated as follows:

$$E_H = \frac{1}{\eta} \cdot \left[\int_{T_0}^{T_t} \left(\sum_{i=1}^{n} m_i^{Tj} \cdot Cp_i^{Tj} \right) \cdot dT + \sum_{i=1}^{n} m_i^{Tk} \cdot L_i^{Tk} \right] \quad (1)$$

where E_H is the energy consumption of the discussed heating (cooling) process (kJ); η is the efficiency of the heat transfer (0.5); T_0 is the initial temperature (°C); T_t is the desired temperature (°C); m is the total mass of the discussed compound (kg); C_p is the heat capacity of the discussed compound (kJ/kg/°C); L is the latent heat of the discussed compound (kJ/kg); the superscripted symbol *Tj* represents the specific temperature of selected heating temperature (°C); *Tk* is the boiling points of the selected compound (°C); subscripted symbol *i* represents the type of compounds in the mixture; and *n* is the total number of the compounds. The calculation neglects the interaction among the components to the heat capacity and latent heat as the mass ratios among different components are all very small in comparing to water in the system (*i.e.*, 75.0-87.5% water versus other compounds).

The calculation of the energy consumption was based on the experiment results and reference values for the water and solvent consumption in the targeted processes, *i.e.*, pretreatment washing, and solid contents during the SSF processes. The key assumptions for the energy balance in the pretreatment process include the liquid-solids ratios. (1 for steam explosion; 3 for sulfite treatment; and 7 for the organosolv process), cooking temperature (165 to 220°C), and cooking time (15 to 75 mins) as the related information [26] (Zhu & Pan, 2010). The distillation energy (E_{dstl}) was calculated based on the ethanol-water equilibrium curve associated with needed liquid/volume streams for heating using district steam as the major heating source, which can be expresses as:

$$E_{Dstl} = L \cdot e_{Steam} = R_C \cdot V \cdot e_{Steam} \quad (2)$$

where L and V are the liquid and vapor flow rate, respectively (kg-mole/h); e_{steam} is the energy content of the city steam (kJ/kg); R_C is the specific constant for L/V ratios (equals to 5 and 6.33 for 10 vt% and 5 vt% ethanol-water solutions, respectively) (Madson, 2003).

Finally, the water consumption and milling energy was referred to the authors previous study on a similar woody biomass (*i.e.*, *Douglas fir*) (Leu et al., 2013) and the related references (Zhu & Pan, 2010). The energy recovery of the high-temperature processes such as pretreatment and distillation was assumed to be 50% for all the processes (Zhu & Zhuang, 2012a).

5.3 Results and discussion

5.3.1 Optimization of the PSSF process

5.3.1.1 Comparison of SP and DA pretreatments for ethanol production

The chemical compositions of the Monterey pine chips are shown in Table 5.2. The untreated Monterey pine chips were composed of 41.8 wt% glucan, 3.7 wt% xylan, 4.6 wt% galactan, 12.1 wt% mannan and 25.2 wt% acid insoluble lignin. Hexosan (58.5 wt%) dominated in the plant cell wall, suggesting the suitability of the feedstock for fermentation using *S. cerevisiae*. Chemical compositions of the SP and DA pretreated substrates are shown in Table 5.2. All the hemicelluloses were completed dissociated by the two pretreatment processes, leaving only the cellulose and lignin in the washed substrate. No significant delignification occurred during the DA pretreatment, and hence

the lignin was accumulated in the substrate (37.5 wt%) after complete dissociation of hemicelluloses. Approximately 22 wt% of cellulose was dissolved in the pretreatment liquor. Higher cellulose (82 wt%) and lower amount of lignin retained in the substrate after the sulfite pretreatment process. The total solids recovery after SP pretreatment reached to 94.8 wt%, among which 79.2 wt% in the unwashed solids fraction and 15.6 wt% in the spent liquor as soluble and suspended solids, respectively. Recoveries of glucan, xylan and mannan were determined as 88.2 wt%, 60.9 wt% and 42.2 wt%, respectively. A total of 82.3 wt% of the glucan was retained in the insoluble solids after pretreatment, whereas almost all the recovered xylan and mannan were solubilized in the liquid fraction. Successful conversion of Monterey pine into cellulosic bioethanol should include co-utilization of insoluble solids, as well as the soluble fraction derived from the pretreatment process or the whole slurry process.

SSF of the washed and whole slurry were conducted at 25% solid loadings and the results were presented in Fig. 5.2. The slurry was prehydrolyzed for 36 h prior to *S. cerevisiae* inoculation, which contributed to the source of the fermentable hexose in the fermentation broth (Fig. 5.2a). The total concentrations of the hexoses were less than 50 g/L in both the washed and whole DA pretreated slurry, while more than 100 g/L hexose was released in the washed and whole SP pretreated slurry. It indicated a higher potential for high efficient ethanol conversion via SSF process with SP substrate than that with DA substrate under the same hydrolysis conditions.

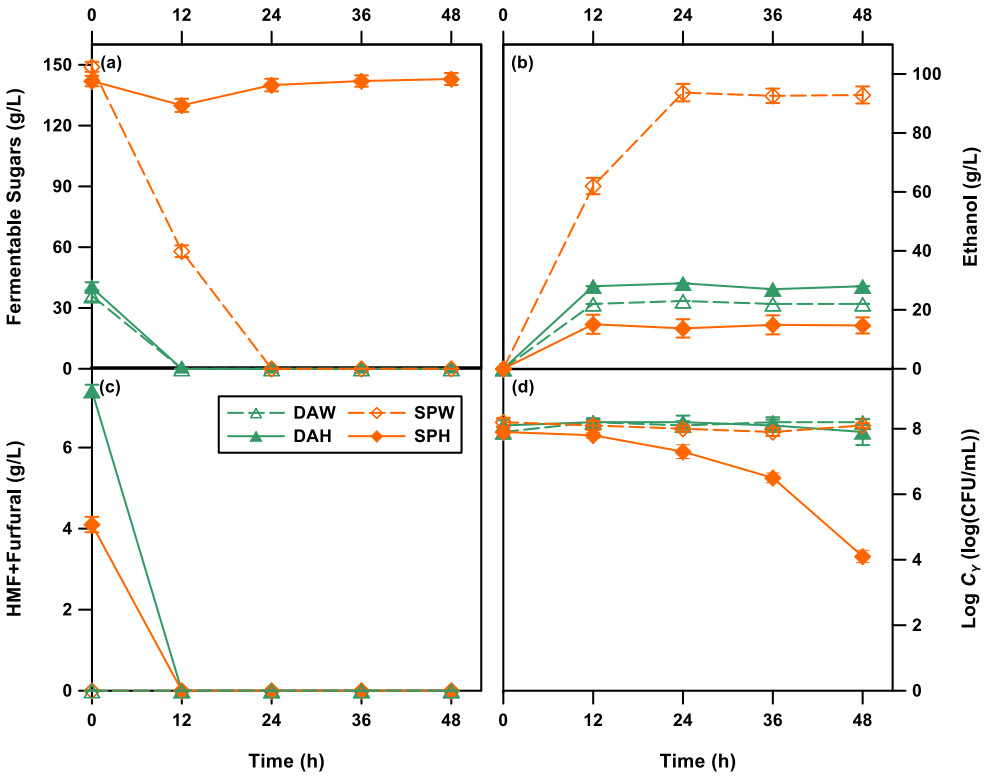


Fig. 5.2 Changes of hexose (a), ethanol (b), HMF+furfural (c) and CFU (d) of SSF over time. DAW: diluted acid pretreated washed slurry; DAH: diluted acid pretreated whole slurry; SPW: sulfite pretreated washed slurry and SPH: sulfite pretreated whole slurry.

Table 5.2 Chemical compositions of untreated, dilute acid pretreated and sulfite pretreated wood chips

Components of substrates (%)	Untreated wood chips	DA washed substrate	DA unwashed substrate	DA unconcentrated liquor	SP washed substrates	SP unwashed substrates	SP unconcentrated liquid
Glucan	41.8	51.9(77.9)	48.5 (81.4)	10.9 (4.5)	59.8 (82.3)	44.8 (84.9)[c]	11.4 (3.3)
Xylan	3.7	ND	1.3 (26.7)	3.7 (17.8)	0.2 (2.7)	1.8 (38.5)	6.9 (22.4)
Galactan	4.6	ND	0.9 (15.1)	2.8 (10.8)	ND[b]	1.0 (17.2)	4.9 (12.8)
Mannan	12.1	ND	5.7 (34.8)	16.3 (23.9)	ND[b]	3.4 (22.3)	20.1(19.9)
Acid insoluble lignin	25.2	37.5(93.3)	32.9 (96.2)	NA[a]	29.2 (66.7)	25.1 (78.9)	NA[a]
HMF	NA[a]	ND[b]	0.7	5.3	ND[b]	0.4	2.4
Fufural	NA[a]	ND[b]	0.4	2.1	ND[b]	0.3	1.7
Total mass[d]	100	62.7	73.7	4.6	57.5	79.2	15.6

[a] Not applicable.
[b] Not detectable.
[c] Numbers in the parenthesis are the percent yields after pretreatment.
[d] Calculated based on the oven dry weight.

Ethanol reached maximum concentrations within 24 h by using washed DA substrate (DAW), DA whole slurry (DAH), and SPW slurry (Fig. 5.2b). Hexose remained low concentrations throughout most of the processes, which indicated that utilization of hexose as readily as it was released. A relative lower ethanol concentration of 13.4 g/L was observed in the SPH slurry. HMF and furfural were considered to be the major inhibitors to the low fermentability, however it was then confirmed that those two inhibitors showed no observable impacts in the fermentation systems at the tested levels (Fig. 5.3b), bottom figure. HMF and furfural were completely metabolized within 12 h after the introduction of yeast in the hydrolysate. Therefore the continuous degradation of cell viability implied that fermentation inhibition might be due to other components (such as aromatic based pretreatment products or organic acids) in the pretreatment liquor.

5.3.1.2 Impacts of fermentation temperatures on ethanol yield

Many studies have demonstrated the difficulty to optimize the SSF process because of the inconsistent operating temperatures for enzymatic hydrolysis (50°C) and ethanol fermentation (28-35°C). Paulová et al. (Paulová et al., 2014) reported that the hydrolysis rates of cellulose can decrease by 40% when the hydrolysis temperature was reduced from 45°C to 30°C. An intermediate temperatures of 35°C were suggested in the SSF process to strike for the balance between the two reactions (Abdel-Banat et al., 2010). It has also been reported that the thermal- and ethanol- tolerant *S. cerevisiae* (same as the one used in this study) can produce the highest ethanol titer (*i.e.*, 60 g/L) at 39°C in the SSF process from steam-exploded corn stover (Liu et al., 2014). To investigate the

effect of operating temperature on the tolerance of *S. cerevisiae* to the inhibitors, SSF experiments were conducted using SPW and SPH slurry at 25°C, 28°C, 31°C, 35°C, 39°C and 42°C (Fig 5.3). It was found that when using SPW slurry the ethanol concentration can be as high as 90 g/L without changing the operating temperature (35°C) throughout the 24 h of fermentation. The ethanol concentration decreased significantly, however, from 83.1 g/L to 50.2 g/L in the SPH slurry when the temperature increased from 28°C to 42°C, respectively. The moderate temperature 28°C was determined to be optimal for the engineered yeast to tolerate the inhibitors in the spent liquor. In this present study, *S. cerevisiae* overcame the toxic effects of the inhibitors at 28°C when it was exposed to the high concentration of ethanol (80 g/L) and pretreatment by-products.

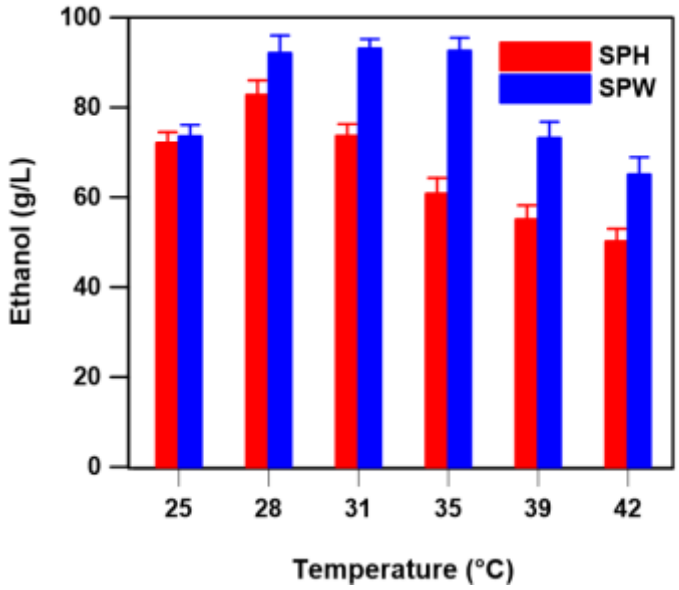


Fig. 5.3 Performance of *S. cerevisiae* in SSF at 25, 28, 32, 35, 38 and 40°C. SPH: sulfite pretreated washed slurry; SPW: sulfite pretreated whole slurry.

5.3.1.3 Effect of fed-batch operation on the efficiency of SSF process

Single stage fed-batch SSF was conducted to test the possibility of increasing sugar contents in the fermentation broth by starting with a whole slurry substrate containing 20 wt% solid, 1 g/L yeast extract, and 2 g/L peptone. The fermentation broth was later fed with 5 wt% solid-containing slurry after 12 h hydrolysis (Fig. 5.4 and Table 5.1). The tested prehydrolysis time were increased from 0 h (W0F35 and H0F28), 12 h (W12F35 and H12F28) and then 24 h (W24P35 and H24P28), respectively. It took more than 72 h to reach maximum ethanol titers in W0F35, H0F28, W12F35 and H12F28 processes, while the maximum ethanol titers was observed in W24P35 and H24P28 process at 48 h (Table 5.3), respectively. Compared with W24P35 and H24P28, *S. cerevisiae* was inoculated earlier to alleviate high sugar inhibition. Meanwhile, temperature was reduced to 28°C or 35°C to strike for a balance between hydrolysis and fermentation. In this way, conversion rates of cellulose into glucose were decreased, which reduce the ethanol productivity consequently. In the W24P35 process, cellulose was concentrated up to 160 g/L and inhibitors were eliminated, resulting in higher ethanol titers (90.1 g/L) than one measured in H24P28 (82.6 g/L) (Fig. 5.4). However, as the pretreatment liquor contains large amount of fermentable sugars H24P28 actually can generate more ethanol than W24P35 process. Fermentation with the pretreatment liquor (whole slurry) resulted in 40.3% more ethanol from 1 ton wood samples.

Table 5.3 Fermentation results

Test Code	Max titer (g/L)	Time to peak (h)	Max ethanol productivity (g/L·h)	Bioconversion efficiency[a] (%)
W24P35	90.1±2.63	48	5.16±0.22	43.2±2.11
H24P28	82.6±2.12	48	4.83±0.27	58.2±1.00
W12F35	90.1±3.32	72	4.18±0.23	43.2±1.53
H12F28	79.1±1.24	>72	4.05±0.27	55.7±0.85
W0F35	77.7±1.24	>72	4.05±0.27	37.2±0.85
H0F28	70.8±1.24	>72	4.05±0.27	49.9±0.85

[a] Bioconversion efficiency (%)= $\frac{\text{Produced ethanol (g)}}{\text{Total initial hexose (g)}\times 1.11\times 0.51}$

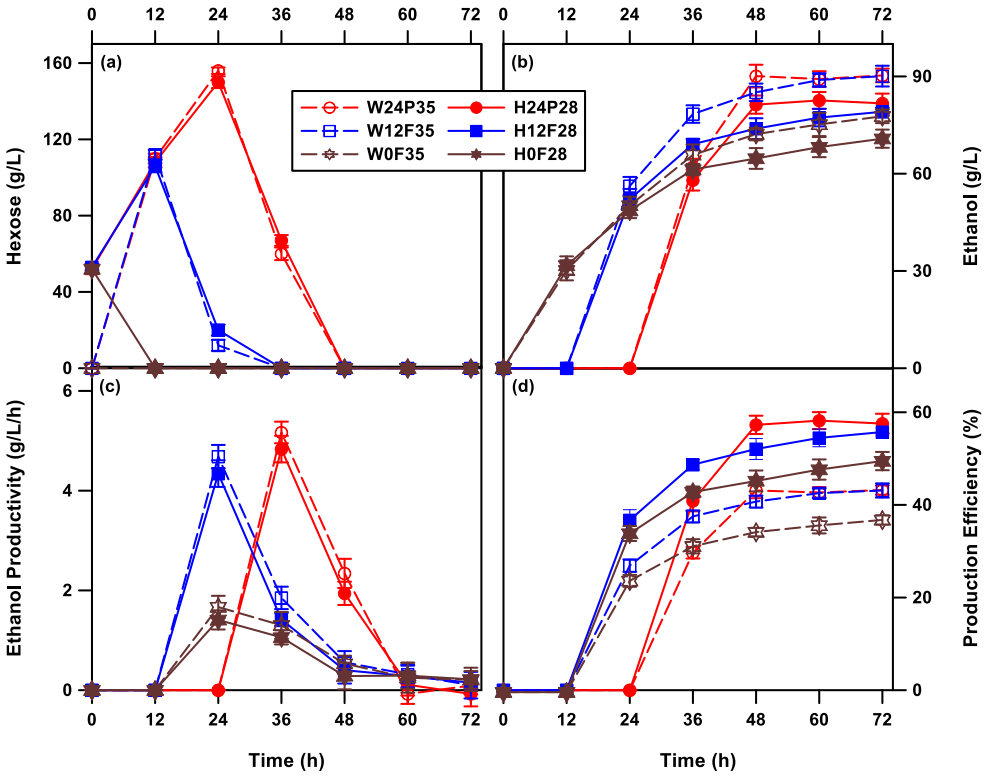


Fig. 5.4 Changes of hexose (a), ethanol (b), ethanol productivity (c) and bioconversion efficiency (d) of different fed-batch PSSF processes over time.

5.3.1.4 Effects of inhibitors in the spent liquor on fermentation

To further evaluate the tolerance of *S. cerevisiae* to inhibitors, fermentation experiments were performed with synthetic medium mixing with different parts of the fractionated pretreatment spent liquor with different molecular weight distribution. The detailed compositions of different fractions of the pretreatment liquors, such as HMF, furfural, acetic acid, and acid soluble lignin, are presented in Table 5.4, and the related ethanol production at 12 h and 24 h are shown in Fig. 5.5. The final ethanol production was not affected by the presence of HMF, furfural and acetic acid, but the addition of the pretreatment spent liquor significantly affected the fermentation performance of *S. cerevisiae*. Only 60.2 g/L ethanol was produced after 24 h fermentation. The result suggested that the inhibitory effects could not be attributed solely to HMF, furfural and acetic acid. Other pretreatment products such as phenolic compounds and aromatics may be responsible for the relatively low fermentability.

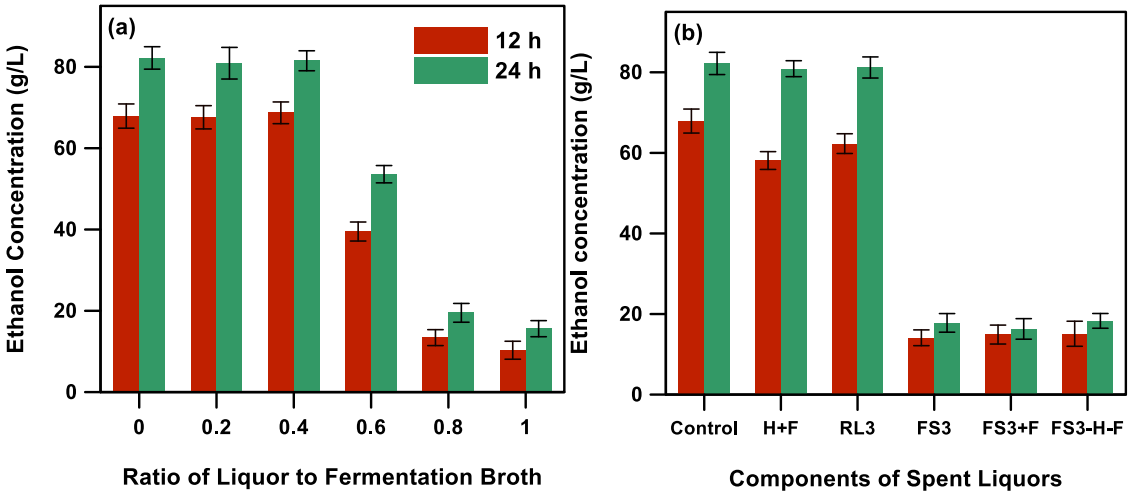


Fig. 5.5 Effects of spent liquor components to fermentation. (a) Concentrated pretreatment spent liquor at different mass ratios, and (b) different spent liquor components were mixed with standard mediums for fermentation. H: HMF; F: Furfural; FS3: <3 kDa filtrate; RL3: >3 kDa retenate; +F: fufural was added into the broth to offset the loss of the concentration process; -H-F: filtrate with no HMF or Furfural.

Table 5.4 Chemical composition of the pretreatment spent liquor (unit: g/L)

Component	DA pretreatment spent liquor	DA concentrated liquor	SP pretreatment spent liquor	SP concentrated liquor[a]	FS3[b]	RL3[c]
Glucose	10.9	20.7	11.4	22.2	21.2	0.02
Xylose	3.7	6.9	6.9	13.4	12.8	0.01
Galactose	2.8	5.4	4.9	9.1	9.0	ND[d]
Mannose	16.3	31.0	20.1	38.2	36.6	0.03
HMF	5.3	7.4	2.4	4.1	3.9	ND[d]
Furfural	2.1	ND[d]	1.7	ND[d]	ND[d]	ND[d]
Acetic acid	6.1	11.1	3.6	5.1	4.8	ND[d]
Acid-soluble lignin	4.2	9.1	59.3	121.6	113.7	5.4
Total solid content	52	102	132	256	228	37

[a] Concentration process removed 50.4% of water and low-boiling point compounds from the liquor.
[b] FS3: <3 kDa filtrate of the first ultrafiltration.
[c] RL3: >3 kDa retentate of the last ultrafiltration.
[d] Not detectable.

Types and concentrations of inhibitors strongly depend on the feedstock and pretreatment methods, whereas the large number and diversity of inhibitors found in different lignocellulosic hydrolysates make identification and quantification of separate compounds extremely difficult (Jönsson et al., 2013b). Furthermore, previous studies also indicated that fermentability of pretreatment substrate mixing with the spent liquor differs significantly from the synthetic medium with the same levels of pure chemicals. Synergistic effects among different inhibitory compounds can occur in the fermentation process (Jönsson & Martín, 2016). Analysis of the composition and types of inhibitors in the pretreatment spent liquor is essential to study the mechanisms of the heat stress induced inhabiting effects to the yeast. Other than HMF and furfural, the most representative inhibitors of fermentation may include organic acid (*i.e.*, formic acid, acetic acid), acid soluble lignin, and other aromatic compounds in the pretreatment liquor (Jönsson et al., 2013b; Jönsson & Martín, 2016). In the current study, however, we focus more on how to reduce the impacts to improve the productivity of ethanol in the proposed system. More sophisticated fractionation processes and analytical tools will be developed and applied to further investigate the sensitivity of the inhibitors in the pretreatment liquor to enzyme hydrolysis and fermentation.

5.4 Remarks on the high yield in a biological system

To achieve a high ethanol titer the solid loading of the total biomass in the fermentation broth must be increased to a considerably high level, which also results in high concentrations of residual lignin and inhibitors in the SSF process. The impacts of lignin to ethanol concentration after different types of bioconversion processes are

summarized in Fig. 5.6 (more detailed numerical results are provided in Table 5.5). It should be noted that the ethanol concentrations is a function of a series of optimized processes with many control parameters, *i.e*., pretreatment severity, solid loadings of the PSSF, hydrolysis conditions, fermentation conditions and strains. The examples shown in Fig. 5.6 present only the summary of different strategies under optimal conditions. The theoretical ethanol titers under different solids contents of the substrate are provided in dashed lines from 10 wt% to 25 wt% for comparison. In general, ethanol concentrations decrease with the lignin contents of the fermentation broth and washing shows certain improvements over the whole slurry process. The major breakthroughs that to overcome the general patterns are mostly related to new pretreatment processes as discussed previously by Chen et al. (Chen et al., 2016) and Zhu et al. (Zhu et al., 2010). Based upon a similar concept, the SSF processes were further improved without the need to remove the residual lignin from the fermentation broth.

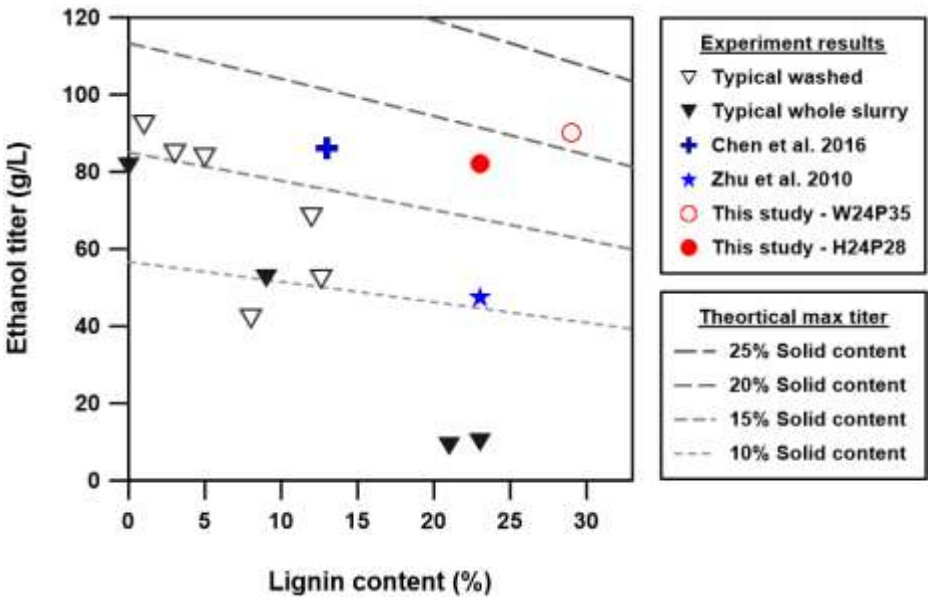


Fig. 5.6 Impacts of lignin residues to ethanol titer of different feedstock and biorefinery processes. The dashed lines show the maximum ethanol titers that can be obtained at different solid loads, and the spots show some examples from literature and this study for comparison.

Table 5.5 Comparison of different fermentation strategies to achieve high ethanol titer

Feedstock	Pretreatment	Pretreatment Temp (°C)	Lignin residues	Substrate	Ethanol titer (g/L)	SSF Temp	Solid Content (%)	Strain	Reference
Cane juice	-	-	0	-	81.4	37	0	*K. marxianus* + *S. cerevisae*	(Eiadpum et al., 2012)
Waste paper	Kraft	-	1	Washed	92	30	65	*S. cerevisiae* NCYC 2826	(Elliston et al., 2013)
Corncob	Acid + alkaline	60	3	Washed	84.7	37	19	*S. cerevisiae*	(Zhang et al., 2010)
Oil Palm Empty Fruit Bunch	Formiline plus Deformylation	120	5	Washed	83.6	37.5	15	*S.cerevisiae* CICC 31014	(Cui et al., 2014)
Miscanthus	Alkaline	150	8	Washed	41.9	42	11.56	*S. cerevisae* mbc 2	(Cha et al., 2015)
Corn stover	Alkaline wet oxidized	195	9	Whole slurry	52.3	30	17	*S. cerevisiae*	(Varga et al., 2004)
Cashew apple bagasse	Acid + alkaline	121	12	Washed	68		20	*K. marxianus* ATCC36907	(de Barros et al., 2017)
Oil palm empty fruit bunches	SPORL	180	12.6	Washed	52	35	18	*S. cerevisae*	(Tan et al., 2013)
Corn Stover	Deacetylation and Mechanical Refining	80	13	Washed	86	33	30	*rZymomonas mobilis* 13-H-9-2	(Chen et al., 2016)
Oil palm empty fruit bunches	Dilute acid	190	21	Whole slurry	9	30	6	*S. cerevisae*	(Jung et al., 2013)
Oil palm empty fruit bunches	Maleic acid	190	23	Whole slurry	10	30	6	*Saccharomyces cerevisae* D5A	(Jung et al., 2014)
Lodgepole Pine	SPORL	180	23	Whole slurry	47.4	35	19.5	*Saccharomyces cerevisae* YRH400	(Lan et al., 2013a)
Momentary pine	Sulfite Pretreatment	165	23	Whole slurry	82	28	25	*Saccharomyces cerevisae*	This study
Momentary pine	Sulfite Pretreatment	165	29	Washed	83	35	23	*Saccharomyces cerevisae*	This study

In addition to lignin, inhibitors can also reduce the final ethanol production in the SSF process, which is one of the major barriers to the development of an economically viable process for cellulosic ethanol production (Jönsson et al., 2013b; Koppram et al., 2014). To overcome this issue, some detoxification techniques have been reported to remove the toxic chemicals from the fermentation broth, including physical (evaporation and membrane separation), chemical (over-liming, activated charcoal treatment, ion exchange, neutralization and organic solvent extraction), and biological (treatment with laccase or peroxidase) techniques (Jönsson et al., 2013b; Jönsson & Martín, 2016). However, these additional detoxification steps increase the overall costs due not only to the capital and chemical costs but also to the loss of sugars (Tian et al., 2010). Recently, "pH profiling" together with low-temperature sulfite pretreatment was used to reduce inhibitor formation (Cheng et al., 2015a). Ethanol yield per ton of wood was increased by 27% in concentration, while the ethanol titer from one fermentation batch was still at 48.9 g/L. In the present work, the final ethanol concentration of the whole slurry SSF was successfully increased to 82.8 g/L by reducing the fermentation temperature to 28°C.

5.5 Energy balances of the new PSSF process

The net energy consumption of different bioconversion processes was calculated in Table 5.6, and the results of this study were compared with a few selected new techniques and presented in Fig. 5.7. The selected processes include steam explosion, dilute acid (DA) (Zhu et al., 2015), ethanol (*i.e.*, organosolv I, (Pan et al., 2006a)), co-solvent process (*i.e.*, THF pretreatment or organosolv II, (Nguyen et al., 2015),

SPORL followed by SSF (Zhu et al., 2010) and the modified PSSF processes developed in this study (*i.e.*, processes W36N35 and H24S28). Pretreatment energy accounted for 38% to 68% of the overall energy consumption in the biorefinery. This part of energy consumption was highly related to the amount of water used for the optimal production of the carbohydrates.

Water consumptions in different unit operations of the selected biorefinery processes are also presented in Fig. 5.7. Water consumptions for biomass pretreatment is a function of many parameters, *i.e.*, the transfer efficiencies of the needed solvent(s); desired mechanisms of delignification for hydrolysis; and the impacts of the pretreatment chemicals to enzyme and fermenting microorganisms. The steam explosion process relies on the physical strength created after the sudden release of vapor pressure therefore requires only little amount of water for pretreatment. It typically consumes an equal amount of water for treating 1 unit mass of woody biomass. DA and SPORL processes are operating under lower temperature (155°C to 180°C) and therefore higher amount of water is needed to rinse all the biomass in the reactor. The water consumptions of those processes are approximately 3:1 water versus woody biomass by mass as lignin removal is not mandatory in those processes when hemicelluloses are completely removed. Organosolv or co-solvent based processes aims to dissolve as much amount of both lignin and hemicelluloses as possible and hence more solvent and water are needed in the processes. The S/L ratios of the processes are approximately 5 to 7. Among those pretreatment processes steam explosion and organosolv processes can completely destroy the cell wall structure, hence do not need any water for milling or grinding, which is essential for DA or SPORL. Meanwhile, organic solvents usually

possess higher toxicity to enzyme and microorganisms the pretreated substrate needs to be thoroughly washed before hydrolysis and fermentation.

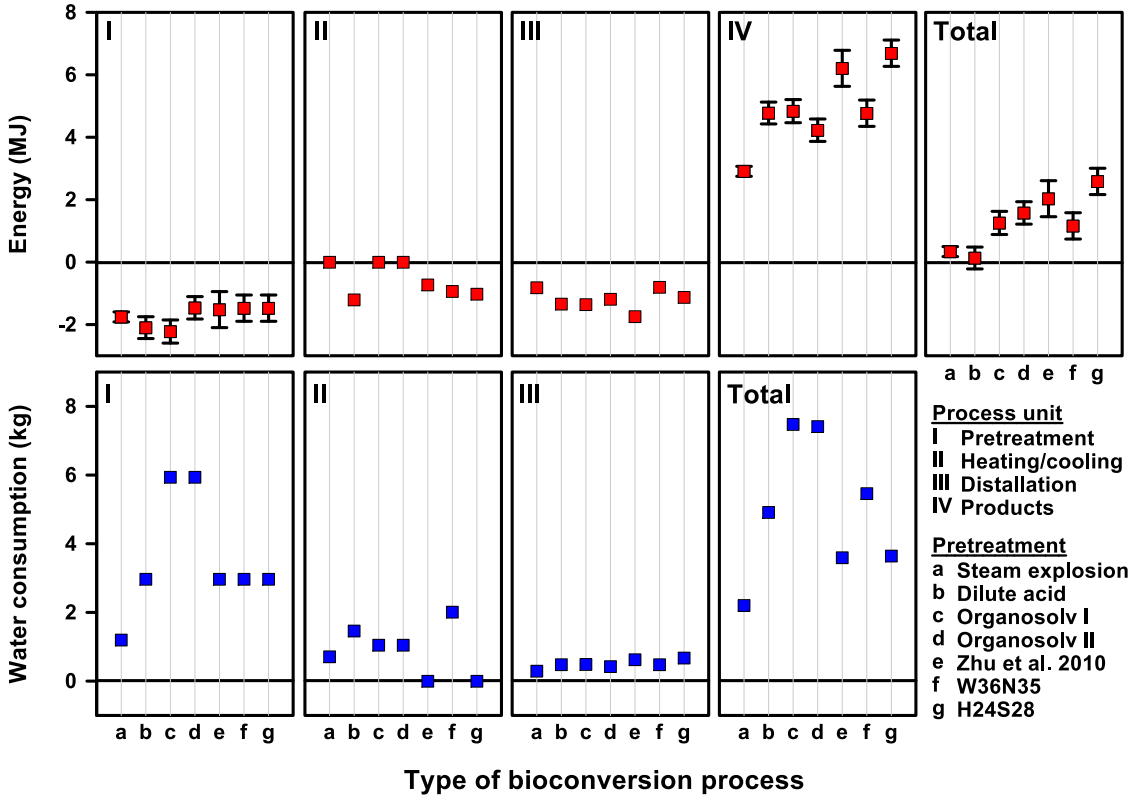


Fig. 5.7 Energy (a) and water footprint (b) of different wood-to-bioethanol techniques. Process I – pretreatment; Process II – milling/concentration/dilution; Process III – distillation; and Process VI shows the heating values of the final products (ethanol).

Table 5.6 Energy and water footprint of different processes

	Steam explode	**DA**	**THF**	**Ethanol**	**Zhu 2010**	**W24P35**	**H24P28**
Temperature (°C)	215	180	150	170	180	165	165
Solid yield (%)	95	42	30	30	75	58	84
Mono sugar (g)	211.0	392.2	397.1	347	496.8	343.9	482.41
EtOH yield (%)	91	57					
Total ethanol (g)	97.9	160.8	162.8	142.3	209	160.6	225.3
Energy (kJ/kg-wood)							
I. Hammer Milling	-0.54	-0.54	-0.54	-0.54	-0.54	-0.54	-0.54
Pretreatment	-1.75	-2.10	-2.22	-1.46	-1.52	-1.48	-1.48
II. Evaporation	0	0	0	0	0	0	-0.40
Size reduction	0	-1.2	0	-0.73	-0.73	-0.73	-0.73
III. Distillation	-0.82	-1.34	-1.36	-1.19	-1.74	-0.80	-1.13
Total Consumed	-3.11	-5.18	-4.12	-3.92	-4.53	-3.55	-4.28
IV. Product	2.91	4.78	4.84	4.23	6.21	4.77	6.69
Net Energy	-0.20	-0.40	0.72	0.31	1.68	1.22	2.41
Water (kg/kg-wood)							
I. Pretreatment	1.2	2.97	5.94	5.94	5.94	2.97	2.97
II-1 Heating/cooling	0	0	0	0	0	0	-0.74
II-2 Milling/dilution	0.71	1.46	1.05	1.05	1.05	0	2.01
III. Distillation	0.29	0.48	0.49	0.49	0.43	0.63	0.48
Total	2.21	4.92	7.48	7.42	3.60	5.46	3.65

A significant amount of energy can be saved in the distillation process by increasing the ethanol concertation through the new PSSF process. Distillation energy was affected by the water-to-solids ratios during pretreatment and the ethanol titer after PSSF, which accounted for approximately 28.9% to 44.8% of the overall bioconversion processes. The highest distillation energy consumption was observed from the whole slurry PSSF process after SPORL pretreatment (Zhu et al., 2010), which is most likely because of the impacts of small molecular weight compounds in the pretreatment liquor. With the same water usage for pretreatment, energy consumption was reduced significantly from 1.74 MJ to 0.80 and 1.13 MJ for washed (W24P35) and unwashed (H24P28) samples before and after temperature reduction, respectively. While cooling and reheating (also including milling energy) can indeed increase the energy consumption (by approximately 41.3%) after temperature modification, the advantages of complete consumption of the fermentable sugars in the biomass and high ethanol concentrations can overcome this limitation, resulting in a significant energy yield from 1.22 MJ (washed samples) to 2.41 MJ (whole slurry).

5.6 Remarks on water consumption

In addition to energy consumption, high water consumption is a critical issue for a biorefinery, which was often neglected in previous studies (*i.e.*, mostly focus only on sugar and ethanol yields). The water consumption shown in Fig. 5.7 was calculated based upon the limiting liquid-to-solids ratios, milling or size reduction, mixing, and washing for detoxification. In summary, the water consumption of the steam explosion

process was approximately 2.2 kg per od wood treated; acid-based pretreatment processes (*i.e.*, DA and SPORL) consume approximately 1.3 to 2.7 more kg of water then steam explosion process; and organosolv processes consume 2.50 to 3.88 kg extra water (total of 7.4-7.5 kg) than the acid-based processes. The newly introduced whole slurry process consumes approximately 3.6 kg water for treating 1 kg oven-dried woody biomass. Based on the requirement of the products, the condition of the biomass and regional conditions, the water consumption can be minimized by properly selecting the biorefinery technologies for optimal energy yield with lowest impacts to the environment. This study considered only the energy and water balance as references for further development of the bioconversion techniques and the extra energy or economic values created from related chemicals (*i.e.*, functional lignin) were not included in our analysis.

5.7 Conclusions

This paper presents three major contributions to obtain the highest ever energy yields in a potential softwood-to-bioethanol biorefinery, including (i) a new temperature profile to maximize the utilization of fermentable hexose and to overcome the inhibiting effects of pretreatment by-products in a PSSF process. By simply reducing the fermentation temperature from 35°C to 28°C, an extremely high cellulosic bioethanol production of 82.6 g/L (or 10 vol%) was achieved from sulfite pretreated Monterey pine whole slurry using commercial enzyme and thermos-tolerant yeast (*S. cerevisiae*). (ii) Through calculating the enthalpy of the pretreatment substrates and liquors the energy

yield of the new process is 2,410 MJ, which is approximately 43.5-234.7% higher than other published processes for treating 1 ton softwood. (iii) Finally, the new process has an extremely low water demand of 3.65 tons/ton-od wood, which is approximately 25.8-51.2% lower than the other processes (except steam explosion process). The findings of this study are somewhat contradictory to the conventional wisdoms and may be useful to full-scale application in a biorefinery.

Chapter 6 Diol pretreatment to fractionate a reactive lignin in lignocellulosic biomass biorefinery

6.1 Introduction

Developing biorefinery to harvest fuels and chemicals from lignocellulosic biomass is a prospective strategy to reduce the reliance of nonrenewable petroleum refinery products (Stocker, 2014). Delignification by pretreatment is a critical procedure to break down plant cell wall and facilitate carbohydrate valorization in the biorefinery. Conventional pretreatment approaches (*e.g.*, steam explosion and dilute acid/alkaline processes) have been carried out at elevated temperature with various chemicals to dissociate lignin. The harsh reaction environment can lead into irreversible lignin degradation and condensation (Fig. 6.1) (Constant et al., 2016; Ragauskas et al., 2014; Renders et al., 2017), especially when the biomass feedstock is formed by a mixture of different plant species and the pretreatment conditions cannot be well-monitored or optimized. Lignin degradation occurs with the cleavage of ether linkage, resulting in formation of phenolic hydroxyls which impact the digestibility of the substrate (Liu et al., 2016b; Yang & Pan, 2016). Lignin condensation is carried out with the formation of carbon-carbon (C-C) linkages, which increases the hydrophobicity of the residual lignin and leads to precipitation of dissolved lignin on the surface of pretreated substrate (Sjostrom, 2013). Compared with native lignin, degraded or condensed lignin is lack of ether linkages and with high amount of C-C linkages, which both affects lignin-to-aromatic conversion. Those influences reduce the yield of fermentable sugars

and lignin monomers in the downstream processes (Galkin & Samec, 2016; Rinaldi et al., 2016; Zhang et al., 2017).

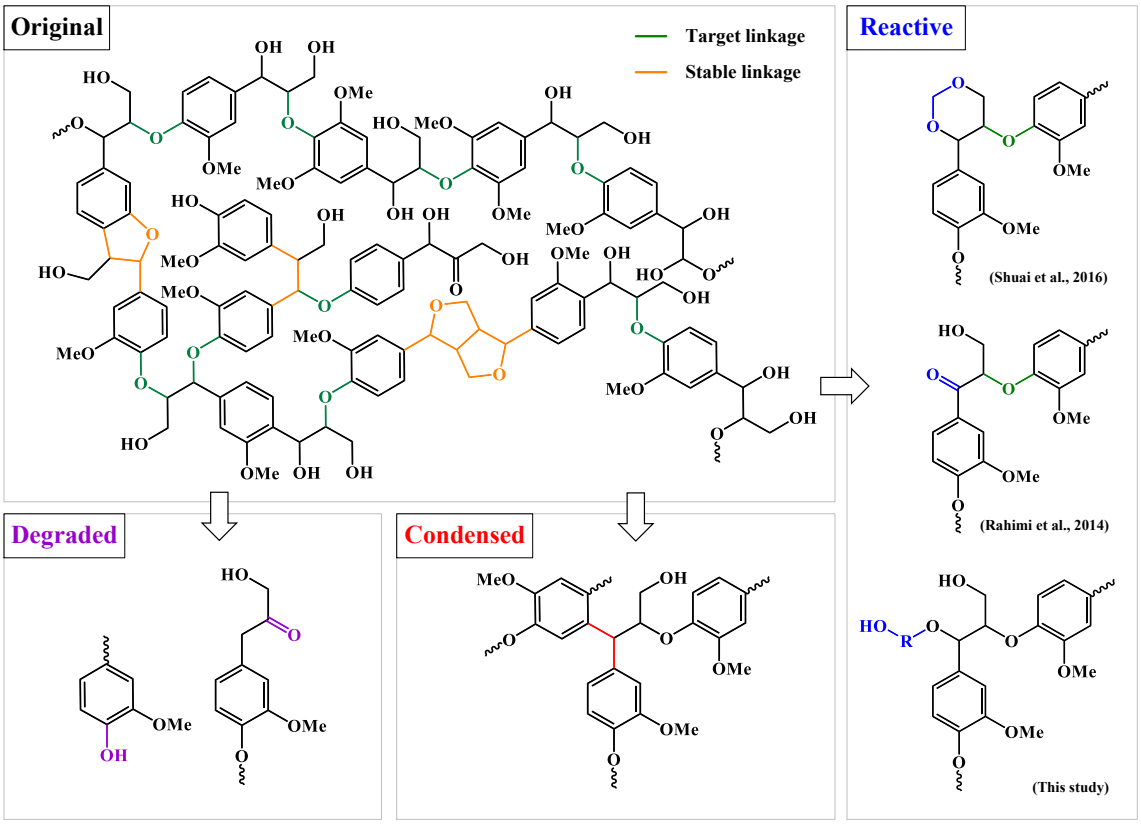


Fig. 6.1 Types of linkages in lignin structure before and after pretreatment.

As ether linkages are directly related to lignin and cellulose valorization, several innovative strategies have been recently disclosed to produce “reactive lignin” (Fig. 6.1, right column). In terms of pretreatment processes, ammonia (da Costa Sousa et al., 2016), ionic liquid (IL) (Brandt et al., 2013; Kim et al., 2017; Sathitsuksanoh et al., 2014), g-valerolactone (GVL) (Luterbacher et al., 2015; Luterbacher et al., 2014) and mild organosolv techniques (<120°C) (Lancefield et al., 2017) have been introduced to promote biomass dissociation under “mild” conditions. Those mild pretreatment

strategies aim to limit the formation of reactive intermediates and hence preserving ether linkages in the lignin structure and decreasing the extent of condensation. In addition to modifying pretreatment medium, catalysts or functional reagents have been applied to quench reactive intermediates (*i.e.*, C_α benzylic carbocation) from dehydroxylation of C_α-OH (Schutyser et al., 2018). Formaldehyde-assisted lignin fractionation encourage the formation of stable acetals (*i.e.*, 1,3-dioxane rings) between lignin's C_α-OH and C_γ-OH, which actively preserve ether linkages and block the formation of C_α benzylic carbocation (Shuai et al., 2016a). Preoxidation of C_α-OH to C_α=O weakens the β-O-4 ether linkage and avoids lignin condensation (Rahimi et al., 2014). *In situ* trapping of aldehydes through reaction with alcohols, hydrogenation, or decarbonylation also suppress lignin condensation (Deuss et al., 2015; Kaiho et al., 2015). Nonetheless, a few studies have addressed the conflicts among maintaining structural integrity of pretreated lignin, extent of delignification, and enzymatic digestibility of cellulose. Deuss et al. (2017) investigated 27 types of lignins and reported the trading between isolation lignin yield and amounts of ether linkages (Deuss et al., 2017; Lancefield et al., 2017). Side-reactions such as grafting of functional groups (*e.g.*, formaldehyde and GVL processes) on the substrate can interfere with the productive binding of cellulase to cellulose (Pan et al., 2006b; Shuai et al., 2016a; Shuai et al., 2016b; Van der Klashorst, 1989). A process to withdraw lignin without causing its condensation has yet to be available.

This study aims to explore the potential of an innovative pretreatment reagent (*i.e.*, diols) to isolate lignin without sacrificing its integrity. Organosolv pretreatment has

shown to be a promising strategy to fractionate lignin and enhance enzymatic saccharification (Pan, 2012; Pan et al., 2005b; Zhang et al., 2016). During organosolv pretreatment the hydroxyl groups carry out nucleophilic reaction with the C_α benzylic carbocation, which minimize lignin degradation and condensation (Luo & Abu-Omar, 2018). However, the extent of delignification in organosolv pretreatment still rely on effective cleavage of ether linkage (*i.e.,* lignin degradation), which conflict with its beneficial effects of nucleophilic reaction. We hypothesized here that 1,4-butandiol (1,4-BDO) can cease this conflict and fractionate reactive lignin in organosolv pretreatment. Systematic experiments on organosolv pulping with diols (e.g., 1,3BDO and 1,4-BDO) were carried out by Kishimoto et al (Hansen, 2002; Kishimoto & Sano, 2001; Kishimoto & Sano, 2002). 1,4-BDO is a very effective delignification solvent for both hardwoods and softwoods even without acid catalyst at 200 °C and 220 °C, respectively (Kishimoto & Sano, 2001). Homolytic cleavage of ether linkage in lignin can be achieved via quinone methide intermediates under the pulping conditions, which eliminate the formation of Hibbert's ketones (Kishimoto & Sano, 2002). Meanwhile, hildebrand solubility theory suggested that 1,4-BDO have smaller relative energy difference (RED) with lignin than other alcohols, implying a better lignin dissolution capability of this organosolv (Hansen, 2002; Schuerch, 1952). We prepared various types of lignin from dilute acid (DA), ethanol (EtOH), 1,4-BDO and other diols at different pretreatment conditions and investigated them through chemical composition analysis, gel permeation chromatography (GPC), and NMR analyses. The potential and mechanisms of diol pretreatment to fractionate reactive lignin were clarified.

6.2 Materials and methods

6.2.1 Materials

Pretreatment solvents and other chemicals were purchased from J&K Acros Organics (Beijing, China). Commercial cellulase Cellic Ctec2 was generously provided by Novozymes Investment Co. Ltd. (Beijing, China) and Pronase was obtained from Sigma Chemical Company (USA).

6.2.2 *Pretreatment*

Eucalyptus were debarked, Wiley-milled, screened (mesh size < 2 mm), and Soxhlet-extracted with ethanol/toluene (1:2, v/v) for 24 h. The extractives-free materials were then pretreated in aqueous organosolv with sulfuric acid as a catalyst using a custom-built, eight-vessel (500 ml each) rotating digester made by Xian Yang Tong Da Light Industry Equipment Co. Ltd. (Shanxi, China). A 50 g (oven-dried weight) batch of wood powder was mixed with 15 mM to 35 mM sulfuric acid and 65%-85% (v/v) organosolv reagents at a liquid-to-solid ratio of 7:1. The digester was heated to 170 ± 3 °C at a rate of 3 °C per minute and maintained at the temperature for 60 min. After digestion, the substrate and spent liquors were then separated using nylon cloth. The substrate was washed three times (75 mL each) with warm (60 °C) aqueous organosolv with the same concentration as the pretreatment liquor. The spent liquor, the organosolv washes were combined and then mixed with three volumes of water to precipitate the

dissolved lignin. The substrate was then washed three times with water at 60 °C, and the washes were discarded.

6.2.3 Chemical compositional analysis

The components of eucalyptus before and after pretreatment were determined according to the procedures established by the National Renewable Energy Laboratory (NERL) (Sluiter et al., 2008). The hydrolyzed monomeric sugar units were quantified via high-performance liquid chromatography (HPLC, Shimadzu) equipped with CHO-782 Transgenomic column and acid insoluble lignin content was calculated gravimetrically.

6.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted at 2% (w/v) solid loading in acetate buffer (50 mM, pH 4.8) with an enzyme loading of 7.5 FPU/g glucan in a 500 mL Erlenmeyer flask. The mixture was incubated in a rotary shaker at 50 °C and 150 rpm for 72 h. A 1.0 ml sampling of supernatant was collected at 3, 6, 9, 24, 48, 72 h for sugar analysis.

6.2.5 Preparation of organosolv soluble lignin and residual lignin

Seven different lignin samples were prepared from the DA, EtOH and 1,4-BDO pretreated eucalyptus based on the experimental procedure presented in Fig. 6.2. The pretreatment conditions were fixed at 15 mM sulfuric acid and 65% organosolv and sample separation procedure was as described in Section 6.2.2.

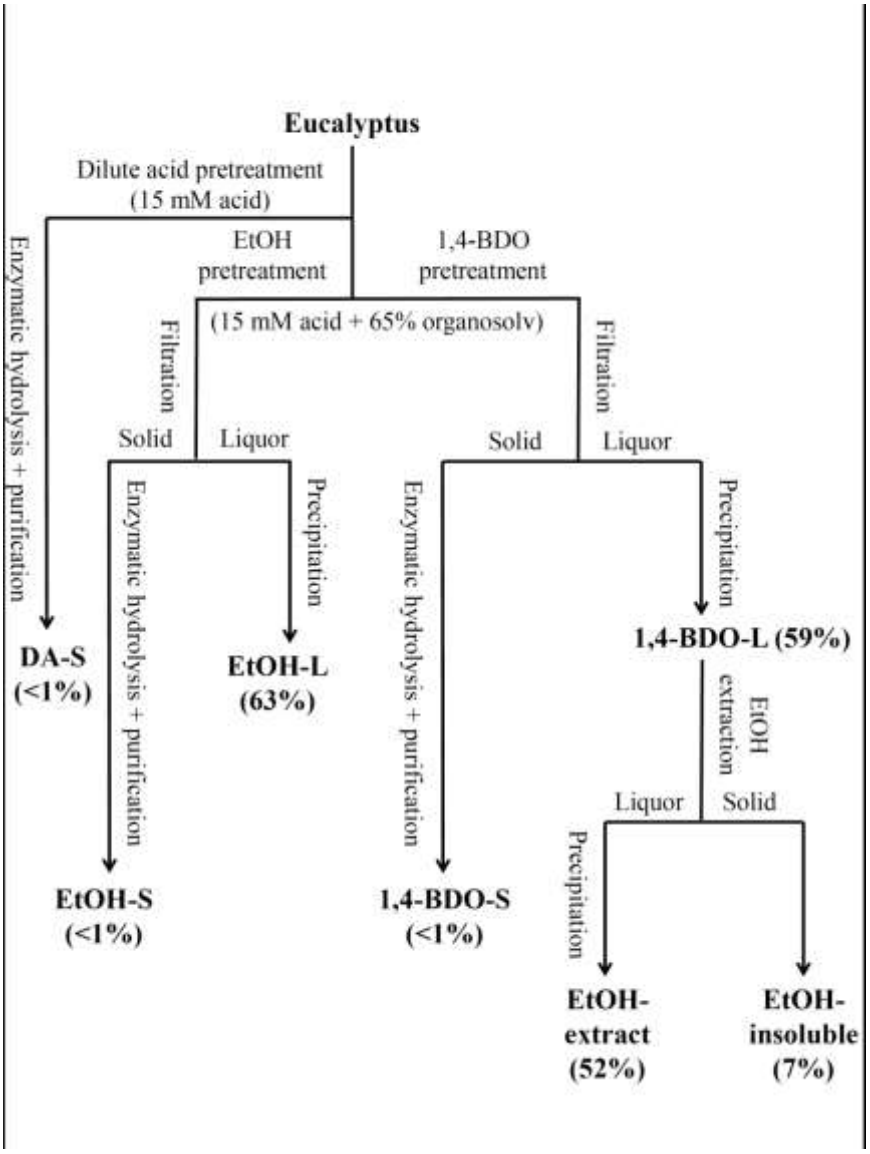


Fig. 6.2 Procedure of lignin fractionation/solubility experiments. Yields of lignin were listed in parentheses.

Residual lignins (DA-S, EtOH-S and 1,4-BDO-S) were prepared and purified according to Meng et al. (2017) (Meng et al., 2017b). The pretreated eucalyptus was hydrolyzed by cellulase at 2% (w/v) solid loading in 50mM sodium citrate buffer (pH = 4.8), 50 °C, 150 rpm for 72 h in a shaker. Enzyme loading was set at 40 FPU/g-substrate

to remove most of the carbohydrates in the pretreated substrate. After hydrolysis, the lignin residues were recovered by centrifugation and rinsed three times with distilled water. The crude lignin was then incubated overnight at 37 °C in 50 mM phosphate buffer (pH=7) with 1 U/mL Pronase. After filtration, the solid residues were extracted twice with 96% (v/v) dioxane/water mixture at room temperature for 48 h. The extracts were combined, rotary evaporated, and freeze-dried to obtain residual lignin for further analysis.

Organosolv soluble lignins (EtOH-L and 1,4 BDO-L) were isolated from the liquid product by room-temperature vacuum distillation according to Pan et al (2012) (Pan, 2012). Three volumes of water were added into the pretreatment spent liquor and the precipitated lignin was rinsed with water to remove soluble impurities. The purified lignin was freeze-dried under vacuum for 24 h and stored in desiccators before analysis. To characterize the unique feature of the 1,4-BDO-L lignin 0.1 g of the lignin sample was extracted with 10 mL ethanol for 24 h. The extract was rotary-evaporated and freeze-dried to obtain EtOH-extract lignin, and the solid residue after the ethanol extraction was freeze-dried to obtain EtOH-insoluble lignin. Organosolv soluble lignin from the other pretreatments in this work was prepared as described above.

6.2.6 Gel permeation chromatographic (GPC)

The weight-averaged molecular weight (Mw) and number-averaged molecular weight (Mn) of the lignin samples were measured by GPC as described previously.(Ringena et al., 2006) Briefly, lignin samples were dissolved in

Dimethylacetamide with addition of 0.11 M LiCl solution, filtered through a 0.45 mm filter and placed in a 2 mL autosampler vial prior to GPC analysis. The molecular weights were estimated by size-exclusion separation performed on the HPLC equipped with Agilent PLgel MIXED-B column.

6.2.7 Nuclear magnetic resonance (NMR) analysis

Two-dimensional Heteronuclear single quantum coherence (2D HSQC) nuclear magnetic resonance (NMR) analyses were conducted to determine the molecular-scale structure of pretreated lignin samples (Meng et al., 2017a). For each test, approximately 50 mg lignin samples were dissolved in 0.5 mL DMSO-d_6. The 2D HSQC ^{1}H-^{13}C experiments were performed using a JEOL ECZR 500 MHz spectrometer equipped with a 5 mm ROYAL probe. A JEOL standard pulse sequence was applied under the following parameters: spectral width of 11 ppm in F2 (^{1}H) with 1024 data points and 190 ppm in F1 (^{13}C) with 256 data points; 64 scans and 1.5 s interscan delay. JEOL's Delta 5.0 software was used for volume integration of contours in HSQC spectra.

For the quantification of hydroxyl groups in the lignin samples, phosphorylation of each sample was performed with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) in a solvent of pyridine/$CDCl_3$ (1.6/1.0, v/v) as described in the previous study of Meng et al. (2018) (Meng et al., 2018). In detail, the stock solution was prepared with pyridine/$CDCl_3$ (500 μL) and 5 mg·mL^{-1} internal standard (cyclohexanol). Approximately 20.0 mg of lignin sample was dissolved in the stock solution, and phosphorylation was conducted by

adding 100 μL of the phosphorylating reagent TMDP. Quantitative ^{31}P NMR spectra were acquired on the JEOL ECZR 500 MHz spectrometer using an inverse-gated decoupling pulse sequence, 90° pulse, 25 s pulse delay with 32 scans. All chemical shifts reported are relative to internal standard at 145 ppm. The quantitative calculation of the hydroxyl groups was based on the amount of the internal standard.

6.2.8 Calculation of relative energy differences and solubility tests

Hansen solubility parameters of various alcohols were calculated to clarify the solvent power dissociating the lignin (Hansen, 2002; Zhang et al., 2016). Thermodynamics requires that the free energy of mixing must be zero or negative for the solution process to occur spontaneously. Hildebrand and Scott pointed out that solubility parameter should be considered as a free energy parameter (Hansen, 2002). Solubility parameters (δ) are derived from the energy required to convert a liquid to a gas, which directly reflects the degree of dispersion forces, permanent dipole-permanent dipole forces, and hydrogen bonding hold to molecules of the liquid. It can be calculated by the following equation:

$$\delta^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \qquad (1)$$

where δ_D is the dispersion component calculated using a homomorph method; δ_P is the polar component; and δ_H is the hydrogen bonding component with the latter parameters determined from best reflecting empirical evidence. The ability of solvents to dissolve

or swell polymer can be predicted by the relative energy difference (RED) as follows (Hansen, 1967):

$$(R_a)^2 = 4 \cdot (\delta_{D2} - \delta_{D1})^2 + (\delta_{P2} - \delta_{P1})^2 + (\delta_{H2} - \delta_{H1})^2 \quad (2)$$

$$RED = R_a/R_o \quad (3)$$

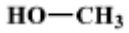
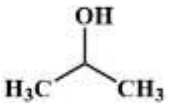
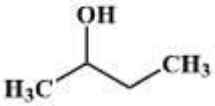
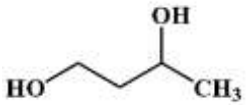
where R_a is a empirically parameter developed by Skaarup. It represents the difference between the Hansen solubility parameters for a solvent (1) and a polymer (2). Smaller RED numbers indicate higher affinity between the solvent and the polymer, indicating higher degree of dissolution of the polymer in the solvent. By plotting δ_D, δ_P, and δ_H of the studied solvent over the same parameters of the solute. The point falling into a boundary sphere with critical distance to the origin was considered a good solvent (Hansen, 1967).

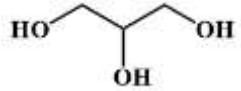
Solubility tests (on 1,4-BDO-L lignin) and organosolv pretreatment (on eucalyptus) were carried out with three types of solvents, including (i) monohydric alcohols, *i.e.*, methanol (MeOH), EtOH, 2-Propanol (2-ProOH), 2-Butanol (2-BuOH); (ii) diols, *i.e.*, ethylene glycol (EG), 1,3-propanediol (1,3-PG), 1,3-butanediol (1,3-BDO), 1,4-butanediol (1,4-BDO), glycerol; and (iii) Tetrahydrofuran (THF).

6.3 Results and discussion

6.3.1 Optimization of pretreatment conditions

Table 6.1 Structure and Hansen solubility parameters ($MPa^{1/2}$) of selected organic solvents

Solvent	Structure	Dispersion component (δ_D)	Polar component (δ_P)	Hydrogen bonding component (δ_H)	Relative energy difference (RED)
Lignin	See Fig.1	21.9	14.1	16.9	0
Methanol (MeOH)		15.1	12.3	22.3	1.08
Ethanol (EtOH)		15.8	8.8	19.4	0.99
2-Propanol (2-ProOH)		15.8	6.1	16.4	1.07
2-Butanol (2-BuOH)		15.8	5.7	14.5	1.10
Ethylene Glycol (EG)		17	11	26	1.00
1,3-Propanediol (1,3-PG)		16.8	13.5	23.2	0.88
1,3-Butanediol (1,3-BDO)		16.6	10	21.5	0.89

1,4-Butanediol (1,4-BDO)		16.6	15.3	21.7	0.85
Glycerol		17.4	12.1	29.3	1.13
Tetrahydrofuran (THF)		16.8	5.7	8	1.16

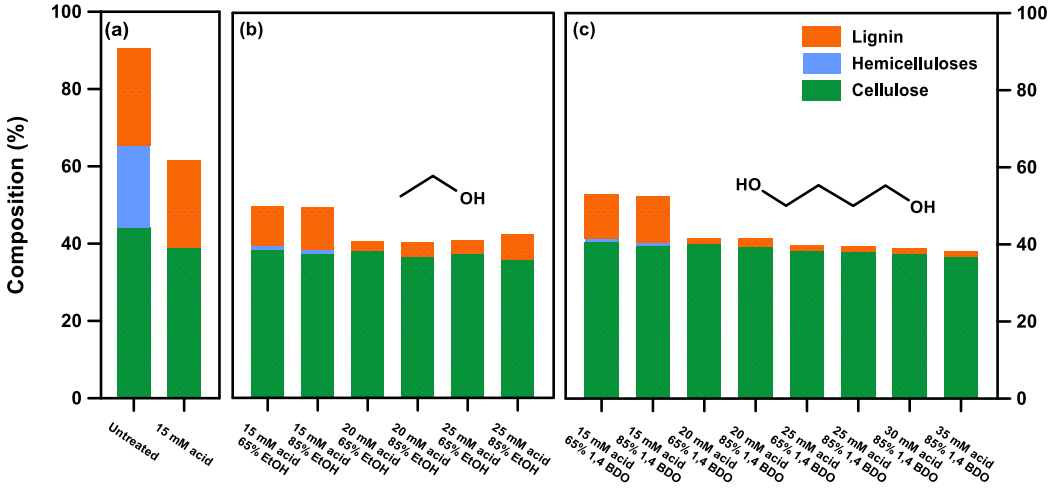


Fig. 6.3 Chemical compositions of DA, EtOH and 1,4-BDO pretreated eucalyptus.

Hansen solubility parameters of various alcohols to lignin were presented in Table 6.1. EtOH and 1,4-BDO showed the lowest REDs among the selected monohydric alcohols and diols, respectively. The two organosolvs were selected for biomass pretreatment and lignin valorization, and DA pretreatment were conducted as a control. The selected hardwood species eucalyptus is a widely used and well-studied woody material. It consists of 42% cellulose, 21% hemicelluloses, and 25% Klason lignin. The optimal processing conditions for eucalyptus in recovering cellulose, hemicellulose, and lignin were defined as 170 °C, 60 min, 15 mM H_2SO_4 and 65% EtOH (Pan, 2012; Pan et al., 2005b). This condition was applied in our pretreatment experiments and followed by more server conditions to fractionate more lignin for valorization.

The chemical compositions of DA, EtOH, and 1,4-BDO pretreated substrates were presented in Fig. 6.3a through Fig. 6.3c, respectively. All hemicelluloses in the eucalyptus were completely removed under acidic conditions, leaving only cellulose and various amounts of lignin in the substrates (Fig. 6.3a). Lignin removal was very limited or even non-detectable in DA pretreated samples and was at high mass content (46% residual lignin) in the substrate. Lignin was subjected to depolymerization reactions during DA pretreatment, but the solubility of depolymerized lignin was limited in acid.(Hansen, 2002; Sun et al., 2015) In EtOH pretreatment, delignification was improved but still limited when acid charge was at 15 mM or lower. The content of lignin residues reduced to 4.6 %when acid and EtOH doses were 20 mM and 65%, respectively (Fig. 6.3b). However, increased doses of acid (25 mM) and/or EtOH (85%)

both resulted in increased residual lignin in the substrates (7.5%), indicating that lignin may be condensed and/or precipitated on the surface of the substrate. In 1,4-BDO pretreatment (Fig. 6.3c), acid soluble lignin reduced to approximately 1.6% when acid and solvent charges increased to 20 mM and 65%, respectively. Lignin residue contents was not increased at higher pretreatment severity in 1,4-BDO pretreatment and was less than 2% even when acid charge was increased to 35 mM. As condensation can be carried out after formation of resonancestabilized benzyl carbocation during organosolv pretreatment, (Pan, 2012) our results suggested that lignin condensation may be alleviated or the condensed lignin (with changed chemical properties after pretreatment) could still dissolve in the solvent 1,4-BDO. As optimal pretreatment conditions are biomass-specific (varying among different plant species and/or parts of plants) the capability of 1,4-BDO in keeping lignin in the liquor can be beneficial to handle complex feedstocks with different amounts and types of lignin in the feedstock. The following section provides more detailed analysis on the change of lignin properties.

6.3.2 Comparison of physicochemical properties of EtOH and 1,4-BDO lignins

The representative 2D HSQC NMR spectra of DA-S, EtOH-S and 1,4-BDO-S (15 mM, 65% organosolv) were demonstrated in Fig. 6.4. In the aliphatic regions of the HSQC NMR spectra, β-O-4, β-5, and β–β were the dominant interunit linkages in lignin samples (Fig. 6.4 upper row). An unique signal was discovered in the HSQC spectra of 1,4-BDO pretreated lignin residues (marked in red in top-right, Fig. 6.4), which indicated the 1,4-BDO related functional group on the α position of β-O-4 linkage (Fig.

6.1). It has been reported that alcohols can act as a nucleophile and react with benzylic carbocation to form α esterified lignin (Luo & Abu-Omar, 2018). Deuss et al. reported that diols could capture resonancestabilized benzyl carbocation and form dioxane structure to prevent lignin condensation under 10 mol% triflic acid (Kaiho et al., 2015), but this structure was not observed in our work. The absence of dioxolanes can be attributed to the relative low concentration of sulfuric acid used in pretreatment. Aromatic compositions in each lignin were presented in the bottom row of Fig. 6.4. The pretreated lignin demonstrated its major aromatic compositions consisting of S and G units for hardwood species (Sjostrom, 2013). Condensed lignin was not observed in organosolv pretreated lignin residues, indicating that both EtOH and 1,4-BDO alleviated lignin condensation. The absence of EtOH related α esterified signal in aliphatic regions of HSQC NMR spectra was possibly due to overlapping of signals from other interunit linkages of the lignin.

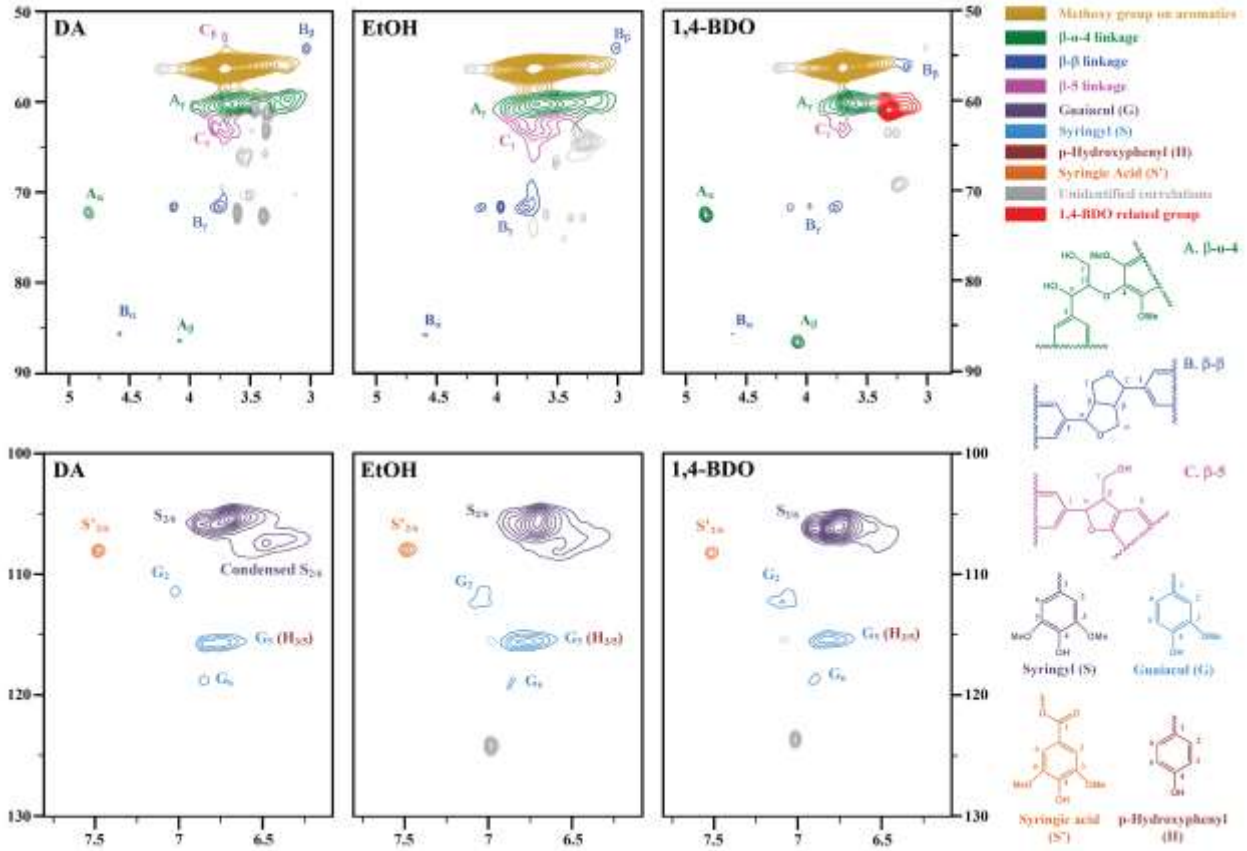


Fig. 6.4 2D HSQC NMR spectra of DA (15 mM acid), EtOH (15 mM acid and 65% EtOH) and 1,4-BDO (15 mM acid and 65% 1,4-BDO) residual lignin.

The approximate ratios of different lignin subunits and inter-unit linkages in each lignin residues were summarized in Table 6.2. The values were calculated from HSQC NMR spectra by integrating the peak volumes of $S_{2/6}$ and G_2 at δC/δH 104.0/6.68 ppm and δC/δH 111.0/6.96 ppm, respectively. The peaks at δC/δH 71.4/4.81 ppm, δC/δH 86.9/5.47 ppm, and δC/δH 85.1/4.65 ppm represented the correlation of α position of β-aryl ether (β-O-4), phenylcoumaran (β-5), and resinols (β–β), respectively. The S/G ratios of lignin subunits were lower in organosolv lignin (3.6-4.4) than DA pretreatment (10). Approximately 10% of β-5 and 2% of β-β were detected in all the lignin samples, indicating that organosolv did not impact on these inter-unit linkages in comparison with DA lignin. Cleavage of β-O-4 linkages was confirmed in all lignin residues from the three pretreatments. The content of β-O-4 linkages over total lignin aromatic subunits in EtOH was only 4%, while 1,4-BDO was 22%. The structure of lignin residues obtained from 1,4-BDO pretreatment was with higher integrity than EtOH lignin. Acid-catalyzed cleavage of β-O-4 interunit linkages has been recognized as the major mechanism of lignin depolymerization and dissolution in organosolv pretreatments (Zhang et al., 2016), but it should be noted that over-cleavage of β-O-4 linkages to yield more dissolved lignin may also damage the lignin structure for valorization under a harsh pretreatment environment.

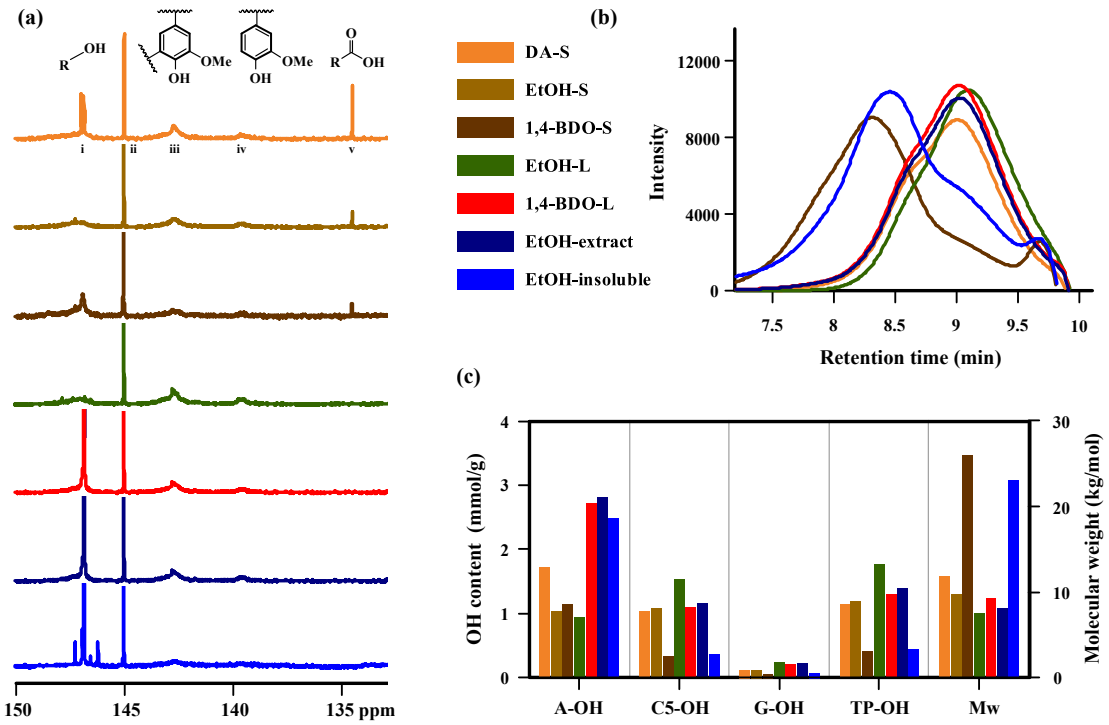


Fig. 6.5 Chemical characteristics pretreated lignin samples after DA (15 mM acid); EtOH; and 1,4-BDO (15 mM acid and 65% organosolv). (a) ^{31}P NMR spectra; (b) GPC chromatogram; and (c) overall comparisons. S: residue lignin; L: organosolv soluble lignin; EtOH-extract and EtOH-insoluble lignin was harvested from 1,4-BDO-L lignin as detailed in Fig. 6.2 and the text.

The results of ^{31}P NMR analyses were demonstrated in Fig. 6.5(a) to Fig. 6.5(c), which was conducted to characterize and quantify the functional OH groups in the fractionated lignin samples. The results of different lignins were colored coded throughout the three subfigures as follows (up to bottom): the solid residues of DA (DA-S), EtOH (EtOH-S), and 1,4-BDO (1,4-BDO-S); the dissolved lignin from EtOH (EtOH-L), 1,4-BDO (1,4-BDO-L); EtOH extractable 1,4-BDO-L (EtOH-extract); and EtOH insoluble 1,4-BDO-L samples (EtOH-insouble). The peaks shown in the NMR spectra (from left to right in Fig. 6.5a) represent the aliphatic OH (i); cyclohexanol as an internal standard (ii); C_5 condensed OH (iii); guaiacyl phenolic OH (iv); carboxyl OH (v); and TMDP hydrolysis product (vi), respectively. Fig. 6.5(b) showed the molecular weight of the seven lignin samples measured by GPC. The content of each OH group was calculated by integrating the area of phosphorylated hydroxyl groups compared to the known concentration of the internal standard cyclohexanol, and the results were presented with the weighted average molecular weight in Fig. 6.5(c). A decrease in aliphatic OH was observed in both the EtOH-L and EtOH-S, indicating that the α position of β-O-4 had been transformed into ether linkages. This reaction pathway is supported by the relatively higher amount of aliphatic OH in 1,4-BDO-L, which also confirmed that dioxane structure was not formed during 1,4-BDO pretreatment. The soluble lignins (EtOH-L and 1,4-BDO-L) contained higher amounts of phenolic OH groups than the insoluble lignins (EtOH-S and 1,4-BDO-S), which can be attributed to cleavage of β-O-4 linkages, allowing low molecular weight lignin to be solubilized. It should be emphasized that EtOH-S and EtOH-L both contained significantly more

phenolic OH than 1,4-BDO-S, which supported our observation in the HSQC NMR analysis that EtOH lignin had less β-O-4 linkage than 1,4-BDO lignin. Hydroxyl groups of soluble lignin at different pretreatment severities were also quantified and presented in Fig. 6.6. The amount of phenolic groups increased with the increasing pretreatment severity. New phenolic OH groups were formed by cleavage of β-O-4 linkage, which were dependent on the increased doses of acids and organosolv reagents as well as the reaction severity of the pretreatment (Yang & Pan, 2016). Meanwhile, formation of phenolic OH was more aggressive in EtOH pretreatment than in 1,4-BDO pretreatment under similar pretreatment conditions.

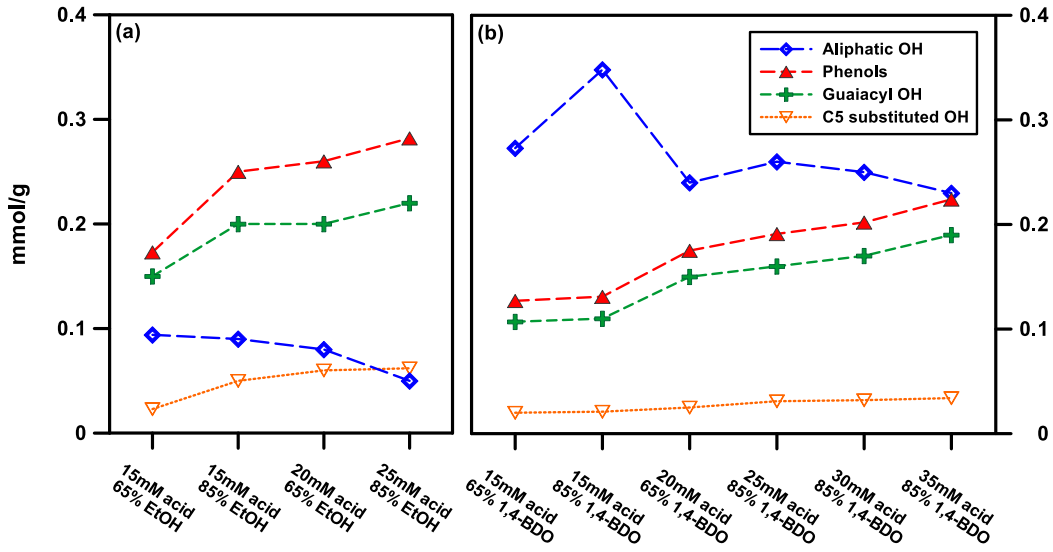


Fig. 6.6 Hydroxyl groups of soluble lignin at different pretreatment severities using EtOH and 1,4-BDO.

In order to understand the structure/functionality of 1,4-BDO-L lignin, EtOH extraction was conducted as described in Section 2,5 (bottom right of Fig. 6.5). EtOH-insoluble lignin was with larger molecular weight, lower amount of phenolic OH groups, and more aliphatic OH groups than EtOH-extract lignin. The results indicated

that aliphatic OH groups can facile dissolution of lignin in 1,4-BDO. Dissociation of lignin without breaking the β-O-4 linkages while increasing its solubility in the pretreatment media is a new biorefinery target aiming at completely utilization of building block chemicals (*i.e.*, cellulose and lignin valorization). During 1,4-BDO pretreatment, 1,4-BDO quenched C_α benzylic carbocation and formed α-etherified β-O-4 linkages on lignin. As 1,4-BDO contains two OH groups, the amount of aliphatic OH groups on lignin would increase after pretreatment (OH on lignin decreased in EtOH pretreatment). This reaction offers 1,4-BDO pretreatment an alternative pathway to increase the solubility of lignin without breaking the β-O-4 linkages, hence implying a potential of higher yield of lignin monomer in the downstream processes. Further investigation on process optimization is expected to promote this pathway and to obtain more reactive lignin.

6.3.3 Solubility of 1,4-BDO-L lignin

Solubility tests and pretreatments were conducted to investigate whether the beneficial effects on lignin dissolution may occur in other organic solvents (Fig. 6.7). 1,4-BDO-L lignin was completely dissolved in all the tested diols (*i.e.*, EG, 1,2-PG, 1,3-PG, 1,3-BDO) and THF, but various amounts of precipitate occurred and were measured in monohydric alcohols. The solubility of 1,4-BDO-L lignin in MeOH, EtOH, 2-ProOH, 2-Bu and Glycerol were 81%, 87%, 75%, 73% and 61%, respectively. Schuerch (1952) confirmed similar solubility of 7 lignins by establishing the first sets of Hansen solubility parameters (Schuerch, 1952). Skaarup (1967) defined the empirical

weighting factor "4" in Equation (2), as dispersion component δ_D was considered a more sensitive parameters than δ_P, and δ_H (Hansen, 1967). The δ_D of diols is closer to lignin than ones of monohydric alcohols, and hence smaller REDs were calculated as well as solubility than other alcohols. Meanwhile, δ_H of lignin is smaller than diols, implying that the solubility of 1,4-BDO-L lignin can be increased by grafting OH group on the lignin.

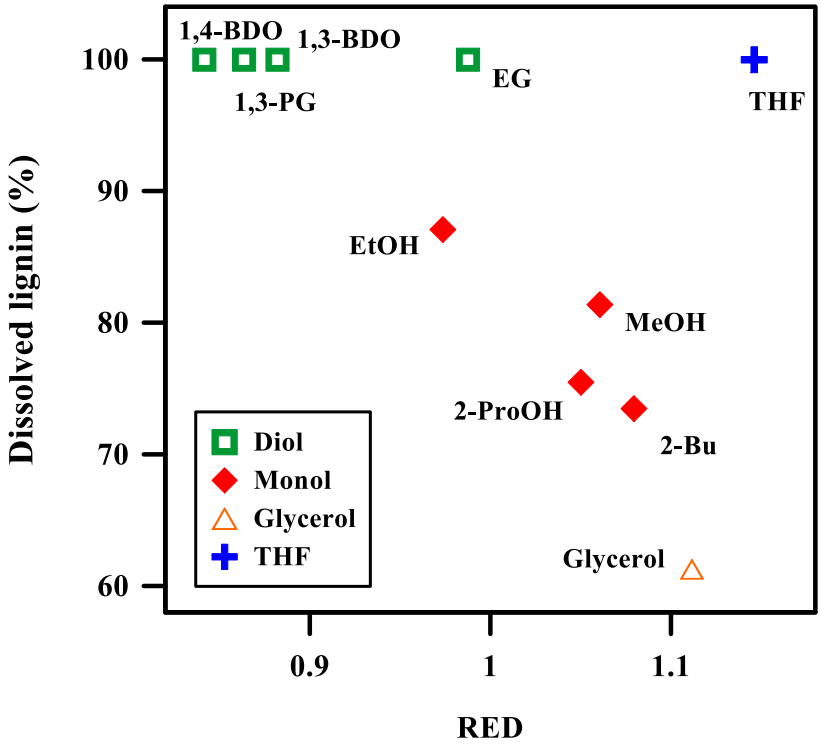


Fig. 6.7 Solubility test with different relative energy difference (REDs) between lignin and solvents under three conditions.

6.3.4 Diol pretreatment

Based on the solubility tests, we hypothesized that all diols can create similar beneficial effects as 1,4-BDO during organosolv pretreatment and designed the related experiments to validate it. Fig. 6.8 showed the levels of lignin dissolution in organosolv

pretreatment at two severities, *i.e*., 20mM 65% and 25mM 85%, respectively. Residual lignin is the percentage of the lignin that was not dissolved during the pretreatment. As expected diols isolated more lignin than monohydric alcohols (in order, methanol, ethanol, 2-Propanol and 2-BuOH) and this difference was more significant with the increase of pretreatment severity. Residual lignin contents of 2-ProOH and 2-BuOH pretreatment were more than 60% at elevated pretreatment severity (25 mM acid, 85% organosolv). As β-O-4 linkages were not observed in the HSQC spectra, the amount of phenolic OH groups was used to estimate the structure integrity of lignin. The amount of phenolic OH groups of lignin from diols pretreated eucalyptus was approximately 20% higher than MeOH and EtOH lignins. 2-ProOH and 2-Bu pretreated samples contained almost same amount of phenolic OH groups (index of preferable structural integrity), but the yield of dissolved lignin was much lower than all the other solvents. The trends of molecular weights of dissolved lignin for different solvents consisted with functional group measurements (from PNMR analysis). Both M_w and M_n of Lignins from diols pretreated eucalyptus were larger than the ones from monohydric alcohols. All the diols showed similar features as 1,4-BDO pretreatment.

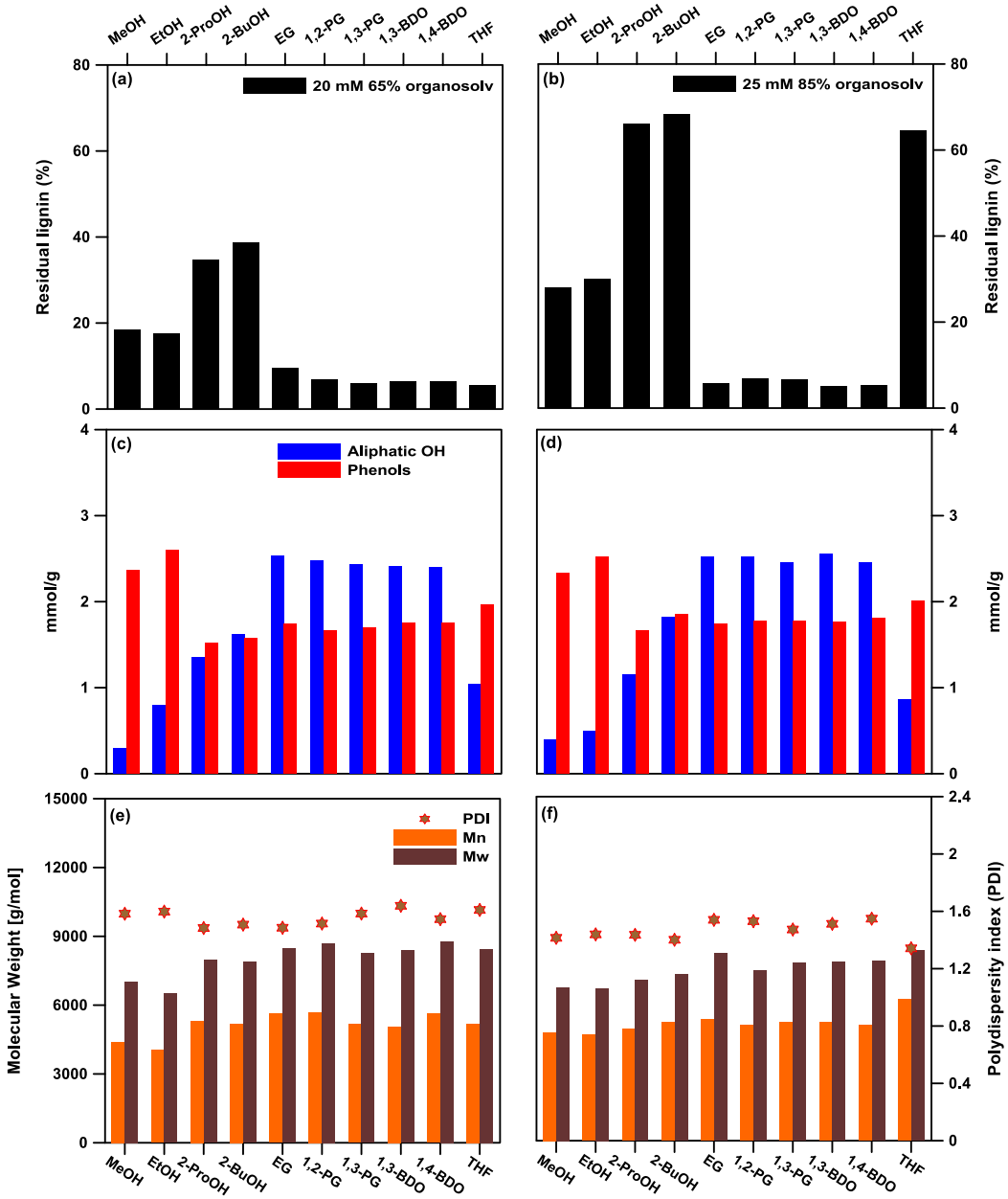


Fig. 6.8 Effects of organosolv pretreatments on isolated lignin yields and lignin structure integrity among different solvents. (a) and (b) percentage of residual lignin after organosolv pretreatments at two severities; (c) and (d) hydroxyl group contents of organosolv soluble lignin; (e) and (f) Molecular weights and polydispersity index of organosolv soluble lignin.

THF pretreatment showed outstanding capacity on lignin removal (95%) at a severity of 15 mM acid and 65% solvent, while the residual lignin was increased to more than 60% when the chemicals charges increased to 25mM acid, 85% solvent. It was reported that THF/water mixture can form a "theta" solvent system, in which the solvent-lignin and lignin-lignin interactions were approximately equivalent in strength, leading to the lignin adopting Gaussian random-coil conformations (Mostofian et al., 2016; Smith et al., 2016). Lignin in this type of coil conformation would not self-aggregate and therefore be more easily broken down into smaller molecular fractions, which facile its dissolution in THF. Unlike monohydric alcohols, aliphatic OH was not reduced in THF pretreatment, which indicated that THF may not quench C_α benzylic carbocation and form α-etherified β-O-4 linkages on lignin. Thus, lignin may be condensed and precipitated on biomass during pretreatment.

6.3.5 Enzymatic hydrolysis

Enzymatic hydrolysis experiments were carried out to evaluate the potential improvements or impacts of diols pretreatment. The digestibilities of cellulose in the pretreated substrates were demonstrated in Fig. 6.9(a) to Fig. 6.9(c). At a relatively low enzyme dose (7.5 FPU/g glucan) cellulose conversion (%) increased rapidly in the first 24 h of hydrolysis and reached a plateau after 24 h operation. At a pretreatment severity of 20 mM acid, 65% organosolv, cellulose conversions of MeOH and EtOH pretreated eucalyptus were more than 90% when the residual lignin content was 20% (Fig. 9(a)). However, cellulose conversion of two same solvents reduced to 80% when pretreatment

severity increased to 20 mM, 85% organosolv (*i.e.*, lignin residue increased to more than 30%). This situation was even worse in 2-ProOH, 2-Bu and THF pretreated substrate. The cellulose conversion of organosolv pretreatment with 2-ProOH, 2-Bu and THF were less than 40% at 20 mM, 85% organosolv (Fig. 9(b)). This phenomenon was not observed in organosolv pretreatment with diols. The lignin residue was still below 10% as pretreatment increased (Fig. 9(c)). Cellulose conversion after 9 h enzymatic hydrolysis was almost proportional to the residual lignin contents when comparing the digestibility of all the organosolv pretreated substrates (Fig. 9(d)).

Lignin inhibits the access of the enzymes to cellulose by forming a physical barrier to restrict cellulose accessibility to enzymes, competing for the enzymes with cellulose by non-productive adsorption, and even deactivating the enzymes (Leu & Zhu, 2013; Li & Zheng, 2017). Organosolv pretreatment is effective to remove lignin and eliminate lignin inhibition on enzymatic hydrolysis. Residual lignin after pretreatment can still pose non-productive adsorption via hydrophobic interaction, electrostatic interactions and hydrogen bond (Liu et al., 2016b). Hydrophobic interaction is expected to play a dominant role in the adsorption of cellulase on lignin, which has been widely proposed (Yang & Pan, 2016). During organosolv pretreatment, lignin unit linkages such as β-O-4 ether bonds could be cleaved by pretreatment chemicals, which would result in lignin depolymerization, while lignin condensation also occurred (Pan, 2012). New carbon-carbon bonds could be formed and C_α-OH served as a leaving group during lignin condensation, which increased hydrophobicity of lignin. Condensed lignin existed in organosolv pretreatment with MeOH, EtOH, 2-ProOH, 2-Bu and THF at higher

pretreatment severity was responsible for the lower cellulose conversion of enzymatic hydrolysis.

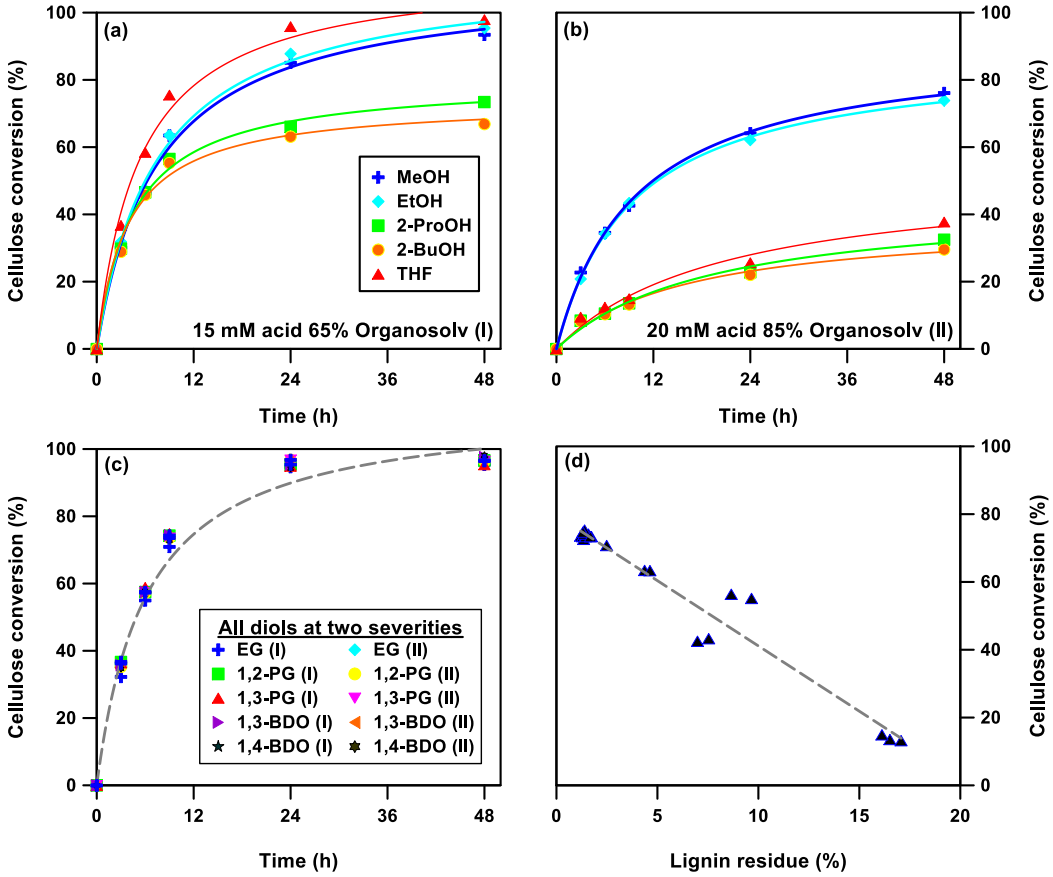


Fig. 6.9 Enzymatic digestibilities of organosolv pretreated eucalyptus. (a), (b) and (c) Cellulose conversion in enzymatic hydrolysis of organosolv pretreated eucalyptus over time, (d) Correlations between lignin content and cellulose conversion at 9 h.

6.4 Conclusions

Diols pretreatment offered a reaction pathway to fractionate a new reactive lignin in biorefinery. During pretreatment, cleavage of α-ether linkage of lignin formed a resonancestabilized benzyl carbocation, resulting in either lignin condensation reaction or cleavage of β-O-4 linkages. The cleavages of β-o-4 linkage increase the solubility of

lignin but affect the structural integrity of dissolved lignin, which may further reduce the yield of lignin monomer in the downstream process. Diols acted as nucleophiles quenching the resonancestabilized benzyl carbocation and formed α-etherified lignin. Unreacted aliphatic OH groups from diols on α-etherified lignin can improve the dissolution of lignin in diols, preserving the β-O-4 linkage for valorization. In addition, due to the high solubility of the pretreated lignin this pretreatment technology without the need of sophisticated process optimization, may be applicable treating complex lignocellulosic biomass such as urban wastes or food processing residues. In the future, pretreatment of model compound need to be conducted to check whether condensation of lignin was reduced by α-etherification.

Conclusions

Surface tension analysis and kinetic modeling are useful techniques elucidating enzyme-substrate interactions. 2D HSQC NMR associated with ^{31}P NMR analysis reveals the complex reactions between catalyzed lignin/hemicellulose degradation and condensation under thermochemical conditions. Through the analytical tools this dissertation provides a few new insights to extend our understanding on wood and enzyme chemistry, which further leads into a few innovative pretreatment and fermentation strategies in the biorefinery. Clarification of the duel effects (enhancing and inhibiting) of lignin based pretreatment by-products (lignosulfonates in this work) in the whole slurry SSF provides a glance of surfactant impacts to enzyme adsorption and activities. Sensitivity tests help us clarify the inhibiting effects of pretreatment by-products to overcome the heat stress and furthermore achieve complete utilization of softwood derived hexoses. By simply changing the temperature profile, the cellulosic ethanol concentration has been increased to from 59.3 g/L to 82.3 g/L, which was only achievable in easily digestible substrate previously. The energy yield of the new process is 2,410 MJ, which is approximately 43.5-234.7% higher than other published processes for treating 1 ton softwood. Meanwhile, water consumption of the strategy was 3.65 tons/ton-od wood, which is approximately 25.8-51.2% less than other processes (except steam explosion pretreatment). Finally, diol pretreatment offers an alternative reaction pathway to fractionate a new type of reactive lignin from hardwood species. Diols can

act as nucleophiles to quench the resonancestabilized benzyl carbocation and form α-etherified lignin. Unreacted aliphatic OH groups from diols on α-etherified lignin can improve the dissolution of lignin in diols, preserving the β-O-4 linkage in lignin for valorization. The findings pioneers a few new directions to the future research and development toward whole biomass biorefinery and worthwhile to be further optimized and investigated.

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