Inhibition Study in Biofuels and Biochemicals Production from Lignocellulosic Biomass

by

Jing Li

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Approved by

Sushil Adhikari, Chair, Associate Professor, Biosystems Engineering
Maobing Tu, Co-chair, Associate Professor, Biomedical, Chemical, and
Environmental Engineering, University of Cincinnati
Yoon Y. Lee, Professor Emeritus, Chemical Engineering
Xinyu Zhang, Associate Professor, Chemical Engineering

Abstract

Developing biofuels and renewable chemicals from lignocellulosic biomass has great potential to reduce the dependence on fossil fuel and address the environmental issues. However, it encounters several techno-economic challenges. One of the major issues is the degradation compounds formed through pretreatment considerably inhibits the subsequent enzymatic hydrolysis and microbial fermentation and thus, severely limits the efficient utilization of lignocellulose. Therefore, the present study focuses on understanding the effects of those potential inhibitors on microbial fermentation and eliminating/reducing their toxicity in biochemical conversion, which is critical to the development of biorefinery.

It is believed that the inhibitory activity of degradation compounds greatly depends on their chemical structures. The effects of carbonyl compounds were examined with lactic acid fermentation and it was observed that their inhibitory severity on cell growth rate and lactic acid yield followed the order: aromatic aldehydes > phenols > acids > furan aldehydes. Moreover, quantitative structure-inhibition relationship (QSIR) was attempted to associate inhibition activity with molecular descriptors. It was found that inhibition constant (K_I) of aldehydes was well correlated with hydrophobicity (log P) and thiol reactivity (log K_{GSH}). It revealed the carbonyl inhibition on lactic acid fermentation was governed by the hydrophobicity and electrophilic reactivity and the target of detoxification should be directed to remove or reduce the hydrophobicity and/or electrophilicity of carbonyl compounds. Since the aromatic aldehydes exhibit the strongest inhibition and they are frequently present in the prehydrolysates and thus,

their inhibitory effects were further examined. The substituent effect of 13 aromatic aldehydes on acetone-butanol-ethanol (ABE) fermentation was assessed with the attempt to reveal the inhibition mechanism and develop effective detoxification method in biofuels production. It was observed that the inhibition activity was affected by the *ortho* substituents (OH > OCH_3 > CHO) and strongly related to the position of hydroxyl group instead of the number of hydroxyl groups. The *ortho*- hydroxyl group significantly contributed to the aromatic aldehyde inhibition. The *ortho*-substituted 2-hydroxybenzaldehyde caused at least 20 fold stronger inhibition than *meta*- and *para*- substituted analogues of 3- and 4-hydroxybenzaldehydes. The presence of ortho-hydroxyl group can form an intramolecular hydrogen bond with carbonyl hydrogen and potentially increase the cell membrane permeability and electrophilicity.

To develop effective detoxification method in butanol production from prehydrolysates is another research interest of this dissertation. Among the six detoxification strategies examined, anion exchange resin treatment was the most effective method but a lag phase of 72 h was observed in fermentation. To alleviate this problem, two-step detoxification strategy ($Ca(OH)_2$+ anion resin) was developed, resulting in a satisfactory ABE fermentation of 11.11 g/L ABE produced within 48 h and a yield of 0.19 g/g sugar. In addition, the mineral salt was found to be toxic to the *Clostridia* and responsible for the long lag phase. The precipitation of salts by $Ca(OH)_2$ could potentially shorten the lag phase.

Finally, the butanol production from ethanol organosolv pretreated loblolly pine was investigated in both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes. SSF was found to be a preferred process configuration compared to SHF in terms of butanol and ABE yield. Surprisingly, we found the addition of lignin into SHF process remarkable enhanced the ABE production. Furthermore, the

supplementation with detoxified prehydrolysates into SSF improved the utilization of sugars present in lignocellulose, giving the butanol and ABE titer of 10.51 g/L and 18.29 g/L, respectively, which were 13% and 16% higher than that from solid only. This indicated the integration of prehydrolysates into the SSF process for butanol production was technically desirable. Our study suggested one tonne of dry wood could produce 46.6 gallons of ABE and 26.5 gallons of butanol in a SSF process, respectively.

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List of Abbreviations

ABE	Acetone-Butanol-Ethanol
AFEX	Ammonia fiber explosion
AIL	Acid soluble lignin
ASL	Acid insoluble lignin
DI water	De-ionized water
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DTNB	5,5-Dithiobis(2-nitrobenzoic acid)
E_{HOMO}	Energy of the highest occupied molecular orbital
E_{LUMO}	Energy of the lowest unoccupied molecular orbital
FPU	Filter paper unit
GC/MS	Gas chromatography/mass spectrometry
GSH	Glutathione
HMF	5-Hydroxymethyl furfural
HPLC	High performance liquid chromatography
LA	Lactic acid
LC/MS	Liquid chromatography/mass spectrometry
MR	Molecular refractivity
NMR	Nuclear magnetic resonance

NREL	National renewable energy laboratory
OD	Optical density
OPA	Ortho-phthalaldehyde
OWD	Oven dry weight
QSAR	Quantitative Structure-Activity Relationship
QSIR	Quantitative Structure-Inhibition Relationship
RCM	Reinforced Clostridial Medium
RPM	Rounds per minute
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and cofermentation
SSF	Simultaneous hydrolysis and fermentation

List of Symbols

C_{GSH}^{o}	Initial GSH concentration
C_{GSH}^{t}	Final GSH concentration
C_{glu}^{0}	Glucose concentration at 0 h
C_{lac}^{0}	Lactic acid concentration at 0 h
C_{lac}^{48}	Lactic acid concentration at 48 h
C_{lac}^{t}	Lactic acid concentration at *t* h
μ	Specific cell growth rate
μ_{m}	Maximum specific cell growth rate
C'_{carb}	Partial charge of the carbonyl carbon in phenolic aldehyde
F	Fisher statistic
I	Inhibitory compound concentration
IC_{50}	50% butanol production inhibition concentration
K_{GSH}	Non-enzymatic rate constants
K_{I}	Inhibition constant
K_{s}	Saturation constant
Log P	Hydrophobicity/Partition coefficient in octanol-water
n	Number of observations
Q	Lactic acid volumetric productivity
s	The standard error of the estimate

t	Time
u	Dipole moment
X	Cell concentration

Chapter 1

Introduction

1.1 Background

Limited availability of fossil fuels, especially petroleum, and the concern of climate change have resulted in great attention in the research of renewable energy. About 60% of the petroleum consumed in the United States is imported, and the number is increasing [1]. Second generation biofuels, derived from lignocellulosic biomass, offer an alternative to traditional energy source. It is superior to starch-based biofuels which is currently the dominant biofuels produced in industry because it addressed the issue of competition between bioenergy and human food [2, 3]. Lignocellulosic biomass is an attractive renewable energy feedstock supplies, due to its abundant availability domestically and globally. The estimated annual potential availability of biomass in the U.S. is more than 1 billion tons by 2030 [4] and the annual worldwide production is 10-50 billion tons [5]. There are various types of lignocellulosic biomass: agricultural residues, wood and herbaceous crops, municipal solid wastes and paper mill sludge wastes etc.

Lignocellulosic biomass is comprised of three main components: cellulose, hemicellulose and lignin. The remainders are minor components, such as ash and extractives. Cellulose is the major constituent in biomass. It has a linear and homogeneous structure which makes it highly stable to enzyme and chemical attack. Hemicellulose is the second most abundant polysaccharides in biomass. Unlike cellulose, hemicellulose is amorphous, and it is branched in structure. So it is very sensitive to heat and chemicals. During chemical treatments, hemicellulose will first release and dissolve into hydrolysate [6-8]. Lignin is the only non-

polysaccharides major components in biomass. It is amorphous in nature and plays a critical role to encrust the cell wall and cements the cell together, giving structural rigidity to hold plant fibers together.

There are mainly three steps in bioconversion of biomass into biofuels and biochemicals. The first step is pretreatment, which breaks the recalcitrance of biomass and increases the accessibility of enzymes to cellulose. The second step is hydrolysis, in which enzymes are commonly used to hydrolyze poly-saccharides into fermentable mono-saccharides. The last step is fermentation; the carbohydrates from biomass are fermented into final products using microorganisms.

Pretreatment is a necessary step to break down the biomass recalcitrance, however, along with producing a cellulose rich solid which is suitable for biofuels production during the process, sugar degradation products and significant amount of solubilization and transformation of lignin-related chemicals are dissolved into the prehydrolysates [9]. These are inhibitory compounds to both enzymatic hydrolysis and fermentation. The formation of inhibitors depends on the types of feedstock and the pretreatment conditions, such as temperature and pH level. The common inhibitors identified during pretreatment are sugar degradations, such as furfural, HMF and levulinic acid; lignin degradations, such as vanillin, syringaldehyde and *p*-hydroxybenzaldehyde [10]. It was demonstrated that the lignin-derived inhibitors are more toxic than sugar degradation compounds [11]. These inhibitors need to be removed before the prehydrolysates could be fermented effectively. So, detoxification step is required in most cases. Various detoxification methods including physical, chemical and biological are aimed to remove and/or modify inhibitors present in the prehydrolysates, thus improve the enzyme activity in enzymatic

hydrolysis process as well as microorganism cell growth and/or metabolism in fermentation process.

1.2 Chemical Composition of Lignocellulosic Biomass

Composition of lignocellulosic biomass varies with types of feedstock as well as species. The age of the plant and the growing conditions, such as climate, soil and fertilizer, also have impact on the composition of biomass. In general, lignocellulosic biomass is composed of three main components: cellulose, hemicellulose and lignin. The composition of representative lignocellulosic biomass is summarized in **Table 1-1**.

Table 1-1 Composition of representative lignocellulosic biomass

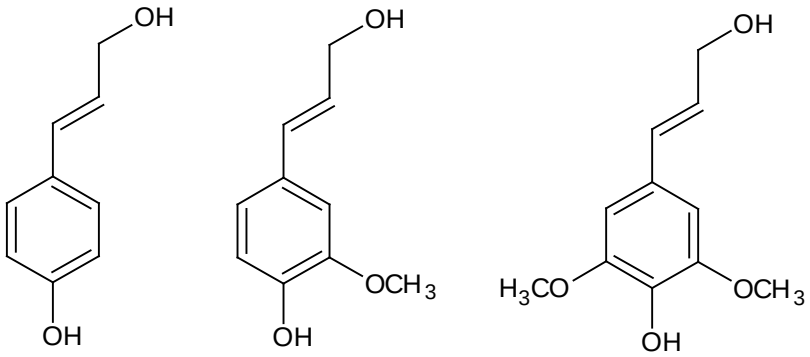
	Composition (% of dry weight)			**References**
	Cellulose	**Hemicellulose**	**Lignin**	
Corn stover	33.4	28.6	17.2	[12]
Corn cob	38.8	36.4	13.1	[13]
Sugarcane bagasse	43.1	27.2	23.2	[14]
Wheat straw	33-40	20-25	15-20	[15]
Rice straw	35.5	24.3	20	[16]
Switchgrass	30.1	15.6	24.5	[17]
Barley hull	33.6	37.2	19.3	[18]
Spruce	43.8	20.8	28.8	[19]
Loblolly pine	41.3	22.0	28.7	[20]
Douglas-fir	43.0	21.0	29.3	[21]
lodgepole pine	47.6	22.9	26.3	[22]
Sweetgum	41.2	22.2	25.8	[20]
Poplar	43.2	26.6	21.3	[23]
Aspen	49.0	18.2	25.6	[24]
Eucalyptus	46.7	14.8	29.2	[14]
Oak	45.2	24.5	24.3	[19]

Cellulose is the primary constituent in biomass. It accounts for 30 - 45 wt% of dry biomass. Cellulose is a polysaccharide polymerizing with glucose as its monomer, which condenses through β (1-4)-glycosidic bonds. The molecular of cellulose has a linear, unbranched structure, which consists 10,000 to 15,000 glucose units [25]. This homogeneous linear chain and intra and inter molecular interactions make cellulose highly stable and resistant to both enzyme and chemical attack. There are two important factors that will affect cellulose digestibility. The first is the degree of crystallinity. It has been shown that the amorphous portion can easily be hydrolyzed while the crystalline portion is more resistant to hydrolysis [26-28]. The second factor is degree of polymerization (DP). DP plays a key role in enzymatic hydrolysis since some fractions of cellulase enzymes cleave the cellulose by an end-wise mechanism [29].

Hemicellulose is the second major component in biomass, which represents 20 - 35 wt% of dry biomass. It is a non-homogeneous and branched polysaccharides made up of various sugars: xylose, arabinose, mannose, glucose and galactose [30, 31]. Hemicelluloses in softwood are mostly glucomannans, while hemicelluloses in hardwood and herbaceous biomass are mainly xylans. The DP of hemicellulose is much lower than cellulose. Due to its amorphous structure and low DP, hemicelluloses are very sensitive to thermal and chemical treatment.

Lignin is the major non-carbohydrate component in biomass and it represents 10 -30 wt% of dry biomass. Lignin is made up of three constituents: *p*-coumaryl alcohol unit, coniferyl alcohol unit, and sinapyl alcohol unit (**Figure 1-1**). The ratios of these three subunits in different types of biomass are listed in **Table 1-2**. Lignin is considered to be one of the main obstacles to enzymatic hydrolysis of cellulose. It can block cellulose from cellulase enzymes and cause non-productive bindings to the enzymes. Also, the lignin degradation products from pretreatment step have strong inhibitory effect on the enzymatic hydrolysis and fermentation. Consequently,

changing lignin chemical structures, lignin distribution and lignin content typically are required during biomass conversion. Alkaline reagents are proven to be effective to remove lignin from biomass [32-34].



I II III

Figure 1-1 Three lignin precursors

(I) p-coumaryl alcohol (H); (II) coniferyl alcohol (G); (III) sinapyl alcohol (S)

Table 1-2 Ratios of the lignin subunits in plant species [35]

	H / G / S ratio (%)
Herbaceous plants	(5-33) / (33-80) / (20-54)
Softwood	<5 / >95 / none or trace
Hardwood	(0-8) / (25-50) / (46-75)

Note: The H / G / S ratio is the relative lignin composition of 4-hydroxybenzyl (H), guaiacyl (G) and syringyl (S) units

1.3 Bioconversion of Lignocellulosic Biomass to Biofuels and Biochemical

A multi-stage biochemical conversion process is needed to convert lignocellulosic biomass to fermentable sugars for the production of advanced biofuels and biochemicals (**Fig. 1-2**). Biomass is pretreated, and then subjected to enzymatic hydrolysis with subsequent microbial fermentation to fuels and chemicals by microorganisms.

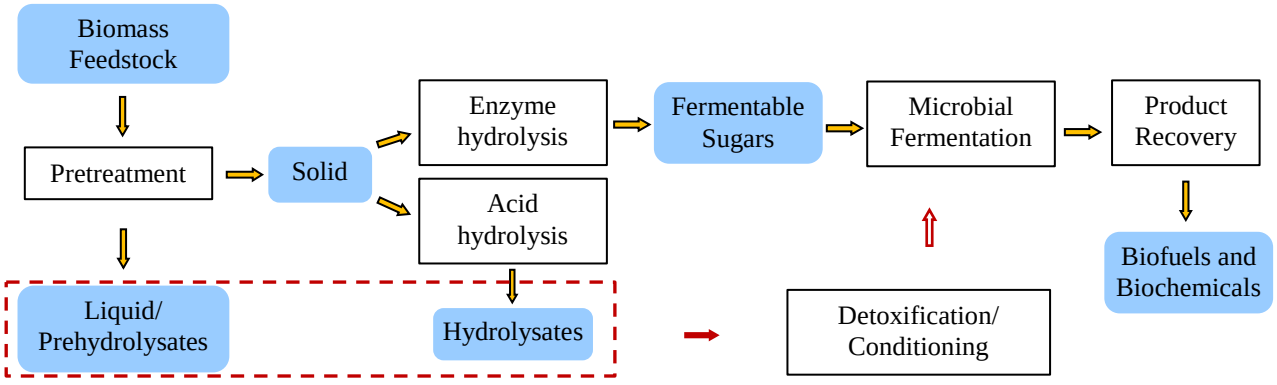


Figure 1-2 Biochemical conversion platform from biomass to biofuels and biochemicals

1.3.1 Pretreatment

Pretreatment is a particularly important step for overall process viability because it is one of the most costly steps in the bioconversion process. The overall purpose of pretreatment is to break down the shield matrix formed by lignin and hemicellulose, to disrupt the crystalline structure and reduce the degree of polymerization of cellulose (**Figure 1-3**). An effective pretreatment is desired to (1) break the lignocellulosic complex and reduce the crystallinity of cellulose with minimizing the hemicellulose sugar loss; (2) minimize the formation of degradation products that are toxic to enzymatic hydrolysis and fermentation; (3) generate value added co-products (e.g. lignin, furfural, etc.); (4) minimize the operating cost and capital cost; (5) minimize the generation of waste such as waste water, solid residues, or, if any, hazardous waste

[36-38]. Although significant effort has been made on pretreatment development, none of them can satisfy all the criteria listed above. Meanwhile, due to the complexity of lignocellulose, the effectiveness of pretreatment greatly depends on the types of biomass. The dilute acid pretreatment was reported to be an efficient treatment on poplar bark and corn leaf but not effective on the bark from sweetgum and corn stalks [38-40].

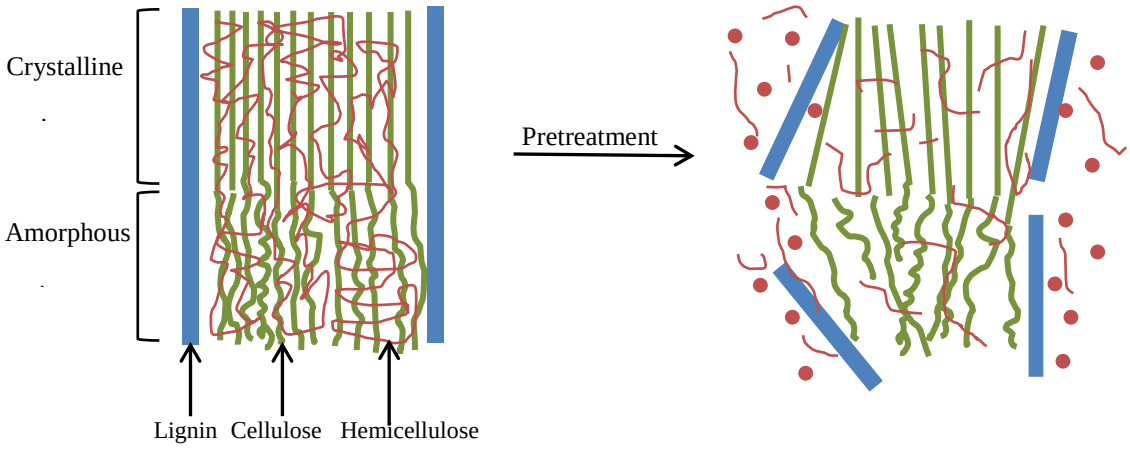


Figure 1-3 Pretreatment effects on lignocellulosic biomass
(Modified from figure by Mosier *et al*., 2005 [36])

A variety of pretreatment methods have been investigated. These technologies basically can be categorized into four groups: physical, physico-chemical, chemical and biological methods. The physical pretreatment process aims to reduce the particle size and crystallinity of raw materials and thus increased specific surface area and reduced degree of polymerization (DP) can be obtained. Physical pretreatment includes milling (e.g. ball milling, two-roll milling, hammer milling, colloid milling), grinding, irradiation, and so forth [41]. Physico-chemical is the process combining both chemical and physical method. The most widely investigated processes include steam explosion (autohydrolysis), steam explosion with addition of SO_2, ammonia fiber

explosion (AFEX), CO_2 explosion, liquid hot-water pretreatment and microwave-chemical pretreatment [38]. Chemical pretreatment technologies typically include ozonolysis, acid, alkaline, and organosolv pretreatment. The ozonolysis is performed by using ozone, and it is effective to remove lignin and part of hemicellulose without forming inhibitory compounds. But the cost is high because of the usage of ozone [38, 42]. The acid pretreatment is recognized as a leading pretreatment process that is currently under industrial employment [43]. It can effectively hydrolyze hemicellulose and decrease the DP of cellulose, but it results in considerable amount of degradation compounds that are inhibitory to subsequent enzymatic hydrolysis and microbial fermentation. Moreover, it cannot remove lignin which deposits on the surface of cellulose and potentially blocks the accessibility of substrate to enzymes [44, 45]. In contrast to acid pretreatment, the alkaline pretreatment can effectively remove lignin from biomass. Both cellulose and hemicellulose are preserved in biomass during alkaline process [46]. On the other hand, it causes less sugar degradation and is proved to be more effective on agricultural residues than woody biomass [41, 47]. Organosolv pretreatment process employs organic solvent (e.g. methanol, ethanol, acetone, ethylene glycol etc.) as the reagent and a catalyst of acid is added to reduce the operating temperature or enhance the delignification effect [41]. Significant amount of lignin and hemicellulose are dissolved in this process while large portion of cellulose remains in the solid fraction. The unique advantage of organosolv pretreatment is that relatively pure, low-molecular-weight lignin is recovered as a by-product [48]. The organic solvent can be recycled after pretreatment to reduce the operational cost, however the simplicity of solvent recovery should be considered. Hence, the alcohol with low boiling point e.g. methanol and ethanol, is a better choice over alcohols with higher boiling point [48, 49]. Biological pretreatment is to degrade lignin and hemicelluloses but very little cellulose

in biomass by using lignin degrading microorganisms such as brown, white and soft-rot fungi [50]. This process rarely produces inhibitors like the chemical process and is carried out in mild conditions with low capital cost, low energy input and no chemical requirement, but it is shown to be very slow compared to other pretreatment processes [51]. Among these four pretreatment categories, chemical methods are most favored for industrial application, since it achieves reasonable high yield with low cost and short reaction time. During the chemical pretreatment process, certain level of sugar and lignin degradation products will be produced and released into the pretreatment liquor. These degradation compounds are inhibitors to the subsequent bioprocesses. So, a detoxification step is usually needed to detoxify the pretreatment liquor.

1.3.2 Enzymatic Hydrolysis

The second step of converting lignocellulose to biofuels and biochemicals is hydrolysis which is normally done by cellulase enzymes. The enzymatic hydrolysis is a relatively slow and complex process. Cellulases and hemicellulases are usually used to hydrolyze cellulose and hemicellulose into fermentable monomeric sugars. Cellulases are usually a mixture of several enzymes and they can be classified into three groups: (1) endoglucanase (EG, endo-1,4- β-D-glucanases, or EC 3.2.1.4.) which randomly attacks the low crystallinity region in the cellulose chain, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which processes along the cellulose chain and cut off the cellobiose units from the free ends; (3) β-glucosidase (BG, EC 3.2.1.21) which hydrolyzes cellobiose to glucose [51-53]. These enzymes work synergistically to hydrolyze polysaccharides into monosaccharides by creating new accessible sites for each other. The synergistic work of cellulolytic enzymes is shown in **Figure 1-4.** Hemicelluloses are heterogeneous with a variety of sugar units and thus the hemicellulytic enzymes are more complex. They at least contain endo-

1,4- β-D-xylanases, exo-1,4- β-D-xylosidases, endo-1,4- β-D-mannanases, β -mannosidases, acetyl xylan esterases, α-glucuronidases, α-L-arabinofuranosidases, and α -galactosidases [53, 54].

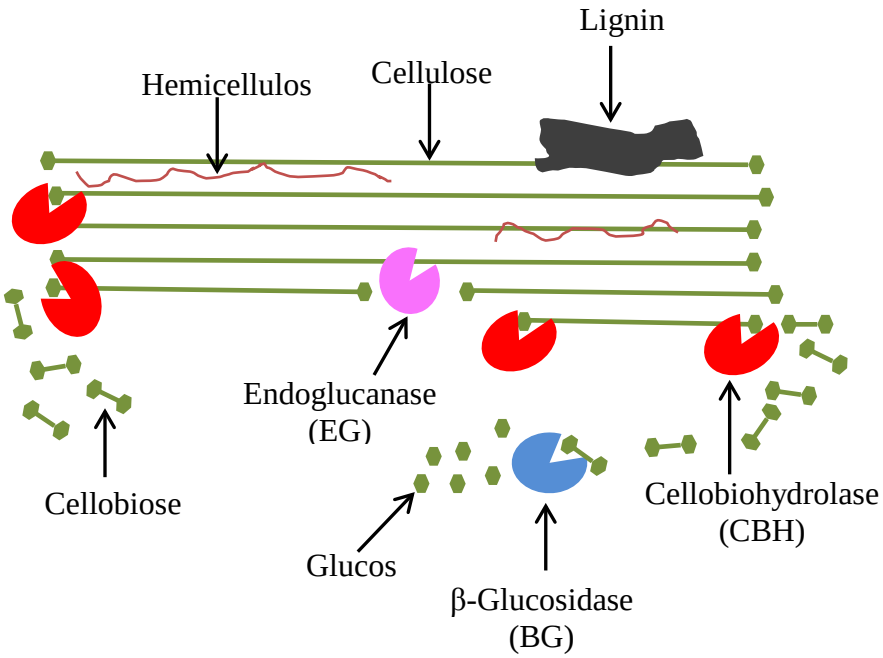


Figure 1-4 Schematic of hydrolysis of cellulose by cellulolytic enzymes (Adapted from Jørgensen *et al*. [53])

Compared to acid hydrolysis, enzymatic hydrolysis is conducted under mild conditions (pH= 4.5-5.0 and T= 40-50 ºC) which require less utility cost and avoid the issue of corrosion and generation of inhibitory compounds [54]. However, the challenges of using enzymatic hydrolysis are the high cost of cellulase enzymes, long hydrolysis duration and inhibition from lignin, hemicellulose and end-product [55, 56]. Numerous effort has been made to address these issues, such as addition of surfactant into the hydrolysis, optimization of cellulase mixture and recycling enzymes [22, 54].

1.3.3 Microbial Fermentation

Fermentation is the phase of the bioconversion process which converts sugars to biofuels and biochemicals. The strategies of conducting hydrolysis and fermentation can be classified into four types: (1) Separate hydrolysis and fermentation (SHF) is to conduct hydrolysis and fermentation sequentially. In this process, both steps can be operated at its optimal temperature and pH. The optimum temperature of enzymatic hydrolysis is usually 40-50 ^{o}C, whereas most microorganisms used in fermentation step requires temperature below 40 ^{o}C [57, 58]. The major drawback of SHF is the accumulation of sugars in hydrolysis step may cause end-product inhibition to enzymes which will decrease the sugar yield. (2) Simultaneous Saccharification and Fermentation (SSF) is to perform the enzymatic hydrolysis and fermentation in one step. The sugars produced by enzymes will be immediately consumed by microorganisms which alleviate the end-product inhibition problem and in turn increase the sugar production rate and yield, but the compromise on optimum conditions for hydrolysis and fermentation has to be considered [49]. (3) Simultaneous Saccharification and Cofermentation (SSCF) is fermenting both hexose and pentose to biofuels and biochemicals. This process is considered potentially enhance the overall economics of bioconversion from lignocellulosic biomass by utilizing all the sugars present in feedstocks [59]. (4) Consolidated bioprocessing (CBP) allows the enzyme production, hydrolysis and fermentation to biofuels occurring in a single bioreactor. Successful application of CBP can significantly reduce the cost for operation and purchasing enzymes [59, 60].

1.4 Identified Inhibitors and Their Inhibition Effect

During pretreatment, significant amount of C5 and C6 sugars are presented in the prehydrolysates. They are coming from the hydrolysis of cellulose and hemicelluloses.

Meanwhile, a large number of degradation compounds are formed (**Fig.1-5**). The furan aldehydes such as HMF and furfural are degradation of carbohydrates including both hexose and pentose, and they further degrade to weak acids. Levulinic acid is formed by degradation of HMF and formic acid is the degraded from HMF and furfural. One the other hand, a large group of phenolic compounds is mainly generated from partial breakdown of lignin [61, 62].

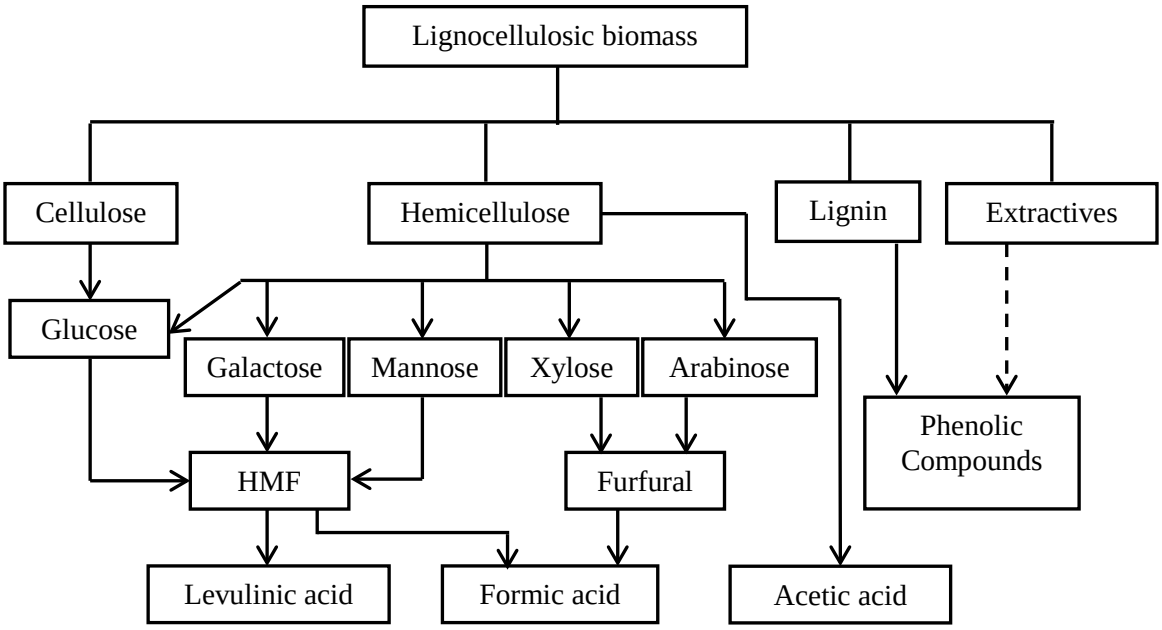


Figure 1-5 Inhibitors formation during pretreatment

The generation of inhibitors from the pretreatment and their concentrations are strongly dependent on the types of feedstock as well as the pretreatment process conditions such as temperature, pH, reaction time, pressure and addition of chemicals. For example, softwood mainly releases guaiacyl lignin compounds while hardwood releases both guaiacyl and syringyl lignin compounds [63, 64]. Salicylic acid was detected much higher in poplar hydrolysate than that in the hydrolysates of corn stover and pine. And 4-hydroxybenzaldehyde was reported to be higher in the corn stover hydrolysates rather than in hydrolysates of poplar and pine [65]. In acid

pretreatment, ketone and phenolic aldehydes tend to be produced, while alkaline pretreatment typically generates phenolic acids [64].

Various analytical techniques, such as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS), have been used to identify inhibitory compounds in the prehydrolysates from various kinds of lignocellulosic feedstocks. The inhibition mechanism of toxic compounds to microorganisms is potentially related to interference with cell maintenance functions [66]. Low molecular weight organic compounds are able to penetrate cell membrane, whereas high molecular weight inhibitors hamper the activity of ion transporters in the cell membrane [63].

The identified inhibitors can be classified according to their chemical structure into furan derivatives such as furfural and HMF; phenolic compounds such as 4-hydroxybenzaldehyde, vanillin and syringaldehyde; carboxylic acids such as acetic, formic, and levulinic acid. Among these inhibitors, phenolic compounds show the most inhibitory effect to microbial fermentation [67].

1.4.1 Furans

The furan aldehydes (furfural and HMF) are common sugar degradation products during thermal or acid pretreatment process. Furfural comes from pentose while HMF is mainly formed by hexose. These two furans have been extensively studied as model inhibitors in previous studies on microbial growth and fermentation [68, 69].

Furan compounds are able to inhibit the glycolysis pathways of many organisms as well as their protein and RNA synthesis [66]. Furfural is a key inhibitor in the prehydrolysates because it is toxic by itself and also acts synergistically with other inhibitors. Furfural was found

to inhibit ethanol production by *S. cerevisiae* at as low as 0.5 g/L and completely inhibit the production at 4 g/L [70]. On the other hand, moderate addition of furfural to the growth medium was found to lead to increasing the alcohol yields [68, 71, 72]. It is likely due to the reduced formation of the undesirable by-product such as glycerol and more sugars can be uses for the formation of alcohol [69, 73]. A mechanistic model involving furfural reduction was developed by Palmqvist *et al*. The model was based on the assumptions: (1) furfural reduction to furfuryl alcohol by NADH- dependent dehydrogenases had a higher priority than reduction of dihydroxyacetone phosphate to glycerol, and (2) furfural caused inactivation of cell replication [61, 74]. HMF inhibits the organisms in the same manner and the threshold concentration is slightly higher [75]. It was found the growth of *C. beijerinckii* BA 101 was increased by 14% in the presence of 2 g/L HMF and an improvement of 7% was observed when furfural was added at the same concentration. HMF could be converted to 2, 5-bis-hydroxymethylfuran [76] by *S. cerevisiae* but the conversion rate is lower than furfural. Similarly, Zhang *et al*. [77] reported the *C. acetobutylicum* ATCC 824 converted furfural and HMF to furfuryl alcohol and 2, 5-bis-hydroxymethylfuran with the specific rates of 2.13 g furfural and 0.50 g HMF per g (biomass) per hour. The difference of conversion rate might be due to lower membrane permeability when HMF is present [78].

1.4.2 Phenolic Compounds

A large number of different phenolic compounds and their derivatives are formed from lignin decomposition and possibly extractives during pretreatment of lignocellulosic biomass [79-81]. They have been identified and quantified in hydrolysates from steam-exploded poplar [82] and birch wood [83], steam and SO_2 pretreated willow [84], dilute acid hydrolyzed pine wood [85], spruce [86], switchgrass [87] and corn stover [88], alkaline wet oxidation pretreated

wheat straw [89], and hot water treated cedar [90]. An overview of these identified phenolic compounds and the derivatives are shown in **Table 1-3**. The species of these aromatic monomers depends on the type of pretreatment and the H/G/S ratio of lignin present in the raw materials. The softwood mostly produces guaiacyl lignin derivatives while hardwood and herbaceous biomass produce H, G and S phenolic compounds [64]. The H (4-hydroxybenzyl) phenols were reported to be of high concentration in poplar acid hydrolysate due to the presence of 4-hydroxylbenaldehyde and 4-hydroxybenzoic acid [63, 82]. The phenolic aldehydes and ketones have been observed to be favored at oxidative acid conditions [89] while the alkaline pretreatment tends to further oxidize the aldehydes/ketones to their corresponding acids. Hibbert's ketone, derived from phenylpropane with one or two keto groups on the 1 or/and 2 position of propyl group, was found in prehydrolysates from dilute acid pretreatment [63, 78, 85]. Moreover, the syringyl unit in lignin is more sensitive to hydrothermal treatment than guaiacyl unit and thus, relative to raw biomass, more syringyl than guaiacyl lignin derivatives were found in hydrolysates [83, 84].

Phenolic compounds can cause a loss of integrity of biological membranes and other hydrophobic targets by partition into membranes. This affects their ability to serve as selective membranes and enzyme matrices, inhibiting cell growth as well as sugar assimilation [91]. Another possible mechanism is that phenolic compounds influence the function of cell membrane by changing its protein-to-lipid ratio [92]. For example, vanillin, a phenolic aldehyde causes partial disruption of potassium gradients in *E. coli*. Membrane destabilization is experienced by 29% of the population after treatment with vanillin [93]. The inhibition effects of phenolic compounds on enzymatic hydrolysis have also been investigated and some reports explained that phenolic compounds affect proteins by inducing precipitation [94-96]. The effects

of phenols and their derivatives on fermentation are different, and can be related to specific functional groups [82, 97]. It has been found the position of hydroxyl group in hydroxybenzaldehydes affected their activity and followed the order: 2-hydroxybenzaldehye > 3-hydroxybenzaldehye > 4-hydroxybenzaldehye [98]. A major group of phenol derivatives are aromatic aldehydes and ketones. They are both carbonyl-containing compounds. It is believed that carbonyl compounds attributed much inhibition to the microorganism and their inhibition effects are ruled by their electrophilic reactivity towards biological nucleophiles [99]. Aromatic aldehydes such as benzaldehyde and *o*-phthaladehyde (OPA) have been used as model inhibitors for their effects on microbial growth and biofuels production in previous studies [82, 100]. It is also found that low molecular weight phenols have shown to be more toxic to microorganism than high molecular weight polyphenols [85, 101].

The mechanism of the inhibitory effect has not been clearly elucidated, largely due to a lack of accurate qualitative and quantitative analysis. The large number and the diversity of the phenolic compounds found in different lignocellulose hydrolysates make identification and quantification very complicated. A method for group analysis of phenols by high-performance liquid chromatography (HPLC) has been used to characterize phenolic compounds [102]. A number of model studies have been performed on the inhibitory action of phenolic compounds using far higher concentrations than actually level presents in the prehydrolysates [85, 103, 104]. When the results of those studies are interpreted, it should be kept in mind that the solubility depends on the composition of the liquid and can be different in hydrolysate and in defined medium. When a high concentration of a certain compound has been used, it is therefore possible that the concentration actually experienced by the microorganism has been lower [68].

Table 1-3 Phenolic compounds identified in hydrolysates

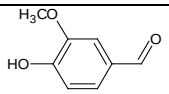
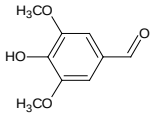
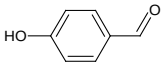
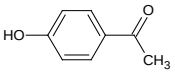
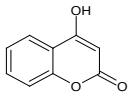
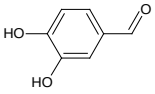
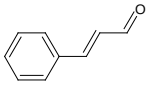
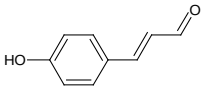
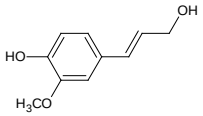
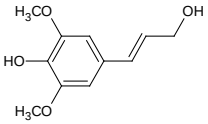
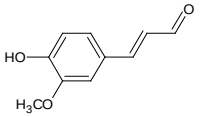
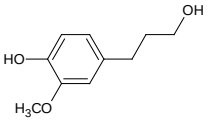
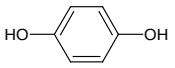
Compound	Formula	Structure	Molecular Weight	Origin	Reference
Vanillin	$C_8H_8O_3$		152.15	Guaiacyl unit	[65, 82]
Syringaldehyde	$C_9H_{10}O_4$		182.17	Syringyl unit	[65, 82]
4-hydroxybenzaldehyde	$C_7H_6O_2$		122.12	*p*-Coumaryl unit	[65, 82]
4-Hydroxyacetophenone	$C_8H_8O_2$		136.05	*p*-Coumaryl unit	[65]
4-Hydroxycoumarin	$C_9H_6O_3$		162.14	Lignin	[65]
3,4-Dihydroxybenzaldehyde	$C_7H_6O_3$		138.12	Lignin	[65]
Cinnamaldehyde	C_9H_8O		132.16	Lignin	[82]
4-Hydroxycinnamaldehyde	$C_9H_8O_2$		148.16	*p*-Coumaryl unit	[82]
Coniferyl alcohol	$C_{10}H_{12}O_3$		180.20	Guaiacyl unit	[82]
Sinapyl alcohol	$C_{11}H_{14}O_4$		210.23	Syringyl unit	[82]
Coniferyl aldehyde	$C_{10}H_{10}O_3$		178.18	Guaiacyl unit	[85, 86]
Dihydroconiferylalcohol	$C_{10}H_{14}O_3$		182.22	Guaiacyl unit	[86]
Hydroquinone	$C_6H_6O_2$		110.11	*p*-Coumaryl unit	[86]

Table 1-3 (Continued)

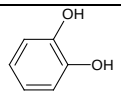
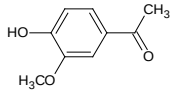
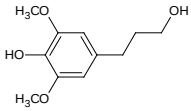
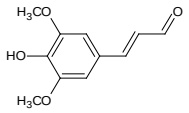
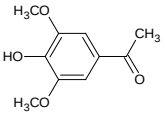
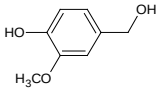
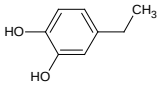
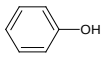
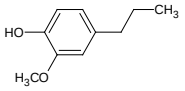
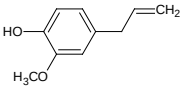
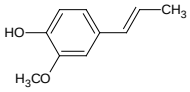
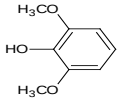
Compound	Formula	Structure	Molecular Weight	Origin	Reference
Catechol	$C_6H_6O_2$		110.11	*p*-Coumaryl unit	[84, 86]
Acetoguaiacone (Acetovanillone)	$C_{10}H_9O_3$		166.17	Guaiacyl unit	[86]
Dihydrosinapyl alcohol	$C_{11}H_{16}O_4$		212.24	Syringyl unit	[105, 106]
Sinapyl aldehyde	$C_{11}H_{12}O_4$		208.21	Syringyl unit	[105, 106]
Acetosyringone	$C_{10}H_{12}O_4$		196.20	Syringyl unit	[105]
Vanillylalcohol	$C_8H_{10}O_3$		154.17	Guaiacyl unit	[107]
Ethylcatechol	$C_8H_{10}O_2$		138.16	*p*-Coumaryl unit	[84]
Phenol	$C_6H_6O_6$		94.11	*p*-Coumaryl unit	[84]
Guaiacol	$C_7H_8O_2$		124.14	Guaiacyl unit	[84]
4-Propylguaiacol	$C_{10}H_{14}O_2$		166.22	Guaiacyl unit	[84]
Eugenol	$C_{10}H_{12}O_2$		164.20	Guaiacyl unit	[97]
Trans-isoeugenol (Trans-2-Methoxy-4-propenylphenol)	$C_{10}H_{12}O_2$		164.20	Guaiacyl unit	[84]
Syringol	$C_8H_{10}O_3$		154.16	Syringyl unit	[89]

Table 1-3 (Continued)

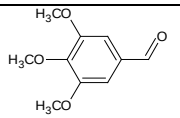
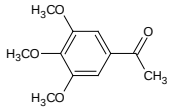
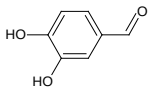
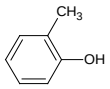
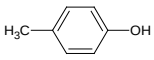
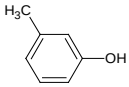
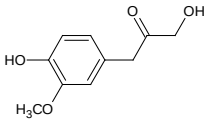
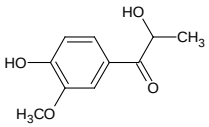
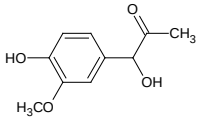
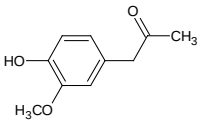
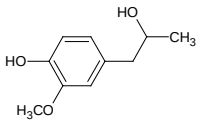
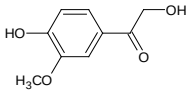
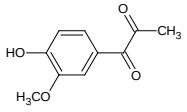
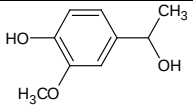
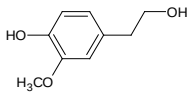
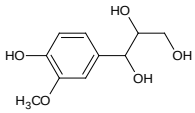
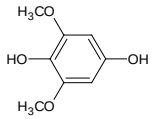
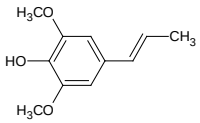
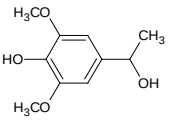
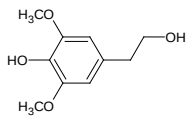
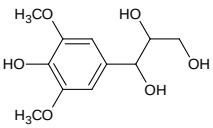
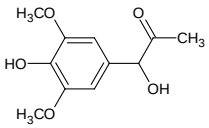
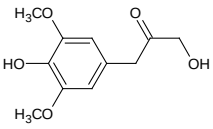
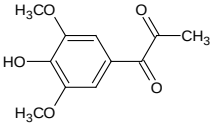
Compound	Formula	Structure	Molecular Weight	Origin	Reference
3,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$		196.20	Not Known	[89]
3,4,5-Trimethoxyacetophenone	$C_{11}H_{14}O_4$		210.23	Not Known	[89]
3,4-Dihydroxybenzaldehyde	$C_7H_6O_3$		138.12	*p*-Coumaryl unit	[88]
o-Cresol	C_7H_8O		108.14	*p*-Coumaryl unit	[84, 108]
p-Cresol	C_7H_8O		108.14	Lignin	[108]
m-Cresol	C_7H_8O		108.14	Lignin	[108]
β-Oxyconiferylalcohol	$C_{10}H_{12}O_4$		196.20	Guaiacyl unit	[85]
α-Hydroxypropiovanillone	$C_{10}H_{12}O_4$		196.20	Guaiacyl unit	[85]
1-Guaiacylacetol	$C_{10}H_{12}O_4$		196.20	Guaiacyl unit	[106]
Guaiacylacetone	$C_{10}H_{12}O_3$		180.20	Guaiacyl unit	[85]
NA	$C_{10}H_{14}O_3$		182.22	Guaiacyl unit	[85]
NA	$C_9H_{10}O_4$		182.17	Guaiacyl unit	[85]
NA	$C_{10}H_{10}O_4$		194.18	Guaiacyl unit	[85]

Table 1-3 (Continued)

Compound	Formula	Structure	Molecular Weight	Origin	Reference
1-Guaiacylethanol	$C_9H_{12}O_3$		168.19	Guaiacyl unit	[106]
2-Guaiacylethanol (Homovanillyl alcohol)	$C_9H_{12}O_3$		168.19	Guaiacyl unit	[106]
Guaiacylglycerol	$C_{10}H_{14}O_5$		214.22	Guaiacyl unit	[106]
2,6-Dimethoxyhydroquinone	$C_8H_{10}O_4$		170.16	Syringyl unit	[106]
4-Propenylsyringol	$C_{11}H_{14}O_3$		194.23	Syringyl unit	[106]
1-Syringylethanol	$C_{10}H_{14}O_4$		198.22	Syringyl unit	[106]
2-Syringylethanol	$C_{10}H_{14}O_4$		198.22	Syringyl unit	[106]
Syringylglycerol	$C_{11}H_{16}O_6$		244.24	Syringyl unit	[106]
1-Syringylacetol	$C_{11}H_{14}O_5$		226.23	Syringyl unit	[106]
β-Oxysinapylalcohol	$C_{11}H_{14}O_5$		226.23	Syringyl unit	[87]
NA	$C_{11}H_{12}O_5$		224.21	Syringyl unit	[87]

1.4.3 Carboxylic Acids

A large number of carboxylic acids were found in lignocellulose prehydrolysates. The commonly identified carboxylic acids are acetic acid, formic acid, levulinic acid, 4-hydroxybenzoic acid and vanillic acid [84, 88, 89]. Acetic acid is a ubiquitous degradation compound formed primarily by hydrolysis of acetyl groups of hemicelluloses. Formic acid is a degradation product of furfural and HMF, while levulinic acid is formed by degradation of HMF. 4-hydroxybenzoic acid and vanillic acid are considered to be lignin degradation products and shown at substantially lower concentration compared to aliphatic acid such as acetic acid. The carboxylic acids identified from the prehydrolysate in previous studies are presented in **Table 1-4**.

The inhibitory effect of carboxylic acids is pH dependent since they are weak acids and exist at both undissociate and dissociate forms. The undissociated acids penetrate the cell membrane and then dissociate in the cytoplasm due to the neutral cytosolic pH. The dissociation of the acids leads to a decrease in the intracellular pH [109]. Maintaining a neutral intracellular pH is crucial for cell viability. Imai and Ohno [110] found that the cell replicative activity decreases linearly with decreasing intracellular pH. The optimal external pH range for growth of *S. cerevisiae* is 5.0-5.5 [111] and the pH is more critical to solvent producing *Clostridia* due to the acid crash occurring [112]. So the high acids concentration in the prehydrolysate may result in cell growth inhibition or cell death.

Table 1-4 Carboxylic acids identified in hydrolysates

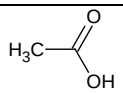
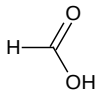
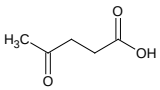
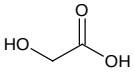
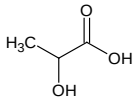
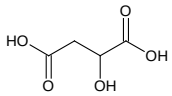
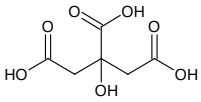
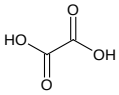
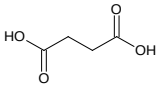
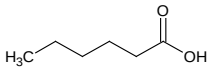
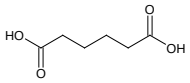
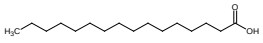
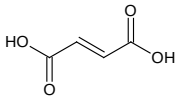
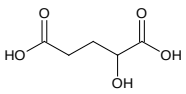
Compound	Formula	Structure	Molecular Weight	Origin	Reference
Acetic acid	$C_2H_4O_2$		60.05	Hemicelluloses	[86]
Formic acid	CH_2O_2		46.03	Sugars	[86]
Levulinic acid	$C_5H_8O_3$		116.11	Sugars	[86]
Glycolic acid	$C_2H_4O_3$		76.05	Sugars	[89]
Lactic acid	$C_3H_6O_3$		90.08	Sugars	[89]
Malic acid	$C_4H_6O_5$		134.09	Not Known	[89]
Citric acid	$C_6H_8O_7$		192.12	Not Known	[89]
Oxalic acid	$C_2H_2O_4$		90.03	Not Known	[89]
Succinic acid	$C_4H_6O_4$		118.09	Not Known	[89]
Hexanoic acid (Caproic acid)	$C_6H_{12}O_2$		116.16	Extractives	[105]
Hexanedioic acid	$C_6H_{10}O_4$		146.14	Extractives /Lignin	[66]
Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$		256.43	Extractives	[66]
2-Butenedioic acid (Fumaric acid)	$C_4H_4O_4$		116.07	Not Known	[66]
2-hydroxypentanedioic acid	$C_5H_8O_5$		148.11	Not Known	[66]

Table 1-4 (Continued)

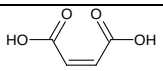
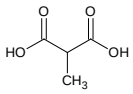
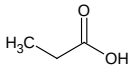
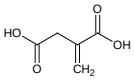
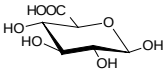
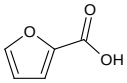
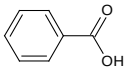
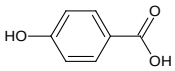
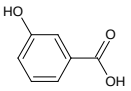
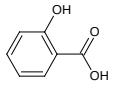
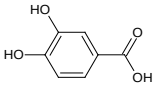
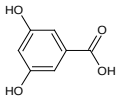
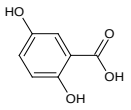
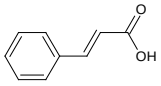
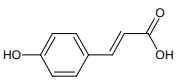
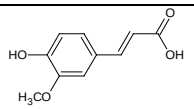
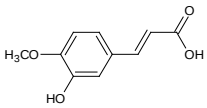
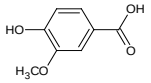
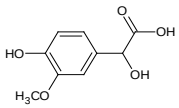
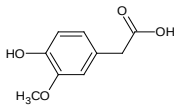
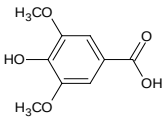
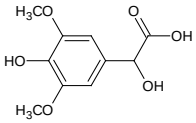
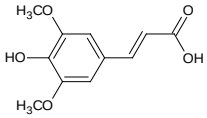
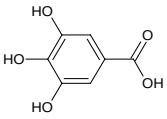
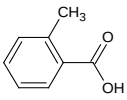
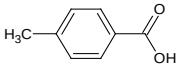
Compound	Formula	Structure	Molecular Weight	Origin	Reference
Maleic acid	$C_4H_4O_4$		116.07	Not Known	[88]
Methylmalonic acid	$C_4H_6O_4$		118.09	Not Known	[88]
Propionic acid	$C_3H_6O_2$		74.08	Not Known	[88]
Itaconic acid	$C_5H_6O_4$		130.10	Not Known	[88]
Glucuronic acid	$C_6H_{10}O_7$		194.14	Sugars	[90]
2-Furoic acid	$C_5H_4O_3$		118.02	Furfural	[89]
Benzoic acid	$C_7H_6O_2$		122.12	*p*-Coumaryl unit	[88]
4-Hydroxybenzoic acid	$C_7H_6O_3$		138.12	*p*-Coumaryl unit	[86]
4-Hydroxybenzoic acid	$C_7H_6O_3$		138.12	*p*-Coumaryl unit	[82]
2-Hydroxybenzoic acid (Salicylic acid)	$C_7H_6O_3$		138.12	*p*-Coumaryl unit	[84]
3,4-Dihydroxybenzoic acid	$C_7H_6O_4$		154.12	*p*-Coumaryl unit	[88]
3,5-Dihydroxybenzoic acid	$C_7H_6O_4$		154.12	Not known	[88]
2,5-Dihydroxybenzoic acid	$C_7H_6O_4$		154.12	*p*-Coumaryl unit	[84]
Cinnamic acid	$C_9H_8O_2$		148.16	Lignin	[82]
4-Hydroxycinnamic acid (p-Coumaric acid)	$C_9H_8O_3$		164.16	*p*-Coumaryl unit	[89]

Table 1-4 (Continued)

Compound	Formula	Structure	Molecular Weight	Origin	Reference
Ferulic acid	$C_{10}H_{10}O_4$		194.18	Guaiacyl unit	[89]
3-Hydroxy-4-methoxycinnamic acid	$C_{10}H_{10}O_4$		194.18	Not known	[88]
Vanillic acid	$C_8H_8O_4$		168.14	Guaiacyl unit	[88]
Guaiacylglycolic acid	$C_9H_{10}O_5$		198.17	Guaiacyl unit	[106]
Homovanillic acid	$C_9H_{10}O_4$		182.18	Guaiacyl unit	[86]
Syringic acid	$C_9H_{10}O_5$		198.17	Syringyl unit	[84]
Syringylglycolic acid	$C_{10}H_{12}O_6$		228.20	Syringyl unit	[106]
Sinapic acid	$C_{11}H_{12}O_5$		224.21	Syringyl unit	[63]
Gallic acid	$C_7H_6O_5$		170.12	Extractives (tannin)	[88]
o-Toluic acid	$C_8H_8O_2$		136.20	Not Known	[88]
p-Toluic acid	$C_8H_8O_2$		136.20	Not Known	[88]

The pKa value of formic acid is 3.75, which is considerably lower than those of acetic acid (4.76) and levulinic acid (4.64). The toxic effect of these three acids on *S. cerevisiae* is pKa value related. Lower pKa value gives higher toxic effect: acetic acid < levulinic acid < formic acid [78]. This may be due to differences in membrane permeability or in toxicity of the anionic form of the acids once they have entered the cell. However, the case in ABE fermentation by *Clostridia* is more complicated. It was reported that the formic acid rather than acetic acid or butyric acid present in the medium triggered the acid crash in ABE fermentation [112]. Although the carboxylic acid has shown to be inhibitory to microbial fermentation, in some cases they could stimulate the cell growth and fermentation. The influence of acetic, formic, and levulinic acid on the ethanol yield has been studied in model fermentations [78]. Low acid concentrations (<100 mM) have shown to boost the ethanol yield at pH 5.5, whereas the yield decreased at higher concentrations.

1.5 Detoxification Methods

As mentioned in previous sections, a wide range of inhibitors are produced in the pretreatment, which can strongly hinder the enzyme and microorganism performance. One way is to genetically modify the microorganisms or make the microorganism adaptive to the toxic environment. For example, adaptation of S. *cerevisiae* to furfural has been reported to increase growth and volumetric ethanol productivity [70, 113, 114]. The other way to solve this problem is to detoxify the pretreatment liquor.

Various physical, chemical and biological methods have been employed to detoxify prehydrolysates by removing or modifying inhibitors. Approaches commonly used include extraction, evaporation, overliming and treatments with other chemicals, and treatments with

microbial and enzymatic biocatalysts. Among those methods, alkaline and ion-exchange are the most effective detoxification methods in terms of removal of potent inhibitors and improvement of fermentation [97, 102]. However, using ion-exchange resulted in over 20% of sugar loss [102]. As a result, alkaline treatment is currently the mostly used approach for detoxifying biomass prehydrolysates.

1.5.1 Physical Detoxification

Physical treatments include vacuum evaporation, extraction, membrane separation and adsorption. Physical detoxification normally tends to remove the inhibitors rather than modify their chemical structures.

Vacuum Evaporation

Volatile inhibitors in prehydrolysates such as acetic acid and furfural can be removed by vacuum evaporation [115]. But the phenolic compounds from lignin degradation, which are usually more inhibitory than the volatiles, cannot be removed using evaporation since they are non-volatile components. As a result, the evaporation method was typically combined with other methods.

Membrane Separation

Electro-dialysis (membrane separation) was reported to remove more than 90% acetic acid with less than 5% loss of sugars [116]. The ABE production from alkaline peroxide wheat straw hydrolysate was remarkably improved (from 2.59 g/L to 22.17 g/L) by employing electro-dialysis [117]. The disadvantage of using membranes is that it is more expensive than other methods of detoxification.

Ion Exchange Resins Extraction

Ion exchange resins can effectively remove 80% of total phenolics, almost all levulinic acid, formic acid, 70% of furfural and HMF [102]. Lu *et al*. demonstrated 68% increasing of ABE production from wood pulping hydrolysate after treatment with ion exchange resins. The main disadvantage of using this method is same as detoxification by membrane which is high cost. In addition, ion exchange resins treatment may lead to up to 20% of sugar loss [102].

Absorption

Activated Carbon and wood charcoal have been studied in detoxification of prehydrolysates due to their excellent absorptivity. It can improve the fermentability of prehydrolysates by removing furans and phenolic compounds but not reduce the fermentable sugar concentrations. Miyafuji *et al*. [118] investigated the factors affecting effect of detoxification by wood charcoal and found the temperature of wood charcoal prepared played an important role in removing inhibitors.

Solvent Extraction

Extraction of inhibitors using chemicals or organic solvents such as ethyl acetate can reduce toxicity of prehydrolysates [115]. Ethyl acetate extraction has been reported to increase the ethanol yield in fermentation by *P. stipitis* from 0 to 93% of reference fermentation due to removal of 56% acetic acid and almost all of furfural, vanillin, and 4-hydroxybenzoic acid [119]. The drawback of this method is the organic solvent is always toxic to downstream fermentation and thus extra effort has to be made to remove the solvent before fermentation.

1.5.2 Chemical Detoxification

Alkaline Detoxification

Alkaline detoxification mainly conducts to convert inhibitors into less toxic compounds at high pH (9-12). Overliming is one of the most common economically viable methods of chemical detoxification. The prehydrolysates are treated with lime (CaO), which becomes $Ca(OH)_2$ when dissolved in liquid, until the pH reaches 10 and the pH is readjusted to 5.0-5.5 with H_2SO_4. The precipitated solids are then removed by centrifuge. This method has been described as early as 1945 by Leonard and Hajny [120]. It is effective in reducing phenolic aldehydes, ketones and furans. Other alkaline such as NH_4OH, KOH and NaOH can also be used as alkaline treatment reagents. The lime treatment has been reported to result in better fermentability than NaOH due to the precipitation of toxic compounds [121]. Preadjustment to pH 10 with alkaline has been reported to decrease the concentration of Hibbert's ketones in a dilute acid hydrolysate of spruce by 20-30%, and the concentration of both furfural and HMF by 20% [122]. Although overliming is proved to be effective in detoxification of numerous hydrolysates, particularly in bioethanol production, it is found no improvement in butanol fermentation [123, 124]. To our knowledge, few studies applying overliming as a single detoxification method has reported to satisfy the butanol fermentation and the reason behind is not clear.

Although the mechanism of overliming is not completely understood, significant progress has been made. The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH [121, 125]. The chemical

conversion of toxic compounds was considered to be more critical, rather than the inhibitor removal by precipitation [125].

One of the problems of alkali detoxification is the sugar loss during the treatment. A glucose loss of 12% at pH 10 was observed with overliming and it increased with increasing of pH [126]. Nilvebrant *et al*. [122] found that during treatment with alkali, xylose was slightly more easily degraded than the other monosaccharides. Under similar conditions (treatment time, temperature, and pH), the sugar loss with lime was greater than that of sodium hydroxide. The other drawback of overliming is the potential to dispose gypsum precipitate ($CaSO_4$) formed though the process [127].

Reducing Agent

A variety of reducing agents including dithionite, hydrogen sulfite and dithiothreitol have been applied into detoxification of lignocellulose hydrolysates. It is performed at low temperature and slightly acidic pH, allowing the development of in-situ detoxification [128]. It was noticed little sugar was degraded by treating with dithionite and sulfite [128]. Moreover, detoxification with reducing agents could also alleviate inhibition effect in enzymatic hydrolysis [129]. The mechanism behind treatment with sulfur oxyanions such as sodium dithionite and odium sulfite was studied; Cavka *et al*. [130] found that the detoxification effect was attributed to reaction or sulfonation of inhibitors, which rendered them unreactive and highly hydrophilic.

Conducting detoxification by reducing agent as single method is not satisfactory in some cases. Therefore, it is always carried out by combining with alkaline detoxification. Qureshi *et al*. [123, 131] observed poor or no ABE fermentation from the hydrolysates from corn stover and barley straw. The detoxification of overliming followed by Na_2SO_3 significantly improved the

ABE yield. Similarly, it was found that when sulfite was introduced to overliming process, the fermentation of willow hemicellulose prehydrolysates was more efficient. The complete fermentation time was shortened to 11 h, comparing to 24% xylose consumption at 40 h [132].

1.5.3 Biological Detoxification

Biologically, many fermenting microorganisms are able to reduce furan aldehydes to the less toxic corresponding alcohols, which are often referred to as in-situ detoxifications [69, 97, 133]. Enzymes, such as laccases and peroxidases, can also detoxify prehydrolysates by oxidizing phenolic compounds to radicals that undergo coupling to larger molecules which are less toxic [84].

Peroxidase and laccase

Treatment with the enzymes peroxidase and laccase obtained from white-rot fungus *Trametes versicolor* has been shown to increase the ethanol productivity in a hemicellulose hydrolysates of willow [84]. This approach specifically removes the phenolic compounds which are considered to be the most toxic. The laccase led to an increase amount of large molecular weight materials and a decrease in small molecular weight materials. Thus, the mechanism of this method was proposed as oxidative polymerization of low molecular weight phenolic compounds [84]. The main disadvantage of this method is the cost of preparing enzyme is higher than most of the other strategies.

Trichoderma reesei

Trichoderma reesei has been reported to significantly increase the ethanol productivity and yield in a hemicellulose hydrolysates obtained after steam pretreatment of willow [134].

Different from the treatment with laccase, treatment with *T. reesei* resulted in a 30% decrease in absorbance at 280 nm whereas no decrease observed after laccase treatment, indicating that the mechanisms of these two detoxifications were different. Acetic acid, furfural and benzoic acid derivatives were removed from the hydrolysate by the treatment with *T. reesei* [134].

The effect of representative detoxification methods is shown in **Table 1-5**. They cannot be simply evaluated because the inhibitory compounds present in hydrolysates are depending on the type of biomass and the pretreatment conditions and the tolerance of microorganism to different inhibitors is various. Therefore, the detoxification is a selective process and it is difficult to find a standard approach to satisfy all the microbial fermentation of lignocellulose hydrolysates. In addition, not all the potential inhibitors have been identified so far. It is possible some degradation compounds at trace amount, or their synergistic inhibitory effect, are dominant to the toxicity of prehydrolysates. Hence, continuing work to identify potential inhibitors and understand their inhibitory profile is still essential for enhancing the improvement of detoxification strategies.

Table 1-5 Effect of detoxification strategies applied to lignocellulose hydrolysates

	Method	Source of hydrolysate	Inhibitor removal	Improvement on fermentation	Reference
Physical	Evaporation	Corn stover	Furfural (100%), acetic acid (78%), Formic acid (60%), HMF (44%), Levullinic acid (10%)	Elimination of 48-hour lag phase	[135]
	Electro-dialysis	Wheat straw	Salts (91%)	Seven-fold increase of ABE production	[117]
	Ion exchange resin extraction	Mixed hardwood	Acetic acid (100%), Formic acid (27%), Levulinic acid (25%), HMF (100%), Phenolics (58%)	68% increase of ABE production	[124]
	Activated carbon adsorption	Mixed hardwood	Acetic acid (74%), Formic acid (30%), Levulinic acid (100%), Furfural (80%), HMF (88%), Phenolics (100%)	33% increase of ABE production	[124]
	Activated carbon adsorption	Switchgrass	Complete removal of HMF, Furfural, Coumaric acid, Syringic acid, Vanillin and Vanillic acid, Cinnamaldehyde (92%)	Ten-fold increase of butanol production (From 1 g/L to 11 g/L)	[136]
	Ethyl acetate extraction	Aspen	Acetic acid(56%) , all furfural, vanillin and 4-hydrolybenzoic acid	Ethanol yield increasing from 0 to 93% of control	[119]

Table 1-5 (Continued)

	Method	Source of hydrolysate	Inhibitor removal	Improvement on fermentation	Reference
Chemical	Overliming	Corncob	HMF (96%), Furfural (98%), Vanillin (60%), Acetic acid (100%), Levunillic acid (34%), Fefulic acid (33%)	Three-fold increase of ABE production (From 3.8 g/L to 16 g/L)	[137]
	Membrane filtration +Overliming	Maple	HMF (100%), Furfural (100%), Formic acid (100%), Acetic acid (92%), Phenol (71%)	Eight-fold increase of butanol production (From 0.8g/L to 7 g/L)	[138]
	Overliming+Na_2SO_3	Barley straw	NA	2.5-fold increase of ABE production (From 7 g/L to 27 g/L)	[131]
Biological	Pervaporation +Laccase	Sweet sorghum bagasse	Furfural (95%) and Phenolics (88%)	12g/L butanol was produced which is comparable to glucose control	[139]
	Trichoderma reesei	Spruce	Furfural (85%), HMF (25%), Phenolics (6%)	34% increase of ethanol production	[86]

1.6 Advances and Challenges of Bioconversion (Lactic Acid and Butanol Production) from Lignocellulosic Biomass

Lactic Acid (LA) is an important industrial commodity with large and fast growing market due to its versatile applications in food industry, cosmetics, pharmaceuticals and textile [140, 141]. Nowadays, its most dominant application is polymerization to biodegradable poly-lactic acid which is an environmental friendly material to manufacture plastic [142, 143]. As a result, lactic acid is of great demand and the annual worldwide production of lactic acid in 2008 was 260,000 tons and it is expected to exceed 1,000,000 tons in 2020 [144]. Currently, 90% of the LA worldwide is produced from fermentation and the rest is from chemical synthesis [145]. The primary microbial sources of LA are lactic acid bacteria such as *L. lactis*, *L. debrueckii*, *L. helveticus* and *L. amylophilus* and some filamentous fungi [140, 146]. In the U.S., most of the commercial lactic acid was produced from fermentation of starch-derived glucose or sucrose [146]. The fermentation of these sugars into lactic acid is well established in terms of both the microbiology and the process. However, the limited starch-based feedstock raises the question about competition between energy and food. Therefore, utilization of lignocellulosic biomass as carbon source for LA fermentation has attracted increasing interest [147, 148].

Exploring biofuels from renewable sources in the past decades has driven by the increasing energy demand over current petroleum storage, concern on CO_2 emission and global climate change, and the opportunities to support local economy [149, 150]. The second generation biofuels from lignocellulosic biomass, the most abundant resources on the planet, holds great potential to replace fossil fuels in the future [151]. Butanol, as a potential alternative to gasoline, is superior to ethanol due to its high energy content, low vapor pressure, low volatility, low flammability and the property of being mixed with gasoline in any proportion

[150, 152]. In addition, butanol has similar characteristics to gasoline and thus it can be blended with gasoline or directly used in vehicle with no engine modification required [153]. Butanol fermentation (ABE fermentation) is one of the oldest technologies discovered by Pasteur in 1861 and industrial production from starch was as early as 1913 [154, 155]. The butanol production in industry reached a peak in the 1950s and lost its competitiveness in 1960s mainly due to the increasing substrate cost and the development of petrochemically derived butanol [156]. Nowadays, considering the large energy demand, limited storage of fossil fuels and climate change, a lot of attention and effort have been put into the field of second generation biofuels. *Clostridium acetobutylicum* is the best known solvent-producing bacteria and also the first one discovered for butanol production [155]. Over the years, a large number of solventogenic clostridia have been investigated, including *C. beijerinckii*, *C. aurantibutyricum*, C. *saccharoperbutylacetonicum*, *C. saccharobutylicum*, *C. puniceum*, and *C. pasteurianum* [155]. They have the capacity to digest a wide range of carbon sources, both hexose and pentose, to produce butanol and thus have the potential to increase the efficiency of sugar utilization in terms of this point. The fermentation by anaerobic clostridia typically undergoes two phases, acidogenic and solventogenic, regulated by different enzymes. In the acidogenic phase, the cell grows exponentially with the accumulation of acetic acid and butyric acid, decreasing the initial pH from 6.7 to 4.5 [157]. The acetate and butyrate is re-assimilated in the late exponential and stationary phase, and more butanol was produced during the solventogenic phase [158].

Although biofuels and biochemicals production from lignocellulose has great advantages and appears to be promising, it encounters huge technical and economic difficulties. (1) The cost of feedstock, contributes significantly to the overall economics of biofuels and biochemicals production. It includes the cost of the substrate itself and also the cost from harvest, storage,

transportation and pre-processing. (2) Effective pretreatment, the goal of pretreatment is to disrupt the recalcitrant structure of lignocellulose and thus increase the enzyme accessibility. However, harsh conditions will cause significant sugar loss (particularly hemicellulose) and formation of degradation compounds. Therefore, to develop a cost-effective pretreatment with less sugar loss and inhibitor is critical to biofuels and biochemicals production. (3) Inhibition, although considerable progress has been made in identifying the inhibitors and reducing/eliminating their inhibition, to fully understand the role of inhibitors and develop an efficient detoxification method is still a bottleneck in commercializing cellulosic LA and butanol production.

The butanol fermentation faces specific challenges except the problem listed above. (1) Severe toxicity of butanol, the solventogenic clostridia exhibits poor tolerance to butanol. The concentration of butanol as low as 10 g/L was found to be inhibitory to cell growth, resulting in low final butanol concentration and yield. It is proposed to be solved in two ways: one is to develop solvent-tolerant strain using genetic and metabolic engineering techniques [159]. Both chemically mutated and genetically engineered solvent producing clostridia have been recorded in literature [160]. The other way is to investigate efficient separation and recovery process. Perstraction, adsorption, gas stripping, liquid-liquid extraction, and pervaporation as separation technologies were extensively studied [158, 161]. Furthermore, process engineering strategies to integrate butanol recovery system into fermentation, which is known in-situ recovery, is successful by controlling the butanol concentration in the reactor not beyond the culture's tolerance. By applying this approach, the total ABE produced was up to 461 g/L [162]. (2) Degeneration of Clostridia, the culture may lose the ability to produce solvent due to the continuous cultivation [163]. The degeneration problem is commonly reported and research has

been conducted to stabilize the strain [156, 163, 164]. (3) Low butanol yield, in general, up to 20-25 g/L ABE is accumulated during the fermentation course of 36-72 h [152]. This problem may be overcome by developing metabolically engineered strain to maximize carbon flux to butanol production [165-167]. (4) High recovery cost, resulting from the low butanol titer, is one of the major economic obstacles. The solutions for addressing the challenges of low butanol yield and poor culture tolerance to solvent may offer the great opportunity to solve this issue.

1.7 Research Objectives

As mentioned previously, to understand the role of inhibitors played in microbial growth and fermentation and develop cost-effective detoxification approach is essential to biochemical conversion of lignocellulosic biomass. It is believed the inhibition effect greatly depends on chemical structure and functional groups of degradation compounds. Extensive studies have been carried out on the effects of model inhibition compounds on ethanol fermentation. It was reported that the inhibition was closely related to their structures: terpenes > aldehydes > polyhydroxy aromatics [120]. Zaldivar *et al*. [168-170] also found the inhibitory activity of furans and phenolic compounds depended on the functional groups on aliphatic sidechain: aldehydes > acids > alcohols. However, few studies have been performed on a different microbial fermentation except ethanol producing strain. So, the first objective of this study was to understand the effect of model inhibitors on lactic acid and butanol fermentation. It will be helpful to reveal the truth from a universal view and add more evidence to the activity-inhibition relationship. In addition, the Quantitative Structure-Activity Relationship (QSAR) analysis will be performed to gain a better understanding towards the mechanism of inhibition.

Although agriculture residue such as corn stover and switchgrass has attracted increasing attention over the years, woody biomass is still a very perspective feedstock in the field of biochemical conversion. It is available in large quantities in the U.S. and worldwide, it is projected to be produced 370 million tons (dry weight) annually in the U.S., accounting for 30% of total biomass available for biofuels production [171]. Moreover, the property of high density reduces transportation cost and lower pentose content over agricultural substrate leads to relative easy bioconversion [172]. Thus, this study will use the loblolly pine as a feedstock to conduct the bioconversion of biomass to biofuels and biochemicals. The second objective was to study the fermentability of prehydrolysates obtained from organosolv pretreated loblolly pine and find effective detoxification method for butanol production.

After knowing how the structure of potent inhibitors affects microbial fermentation, developing effective detoxification strategies of prehydrolysates, the study of production of biofuels from loblolly pine is needed. Therefore, the third objective was to investigate the butanol production from ethanol organosolv pretreated loblolly pine with both SHF and SSF process. Furthermore, the performance of supplementation of detoxified prehydrolysates to solid fraction is also studied.

Chapter 2

Effect of Carbonyl Inhibitors and Their H_2O_2 Detoxification on Lactic Acid Fermentation

2.1 Abstract

Biomass degradation compounds significantly inhibit biochemical conversion of biomass prehydrolysates to biofuels and chemicals, such as lactic acid. To characterize the structure-activity relationship of carbonyl inhibition on lactic acid fermentation, we examined effects of eight carbonyl compounds (furfural, 5-hydroxymethyl furfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, phthalaldehyde, benzoic acid and pyrogallol aldehyde) and creosol on lactic acid production by *Lactobacillus delbrueckii*. Pyrogallol aldehyde reduced the cell growth rate by 35% at 1.0 mM and inhibited lactic acid production completely at 2.0 mM. By correlating the molecular descriptors to the inhibition constants in lactic acid fermentation, we found a good relationship between the hydrophobicity (Log *P*) of aldehydes and their inhibition constants in fermentation. The inhibitory effect of carbonyl inhibitors appeared to correlate with their thiol reactivity as well. In addition, we found that H_2O_2 detoxified pyrogallol aldehyde and phthalaldehyde inhibitory activity. H_2O_2 detoxification was applied to real biomass prehydrolysates in lactic acid fermentation.

2.2 Introduction

Developing renewable chemicals from lignocellulosic biomass has great potential to reduce the dependence on fossil fuel and address the environmental issues. Lactic acid is a useful chemical; it has been widely used in food, cosmetic and pharmaceutical industries. However, production of lactic acid from biomass has encountered at least two major challenges. One is that most microbial strains cannot ferment xylose effectively [173]. The other is the fermentation

inhibition by compounds generated from the degradation of cellulose, hemicellulose, lignin and extractives [174]. Many of these degradation compounds significantly inhibit the microbial growth and fermentation productivity [175].

Although various inhibitors in biomass hydrolysates have been identified with analytical tools, the most potent inhibitors have not been elucidated yet [174, 175]. Common identified inhibitors include aliphatic acids (such as acetic, levulinic and formic acids), furans (furfural and 5-hydroxymethyl furfural), phenolic aldehydes (vanillin, 4-hydroxybenzaldehyde and syringaldehyde), aromatic acids (ferulic, syringic, 4-hydroxybenzoic and protocatechuic acids) and phenols (catechol and conifery alcohol). Of these inhibitors, the inhibitory effect of acetic and benzoic acid on ethanol fermentation by *S. cerevisiae* appeared to pH dependent [176, 177]. Acetic, ferulic, syringic and 4-hydroxybenzoic acids have been evaluated for their inhibitory effects on *Z. mobilis* [178] and *E. coli* LY01, and it was reported that their inhibition was strongly correlated to hydrophobicity [170]. Aromatic aldehydes showed more toxic than furfural and HMF on *E. coli* KO11 [168]. Catechol and conifery alcohol have also been tested for their inhibition on *S. cerevisiae* and *E. coli.* [169, 179]. It was found that phenols were less toxic than aldehydes and aliphatic/aromatic acids [169]. Aldehydes and aliphatic/aromatic acids are carbonyl-containing compounds. The carbonyl group can react with amine group of the proteins by nucleophilic carbonyl addition or by Michael addition [180, 181]. It is believed that the inhibitory effects of carbonyl compounds are ruled by their electrophilic reactivity towards biological nucleophiles [180]. The electrophilic reactivity can be characterized by some molecular descriptors such as hydrophobicity, molar refractivity, dipole moment, energy of the lowest unoccupied molecular orbital (E_{LUMO}), energy of the highest occupied molecular orbital (E_{HOMO}) and electrophilicity index (ω) [182, 183]. Previous studies revealed that there was a

strong correlation between E_{LUMO} and carbonyl inhibition on ethanol fermentation by *S. cerevisiae* [177].

However, little is known about the structure-activity relationship of carbonyl inhibition on lactic acid production by *L. delbrueckii*, although a few preliminary studies have investigated the inhibitory effects of degradation compounds on lactic acid fermentation [184].

In this study, to characterize the structure-activity relationship of carbonyl inhibition on lactic acid fermentation, we examined effects of eight carbonyl compounds (furfural, 5-hydroxymethyl furfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, phthalaldehyde, benzoic acid and pyrogallol aldehyde) and creosol on cell growth rates and lactic acid yields by *L. delbrueckii*. The electrophilic reactivity of test carbonyl compounds was determined based on the reaction rate between inhibitory compounds and glutathione (GSH) [181]. Quantitative structure-inhibition relationship (QSIR) of carbonyl aldehydes between the inhibition constants and molecular descriptors was established. We further investigated the H_2O_2 detoxification for model compounds (pyrogallol aldehyde and phthalaldehyde) and real biomass prehydrolysates in lactic acid fermentation.

2.3 Materials and Methods

Chemicals and medium

4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin) and 2, 3, 4-trihydroxybenzaldehyde (pyrogallol aldehyde) were purchased from Acros Organics (Morris Plains, NJ). Furfural and 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde) were purchased from Aldrich (Milwaukee, WI). 5-hydroxymethylfurfural (HMF) was purchased from Toronto Research Chemicals (North York, ON, Canada). *o*-Phthalaldehyde was purchased from

Pickering Laboratories (Mountain View, CA). Benzoic acid, 2-methoxy-4-methylphenol (creosol) and glucose (anhydrous) were purchased from Alfa Aesar (Ward Hill, MA). Reduced glutathione (GSH) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Alfa Aesar (Heysham, England). Catalase (from bovine liver) and ethanol were purchased from sigma-Aldrich (St. Louis, MO). Dimethyl Sulfoxide (DMSO) and hydrogen peroxide (30% w/w) were purchased from VWR (West Chester, PA). Lactobacillus MRS Broth was purchased from Himedia Laboratories. Soy peptone (Bacteriological grade) was purchased from Amresco (Solon, Ohio). K_2HPO_4, KH_2PO_4, NaAc, sodium citrate, $MgSO_4$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot H_2O$, NaOH and H_2SO_4 were purchased from Fisher Scientific (Fair Lawn, NJ). $CaCO_3$ was purchased from EMD chemicals (Gibbstown, NJ).All chemical reagents were of chromatographic grades.

Microorganisms and fermentation

Lactobacillus delbrueckii was the strain for lactic acid production. It was stored in 50% glycerol at -25°C. The *Lactobacillu*s MRS broth was used as the medium for the seed culture, which contains 20 g/L glucose, 10 g/L protease peptone, 10 g/L beef extract, 10 g/L yeast extract, 1.0 g/L polysorbate 80, 2.0 g/L ammonium citrate, 5.0 g/L CH_3COONa, 0.1 g/L $MgSO_4$, 0.05 g/L $MnSO_4$ and 2.0 g/L KH_2PO_4. The medium for lactic acid fermentation contained 20 g/L glucose, 10 g/L soy peptone, 2 g/L K_2HPO_4, 2 g/L KH_2PO_4, 2 g/L NaAc, 2 g/L sodium citrate, 0.5 g/L $MgSO_4$, 0.12 g/L $MnSO_4 \cdot H_2O$ and 0.05 g/L $FeSO_4 \cdot H_2O$. $CaCO_3$ (12 g/L) was also added into medium to keep the pH at neutral. All the media were autoclaved at 121°C for 15 min.

L. delbrueckii from agar plate was inoculated to 50 mL medium and cultured for 24 h, then transferred to 100 mL of MRS broth and cultivated for 18 h as seed inoculum. The cultured bacteria (2 mL, OD_{600}=0.7) was inoculated into 50 mL fermentation medium. All the

fermentations were carried out at 37 °C with shaking at 150 rpm. Testing compounds (**Fig. 2-1**) were dissolved into 50% alcohol individually except for furfural, 2-methoxy-4-methylphenol and benzoic acid which were added into media directly. All stocks were kept at dark and stored at 4°C for no longer than a month. Various concentrations of inhibitory compound were added into fermentation broth prior to inoculation. All fermentations were carried out in duplicates.

To quantify and assess the inhibitory effects of these compounds on microbial growth and fermentation, we studied the growth kinetics of *L. delbrueckii* in batch fermentation. The specific cell growth rate (μ) was estimated from the beginning of the fermentation to the time when the cell biomass reached maximum by the following equation:

$$\frac{dX}{dt} = \mu X \qquad \text{Eq. 2-1}$$

Where X is the cell concentration and t is time. The integration form of above equation is:

$$X = exp^{(\mu t + a)} \qquad \text{Eq. 2-2}$$

Where a is a constant. A natural logarithm transformation was applied to equation 2-2 and then the specific cell growth rate was calculated by a liner-regression on log X and t. A noncompetitive inhibition model was used to estimate inhibition constant (K_I) which is able to indicate the inhibition effect of each compound on cell growth [185]. The K_s (saturation constant) typically is about 0.1-0.3 g/L [186], which was much less than S (20 g/L) in this fermentation. Subsequently, K_I could be calculated by non-linear regression of the following equation 2-3 ($S >> K_s$).

$$\mu = \frac{\mu_m}{(1+\frac{K_S}{S})(1+\frac{I}{K_I})} \approx \frac{\mu_m}{1+\frac{I}{K_I}} \quad \text{Eq. 2-3}$$

Where μ_m is maximum specific cell growth rate, I is the inhibitory compound concentration and K_I is the inhibition constant. Software SAS 9.3 (SAS Institute Inc., Cary, NC) was used to carry out the regression and statistical analysis.

We also calculated volumetric productivity of lactic acid (Q) and the final lactic acid yield (Y) to assess the inhibitory effect on lactic acid production.

$$Q = (C_{lac}^{t} - C_{lac}^{0})/t \quad \text{Eq. 2-4}$$

$$Y = (C_{lac}^{48} - C_{lac}^{0})/C_{glu}^{0} \quad \text{Eq. 2-5}$$

Where $C_{lac}^{0}\ and\ C_{lac}^{t}$ were the lactic acid concentration at 0 and t h, respectively, t was the least time when lactic acid concentration reached the maximum. Where $C_{lac}^{48}\ and\ C_{glu}^{0}$ were the lactic acid concentration at 48 h and the glucose concentration at 0 h, respectively.

HPLC analysis

L. delbrueckii cell mass was measured by an UV-vis spectrometry at 600 nm. Glucose and lactic acid concentration were quantitated by a Shimadzu (LC-20A) HPLC system consisting of an autosampler, LC-20AD pump, and RID-10A detector, with a 300 mm × 7.8 mm i.d., 9 µm, Aminex HPX-87H column, and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio-Rad, Hercules, CA). The mobile phase was composed of 5 mM sulfuric acid running isocratic at 0.6 mL/min. The column temperature was maintained at 45 °C throughout the run. The sugars concentration of pretreated biomass hydrolysates was quantitated by the same HPLC system with

the Aminex HPX-87P column. The mobile phase used nanopure water running at 0.6 mL/min. The column temperature was kept at 85 ºC.

Glutathione reactivity assay

To quantify the thiol reactivity of potential inhibitory compounds, the non-enzymatic reaction rate of glutathione (GSH) with model compounds was measured as described previously [187]. Briefly, the GSH solution was prepared freshly for each experiment and all reactions were performed at 40°C except the reaction with *o*-phthalaldehyde at 25°C. The final concentrations of GSH and test inhibitors were 0.084 mM and 5.0 mM, respectively. The total reaction volume was 9.8 mL. After 2-60 min reaction, 0.2 mL of DTNB was added to the reaction and the absorbance at 412 nm was determined. Free GSH was quantified based on the standard curve. The reaction without GSH/inhibitors was used as a blank and the reaction with only GSH was used as a control. To remove the potential interference from inhibitors, a negative control (only inhibitors) was also used. For the reaction with *o*-phthalaldehyde, the final concentration of GSH was 0.05 mM and *o*-phthalaldehyde was 1 mM.

To estimate the thiol reactivity of inhibitors with GSH, the reaction rate (k_{GSH}) was calculated using the following equation [180, 187, 188].

$$k_{GSH}^{pseudo} = (lnC_{GSH}^{0} - lnC_{\mathrm{GSH}}^{t})/t \qquad \text{Eq. 2-6}$$

$$k_{GSH} = k_{GSH}^{pseudo}/C_{I}^{0} \qquad \text{Eq. 2-7}$$

where C_{GSH}^{o} is the initial GSH concentration, C_{GSH}^{t} is the final concentration of GSH, t is the reaction time and C_{I}^{o} is the initial concentration of inhibitors. All experiments were run in duplicates.

Calculation of physicochemical descriptors

The physicochemical properties of model inhibitory compounds were calculated using open software MarvinSketch for the hydrophobicity (log *P*) and steric parameter, molecular refractivity. The E_{LUMO}, E_{HOMO} and dipole moment (*u*), were estimated through Density Functional Theory (B3LYP/6-31G) calculations (Gauss 9.0 and GaussView 5.0). Together, the molecular electrophilicity index (ω) was calculated by the following equation [182, 189]:

$$\omega = \frac{\mu^2}{2\eta} = \frac{E_{HOMO}{}^2 + 2E_{HOMO}E_{LUMO} + E_{LUMO}{}^2}{4(E_{LUMO} - E_{HOMO})} \qquad \text{Eq. 2-8}$$

where μ is the molecular chemical potential and η is the molecular hardness.

Statistical analysis and structure-inhibition relationships

Statistical calculation of correlations between physiochemical parameters and inhibition constant was carried out by regression analysis using SAS 9.3 (SAS Institute Inc., Cary, NC). The statistical values include: *n*, the number of observations; *s*, the standard error of the estimate; r^2, the coefficient of determination; *F*, Fisher statistic; and *p*, the significance. A value of $p < 0.05$ indicated that the correlation was significant.

Dilute acid pretreatment and biomass prehydrolysates detoxification

Loblolly pine wood chips were collected and those with size of 1cm ×1cm were used for acid pretreatment. They were soaked in 1 % (w/w) sulfuric acid overnight (7:1 liquor/solid ratio) and then loaded into Parr reactor to be treated at 170 °C for 60 min. Prehydrolysates were stored at 4 °C before use. To detoxify model compound, 0.2% H_2O_2 (w/w) was used to oxidize the *o*-phthalaldyde (5.0 mM) and pyrogallol aldehyde (3.0 mM) at pH 2.0. Briefly, 1-2 mL of

phthalaldehyde or pyrogallol aldehyde stock solution (0.5 M) was mixed with 120 mL of DI water, then added 0.45 mL of H_2O_2 (50% w/w). The pH of resulting solution was adjusted to 2. The detoxification reaction was kept at 80°C for 2 h. After the reaction, the solution was cooled to room temperature and adjusted to pH 7.0. Then, 2.0 mg of catalase was added to remove any remaining H_2O_2 and incubated overnight. After that, the detoxified solution was supplemented with glucose (20 g/L) and other nutrients. This prepared solution was sterilized through a 0.2 μm membrane filter into a serum bottle.

For the real biomass prehydrolysates, H_2O_2 detoxification was done at pH 2.0 and pH 6.0 as described above. For alkaline detoxification, the prehydrolysates was conditioned pH at 10 with NaOH and reacted at 80 °C for 2 h.

2.4 Results and Discussion

2.4.1 Effects of Carbonyl Compounds on Lactic Acid Fermentation and Cell Growth

To examine the inhibitory effects of carbonyl aldehydes on bacteria growth and lactic acid fermentation, furfural, HMF, vanillin, 4-hydroxybenzaldehyde, *o*-phthalaldehyde, syringaldehyde, and pyrogallol aldehyde (**Fig. 2-1**) were added into *L. delbrueckii* fermentation. Furfural and HMF at 2.5 - 15 mM did not inhibit the lactic acid yield (~0.76 g/g), but reduced it by 75-83% at 25 mM (**Table 2-1**). Likewise, the productivity (0.61 g/L/h) was not affected by furfural and HMF at 2.5-7.5 mM, but reduced by 31% at 15 mM, by 88% and 92% at 25 mM. Similarly the cell specific growth rate (0.17 h^{-1}) was not inhibited by furfural and HMF at 2.5 - 7.5 mM (**Fig. 2-2**). However at 15 mM, they inhibited the cell growth rate by 35% and 24%, respectively, and at 25 mM, by 82% and 88%, respectively. Based on the inhibition of carbonyl compounds at various concentrations on the cell specific growth rate, the inhibition constant for

each compound can be estimated using equation 2-3. For furfural and HMF, they are 17.63 and 17.26 mM^{-1}. This indicated that furfural and HMF had similar low inhibitory activity on cell growth in lactic acid fermentation.

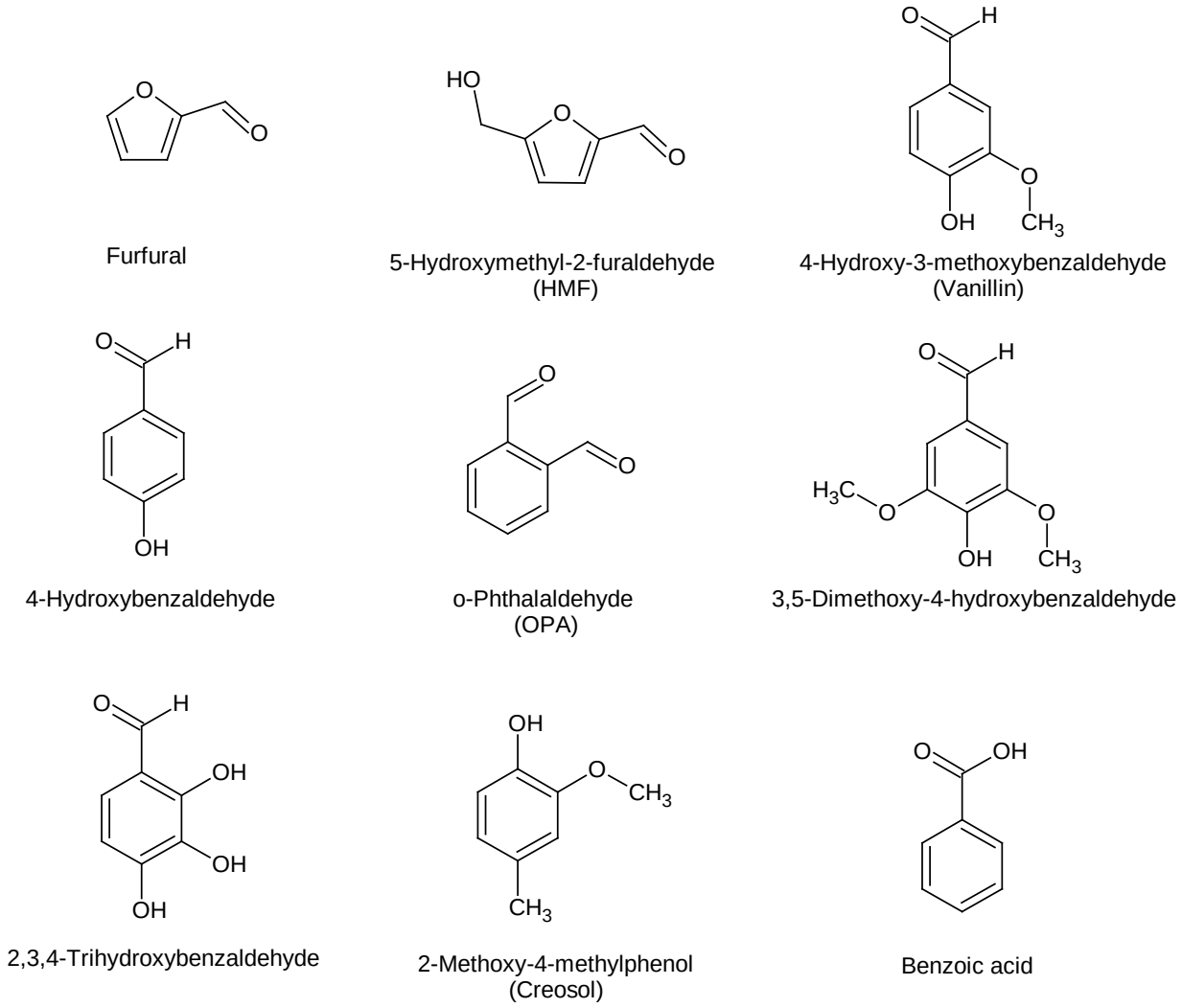


Figure 2-1 Structure of tested carbonyl compounds

Table 2-1 Effects of model inhibitory compounds on lactic acid fermentation

Compounds	**Concentration (mM)**	***C*[a] (g/L)**	***Q*[b] (g/L/h)**	***Y*[c] (g/g)**	***μ*[d] (h^{-1})**	***K_I*[e] (mM^{-1})**
Glucose control	0.0	14.96±0.47	0.61±0.02	0.76±0.04	0.17±0.04	
Furfural	2.5	14.87±0.24	0.61±0.00	0.75±0.02	0.17±0.02	17.63
	7.5	14.47±0.61	0.59±0.01	0.73±0.04	0.17±0.01	
	15.0	15.65±0.14	0.42±0.00	0.80±0.00	0.11±0.01	
	25.0	4.42±0.66	0.08±0.01	0.19±0.04	0.03±0.01	
HMF	2.5	14.47±0.02	0.61±0.01	0.73±0.01	0.17±0.02	17.26
	7.5	14.93±0.23	0.61±0.01	0.76±0.01	0.16±0.02	
	15.0	15.68±0.25	0.42±0.01	0.79±0.01	0.13±0.02	
	25.0	3.40±0.31	0.05±0.01	0.13±0.02	0.02±0.01	
Vanillin	2.5	15.45±0.36	0.62±0.01	0.79±0.01	0.17±0.03	12.95
	5.0	15.41±0.29	0.61±0.02	0.79±0.01	0.17±0.02	
	10	15.76±0.35	0.41±0.00	0.81±0.01	0.12±0.01	
	25	2.44±0.33	0.03±0.00	0.09±0.00	0.02±0.00	
4-Hydroxybenzaldehyde	2.5	15.37±0.11	0.62±0.01	0.75±0.00	0.17±0.02	7.15
	5.0	15.41±0.07	0.50±0.00	0.77±0.01	0.14±0.01	
	10.0	4.21±0.11	0.07±0.00	0.18±0.01	0.03±0.01	
	25.0	2.10±0.04	0.03±0.00	0.07±0.00	0.03±0.01	
o-Phthalaldehyde	0.25	15.17±0.11	0.61±0.00	0.76±0.01	0.17±0.06	6.42
	0.75	15.28±0.16	0.61±0.01	0.76±0.00	0.17±0.05	
	2.5	15.24±0.03	0.61±0.01	0.77±0.01	0.17±0.02	
	5.0	6.92±0.89	0.12±0.02	0.32±0.04	0.06±0.02	
Syringaldehyde	0.25	15.12±0.13	0.62±0.00	0.75±0.00	0.17±0.04	6.33
	1.0	14.73±0.03	0.60±0.02	0.74±0.01	0.17±0.03	
	3.0	14.77±0.21	0.48±0.01	0.75±0.03	0.13±0.01	
	5.0	14.87±0.31	0.30±0.01	0.76±0.00	0.08±0.01	
Pyrogallol aldehyde	0.25	16.11±0.89	0.60±0.01	0.80±0.03	0.17±0.01	1.97
	1.0	15.91±0.10	0.30±0.00	0.79±0.03	0.11±0.02	
	2.0	1.14±0.01	0.00±0.00	0.00±0.00	0.00±0.00	
	3.0	1.16±0.06	0.00±0.00	0.00±0.00	0.00±0.00	
Benzoic acid	2.5	14.81±0.00	0.60±0.00	0.76±0.00	0.13±0.03	16.12
	15.0	15.14±0.13	0.48±0.02	0.78±0.00	0.10±0.02	
	25.0	10.73±1.10	0.21±0.02	0.55±0.05	0.05±0.01	
Creosol	2.5	15.41±0.23	0.60±0.01	0.77±0.02	0.16±0.01	7.49
	5.0	15.57±0.15	0.40±0.01	0.78±0.01	0.11±0.01	
	7.5	15.41±0.10	0.29±0.00	0.78±0.02	0.08±0.01	
	10.0	7.58±0.42	0.13±0.01	0.35±0.02	0.06±0.00	

[a] The lactic acid concentration was determined at 48 h.
[b] The lactic acid productivity was calculated when lactic acid concentration reached the maximum.
[c] The lactic acid yield was determined at 48 h.
[d] The specific growth rate was calculated the beginning of the fermentation to the time when the cell mass reached maximum.
[e] The inhibition constant was estimated by non-linear regression

Similarly at low concentration, vanillin (2.5-10 mM), 4-hydroxybenzaldehde (2.5-5.0 mM), *o*-phthalaldehyde (0.25-2.5 mM), syringaldehyde (0.25-5.0 mM), and pyrogallol aldehyde (0.25-1.0 mM) did not present inhibition on lactic acid yield. However at high concentration, vanillin at 25 mM inhibited the lactic acid yield by 88%, *o*-phthalaldehyde at 5.0 mM by 58%. Syringaldehyde did not inhibit the lactic acid yield within 0.25-5.0 mM. Pyrogallol aldehyde at 2.0-3.0 mM inhibited the lactic acid yield completely.

In cell growth, vanillin reduced the cell specific growth rate by 29% at 10 mM, *o*-phthalaldehyde by 65% at 5.0 mM, syringaldehyde by 24% at 3.0 mM, pyrogallol aldehyde by 35% at 1.0 mM (**Fig. 2-2**). More directly, we observed the inhibitory effects of carbonyl compounds on fermentation based on their inhibition constants. The results indicated the most inhibitory compound was pyrogallol aldehyde with a lowest inhibition constant at 1.97 mM^{-1}. Thus, the inhibition by carbonyl aldehydes can be ranked as: Pyrogallol aldehyde > syringaldehyde > *o*-phthaldehyde > 4-hydroxybenzaldehyde > vanillin > HMF > Furfural.

Interestingly, it was noticed that furfural (15 mM), HMF (15 mM), vanillin (10 mM), and pyrogallol aldehyde (0.25 mM) could slightly increase the lactic acid yields by 5%, 4%, 6% and 5% respectively, which were consistent to our previous findings [190], and also agree with that in the literature that ethanol or butanol yields could be enhanced by the addition of weak inhibitors [72, 74, 191, 192].

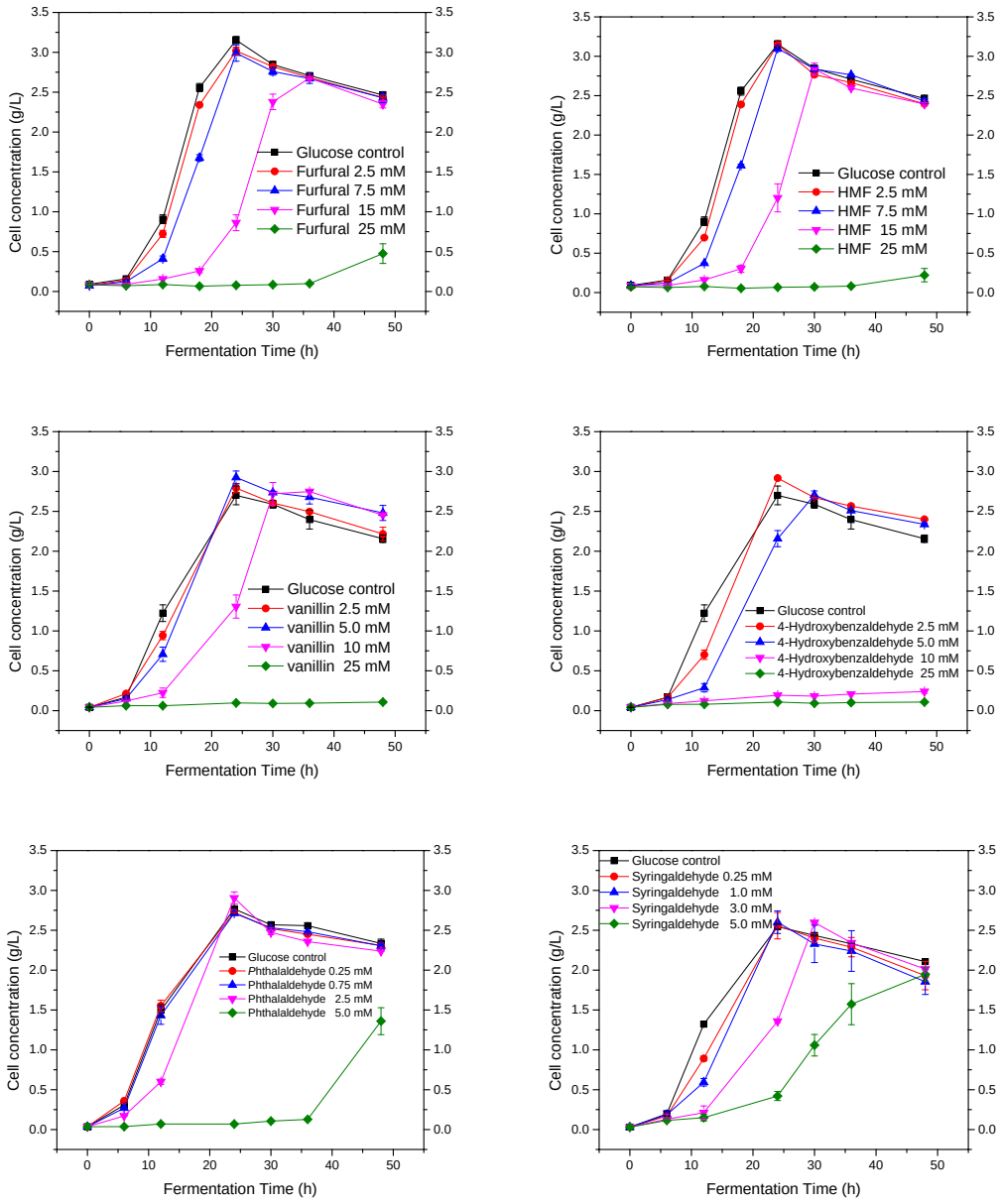


Figure 2-2 Effects of furfural, HMF, vanillin, o-phthalaldehyde and syringaldehyde on cell growth in lactic acid fermentation

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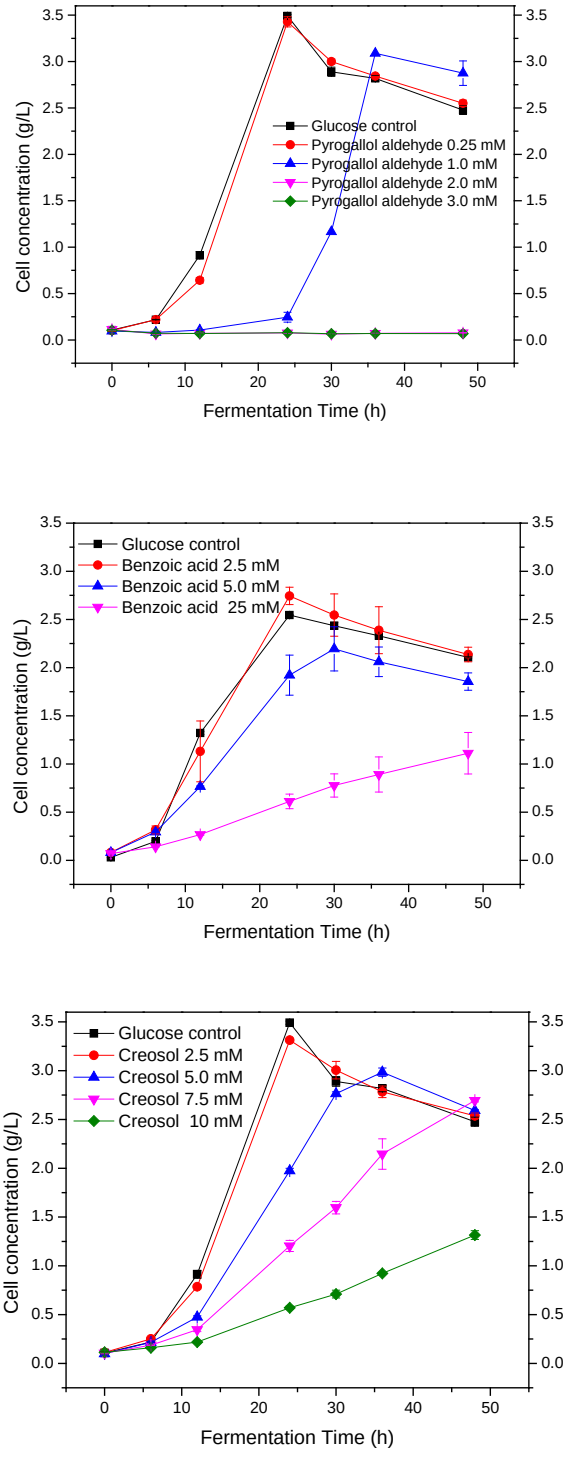


Figure 2-3 Effects of pyrogallol aldehyde, benzoic acid and creosol on cell growth in lactic acid fermentation

Subsequently, carbonyl acid (benzoic acid) and non-carbonyl compound (creosol) were tested for their effects on lactic acid fermentation, which may shed light at a different angle by having a different mode of action. At 25 mM, benzoic acid reduced the lactic acid yield by 28% and decreased the cell specific growth rate by 71%. At 15 mM, it did not change the lactic acid yield, but reduced the productivity by 21% and the cell specific growth rate by 41%. At 2.5 mM, it did not exhibit any inhibition on fermentation. For creosol, it reduced the lactic acid yield by 54% at 10.0 mM; at 2.5, 5.0 and 7.5 mM, it decreased the productivity by 2%, 34% and 52% respectively, and decreased the cell specific growth rate by 6%, 35% and 53% respectively (**Fig. 2-3**).

We believe that the inhibitory effects of carbonyl and phenolic compounds are related to their electrophilic reactivity, which typically governs covalent binding between reactive electrophiles and biological nucleophiles (such as amino acid residues in proteins) [180].

2.4.2 Electrophilic Reactivity of Inhibitory Compounds

To determine the electrophilic reactivity of these inhibitory compounds and to understand the relationship between their reactivity and inhibition, the non-enzymatic rate constants (K_{GSH}) of the reactions between inhibitory compounds and glutathione (GSH) were measured and presented in **Table 2-2**. *o*-Phthalaldehyde has the highest thiol reactivity (K_{GSH} = 557.44 M^{-1} min^{-1}). Syringaldehyde has higher thiol reactivity (K_{GSH} = 11.87 M^{-1} min^{-1}) than any other tested compounds. Vanillin, 4-hydroxybenzaldehyde and benzoic acid displayed similar thiol reactivity (K_{GSH} = 0.6 - 0.7 M^{-1} min^{-1}). The lowest thiol reactivity was from furfural and HMF (K_{GSH}=0.08-0.13 M^{-1} min^{-1}). The K_{GSH} of pyrogallol aldehyde was not measured because the reaction was rapidly turned into dark yellow color after addition of the compound, not suitable for the

Table 2-2 Physicochemical descriptors of inhibitory compounds and inhibition constants

Inhibitors	**Log *P***	**E_{LUMO} (eV)**	**E_{HOMO} (eV)**	**Dipole (Debye)**	**MR**	**ω**	**Thiol reactivity K_{GSH} (M^{-1} min^{-1})**	**Inhibition constant K_I (mM^{-1})**
Furfural	0.75	-2.01	-7.05	3.50	5.03	4.07	0.08	17.63
HMF	-0.10	-2.15	-7.14	4.50	31.73	4.32	0.14	17.26
Vanillin	1.22	-1.68	-6.20	4.05	41.09	3.43	0.71	12.95
4-Hydroxybenzaldehyde	1.38	-1.71	-6.65	2.77	34.62	3.54	0.67	7.15
o-phthalaldehyde	1.44	-2.77	-6.83	5.21	39.23	5.67	557.44	6.42
Syringaldehyde	1.07	-1.66	-5.93	5.50	47.55	3.38	11.87	6.33
Pyrogallol aldehyde	1.43	-1.59	-6.60	5.66	38.58	3.35	N/A	1.97
Benzoic acid	1.63	-0.46	-7.06	2.33	33.31	2.14	0.61	16.12
Creosol	2.03	0.22	-5.50	3.48	39.54	1.22	0.32	7.49

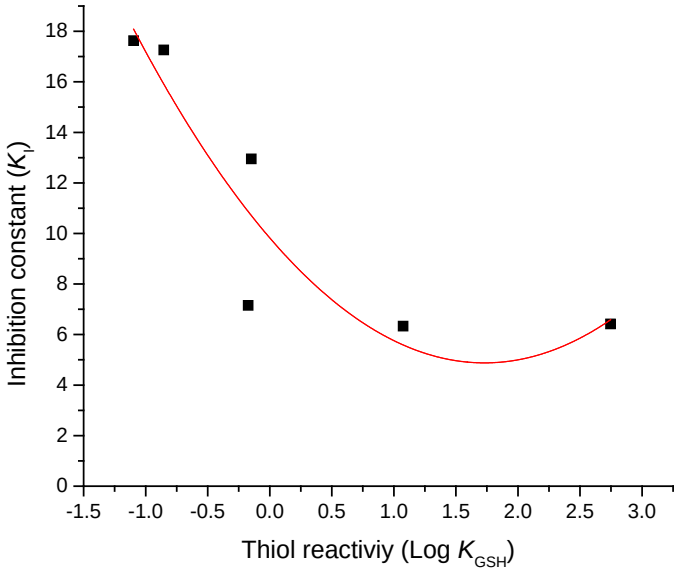


Figure 2-4 Correlation between the thiol reactivity of aldehydes and their inhibition constants

measurement. Shown in **Figure 2-4,** the inhibition of carbonyl aldehydes on lactic acid fermentation was correlated with thiol reactivity. Strong correlation (K_I = 1.65 log K_{GSH}^2-5.72 log K_{GSH} + 9.83, r^2=0.85) has been found between inhibition constant (K_I) and electrophilic reactivity (K_{GSH}) of carbonyls. If benzoic acid and creosol were included in the regression analysis, a weaker correlation (r^2=0.59) was observed.

The electrophilic activities of the aldehydes appear associated with their physicochemical descriptors. Various molecular descriptors have been successfully used to depict chemical reactivity of quinones, unsaturated esters and phenols [180, 193, 194]. The molecular descriptors typically include energy of the lowest unoccupied molecular orbital (E_{LUMO}), energy of the highest occupied molecular orbital (E_{HOMO}) and electrophilicity index (ω), hydrophobicity (Log *P*, octanol/water partition coefficient), molar refractivity, dipole moment [182, 183]. Among those, the electrophilic reactivity (K_{GSH}) of tested aldehydes was correlated well to E_{LUMO} (K_{GSH} = -465.05 E_{LUMO} - 833.39, r^2=0.77, p=0.021). It indicated that thiol reactivity of tested aldehydes probably was governed by the $E_{LUMO,}$ which agrees with the previous report on the electrophilic reactivity of unsaturated esters and acrylates [180].

2.4.3 Quantitative Structure-Inhibition Relationship (QSIR) of Tested Aldehydes

The inhibition of carbonyl aldehydes on lactic acid fermentation was correlated with several molecular descriptors as shown in **Table 2-2 and 2-3**. Good correlation (r^2 = 0.60, p = 0.039) has been found between Log *P* and inhibition constant (K_I) of aldehydes (*Regression equation 1*). Log *P*, which measures the hydrophobicity of a molecule [195], indicates the ratio of a compound partitions between lipid and water phases. The Log *P* of pyrogallol aldehyde has the second highest value at 1.43 among tested aldehydes, which was the most inhibitory

compound in the lactic acid fermentation. The higher hydrophobicity of the inhibitory compounds probably means higher affinities to the cell membranes, thus more binding (or reaction) with enzymes inside cells. This agrees well with the earlier reports on effects of aldehydes on ethanol fermentation [168]. Previously, Zaldivar *et al.* [168, 170] reported that the toxicity of aldehydes and organic acids on ethanolic fermentation by *Escherichia coli* was directly related to hydrophobicity. E_{LUMO} was a global parameter which generally indicates the electrophilic reactivity of a molecule. The E_{LUMO} of *o*-phthalaldehyde has the most negative value at -2.77 eV, but it was not the most inhibitory compound (**Table 2-2**). Not as tight correlation between E_{LUMO} and K_I indicated that the inhibition of aldehydes on lactic acid fermentation is loosely dependent on this molecular descriptor. Previously we have found a strong correlation between E_{LUMO} and inhibition of carbonyls on yeast fermentation [177]. The discrepancy is not clear and probably due to different microorganisms (*S. cerevisiae* vs. *L. delbrueckii*) or different media (basic medium vs soy peptone) where enriched medium in this study have an impact on aldehydes inhibition.

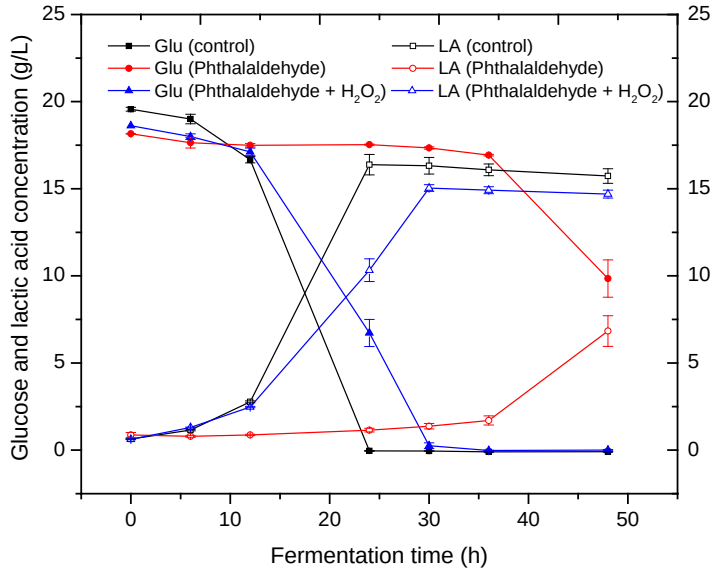
Table 2-3 Regression analysis between inhibition constants and molecular descriptors

Equation#	**Regression**	***n***	r^2	***s***	***F***	***p***
1	K_I = -8.46 log P + 18.65	7	0.60	4.16	7.64	0.039
2	K_I = -2.65 E_{LUMO} + 4.82	7	0.03	7.44	0.18	0.692
3	K_I = -6.66 E_{HOMO} - 34.17	7	0.23	5.79	1.53	0.272
4	K_I = -2.78 *Dipole* + 22.36	7	0.24	5.73	1.66	0.254
5	K_I = -0.29 *MR* + 19.71	7	0.43	5.01	3.70	0.113
6	K_I = 0.94 ω + 6.23	7	0.02	6.55	0.09	0.780
7	K_I = -8.35 log P - 1.09 E_{LUMO} + 16.41	7	0.61	4.61	3.13	0.151
8	K_I = -7.77 log P - 1.75 E_{HOMO} + 6.29	7	0.62	4.57	3.22	0.147
9	K_I = -6.77 log P - 0.19 *MR* + 23.25	7	0.76	3.62	6.32	0.058

Linear regression analysis showed that the dipole moment, ω, E_{HOMO}, and molecular refractivity were weakly correlated to the inhibition constants (**Table 2-3**). The pyrogallol aldehyde showed the highest dipole moments (5.66 Debye), and it was the most inhibitory compound among tested carbonyl compounds. The electrophilicity index (ω) was the square of its electronegativity divided by its chemical hardness [189]. *o*-Phthalaldehyde showed the highest electrophilicity index (5.67), but its correlation with inhibition constants was weak. E_{HOMO} is a global parameter which represents the electron donor capacity of a molecule, but it appeared to be a weak than expected parameter for inhibitory effect of tested aldehydes in the system.

2.4.4 Detoxification of Model Carbonyl Compounds and Prehydrolysates by H_2O_2

To neutralize carbonyl reactivity, H_2O_2 was added (0.2%, w/w) to detoxify model compounds (pyrogallol aldehyde and *o*-phthalaldehyde) and real softwood prehydrolysates (**Fig. 2-5, 2-6 and 2-7**). The results showed that *o*-phthalaldehyde at 5 mM significantly inhibited the lactic acid yield by 58%, while after H_2O_2 detoxification, the growth rate was changed from 0.06 h^{-1} to 0.12 h^{-1} and the final lactic acid concentration was increased from 7.2 g/L to 14.1 g/L, close to that in control (15.1 g/L) (**Fig. 2-5**). This indicated the H_2O_2 could detoxify the *o*-phthalaldehyde effectively. Similar result was observed for pyrogallol aldehyde, after treatment, the growth rate was increased from 0.02 to 0.11 h^{-1} and lactic acid yield increased from 0 to 0.7 g/g (**Fig. 2-6**). The reaction between phthalaldehyde/pyrogallol aldehyde and H_2O_2 was an aldehyde oxidation which converts aldehydes to carboxylic acids.



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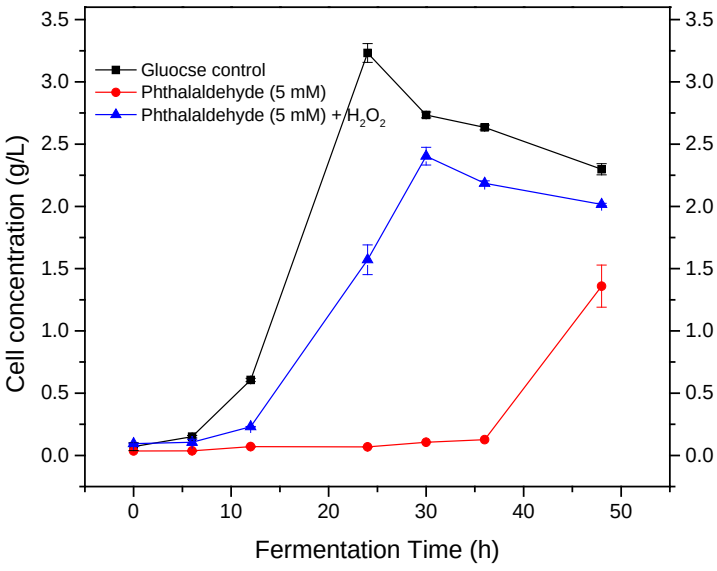
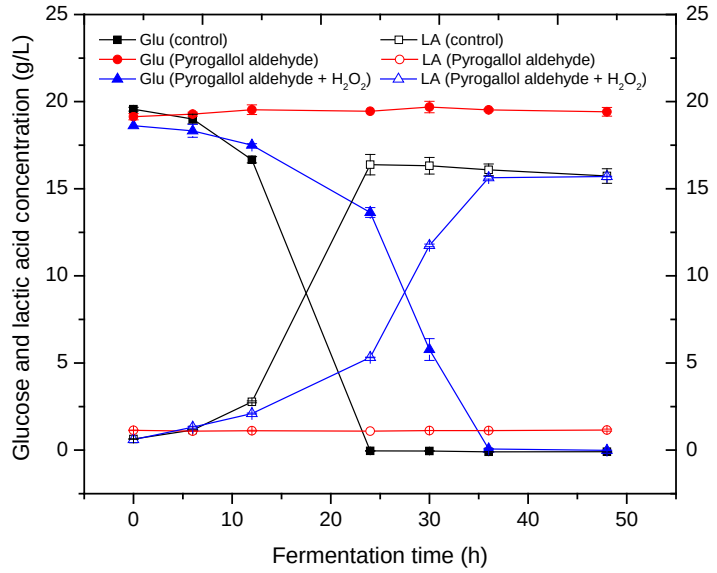


B

Figure 2-5 Effect of H_2O_2 detoxification of o-phthalaldehyde (5 mM) on cell growth and lactic acid production in batch fermentation



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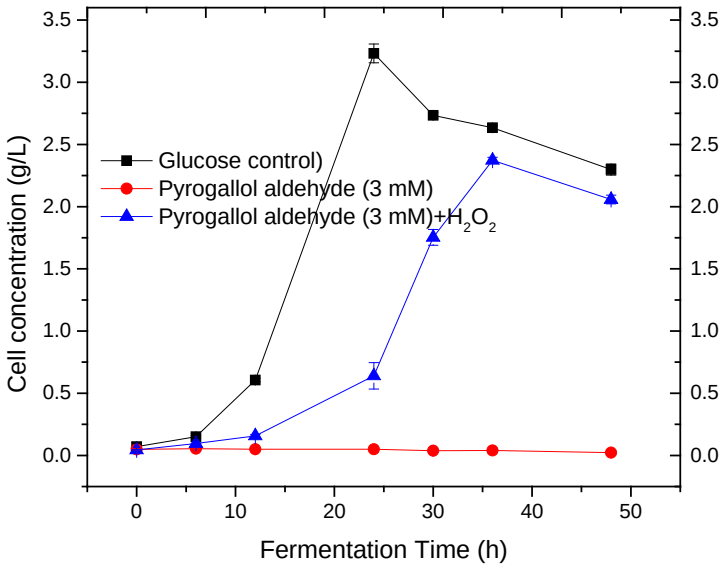

B

Figure 2-6 Effect of H_2O_2 detoxification of pyrogallol aldehyde (3 mM) on cell growth and lactic acid production in batch fermentation

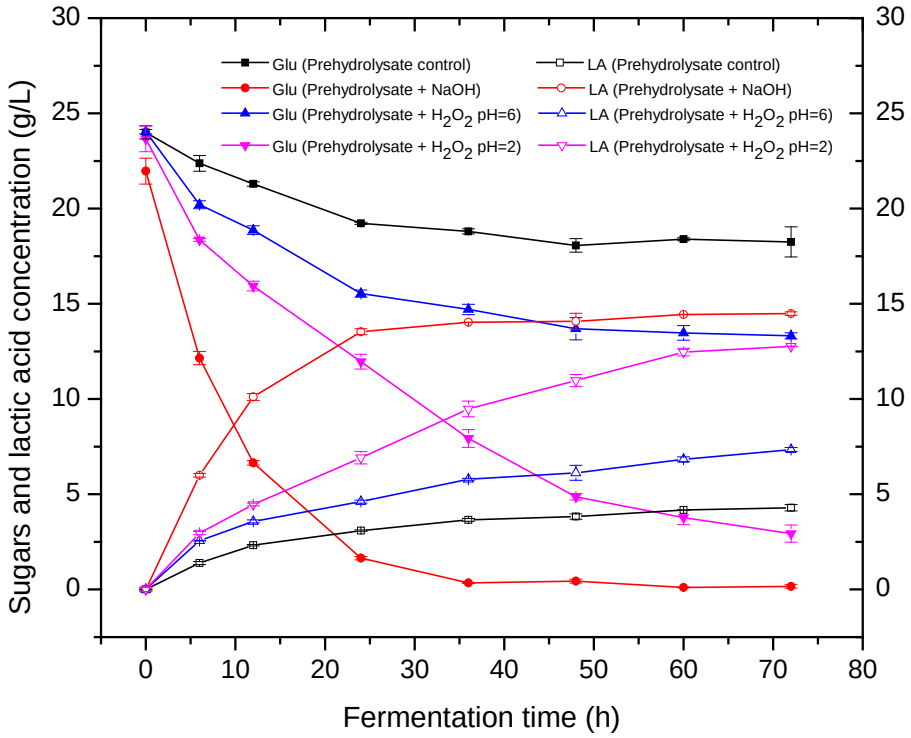

Figure 2-7 Effect of H_2O_2 detoxification on biomass prehydrolysates fermentation (inoculum size, 1.0 g/L)

Biomass prehydrolysates were treated by H_2O_2, the results (**Fig. 2-7**) indicated that H_2O_2 detoxified real biomass prehydrolysates and more than 90% of sugars were fermented at pH 2. Although the detoxification was not as effective as compared to that by NaOH detoxification at pH 10 (produced 14 g/L of lactic acid), but without detoxification, only 5 g/L of fermentable sugars (glucose and mannose) in biomass prehydrolysates were consumed at 72 h, and total 4 g/L of lactic acid was produced at the end of fermentation. When the prehydrolysates were detoxified by H_2O_2 at pH 6.0, around 50% of sugars were fermented and 7.5 g/L of lactic acid was produced. The lactic acid yield was 0.62 g/g under the H_2O_2 detoxification at pH 2.0, which was slightly lower than that (0.66 g/g) from alkaline detoxification. Removal of degradation

inhibitors in biomass prehydrolysates by chemical methods have been extensively studied in the literature [196-198]. For example, alkaline detoxification has been identified as one of the most efficient detoxification methods [199], it improved the ethanol yield by 30-40% and significantly decreased the phenolic compounds [197]. Treatment with $Ca(OH)_2$, known as overliming, resulted in a better ethanol fermentation than by NaOH [197], but the formation of calcium sulfate precipitate (gypsum) generated considerable amount for disposition. Our results showed the sugars loss was much higher in alkaline detoxification (8.5%) than by H_2O_2 detoxification (1.4%). Hodge *et al*. also reported the total sugar loss was up to 11% by overliming treatment [200]. This would make the alkaline treatment less economic for inhibitor removal. Another detoxification method used anion exchange resin (AG 1-X8) at pH 10 was found to increase the ethanol yield and productivity by 50% and 34 times, respectively, but with a significant sugar loss (26%) [197]. Yet another detoxification of hardwood hydrolysates by activated carbon produced 100% of the theoretical ethanol yield with a moderate fermentable sugar loss (8.9%) [201]. These detoxification methods aimed at removal of furfural, HMF, phenolic compounds and weak acids degraded from sugars and lignin. Our study indicated that H_2O_2 detoxification consumes little acid or base as comparing to the alkaline detoxification. It suggested that H_2O_2 detoxification could be an environmentally friendly detoxification method, because no considerable amount of salts are produced in the detoxification process and H_2O_2 can be easily decomposed to water and oxygen by enzyme that leaves no residues behind.

2.5 Conclusions

Carbonyl aldehydes and acids on lactic acid fermentation have been examined based on cell growth rate and lactic acid yield. A good correlation between the inhibition constants of aldehydes and the hydrophobicity (Log *P*) was observed. The study revealed that the inhibitory effects of carbonyl aldehydes on lactic acid fermentation were controlled by their electrophilic reactivity and hydrophobicity. It suggests the removing the electrophilic reactivity of carbonyl compounds or changing the hydrophobicity by H_2O_2 oxidation can detoxify the carbonyl inhibition. Future work is needed to build the QSAR model for various microorganisms with a larger data set.

Chapter 3

Inhibition Effect of Aromatic Aldehydes on Butanol Fermentation

3.1 Abstract

A large number of degradation compounds are formed during pretreatment and they significantly inhibit the efficiency of biomass conversion to biofuels. Of those identified potential inhibitors, aromatic aldehydes played an important role in inhibition activity. Hence the effect of 13 aromatic aldehydes on ABE fermentation was assessed at four concentrations in the present work. It was found that the inhibition severity was affected by the *ortho* substituents (OH > OCH_3 > CHO) and closely related to the position of hydroxyl group instead of the number of hydroxyl groups. The compounds containing *ortho*-hydroxyl group showed the most inhibitory impact on butanol production, indicating the presence of *ortho*-hydroxyl group played an important role in aromatic aldehyde inhibition. The *ortho*- substituted hydroxyl group can form an intramolecular hydrogen bond within the aromatic aldehydes and potentially increased the cell membrane permeability and electrophilicity. Quantitative structure-activity relationship (QSAR) analysis was attempted to establish a correlation between inhibition concentration (IC_{50}) and physicochemical descriptors. A strong linear relationship was observed between IC_{50} and energy of the highest occupied molecular orbital E_{HOMO}.

3.2 Introduction

Lignocellulosic biomass as the most abundant sustainable resource on earth has great potential to produce biofuels. But the degradation compounds derived from sugars and lignin during pretreatment are detrimental to subsequent enzymatic hydrolysis and microbial fermentation and severely limit the efficient utilization of lignocellulose [63, 202]. Due to the

large number and diversity of degradation products, it is challenging to identify toxic compounds and clearly understand which individual components are responsible for low conversion efficiency and whether or not they have synergistic effects towards inhibition.

The species and amount of degradation compounds are feedstock and pretreatment specific [63, 65, 203]. Thirty two degradation products including organic acid and phenolic compounds were found in dilute sulfuric acid hydrolyzed corn stover aqueous phase as mentioned by Chen *et al*. [88]. Luo *et al*. [66] reported more than 35 degradation products in the prehydrolysates from dilute nitric acid treated hybrid poplar and most of them were aromatic aldehydes and acids, aliphatic aldehydes and acids and furan compounds. Du *et al*. [65] applied eight pretreatment methods/conditions on three feedstocks (corn stover, poplar and pine) and quantified 40 potentially inhibitory degradation compounds resulting from these processes. Aromatic monomers (including vanillin, syringaldehyde, cinnamaldehyde, p-hydroxybenzoic acid etc.) were quantitatively identified in steam-exploded poplar by Ando *et al*. [82]. It was also suggested most of these compounds were inhibitory to ethanol fermentation by *S. cerevisiae* and their inhibition severity greatly depended on functional groups (CH=CH, CHO, OH and OCH_3) attached to the benzene ring. Several of these identified compounds were selected added into model fermentation aims to determine these potential inhibitors' contribution to the toxicity of prehydrolysates and the mechanism of their inhibition. Ezeji *et al*. [72] reported 3 g/L furfural or HMF was not inhibitory to *C. beijerinckii* BA101, instead they had a stimulation effect on cell growth and an improvement on ABE production was observed when furfural and HMF was up to 2 g/L. They found ferulic acid and *p*-coumaric acid higher than 1 g/L exhibited complete inhibition on cell growth and ABE production. Cho *et al*. [204] investigated 6 phenolic compounds (*p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, vanillic acid, syringaldehyde,

and vanillin) on butanol fermentation by *Clostridium beijerinckii*. They found little or no butanol was produced in the presence of 1 g/L of each compound. Cao *et al*. [205] found the growth of *T. thermosaccharolyticum* W16 and hydrogen production were stimulated by 5 g/L sodium acetate and negatively affected by further increasing concentration. On the contrary, the fermentation was completely inhibited by adding 1.8 g/L syringaldehyde. Of these studies, the phenolic compounds had a significant inhibition on microbial growth and fermentation. Phenolic aldehydes and ketones mainly generated from lignin were considered more inhibitory than sugar-derived inhibitors [168]. Our previous work presented in Chapter 2 found the aromatic aldehydes resulted in the most inhibitory impact on lactic acid fermentation. That is one of the reasons why we selected aromatic aldehydes to further assess their influence on butanol fermentation.

Considerable effort has been made to evaluate the impact of potential inhibitors but little is known about the mechanism of inhibition. Effective evidence is lacking although some researchers reported the hydrophobicity might be involved in phenol toxicity. The phenols were most likely responsible for increasing the fluidity of membrane and consequently affected the membrane permeability [91, 168, 206]. The complexity of prehydrolysates made the study of mechanism much difficult. Quantitative structure-activity relationships (QSAR) analysis may be helpful to address this issue. Although it was used in pharmacology in 1964 for the first time and then successfully used in environmental toxicity assessment [207-210], little attention was paid in the field of biofuels production from lignocellulose. The biological toxicity can be understood from a chemical-class perspective by association chemical structural properties with their inhibition activities, through which we can also predict their inhibition towards microbial fermentation. The molecular descriptors typically used for QSAR includes octanol/water partition coefficient (log P), energy of the lowest unoccupied molecular orbital (E_{LUMO}), energy

of the highest occupied molecular orbital (E_{HOMO}), molar refractivity (MR), dipole moment (u), and electrophilicity index (ω) [211, 212].

In the present study we selected 13 aromatic aldehydes and added them individually into butanol fermentation by *C. acetobutylicum*. The objectives of this chapter are (1) to understand the effects of aromatic aldehydes structure (substitution group, hydroxyl group position and amount of hydroxyl group) on ABE fermentation; and (2) to establish relationship between physicochemical properties and inhibition towards butanol fermentation.

3.3 Materials and Methods

Chemicals

Glucose (anhydrous) 2, 4-dihydroxybenzaldehyde and thiamine were purchased from Alfa Aesar (Ward Hill, MA). 4-hydroxybenzaldehyde, vanillin and 2, 3, 4-trihydroxybenzaldehyde were purchased from Acros Organics (Morris Plains, NJ). *o*-phthalaldehyde was purchased from Pickering Laboratories (Mountain View, CA). 2, 3-dihydroxybenzaldehyde, 3, 4, 5-trihydroxybenzaldehyde, *o*-vanillin and 2-methoxybenzaldehyde were obtained from TCI America (Portland, OR). 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, NH_4Ac and *p*-aminobenzoic acid were purchased from Alfa Aesar (Heysham, England). NaCl was purchased from VWR (Radnor, PA), 3, 5-dihydroxybenzaldehyde, benzaldehyde and biotin were purchased from Sigma-Aldrich (St. Louis, MO). K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ were obtained from Fisher Scientific (Fair Lawn, NJ). $CaCO_3$ was supplied from EMD chemicals (Gibbstown, NJ). All chemical reagents were of chromatographic grades. DI-water was produced by the Barnstead Nanopure UV Ultrapure Water System (Thermo Fisher Scientific, Marietta, OH).

Microorganism and culture

Clostridium acetobutylicum (ATCC 824) was kindly provided by Dr. Y.Y. Lee of Chemical Engineering, Auburn University. It was stored as spores at 4°C and treated by heat shock at 75°C for 10 min and then cooled down in ice bath prior to cultivation. Reinforced Clostridia Medium (RCM) was used to cultivate the strain. It was bubbled through nitrogen for 15 min to remove oxygen and then autoclaved at 121 °C for 15 min. The heat-shocked cells were grown in RCM medium as seed inoculum until the optical density (OD) reached 1.30 determined by an UV-vis spectrometer at 600nm.

Fermentation and inhibition study

Fermentation was carried out in 125 ml serum bottle with working volume of 50 mL at 35 °C and 80 rpm. To evaluate the effect of aromatic aldehydes, each inhibitor at four concentrations was added into P2 medium sterilized by filtration through a 0.2 μm membrane filter. 0.25 M stock of 2, 3-dihydrdoxybenzaldehyde, 2, 4-dihydrdoxybenzaldehyde, 2, 3, 4-trihydroxybenzaldehyde and *o*-vanillin were prepared and the other inhibitors tested were used as received. The P2 medium stock was prepared at high concentration. It contained mineral ($MgSO_4 \cdot 7H_2O$ 40 g/L, $MnSO_4 \cdot H_2O$ 2 g/L, $FeSO_4 \cdot 7H_2O$ 2 g/L and NaCl 2 g/L), buffer (KH_2PO_4 50 g/L, K_2HPO_4 50 g/L and NH_4Ac 220 g/L) and vitamin (*p*-aminobenzoic acid 1 g/L, thiamine 1 g/L and biotin 0.01 g/L). 0.25 mL mineral, 0.5 mL buffer and 0.05 mL vitamin solution were added into 45 mL water along with glucose at final concentration of 60 g/L. 0.25 g $CaCO_3$ was supplemented to well control the pH during fermentation. The oxygen in medium was then purged out by a Nitrogen purging system. The schematic of purge valve was shown in **Figure 3-1**. The purging circle was repeated 7 times with each circle lasting 5 minutes. A 10%

(*v*/*v*) seed inoculum was transferred into P2 medium throughout the inhibition studies. Glucose control without adding any inhibitor was performed with every batch as reference fermentation. All the fermentation experiments were conducted in duplicates.

50% butanol production inhibition concentration (IC_{50}) was calculated to quantify the inhibition effect of the thirteen compounds. Butanol production was linearly related to the concentrations of tested aromatic aldehydes. IC_{50} represents the concentration of aromatic aldehydes resulting in final butanol concentration 50% of control and the four concentrations of test compounds were selected to cover this point. The lower the value of IC_{50}, the higher is the inhibition of aromatic aldehydes.

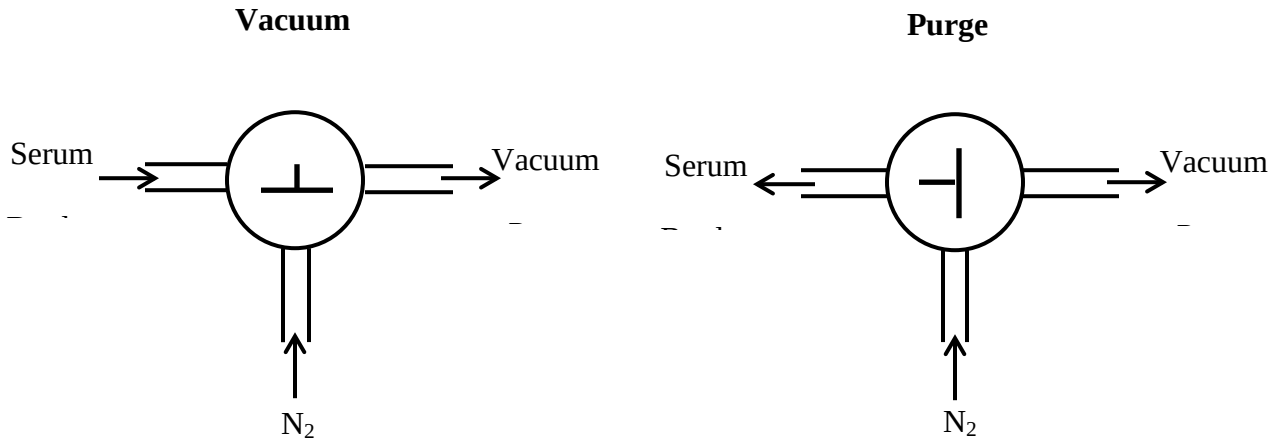

Figure 3-1 Schematic of purge valve

Calculation of physicochemical descriptors and statistical analysis

Hydrophobicity (Log *P*) and molecular refractivity were calculated by MarvinSketch. E_{LUMO}, E_{HOMO}, and dipole moment (*u*) and partial charge of the carbonyl carbon in phenolic aldehyde (C'_{carb}) were estimated by Gauss 9.0 and GaussView 5.0 with Density Functional

Theory (B3LYP/6-31G). The molecular electrophilicity index (ω) was calculated as described previously [213]. Correlations between physiochemical parameters and inhibition activity were carried out by regression analysis using Origin 8.5. The statistical values include: *n* the number of observations, *s* the standard error of the estimate, r^2 the coefficient of determination, *F* Fisher statistic, and p the significance. A value of $p < 0.05$ indicated that the correlation was significant.

HPLC analysis

Glucose, acetic acid, butyric acid, ethanol, acetone and butanol were quantified by a HPLC system (Shimadzu LC-20A) equipped with an autosampler, LC-20 AD pump, and RID-10A detector, with a 300×7.8 mm i.d., 9 μm, Aminex HPX-87H column, and a 30×4.6 mm i.d. guard column of the same material (Bio- Rad, Hercules, CA). The mobile phase was composed of 5 mM of sulfuric acid running isocratic at 0.6 mL/min. The column temperature was maintained at 45 °C throughout the run.

3.4 Results and Discussion

3.4.1 Inhibition Effects of Aromatic Aldehydes on Butanol Production

To examine the inhibition effect of aromatic aldehydes on butanol fermentation, thirteen aromatic aldehydes with different substitution group (OH, CHO and OCH_3) were added into fermentation by *C. acetobutylicum*. The structures are shown in **Figure 3-2**. Overall, the inhibition activities of these compounds were dose dependent but the range at which the compounds showed their inhibition were quite different. Some aldehydes exhibited their inhibition at extremely low concentrations, while some of the others did not show any inhibition at very large amount (**Table 3-1**). Moreover, these aldehydes delayed the fermentation initial

time but not affecting the butanol yield at lower concentration and inhibited the butanol production only when the concentration further increased.

The glucose control without adding any inhibitors produced 9.8 g/L butanol at 96 h with butanol yield of 0.17 g/g glucose and the butanol production rate at the exponential phase was 0.25 g/L/h. The benzaldehyde did not inhibit the butanol production at 5.0 and 7.5 mM, but resulted in a delay on fermentation starting time from 0 h (control) to 12 and 24 h respectively. When it increased to 10.0 and 12.5 mM the final butanol yield was reduced by 28% and 94% and the fermentation initial time delayed to 36 and 72 h respectively. Meanwhile, the butanol

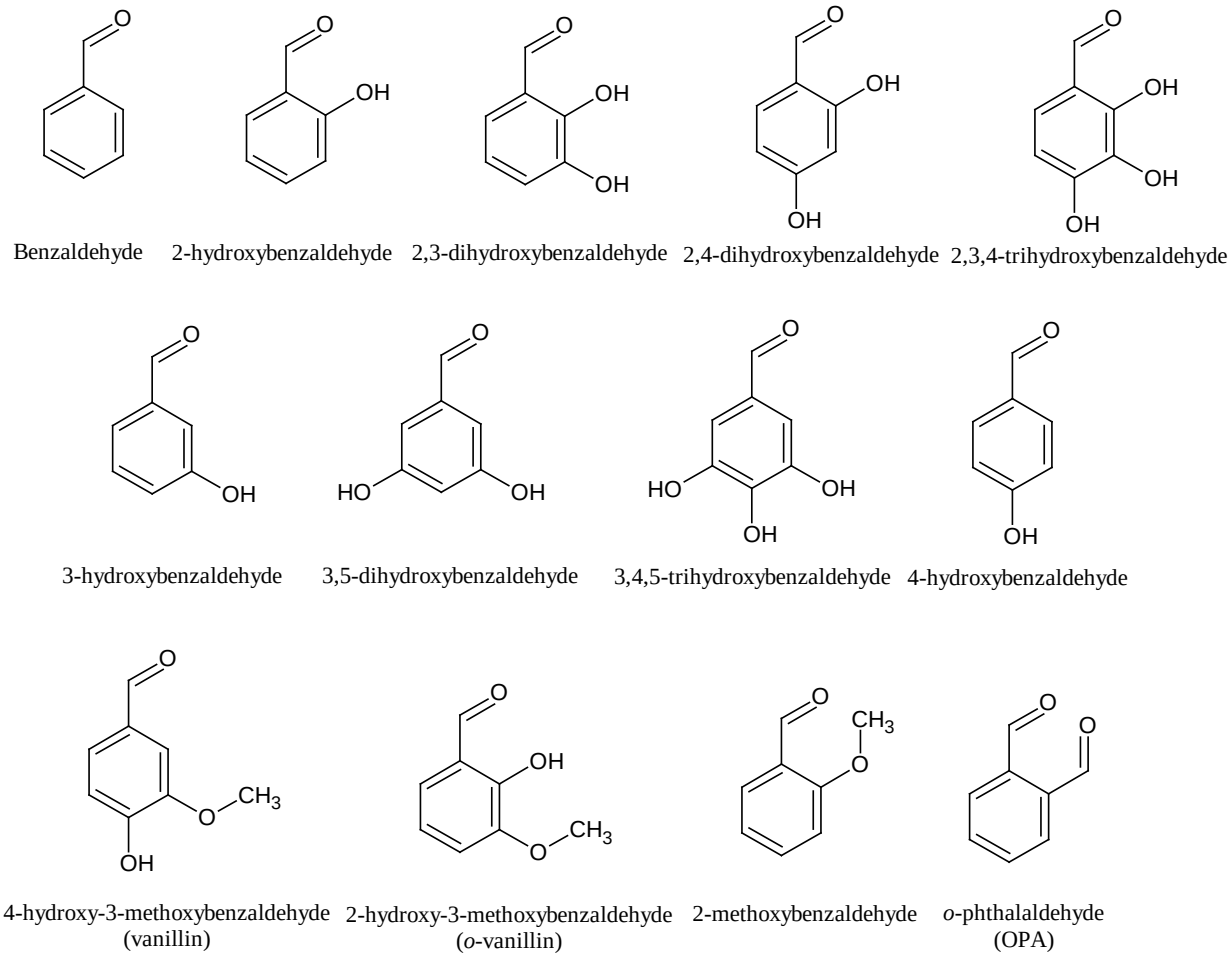

Figure 3-2 Structure of tested aromatic aldehydes

Table 3-1 Effects of aromatic aldehydes on butanol fermentation

Compounds	Concentration (mM)	$C_{butanol}$ [a] (g/L)	C_{ABE} [b] (g/L)	$Y_{butanol}$ [c] (g/g)	Y_{ABE} [d] (g/g)	$Q_{butanol}$ [e] (g/L/h)	T_i [f] (h)
Control	0.00	9.80±0.36	13.74±0.45	0.17±0.01	0.24±0.01	0.25±0.02	0
Benzaldehyde	5.0	10.59±0.34	15.15±0.55	0.18±0.00	0.25±0.00	0.23±0.01	12
	7.5	9.85±0.50	14.21±0.52	0.18±0.01	0.25±0.01	0.21±0.01	24
	10.0	7.52±0.74	10.86±0.78	0.13±0.01	0.18±0.01	0.16±0.02	36
	12.5	0.79±0.17	0.91±0.31	0.01±0.00	0.02±0.01	0.07±0.01	72
2-hydroxybenzaldehyde	0.1	9.61±0.06	13.12±0.13	0.17±0.00	0.24±0.01	0.25±0.01	12
	0.25	9.90±0.14	13.55±0.15	0.18±0.01	0.25±0.01	0.25±0.00	12
	0.35	5.98±0.60	8.19±0.61	0.11±0.01	0.15±0.02	0.10±0.00	36
	0.5	2.76±0.60	3.85±0.91	0.05±0.01	0.07±0.02	0.08±0.00	48
2,3-dihydroxybenzaldehyde	0.1	9.54±0.02	13.60±0.12	0.17±0.00	0.24±0.01	0.19±0.01	12
	0.25	8.25±0.56	11.65±0.79	0.15±0.02	0.21±0.02	0.13±0.00	24
	0.35	1.17±0.08	1.48±0.01	0.02±0.00	0.03±0.00	0.05±0.00	60
	0.5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	>96
2,4-dihydroxybenzaldehyde	1.0	9.74±0.15	13.78±0.20	0.16±0.00	0.23±0.00	0.15±0.00	0
	1.25	8.94±0.03	12.86±0.18	0.15±0.01	0.21±0.00	0.10±0.00	0
	1.5	2.50±0.08	2.86±0.19	0.04±0.00	0.05±0.01	0.11±0.01	12
	2.0	1.18±0.05	1.30±0.04	0.02±0.00	0.02±0.00	0.03±0.00	48
2,3,4-hydroxybenzaldehyde	0.25	9.86±0.07	13.74±0.07	0.17±0.00	0.23±0.00	0.19±0.01	12
	0.5	8.83±0.03	12.03±0.05	0.15±0.00	0.20±0.00	0.12±0.00	12
	1.0	4.41±0.18	6.13±0.15	0.08±0.00	0.10±0.00	0.07±0.00	36
	1.5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	>96
3-hydroxybenzaldehyde	5.0	9.75±0.27	13.80±0.30	0.17±0.00	0.24±0.00	0.20±0.01	12
	7.5	9.41±0.47	13.90±0.54	0.16±0.00	0.24±0.00	0.16±0.01	24
	8.5	5.44±0.06	7.48±0.00	0.09±0.00	0.13±0.00	0.12±0.00	36
	10.0	2.88±0.41	3.62±0.62	0.05±0.01	0.06±0.01	0.08±0.01	48
3,5-hydroxybenzaldehyde	2.5	9.40±0.41	13.76±0.43	0.16±0.01	0.23±0.01	0.16±0.00	12
	5.0	9.05±0.32	13.37±0.39	0.15±0.01	0.22±0.01	0.10±0.00	12
	7.5	0.00±0.00	0.02±0.00	0.00±0.00	0.00±0.00	0.00±0.00	72
	8.5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	>96
3,4,5-hydroxybenzaldehyde	1.0	7.81±0.43	11.08±0.68	0.14±0.01	0.19±0.02	0.16±0.01	24
	2.5	6.65±0.21	9.37±0.30	0.12±0.01	0.17±0.01	0.14±0.00	36
	5.0	3.16±0.10	4.07±0.16	0.06±0.00	0.07±0.00	0.11±0.00	48
	10.0	0.15±0.20	0.16±0.22	0.003±0.00	0.003±0.00	0.02±0.00	84
4-hydroxybenzaldehyde	2.5	9.23±0.05	13.26±0.00	0.16±0.00	0.23±0.00	0.16±0.00	0
	5.0	6.64±0.05	9.19±0.18	0.11±0.00	0.16±0.00	0.08±0.00	0
	10.0	5.74±0.08	8.20±0.37	0.10±0.00	0.14±0.00	0.08±0.00	12
	15.0	4.40±0.81	6.02±0.26	0.08±0.01	0.10±0.02	0.07±0.01	24
Vanillin	2.5	7.00±0.21	9.43±0.58	0.12±0.00	0.17±0.01	0.17±0.01	0
	5.0	6.00±0.52	8.23±0.79	0.10±0.01	0.14±0.01	0.07±0.01	0
	15.0	4.85±0.30	6.69±0.73	0.09±0.01	0.12±0.00	0.06±0.00	12
	25.0	0.25±0.07	0.24±0.07	0.004±0.00	0.004±0.01	0.01±0.01	36

Table 3-1 (continued)

Compounds	Concentration (mM)	$C_{butanol}$ [a] (g/L)	C_{ABE} [b] (g/L)	$Y_{butanol}$ [c] (g/g)	Y_{ABE} [d] (g/g)	$Q_{butanol}$ [e] (g/L/h)	T_i [f] (h)
o-vanillin	0.5	10.77±0.43	15.45±0.68	0.19±0.00	0.27±0.01	0.20±0.01	12
	0.65	3.77±0.05	4.62±0.04	0.07±0.00	0.09±0.00	0.10±0.00	36
	0.7	1.05±0.53	1.43±0.56	0.03±0.01	0.02±0.01	0.08±0.04	72
	0.8	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	>96
2-methoxybenzaldehyde	2.5	9.74±0.06	13.75±0.10	0.17±0.01	0.25±0.01	0.21±0.00	12
	3.5	9.86±0.14	13.97±0.34	0.18±0.00	0.25±0.00	0.20±0.01	12
	5.0	7.80±0.82	10.94±0.67	0.14±0.01	0.19±0.01	0.17±0.02	48
	6.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	84
o-phthalaldehyde(OPA)	12.5	8.72±0.32	12.33±0.90	0.15±0.01	0.21±0.02	0.17±0.00	12
	15.0	7.48±0.25	11.11±0.54	0.13±0.00	0.19±0.01	0.12±0.00	24
	17.5	5.36±0.32	8.04±0.53	0.09±0.01	0.14±0.01	0.08±0.01	24
	20.0	3.59±0.01	5.31±0.00	0.06±0.00	0.09±0.00	0.07±0.00	36

[a] $C_{butanol}$ Butanol concentration at 96h

[b] C_{ABE} ABE concentration at 96h

[c] $Y_{butanol}$ Butanol yield at 96h (g butanol /g glucose)

[d] Y_{ABE} ABE yield at 96h (g butanol /g glucose)

[e] $Q_{butanol}$ Volumetric butanol productivity at exponential phase

[f] T_i Fermentation initial time

The data was presented as mean value ± standard deviation

production rate was decreased accordingly (30% and 70% at 10.0 and 12.5 mM respectively). Similarly, addition of 3- hydroxybenzalde and 4-hydroxybenzaldehyde did not or lightly inhibit butanol yield at low concentration (3- hydroxybenzalde at 5.0 and 7.5 mM and 4-hydroxybenzaldehyde at 2.5 and 5.0 mM), but greatly reduced butanol yield and productivity when the concentration reached high level (3-hydroxybenzalde at 8.5 and 10.0 mM and 4-hydroxybenzaldehyde at 10.0 and 15.0 mM) and gradually delayed fermentation starting time. In the case of vanillin, 3,5-dihydroxybenzaldehyde, 3,4,5-trihyroxybenzaldehyde and 2-methoxybenzaldehyde, butanol yield and productivity were decreased at lower concentration and fermentation initial time was delayed dramatically. Moreover, addition of 2, 4-dihydroxybenzaldehyde at 1.0 and 1.25 mM and 2, 3, 4-trihydroxybenzaldehyde at 0.25 and 0.5

mM did not lead to reduction on butanol yield but lightly drop on productivity. While when the concentration of them slightly increase to 2.0 and 1.5 mM both butanol yield and butanol production rate considerable dropped by 88% and 100% of control respectively, indicating a strong inhibition of these two hydroxybenzaldehydes. An even more strong inhibition was observed by addition of 2-hydroxybenzaldehyde, 2, 3-dihydroxybenzaldehyde and *o*-vanillin. At 0.1 and 0.25 mM 2-hydroxybenzaldehyde and 2, 3-dihydroxybenzaldehyde did not inhibit final butanol yield, but the butanol productivity was found to be reduced (17% reduction at 0.1 mM and 48% reduction at 0.25 mM) as well as fermentation started as late as 12 h and 24 h by adding 2, 3-dihydroxybenzaldehyde at 0.1 mM and 0.25 mM. When the concentration was only further increased to 0.5 mM, 2-hydroxybenzaldehyde inhibited the fermentation by decreasing butanol yield to 29% of control and butanol productivity to 32% of control; 2, 3-dihyroxybenzaldehyde terminated the fermentation with no butanol produced. Likewise, *o*-vanillin partially inhibited the fermentation at 0.65 and 0.7 mM but completely stopped the butanol production by concentration increasing to 0.8 mM. Interestingly, *o*-vanillin and benzaldehydehyde were observed to slightly improve butanol yield by 12% and 6% at 0.5 and 5.0 mM respectively. The similar results were reported by our previous work and literature [72, 74, 77]; it was found the fuels and chemicals production could be enhanced by furfural, HMF and vanillin at low concentrations. In the case of *o*-phthalaldehyde (OPA), it decreased the butanol yield by 12% when the concentration was as high as 12.5 mM and further decreased it by 65% with concentration increasing to 20 mM. It was different from that in lactic acid fermentation which resulted in a very strong inhibition. This difference was probably caused by the fact that *C. acetobutylicum* is more tolerant to *o*-phthalaldehyde (OPA).

3.4.1.1 Influence of *Ortho* Substituents on Butanol Production

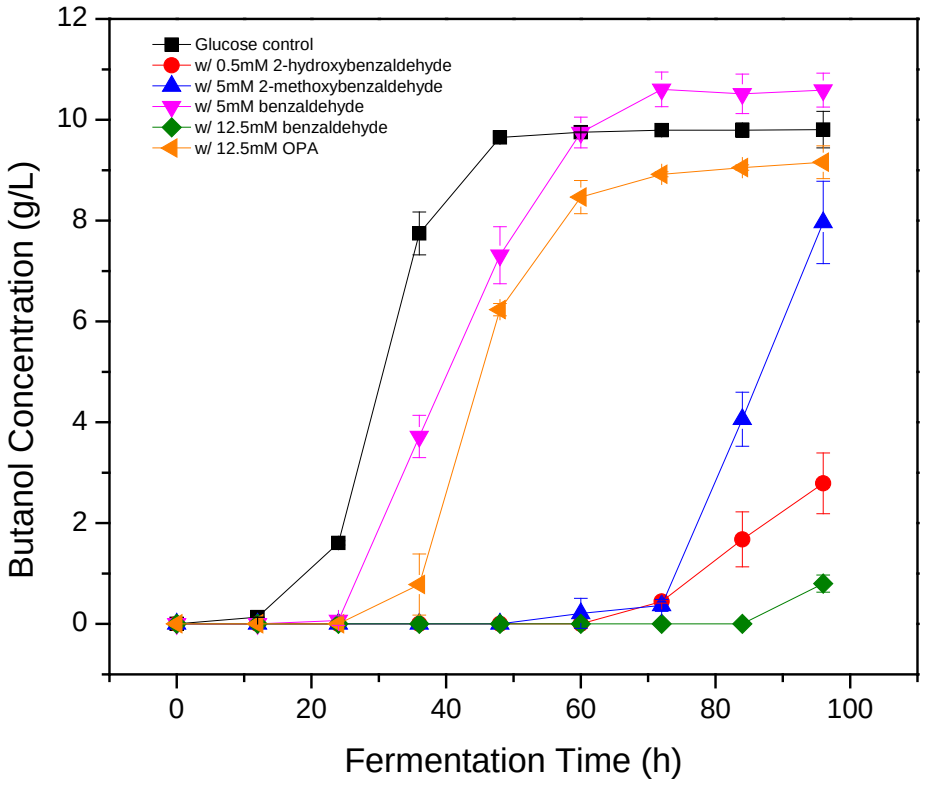

Figure 3-3 Effect of ortho substituents on butanol production

Since hydroxyl (OH), methoxyl (OCH_3) and aldehyde (CHO) groups are commonly present in the prehydrolysates, they were selected for the purpose to understand how they affect the fermentation by *C. acetobutylicum (***Table 3-1 and Fig. 3-3***)*. The benzaldehyde at 5 mM did not inhibit butanol yield compared to control but improved butanol concentration by 8% instead. While it decreased butanol production rate and delayed the fermentation by 12h. At the same concentration, 2-methoxybenzaldehyde inhibited final butanol concentration and yield by 20% and 18%, respectively **(Fig. 3-3**). Moreover, at 0.5 mM, a tenth of their concentration, 2-hydroxybenzaldehyde considerably reduced final butanol concentration by 72% and delayed the

fermentation by 48 h **(Fig. 3-3)**. On the other hand, we compared *o*-phthalaldehyde (OPA) with benzaldehyde at 12.5 mM. It was shown that the benzaldehyde at this concentration significantly inhibited butanol yield to 0.02 g/g and delayed the fermentation by 72 h while the butanol yield only dropped by 12% and the fermentation was just 12 h behind of control with addition of *o*-phthalaldehyde (OPA) **(Fig. 3-3)**. Therefore, these results suggested the inhibition of *ortho*-substituents followed: *ortho*-OH > *ortho*-OCH_3 > no *ortho* substituent > *ortho*-CHO.

3.4.1.2 Influence of Hydroxyl Group Positions on Butanol Production

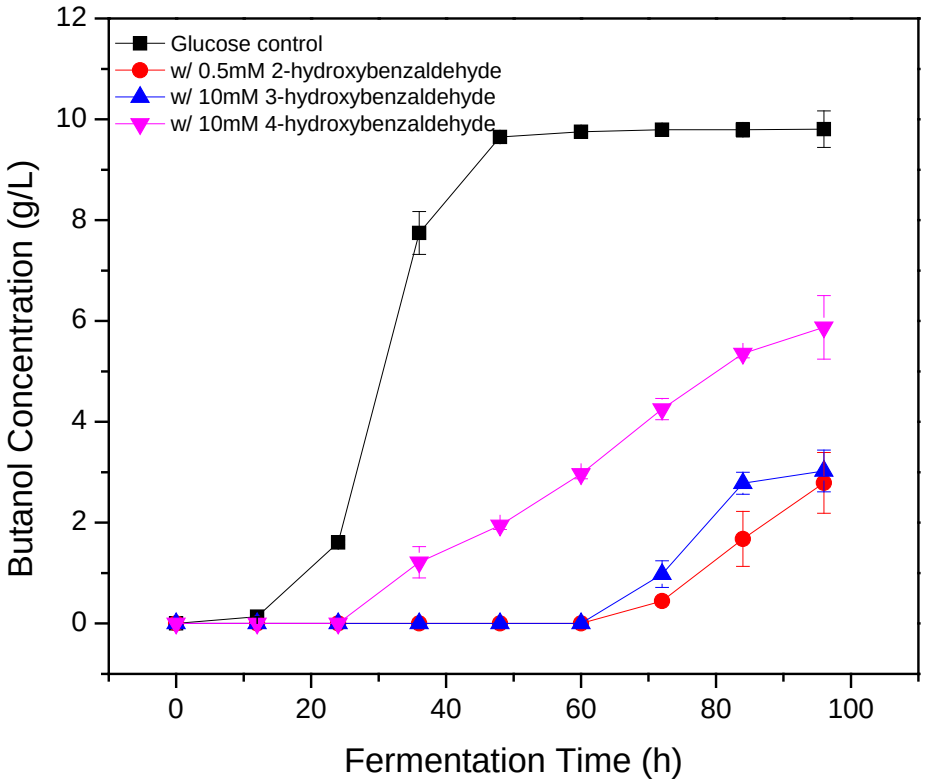

Figure 3-4 Effect of hydroxyl group positions in benzaldehyde on butanol production

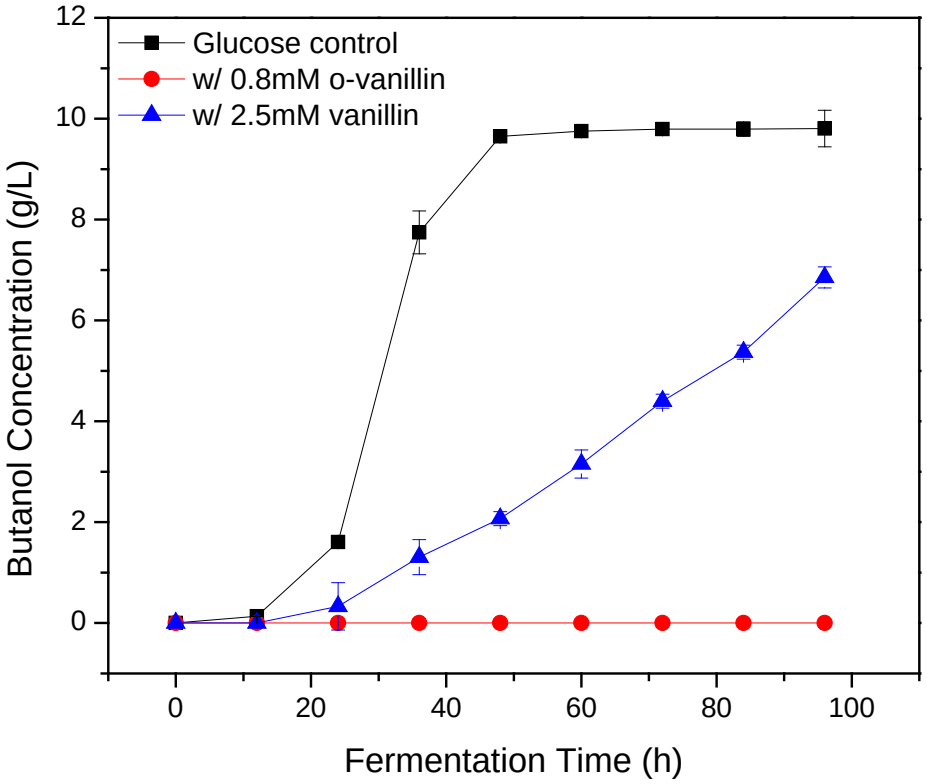

Figure 3-5 Effect of hydroxyl group positions in methoxy-benzaldehyde on butanol production

Phenolic compounds are frequently present in the prehydrolysates after pretreatment [63, 87, 214], so it is essential to know the how the hydroxyl groups in aromatic compounds affect their inhibition including the positions and amount of hydroxyl group. It was found that 2-hydroxybenzaldehyde showed strong inhibition even at only 0.5 mM in the previous section, which postponed fermentation by 48 h and decreased final butanol concentration to 2.76 g/L equaled to 28% of the control (**Fig.3-4**). While 3-hyroxybenzaldehyde and 4-hydroxybenzalde in which the hydroxyl group occupied *meta*- or *para*- position, lost the inhibition significantly. At twenty times higher concentration (10 mM), 3-hydroxybenzalde exhibited a similar butanol production inhibition with 2-hydroxybenzladehyde and 4-hydroxybenzaldehyde resulted in only 32% reduction in final butanol concentration and 35% decrease in butanol yield (**Fig. 3-4**). These

results indicated the hydroxyl group in *ortho-* position caused at least 20 fold stronger inhibition than *meta-* and *para-* position, and *meta-* positon was more inhibitory than *para-* positon. The same effect was noticed with vanillin and *o*-vanillin. Vanillin at 2.5 mM reduced both final butanol concentration and yield by 29% and delayed fermentation by 12 h, while *o*-vanillin at 0.8 mM completely inhibited fermentation with no butanol produced (**Fig. 3-5**). Therefore, by examining the effect of hydroxyl group positions in both benzaldehyde and methoxy-benzaldehyde on butanol production, it was found the inhibition of hydroxyl group followed the order of *ortho-* > *meta-* > *para-* and the *ortho* hydroxyl group inhibited butanol fermentation significantly.

3.4.1.3 Influence of Hydroxyl Group Number on Butanol Production

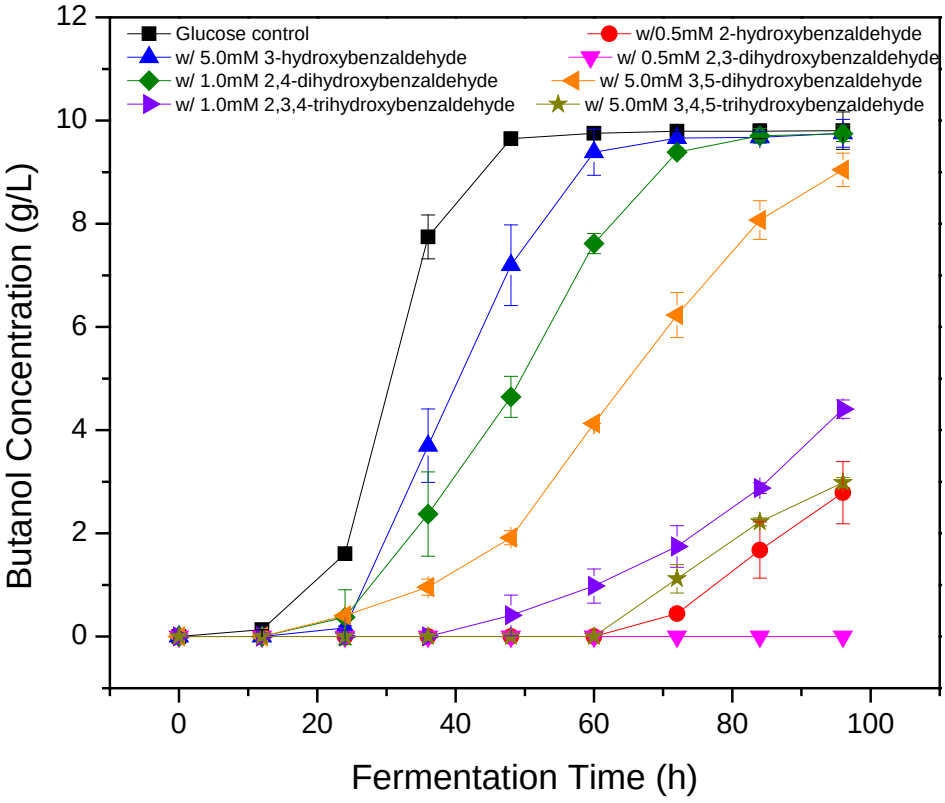

Figure 3-6 Effect of hydroxyl group number on butanol production

After knowing the positions of hydroxyl group had an effect on butanol fermentation, we further investigated whether the hydroxyl group number had an influence on butanol production. Our study covered aromatic aldehydes with one, two and three hydroxyl groups, including 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 2, 3-dihydroxybenzaldehyde, 2, 4-dihydroxybenzaldehyde, 3, 5-dihydroxybenzaldehyde, 2, 3, 4-trihydroxybenzaldehyde and 3, 4, 5-trihydroxybenzaldehyde. It was observed that 2-hydroxybenzaldehyde and 2, 3-dihydroxybenzaldehyde at 0.5 mM reduced final butanol concentration by 72% and 100%, respectively (**Fig.3-6**), indicating 2, 3-dihydroxybenzaldehyde which had two hydroxyl groups was more inhibitory. However 2, 4-dihydroxybenzaldehyde and 3, 5-dihydroxybenzaldehyde also contained two hydroxyl groups, they decreased final butanol concentration by 1% and 8% at 1 mM and 5 mM, respectively (**Fig. 3-6**). Moreover, addition with 2, 3, 4-trihydroxybenzaldehyde and 3, 4, 5-trihydroxybenzaldehyde at 1.0 mM and 5.0 mM had a higher final butanol concentration and yield than that with 2-hydroxybenzaldehyde at 0.5 mM (**Fig. 3-6**). It appeared that more hydroxyl groups resulted in lower inhibition except 2, 3-dihydroxybenzaldehyde. While in contrast, 3-hydroxybenzaldehyde at 5 mM almost showed no inhibition, which had the higher final butanol concentration, yield and productivity than all the other aldehydes with two or three hydroxyl groups at same or low concentrations (**Fig. 3-6**). Hence, the number of hydroxyl groups did not contribute to the phenolic inhibition on butanol fermentation.

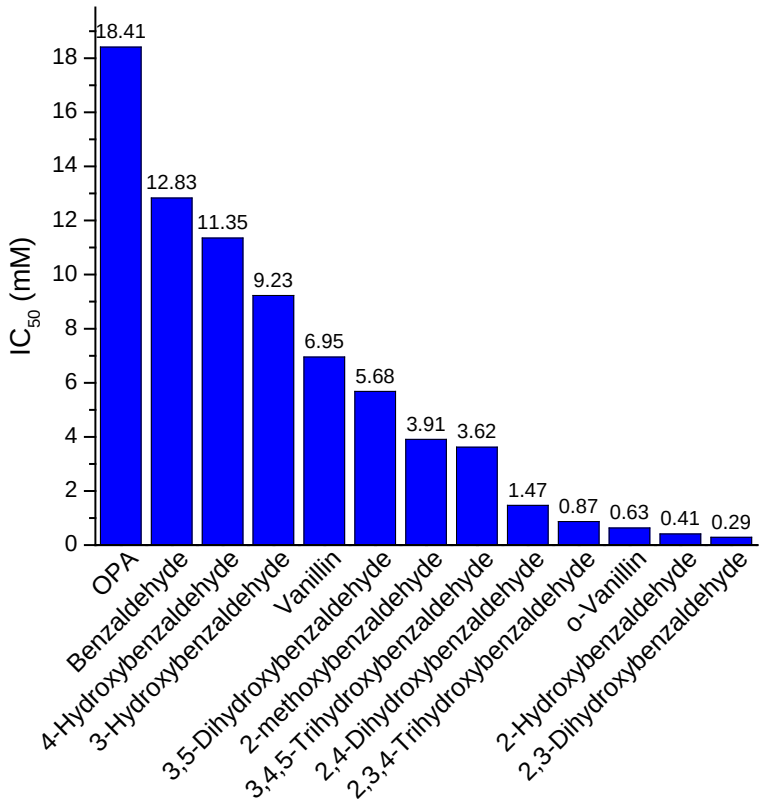

Figure 3-7 IC_{50} of examined phenolic benzaldehydes

In order to quantify the inhibition effect of aromatic aldehydes, 50% butanol production inhibition concentration (IC_{50}) was calculated. It was defined as the inhibitor concentration at which the final butanol concentration is 50% of glucose control. **Figure 3-7** showed the IC_{50} value of tested compounds. The lower the value, the higher is the inhibitory effect. Interestingly, it was observed that all the compounds contained *ortho*- hydroxyl group had a very low IC_{50} value ranging from 0.29 to 1.47 mM, which were lower than any other aromatic aldehydes, indicating fairly high inhibition activity. This severe inhibition was probably due to the *ortho*-hydroxyl group forming intramolecular hydrogen bond within the aromatic aldehydes and thus, it potentially increased cell membrane permeability and electrophilicity. The strong inhibition caused by *ortho* –OH is also observed by other researchers. Friedman *et al*. investigated the

activity of 35 benzaldehydes, 34 benzoic acids and 1 benzoic acid methyl ester on *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica* and they found nine of the ten compounds which were most active against four microbes contained *ortho* hydroxyl group. In addition, it was found compounds with OH were more active than that with OCH_3 [98]. A similar finding was reported by Larsson *et al*. and they noticed vanillin at 0.2 g/L was slightly inhibitory to ethanol formation and cell growth while o-vanillin at the same concentration resulted in complete inhibition [97].

3.4.2 Inhibition Effects of Aromatic Aldehydes on ABE and Butyric Acid Production

Butanol fermentation is also known as ABE fermentation since two other solvents, acetone and ethanol were produced along with butanol by *C. acetobutylicum*. Thus, it is necessary to know how these phenolic compounds affect their production. It was found that the ABE final concentration at 96 h was dose dependent (**Table 3-1 and Figure 3-8**). The glucose control without any inhibitors generated 13.74 g/L ABE with a yield of 0.24 g/g glucose. Interestingly, similar to butanol production, benzaldehyde at 5.0 and 7.5 mM and *o*-vanillin at 0.5 mM improved ABE final concentration by 10%, 3% and 12%, respectively. Particularly, it was observed that the aromatic aldehydes suppressed acetone, butanol and ethanol production proportionally. The distribution of acetone, butanol and ethanol was not changed by different compounds at various concentrations (**Fig.3-8**). Butanol was the most one accounting for 67%-73%, acetone and ethanol took 24%-27% and 5%-8% respectively. It was agreed with the previous report that the ratio of butanol, acetone, and ethanol was 6:3:1 [215]. On the other hand, the acid production was not found in any trend with compounds at different concentrations. This is caused by the two phases involved in ABE fermentation, named acidogenic phase and solventogenic phase. The butyric acid accumulated during acidogenic phase and then re-entered

into cells to form butanol at solventogenic phase [159, 216], resulting in a peak concentration of butyric acid. The final acid concentration recorded at 96 h in this assay could be in acidogenic phase or solventogenic phase, the acid production consequently appeared not to be related to any parameters.

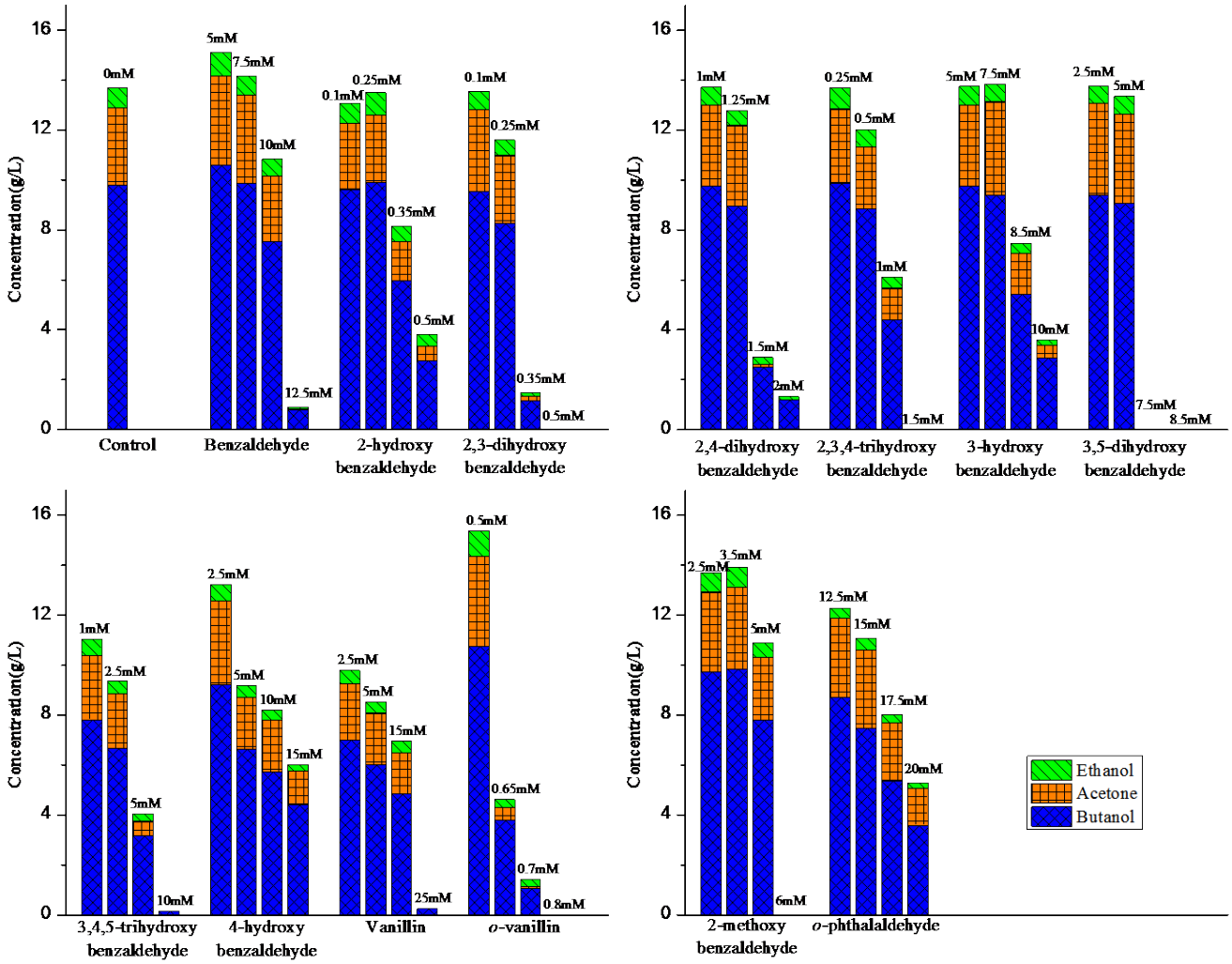

Figure 3-8 Effect of phenolic benzaldehydes on ABE production

3.4.3 Quantitative Structure-Activity Relationship (QSAR) Analysis

The inhibition activity (IC_{50}) of aromatic aldehydes on butanol fermentation was correlated with molecular descriptors as summarized in **Tables 3-2** and **Table 3-3**. Among these calculated physicochemical descriptors a significant linear relationship ($r^2=0.76$, $p<0.001$) was found between IC_{50} and E_{HOMO} (energy of the highest occupied molecular orbital) (Equation 3 in **Table 3-3 and Fig.3-9**). E_{HOMO} is a global parameter that represents the tendency to release electrons. The smaller the absolute value of E_{HOMO}, the stronger is the compounds' electron donor capacity. IC_{50} was negatively correlated to E_{HOMO} suggesting the aromatic aldehydes with high E_{HOMO} value resulted in high inhibition activity. 2, 3-dihydroxybenzaldehyde had the highest E_{HOMO} value and was observed to have the highest inhibition on butanol fermentation. The E_{HOMO} of *o*-phthalaldehyde was the lowest among all the test aromatic aldehydes and showed the lowest inhibition. This correlation suggested E_{HOMO} could be used to predict the inhibition of phenolic compounds on butanol fermentation and also revealed the possible mechanism of inhibitors' toxicity.

Table 3-2 Calculated physicochemical descriptors of aromatic aldehydes and inhibition

Compounds	Log *P*	E_{LUMO} (eV)	E_{HOMO} (eV)	Dipole (debye)	MR	ω	C'_{carb}	IC_{50} (mM)
Benzaldehyde	1.69	-0.88	-10.09	4.53	32.64	3.27	0.431	12.83
2-hydroxybenzaldehyde	2.03	-0.91	-9.61	6.53	34.62	3.18	0.471	0.41
2,3-dihydroxybenzaldehyde	1.73	-1.09	-9.23	6.75	36.6	3.27	0.458	0.29
2,4-dihydroxybenzaldehyde	1.73	-0.83	-9.7	5.91	36.6	3.13	0.485	1.47
2,3,4-hydroxybenzaldehyde	1.43	-1.11	-9.25	0.79	38.58	3.30	0.469	0.87
3-hydroxybenzaldehyde	1.38	-1.06	-9.47	4.65	34.62	3.30	0.414	9.23
3,5-hydroxybenzaldehyde	1.08	-0.92	-9.44	6.04	36.6	3.15	0.404	5.68
3,4,5-hydroxybenzaldehyde	0.78	-1.17	-9.56	5.05	38.58	3.43	0.410	3.62
4-hydroxybenzaldehyde	1.38	-0.85	-9.62	5.99	34.62	3.12	0.452	11.35
Vanillin	1.22	-1.01	-9.14	6.41	41.09	3.17	0.435	6.95
o-vanillin	1.87	-1.05	-9.1	7.76	41.09	3.20	0.458	0.63
2-methoxybenzaldehyde	1.53	-0.87	-9.45	7.09	39.11	3.10	0.471	3.91
o-phthalaldehyde	1.4	-1.4	-10.31	7.11	39.23	3.85	0.419	18.41

Table 3-3 Regression analysis between IC_{50} and molecular descriptors

Equation No.	Regression	*n*	r^2	*s*	*F*	*p*
1	IC_{50} = -1.766 log *P* + 9.1927	11	0.01	6.05	0.08	0.78
2	IC_{50} = -6.9183E_{LUMO} - 0.4703	11	0.04	5.97	0.35	0.57
3	IC_{50} = -13.169E_{HOMO} - 118.59	11	0.76	3.00	27.96	<0.001
4	IC_{50} = 0.4159Dipole + 4.3555	11	0.02	6.02	0.18	0.68
5	IC_{50} = -0.7756MR + 35.809	11	0.14	5.65	1.42	0.26
6	IC_{50} = 14.813ω - 41.978	11	0.29	5.13	3.64	0.09
7	IC_{50} = -110.93C_{carb} + 55.322	11	0.22	5.37	2.55	0.14

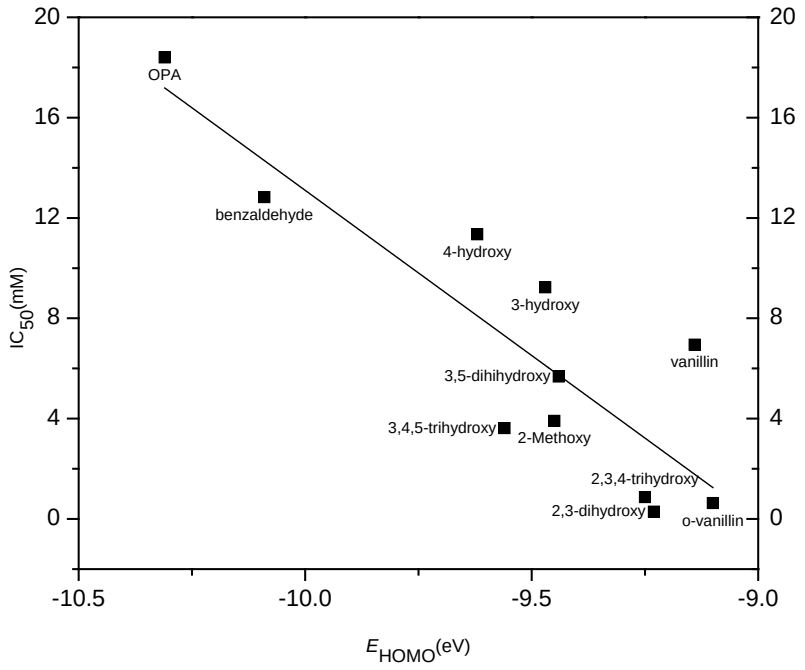

Figure 3-9 Plot of IC_{50} versus E_{HOMO}

Regression analysis showed log *P*, E_{LUMO}, Dipole moment, molecular refractivity (MR), ω, C'_{carb} did not have a linear relationship to inhibition (**Table 3-3**). Log *P* is a global parameter which measures the hydrophobicity of a molecule [195]. We found a good correlation between inhibition constant and log *P* in lactic acid fermentation in the previous chapter. However, it was not the case in butanol fermentation, and 2, 3-dihydroxybenzaldehyde exhibited the highest

inhibition with log *P* value of 1.73, which did not show the highest hydrophobicity. This difference was probably caused by the different microorganisms and fermentation media, and the diffusion of compounds through cell membrane might not be the dominant step when the inhibitors interact with biological objects in butanol fermentation process.

3.5 Conclusions

The influence of thirteen aromatic aldehydes on ABE fermentation by *C. acetobutylicum* was evaluated in this chapter. We found their inhibition activity on butanol production was related to the *ortho* substituents (OH > OCH_3 > CHO) and it was also affected by the position of hydroxyl group instead of the number of hydroxyl group. By examining the effect of hydroxyl group positions in both benzaldehyde and methoxy-benzaldehyde on butanol production, it was observed the inhibition of hydroxyl group followed the order of *ortho-* > *meta-* > *para-*. The *ortho*-hydroxyl group played an important role in the inhibition severity. It caused at least 20 fold stronger inhibition than *meta-* and *para-* position. Perticularly, it was noticed that the *ortho-* hydroxyl group was present only in the top five most inhibitory compounds. The presence of *ortho-* hydroxyl group can form an intramolecular hydrogen bond with carbonyl hydrogen and potentially increase the cell membrane permeability and electrophilicity. In addition, the distribution of acetone, butanol and ethanol was not affected by these aromatic aldehydes. Butanol acetone and ethanol accounted for 67%-73%, 24%-27% and 5%-8%, respectively. Quantitative structure-activity relationship (QSAR) analysis suggested a strong linear relationship ($r^2=0.76$, $p<0.001$) between inhibition activity (IC_{50}) and energy of the highest occupied molecular orbital (E_{HOMO}).

Chapter 4

Detoxification of Biomass Prehydrolysates in Butanol Fermentation

4.1 Abstract

Bioconversion of lignocellulose to biofuels suffers from the degradation compounds formed through pretreatment and acid hydrolysis. In order to achieve an efficient conversion, detoxification is required in most cases. Six detoxification approaches were assayed in the present study, including treatment by overliming, anion exchange resin, nonionic resin, laccase, activated carbon and cysteine. It was found that detoxification by anion exchange resin was the most effective method and the combination of $Ca(OH)_2$ and anion exchange resin was carried out to improve the fermentation efficiency, resulting in completion of fermentation within 48 h and ABE production of 11.11 g/L, corresponding to yield of 0.19 g/g sugar. The effect of salt on ABE fermentation was assessed and the possible role of $Ca(OH)_2$ was assumed to remove the salts in the prehydrolysates by precipitation.

4.2 Introduction

Exploring biofuels from renewable sources in the past decades has driven by the increasing energy demand over current petroleum storage, concern on CO_2 emission and global climate change, and the opportunities to support local economy [149, 150]. The second generation biofuels from lignocellulosic biomass, the most abundant resources on the planet, holds great potential to replace fossil fuels in the future [151]. However, the bioconversion of lignocellulose encounters huge technical and economic difficulties. One of these issues is the degradation compounds formed through pretreatment severely limits the efficiency of converting sugars present in biomass to biofuels via biological strategy [85, 115, 217]. Furans and aliphatic

acids derived from cellulose and hemicellulose have been extensively studied as fermentation inhibitors; phenolic aldehydes/ketones and acids mainly degraded from lignin have attracted increasing attention due to their more severe toxicity [78, 85, 203]. A number of efforts have been made to minimize the inhibitory effect of these compounds. The detoxification methods including physical, chemical and biological treatment were employed to enhance the microorganism growth and bioproducts yield [68, 115]. Among all those detoxification methods, alkaline treatment, especially overliming, was most widely investigated. Detoxification by $Ca(OH)_2$ at high pH (overliming) was found to significantly improve fermentation yield [126, 218-220]. Impact of different cations (NaOH, $Ca(OH)_2$, NH_4OH and KOH) in alkaline treatment was investigated; $Ca(OH)_2$ and ammonia were found to be able to result in the best fermentability [125]. Large portion of the potential inhibitors could be removed by alkaline treatment, nevertheless the overliming mechanism is not yet fully understood [125, 220-222]. Activated charcoal and ion exchange resins were another effective approaches in detoxification of hydrolysate to facilitate the fermentation. They were reported to greatly reduce the furans and phenolic compounds in sugarcane bagasse hydrolysates and dramatically increase ethanol production [223]. Similarly, activated carbon was found to be effective in detoxification of the prehydrolysates from hydrothermolysis-pretreated switchgrass which raise the ABE production from 1g/L to 17 g/L. The activated carbon was able to remove furanic and phenolic compounds but not cinnamaldehyde [136]. Larsson *et al.* [86] evaluated different detoxification approaches on spruce hydrolysate including alkaline treatment, addition of sulfite, evaporation, adsorption on anion exchange resin and laccase treatment. The highest ethanol yield was obtained after the anion exchange resin treatment at pH 10 and followed by laccase treatment.

Ethanol organosolv pretreatment is to treat biomass at high temperature in ethanol-water mixture with dilute acid as a catalyst. Ethanol organosolv pretreatment was first developed to produce a clean biofuel for turbine generators and then adapted in lignocellulose biorefinery platform known as Lignol process. In the Lignol process, high quality of lignin and byproducts such as hemicellulose sugars, furfural and acetic acid could be recovered [224]. Ethanol organosolv process is currently being assessed as a promising pretreatment for efficient fractionation of lignocellulosic biomass [224, 225]. The ethanol organosolv pretreatment has been investigated on hardwood and mixed softwood (spruce, pine and Douglas fir) by pan *et al.* [224, 226-228]. They evaluated the efficiency of organosolv pretreatment based on substrate characteristics and enzyme digestibility, carbohydrate recovery and lignin properties. The benefits of applying organosolv process to pretreat biomass for biofuels production are ascribed to high fermentable sugar production, low energy input, higher carbohydrate sugar recovery and less inhibitors generation [172, 229]. Production of high quality lignin and other valuable byproducts in the biorefinery process of lignocellulosic feedstock is critical to cost effective biofuels production, ensuring the ability to overcome the economic challenge of lignocellulose bioconversion.

Investigation on ethanol fermentation has been mostly performed in the field of detoxification of hydrolysate to produce biofuels. However, biobutanol production by clostridia received less attention and it is essential to study the detoxification efficiency in butanol production. Since the clostridia are more sensitive to fermentation environment and the cells undergoing biphasic pathways, acidogenic phase and solventogenic phase, lead to a complicated metabolism, resulting in difficulties in improvement on solvent production. It will be interesting to compare the different performance between yeast and bacteria fermentation with the typical

detoxification methods. The objective of this chapter is to (1) examine the detoxification effectiveness of different methods including overliming, ion-exchange resin, activated carbon, laccase and cysteine on butanol fermentation with the prehydrolysates obtained from organosolv pretreatment; (2) characterize the potential inhibitors to *C. acetobutylicum* before and after detoxification treatment.

4.3 Materials and Methods

Chemicals and Microorganisms

Glucose and NaOH were purchased from VWR (West Chester, PA). $Ca(OH)_2$, p-aminobenzoic acid and CH_3COONH_4 were purchased from Alfa Aesar (Heysham, England). Activated carbon was purchased from J.T.Baker (Phillipsburg, NJ). L-cysteine was purchased from Acros Organics (Morris Plains, NJ). Thiamine was purchased from Alfa Aesar (Ward Hill, MA). Amberlite XAD4 resin, Dowex 1X4 resin (chloride form), biotin and laccase (from Trametes versicolor) were purchased from Sigma-Aldrich (St. Louis, MO)**.** H_2SO_4 and NaCl were purchased from VWR (West Radnor, PA). K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ were purchased from Fisher Scientific (Fair Lawn, NJ). $CaCO_3$ was purchased from EMD chemicals (Gibbstown, NJ). Tryptone glucose yeast extract (TGY) medium was purchased from HIMEDIA laboratories (Mumbai, India) with the composition of 10g/l casein enzymic hydrolysate, 1 g/L yeast exact, 5 g/L glucose and 1.25 g/L dipotassium phosphate.

Clostridium acetobutylicum ATCC 824 (obtained from Dr. Y. Y. Lee's lab at Auburn University) was used for butanol production. It was maintained as spores at 4 °C and treated with heat shock at 75 °C for 10 min before cultivation. Tryptone glucose yeast extract (TGY) medium

was used to cultivate the strain and the anaerobic environment was achieved by bubbling through nitrogen prior to autoclave at 121 °C for 15 min. The strain was cultivated until the OD_{600} reached near 1.0 and then used as seed culture.

Organosolv Pretreatment

Loblolly pine wood chips were collected by Forest Products Laboratory at Auburn University and those with bark free and size of 1.0 × 1.0 cm (L × W) were selected for organosolv pretreatment. Wood chips (400 g, oven-dry weight) were soaked in 65% (*v/v*) ethanol solution with 1% (*w/w*) sulfuric acid (on the basis of dry biomass) overnight (7:1 liquor/solid ratio) and then loaded into a 2- gallon Parr reactor (Parr Instrument Co., Moline, IL) to be treated at 170 °C for 60 min. The aqueous solution named as prehydrolysates was separated from solid by vacuum filtration upon the completion of pretreatment. Ethanol in prehydrolysates was evaporated at 40 °C in a rotary evaporator (IKA RV10 basic) and the pH was adjusted to 4.0 before evaporation. The total concentration of reducing sugars in the concentrated prehydrolysates was found to be 74.3 g/L. The concentrated prehydrolysates were stored at 4 °C until use.

Detoxification and fermentation

The concentrated prehydrolysates were diluted by three times before detoxification and additional glucose was supplemented to make the sugar concentration in the final hydrolysates to be 74.7 g/L. Overliming detoxification was conducted by adjusting pH of prehydrolysates to 10 with $Ca(OH)_2$ and then incubated at 60 °C and 100 rpm for 2 hours. Detoxification by activated carbon was performed by adding 3 g carbon into 100 ml prehydrolysates at 25 °C and 100 rpm for 1 hour. Detoxification by laccase was done at pH=5 and 0.2 g laccase was added into 100 ml

prehydrolysates and followed by incubation at 30 °C and 100 rpm for 4 hours. For detoxification by resins, activation was performed prior to the treatment. Briefly, Amberlite XAD4 resin (10 g) was washed with 200 ml DI water. Dowex 1X4 resin (10 g) was washed wth 100 ml saturated NaCl solution and then washed with DI water for three times, followed by 200 ml NaOH (1 M). Finally, it was washed with DI water until the pH became neutral. Upon the activation completed, the resin was added into 100ml prehydrolysates for detoxification. It was kept at 25 °C and 100 rpm for 1 h. For detoxification by cysteine, 0.2% (*w/v*) cysteine was added into 100 ml prehydrolysates and then incubated at 60 °C and pH 6 for 2 hours.

Two-step detoxification combined two detoxification methods. The first step was to adjust pH of prehydrolysates to 7 or 10 by adding Ca $(OH)_2$ and incubate the liquid at 25 °C (for pH=10 and 7) or 90 °C (for pH=10) and 100 rpm for 30 min. The second step was done by Dowex 1X4 resin and cysteine respectively and the procedures were same as resin detoxification and cysteine detoxification described above.

After all treatments, the pH was adjusted to 7 and the precipitates were removed by centrifuge at 4000 rpm for 15 min. All detoxified prehydrolysates were sterilized by filtration through a 0.2 μm membrane filter. The ABE fermentation was carried out in 125ml serum bottles. Each serum bottle contains 45ml prehydrolysates and 50 μl vitamin solution (p-aminobenzoic acid 1 g/L, thiamine 1 g/L, biotin 0.01 g/L), 0.25 ml mineral solution ($MgSO_4 \cdot 7H_2O$ 40 g/L, $MnSO_4 \cdot H_2O$ 2 g/L, $FeSO_4 \cdot 7H_2O$ 2 g/L, NaCl 2 g/L) and 0.5 ml buffer solution (K_2HPO_4 50 g/L, KH_2PO_4 50 g/L, CH_3COONH_4 220 g/L). Yeast extract (1 g/L) was added to provide nutrients for the microbes and $CaCO_3$ (0.25 g) was added to control the pH during fermentation. The medium in serum bottle was vacuumed and then flushed with nitrogen. Each vacuum and flushing cycle took about 5 minutes and totally 7 cycles were applied to

remove oxygen. Schematic purge valve was shown in **Figure 3-1**. The inoculation volume was 10% (*v/v*), which makes the total working volume to be 50ml, and the fermentation was carried out at 35 °C and 80 rpm. All fermentation experiments were performed in duplicates. Samples were taken periodically for analyzing sugar consumption and ABE production.

Butanol yield was calculated as butanol produced divided by the available sugars in medium and is expressed as g/g. ABE yield was calculated as the total ABE produced divided by the available sugars in medium and is expressed as g/g. ABE productivity was calculated as total ABE produced divided by the fermentation time and is expressed as g/L/h. The fermentation time was defined as the period from the inoculation induced to the products stopped increasing.

Effect of salt on fermentation

Various concentrations of sodium sulfate (1 g/L, 3 g/L, 5 g/L and 7 g/L) were added into P2 medium to study the effect of salt on ABE fermentation. The inoculum preparation, sterilization and fermentation were performed same as described above.

HPLC and GC/MS Analysis

The fermentation products, furfural, HMF, formic acid and levunilic acid were quantitated by a Shimadzu (LC-20A) HPLC system consisting of an autosampler, LC-20 AD pump, and RID-10A detector, with a 300 × 7.8 mm i.d., 9 μm, Aminex HPX-87H column, and a 30 × 4.6 mm i.d. guard column of the same material (Bio-Rad, Hercules, CA). The mobile phase was composed of 5 mM of sulfuric acid running isocratic at 0.6 mL/min. The column temperature was maintained at 45 °C throughout the run. The sugar concentration was quantitated by the same HPLC system with the Aminex HPX-87P column. The mobile phase was nano-pure water running at 0.6 mL/min. The column temperature was kept at 85 °C.

In order to determine the degradation compounds present in the prehydrolysates, the prehydrolysates were dissolved in anhydrous ethyl acetate with the ratio of 2:1. The ethyl acetate phase was then subjected to analysis by an Agilent GC/MS system (Agilent 7890A GC/5975 MS) equipped with a DB- 1701 column (30 m×0.25 mm, 25 micron). The inlet temperature was set at 280 ºC and split ratio was 20:1. The injection volume was 1.0 µm. The analysis was run with a constant flow mode at 1.0 mL/min using helium as carrier gas. The program started at 40 ºC for 2 minutes, with a heating rate of 6 ºC /min to 280 ºC and held for 10 minutes for column cleaning. The MS scans m/z ranging from 29 to 500 Da.

4.4 Results and Discussion

4.4.1 Single Detoxification Strategy

The prehydrolysates after three times dilution consisted glucose 3.5 g/L, xylose 9.3 g/L, galactose 3.9 g/L, arabinose 1.64 g/L, mannose 6.31 g/L, and sugar degradation compounds including acetic acid 3.39 g/L, HMF 0.73 g/L, furfural 1.31 g/L, formic acid 0.56 g/L and levunilic acid 0.06g/L and phenolic compounds derived from lignin. The untreated prehydrolysates could not be fermented by *Clostridium acetobutylicum* ATCC 824, indicating the detoxification was required to remove the inhibitors. Different detoxification methods were performed on loblolly pine prehydrolysates (**Table 4-1**). Of these detoxification approaches investigated, treatment by overliming, anion resin (Dowex 1X4) and cysteine improved the fermentability. It was found that overliming and anion resin detoxification increased butanol production to 4.26 g/L and 6.9 g/L and ABE production to 6.77 g/L and 10.38 g/L, respectively **(Table 4-1, Fig. 4-1 and Fig. 4-2)**. They partially detoxified the prehydrolysates considering the sugars were not fully utilized and anion resin detoxification had a better effect than overliming in

terms of sugar utilization. While the resin detoxification resulted in a higher sugar loss (17.6%) compared to overliming detoxification (7.6%). Overliming and anion exchange resin have been extensively studied in detoxification of hydrolysate. *Larsson et al*. [86] observed better ethanol yield and productivity by anion exchange resin than that by $Ca(OH)_2$ and a significant sugar loss (26%) was reported by anion exchange resin at pH 10. They found the treatment by resin removed the most potential inhibitors. The ethanol yield and productivity after overliming treatment in their case are much closer to reference fermentation, but it is not the case in this study. It could be ascribed to the different tolerance to certain inhibitors between baker's yeast and *C. acetobutylicum*. Similarly, Lu *et al.* [124] observed ABE production of 6.73 g/L from untreated wood pulping hydrolysates, and treatment by overliming did not improve its fermentability. $Ca(OH)_2$ treatment appeared not to change the peaks corresponding to phenolic compounds [230] and some phenolic compounds were found to be far much more inhibitory than inhibitors derived from sugars even in trace amount [85, 168, 203].

Table 4-1 ABE fermentation on prehydrolysates detoxified by different methods

Treatment	Utilized Sugar (Percentage of fermentable sugar)(g/L)	Butanol (g/L)	Butanol Yield (g/g sugar)	ABE (g/L)	ABE Yield (g/g sugar)	Acid (g/L)
Glucose Control	49.55±0.31 (95.60%)	9.07±0.08	0.18±0.01	13.85±0.21	0.26±0.02	3.59±0.11
Untreated	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Cysteine	27.72±0.35 (40.27%)	0.44±0.07	0.01±0.00	0.44±0.07	0.01±0.00	8.9±0.47
Overliming	30.35±0.16 (46.94%)	4.26±0.20	0.07±0.00	6.77±0.28	0.10±0.01	6.06±0.33
Anion Resin (1X4)	48.57±0.28 (84.19%)	6.90±0.32	0.12±0.01	10.38±0.19	0.18±0.01	6.80±0.18
Nonionic Resin(XAD4)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Carbon	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Laccase	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Note: the format was presented as mean value ± standard deviation

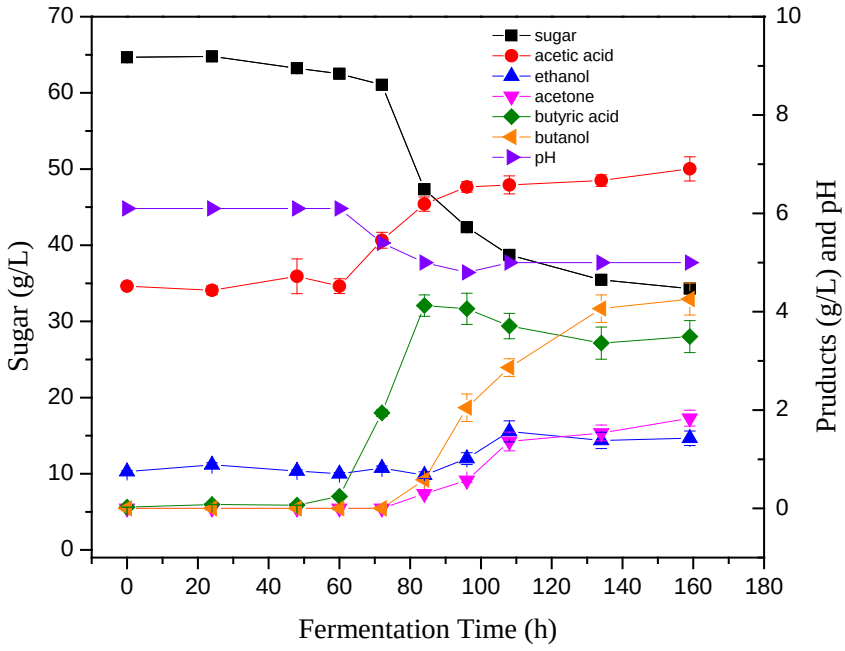

Figure 4-1 Detoxification by overliming

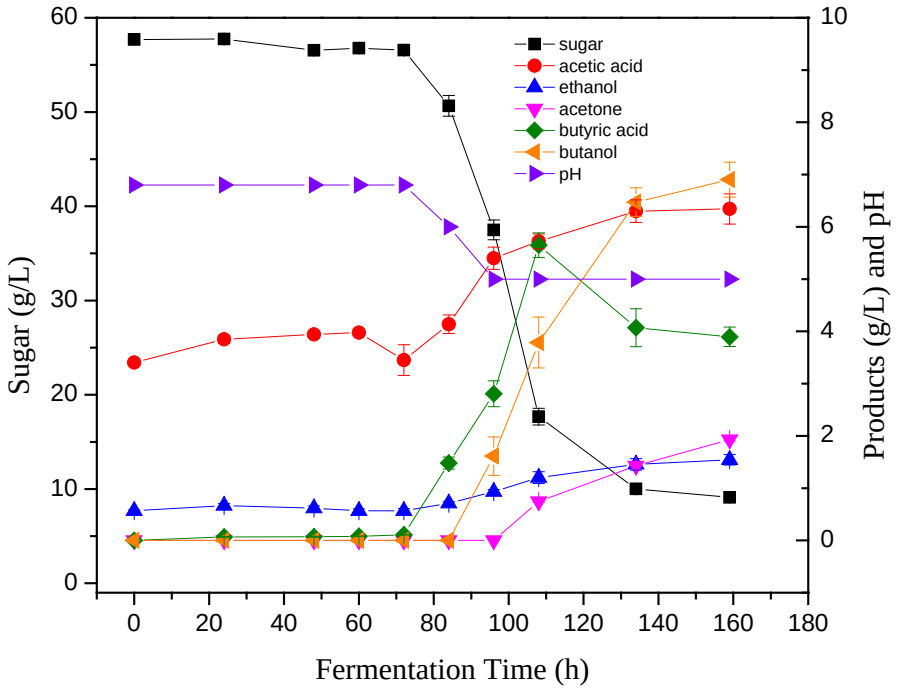

Figure 4-2 Detoxification by anion resin

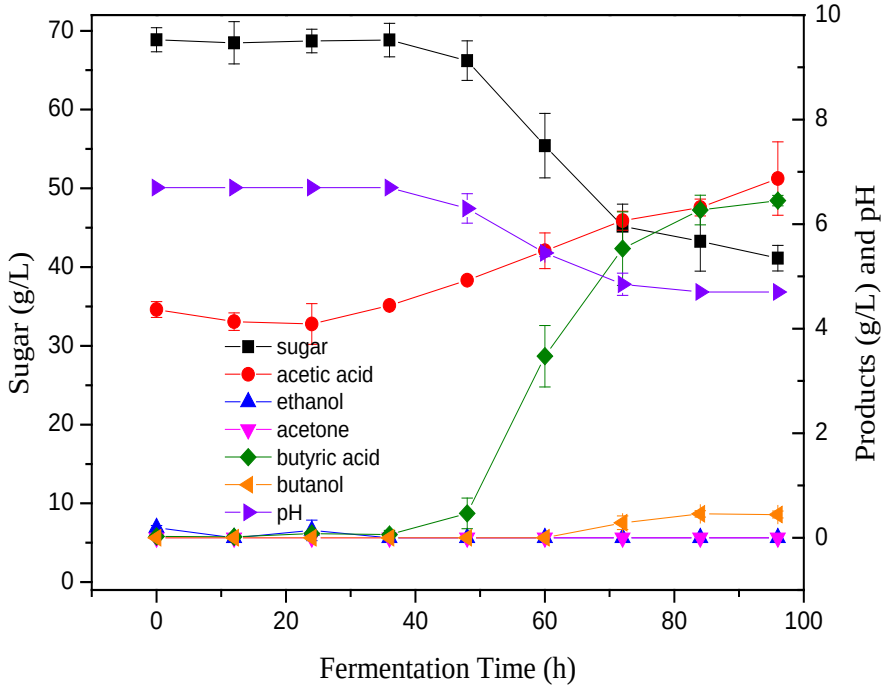

Figure 4-3 Detoxification by cysteine

On the other hand cysteine detoxification improved sugar utilization to 40.27% but led to a considerable acid production (2.5 times of control) and little amount of solvent (**Table 4-1 and Fig. 4-3**). It was reported by Xie *et al.* [231] that amino acids could detoxify prehydrolysates from acid treated loblolly pine and increased both ethanol productivity and yield, among the tested amino acids, cysteine was the most effective. In this case, it was effective on improving the fermentability of prehydrolysates but produced a negligible amount of acetone-butanol-ethanol. It was probably due to cysteine stimulating the metabolic pathway towards acid production. Detoxification by nonionic (Amberlite XAD4) resin, activated carbon and laccase did not show any improvement on butanol fermentation with the prehydrolysates although they have been successfully applied in this field (**Table 4-1**). The inhibition effect was remarkably reduced after specifically removing phenolic compounds by laccase and more than 80% of phenolic compounds in hydrolysate were reported to be removed [84, 139, 232]. Moreover, detoxification by laccase caused 80% reduction in phenolic compounds but no impact on weak acid and furans, indicating phenolic compounds are important inhibitors resulting in microbial inhibitions [86]. However, this method was not effective in the present work. It is possible that residual phenolic compounds interacting with other toxins retained the toxicity to fermentation by *C. acetobutylicum*.

4.4.2 Two-step Detoxification

Although overliming and anion resin treatments are able to improve ABE production (**Fig.4-1 and Fig.4-2**), it was noticed that a lag phase of approximately 60 h occurred before the fermentations started after these treatments. This lag phase made the fermentation less efficient and thus two-step detoxification was developed to seek better fermentation efficiency. Adjusting pH to 10 by $Ca(OH)_2$ and holding for 30 min at 90 ºC was performed as the first step and

followed by anion resin treatment as the second step. It was found that this strategy could remove the lag phase and shorten the fermentation time to 48 h (data not shown). To optimize the condition in first step, lower pH and temperature (pH=7 at 90 ºC and pH=7 at 25 °C) were investigated. Conducting the first step at pH=7 and 25 °C obtained a pretty similar butanol production to that at pH=10 and 90 °C. So, the condition of pH=7 and 25 °C was considered to be the best condition in terms of chemical consumption and energy input. Addition of first step did not increase sugar loss (17.3%), which was comparable to anion resin detoxification. But it did improve the fermentation efficiency, the fermentation could complete before 48 h with a sugar conversion of 92.0% and butanol and ABE production of 7.52 g/L and 11.11 g/L respectively, corresponding to a butanol yield of 0.13 g/g sugar and ABE yield of 0.19 g/g sugar. (**Fig.4-4**). This result suggested that treatment by $Ca(OH)_2$ at neutral pH was able to further detoxify the inhibitors in the prehydrolysates and enhance the fermentability on the basis of anion detoxification. Various detoxification methods have been applied in the prehydrolysates and reported in previous research. However, to the best of our knowledge, detoxification on wood prehydrolysates from organosolv pretreatment has never been reported. Liu *et al.* [136] reported detoxification of switchgrass hydrolysate by activated carbon increased the butanol production from 0.4 to 11.0 g/L and total ABE from 0.9 to 16.8 g/L. Prehydrolysates generated from a mixture of aspen (60%) and maple (40%) wood was detoxified by Coagulation–flocculation using ferric sulfate ($Fe_2(SO_4)_3$) followed by 13-fold dilution and the ABE production was increased from 0.8 g/L to 6.4 g/L [233]. An evaluation of three detoxifications (overliming, activated carbon and ion exchange resin) of hydrolysate from mixed hardwoods (maple, beech and birch) was conducted and the best improvement (butanol production of 9.14 g/L, 104% increase of untreated hydrolysates) was achieved by ion exchange resin, while the

sugars were not completely utilized, with a conversion of 65.6% [124]. A comparison between this study and other works is summarized in **Table 4-2**. To compare the data from this study with the literature, the ABE yield of this work presented in the table was recalculated as total ABE produced divided by the total sugar utilized, which is slightly higher than the one (0.19 g/g) mentioned in above text. This difference is caused by the residual unused sugar after fermentation. The detoxification effectiveness is not able to be directly compared considering the difference of strain and the various inhibitors generated from different biomass treated at certain conditions. For instance, Qureshi *et al*. reported switchgrass hydrolysates treated by overliming and Na_2SO_3 exhibited poor ABE production, on the contrast, corn stover treated by the same detoxification process received remarkable improvement, resulting in higher ABE production and yield than the control experiment [123].

We applied the same strategy to cysteine detoxification as well. A similar effect was observed and the fermentation started upon the inoculation was induced. However, the treatment by $Ca(OH)_2$ seemed to shorten the bacteria adaption time instead of improving solvent production (**Fig. 4-5**). Like the treatment by cysteine alone, it formed significant amount of acid (6.75 g/L) instead of butanol.

Table 4-2 ABE production from detoxified prehydrolysates

Substrate	Pretreatment Process	Detoxification Method	Strain	Butanol Production (g/L)	ABE Production (g/L)	ABE yield [a] (g/g)	ABE Productivity [b] (g/L/h)	Reference
Loblolly pine	Organosolv	$Ca(OH)_2$+Anion exchange resin	*C. acetobutylicum* ATCC824	7.52	11.11	0.21	0.23	This study
Switchgrass	Hydro-thermolysis	Activated carbon	*C. acetobutylicum* ATCC824	11.03	16.80	0.30	NA	Liu *et al*. [136]
Hardwood mixture	Steam and hot water	Flocculation+dilution	*C. acetobutylicum* ATCC824	NA	6.40	0.17	0.044	Mechmech *et al*. [233]
Hardwood mixture	Alkaline	Overliming	*C. beijerinckii* CC101	4.41	5.83	0.28	0.08	Lu *et al*. [124]
Hardwood mixture	Alkaline	Activated carbon	*C. beijerinckii* CC101	6.27	8.98	0.28	0.13	Lu *et al*. [124]
Hardwood mixture	Alkaline	Ion exchange resin	*C. beijerinckii* CC101	9.14	11.35	0.39	0.16	Lu *et al*. [124]
Barley straw	Dilute sulfuric acid	Overliming+Na_2SO_3	*C. beijerinckii* P260	18.01	26.64	0.43	0.39	Qureshi *et al*. [131]
Corn stover	Dilute sulfuric acid	Overliming+Na_2SO_3	*C. beijerinckii* P260	14.50	26.27	0.44	0.31	Qureshi *et al*. [123]
Switchgrass	Dilute sulfuric acid	Dilution with water	*C. beijerinckii* P260	9.55	14.61	0.39	0.17	Qureshi *et al*. [123]

a ABE yield: Total ABE produced divided by the total sugar utilized
b ABE productivity : Total ABE produced divided by the fermentation time
NA: Not available

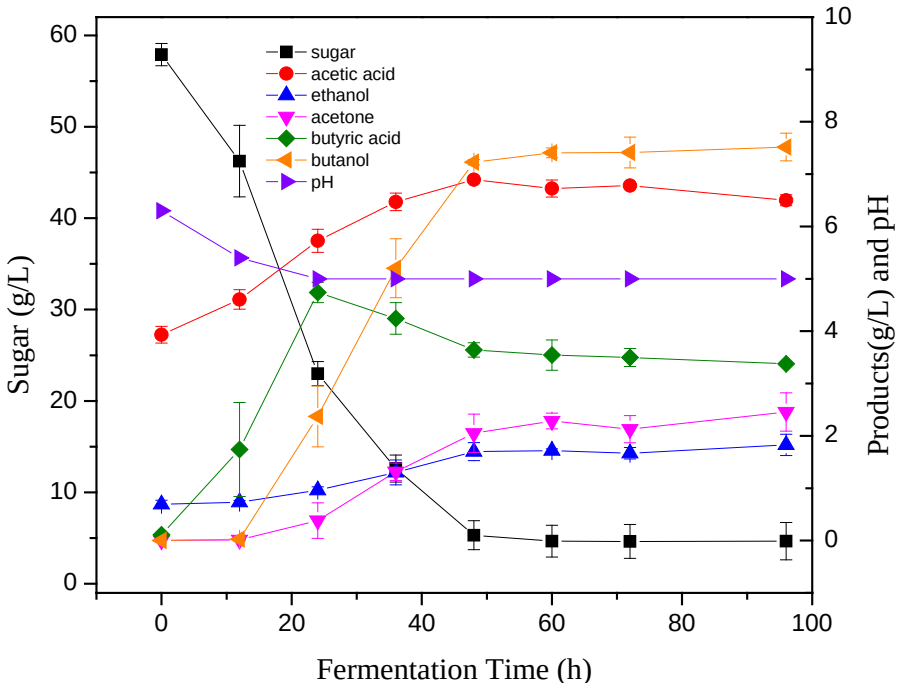

Figure 4-4 Two-step detoxification ($Ca(OH)_2$ + anion resin)

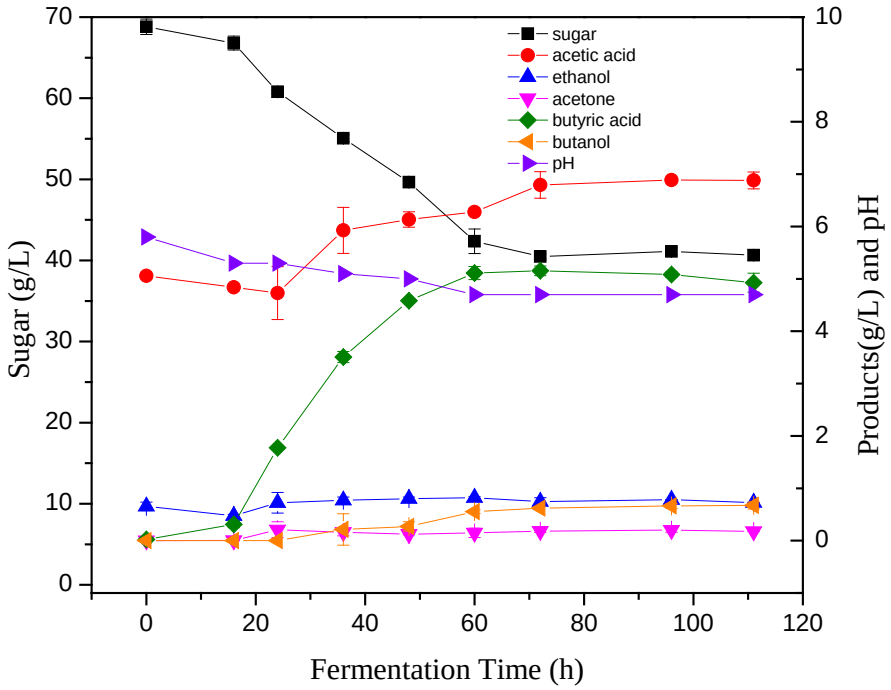

Figure 4-5 Two-step detoxification ($Ca(OH)_2$ + cysteine)

4.4.3 Effect of Salts on ABE Fermentation

$Ca(OH)_2$ in the two-step detoxification strategy played an important role in eliminating the lag phase, it was speculated that the precipitation of salts in prehydrolysates could be one of the reasons for this improvement. In order to prove this hypothesis, fermentation with various concentrations of sodium sulfate (1 g/L, 3 g/L, 5 g/L and 7 g/L) was investigated. The ABE and acid production appeared to be normal comparing with control experiment with the concentration of sodium sulfate between 1 to 5 g/L (**Fig.4-6**). When the sodium sulfate reached 7 g/L, a lag phase of 36 hours occurred (**Fig. 4-7**). It suggested that the sodium sulfate resulting from sulfuric acid induced at pretreatment could be one of the factors to delay butanol fermentation with the prehydrolysates.

This result was in agreement with previous findings. It has been elucidated that the presence of mineral salts was toxic to bacterium growth and ABE fermentation [72, 117, 234]. Acetate is formed from hydrolysis of hemicellulose during acid pretreatment and sulfate is generated due to the use of sulfuric acid to pretreat substrate. Ezeji *et al.* [72] reported 13.3 g/L sodium sulfate resulted in 53% reduction in cell growth and much less ABE produced than control while 8.9 g/L sodium acetate was observed to have a promotion effect on both cell growth and ABE fermentation. However, the combination of these two caused even lower ABE production than that with sodium sulfate alone. Maddox *et al.* [234] selected sodium chloride and magnesium chloride as representative salts and it was demonstrated that they both inhibited growth of *C. acetobutylicum* and ABE production in the presence of sodium chloride. It was found that the ABE production was dramatically increased to 22.17 g/L (comparable to control at 21.31 g/L) when salts were removed from alkaline peroxide wheat straw hydrolysates by electrodialysis [117].

The sodium sulfate was estimated to be 2.07 g/L based on the sulfuric acid input during pretreatment. The inhibition effect was noticed until it was as high as 7 g/L, probably resulting from the synergistic inhibitory effect which was already found by Ezeji *et al.* [72]. Considering the impact of $Ca(OH)_2$ on two-step detoxification method and the inhibition effect of sodium sulfate at 7g/L, we speculated the reason why $Ca(OH)_2$ could accelerate the fermentation process might be the precipitation of sulfate by $Ca(OH)_2$.

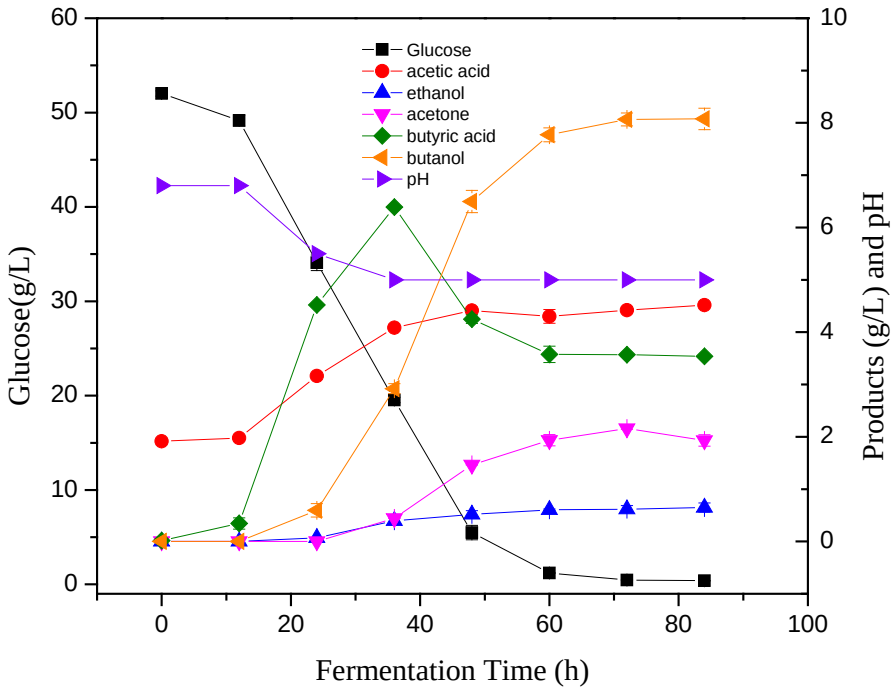

Figure 4-6 Glucose fermentation with 5 g/L Na_2SO_4

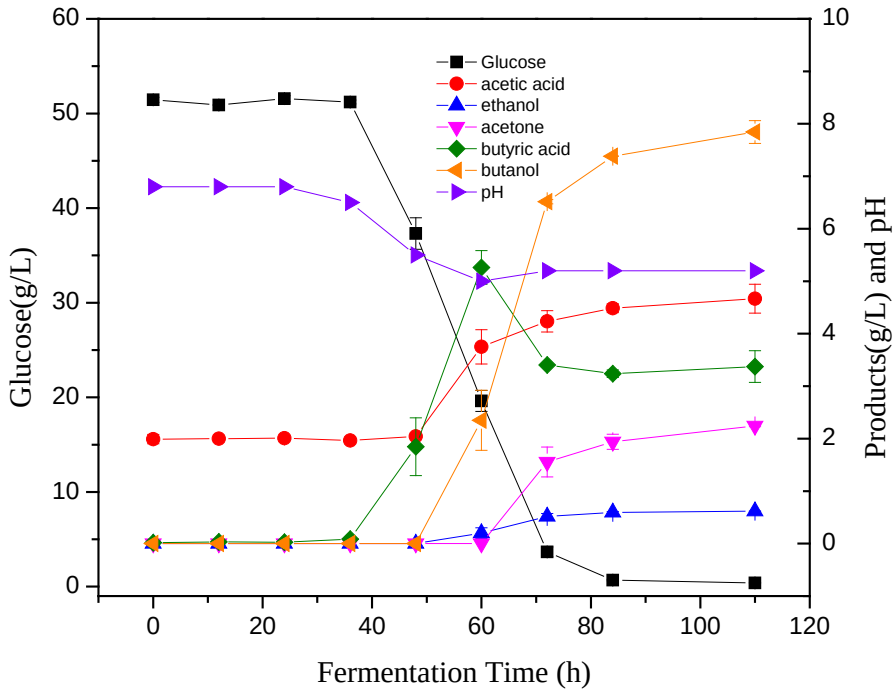

Figure 4-7 Glucose fermentation with 7 g/L Na_2SO_4

4.4.4 Potential Inhibitors in Organosolv Pretreated Loblolly Pine Prehydrolysates

The degradation compounds in prehydrolysates were determined by GC/MS (**Table 4-3**). Some phenolic compounds including vanillin, 4-propylguaiacol and guaiacylacetone, coniferyl alcohol, homovanillic acid, and homovanillic acid methylester were identified and they are all decomposed from guaiacyl lignin unit. The species of aromatic monomers depends on the type of pretreatment and the H/G/S ratio of lignin present in the raw materials. The softwood mostly produces guaiacyl lignin derivatives while hardwood and herbaceous biomass produce H, G and S phenolic compounds [64]. Similar to this work, Clark *et al*. found vanillin and guaiacylacetone in hydrolysate derived from dilute acid treated softwood [85]. Larsson *et al*. identified homovanillic acid from dilute acid hydrolysate of spruce [86]. It was found the two-step detoxification was effective on eliminating the inhibitors in the prehydrolysates (**Table 4-3**

Table 4-3 Compounds determined in untreated and detoxified prehydrolysates (ethyl acetate extract)

Compound	Untreated (%)	$Ca(OH)_2$ (%)	Anion Resin (%)
HMF	100	73	0
Vanillin	100	33	0
4-Propylguaiacol	100	4	0
Guaiacylacetone	100	28	0
Coniferyl alcohol	100	0	0
Homovanillic acid	100	78	0
Homovanillic acid methylester	100	78	0

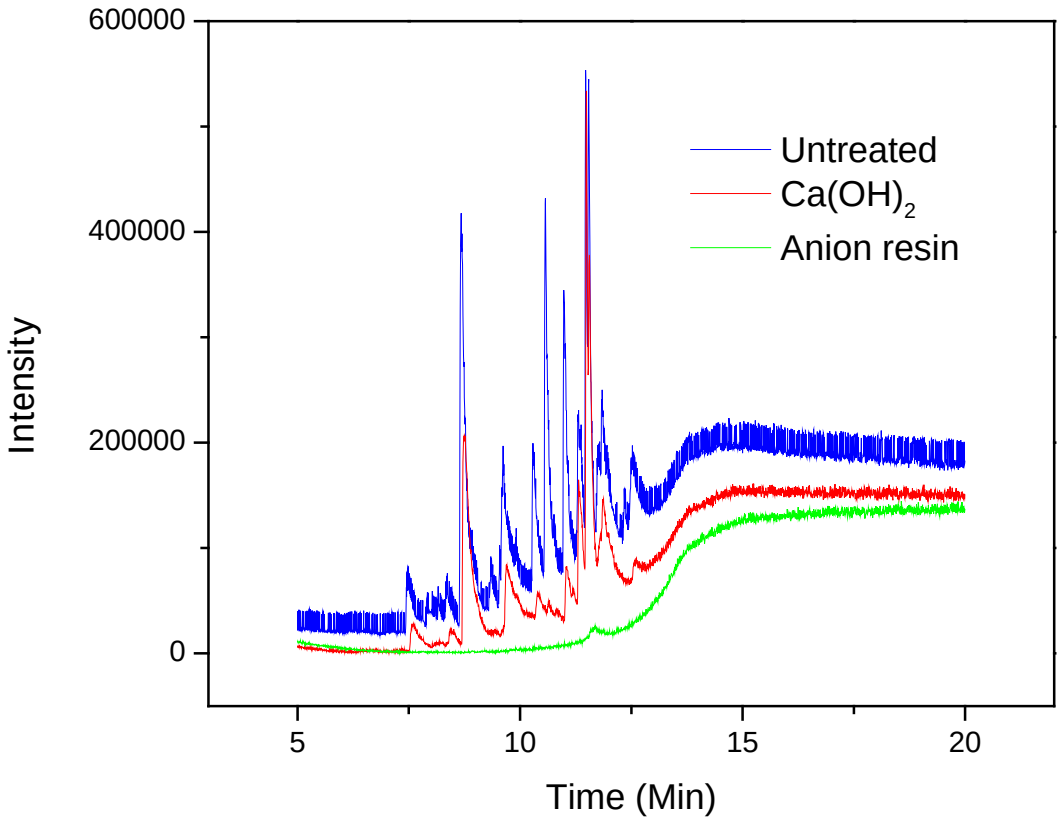

Figure 4-8 Gas chromatography of untreated and detoxified prehydrolysates

and Fig.4-8). The first step (treated by $Ca(OH)_2$) partially removed the degradation compounds. HMF, vanillin, 4-propylguaiacol and guaiacylacetone, coniferyl alcohol, homovanillic acid, and homovanillic acid methylester were reduced by 27%, 67%, 96%, 72%, 100%, 22% and 22%, respectively. The second step (treatment by anion resin) substantially removed the potential inhibitors. The gas chromatography in **Fig. 4-8** showed a small peak of prehydrolysates after resin treatment, indicating HMF, vanillin, 4-propylguaiacol, guaiacylacetone, coniferyl alcohol, homovanillic acid, homovanillic acid methylester and the unidentified compounds were completely removed, except the only one represented by the peak.

4.5 Conclusions

Due to the inhibitors generated from the organosolv pretreatment process, prehydrolysates obtained from pretreated loblolly pine was not fermentable by *C. acetobutylicum* ATCC 824. Among the six detoxification approaches examined, anion exchange resin was found to be the best method to detoxify the prehydrolysates which gave 84.19% sugar utilization with 10.38 g/L ABE production and a yield of 0.18 g/g sugar. Overliming detoxification process showed the second best result which gave 46.94% sugar utilization with 6.77 g/L ABE production and 0.10 g/g sugar yield. However, a lag phase of approximately 60 h was observed in fermentations by these two methods. In order to alleviate this problem, a two-step detoxification strategy was developed. $Ca(OH)_2$ was firstly used to treat the prehydrolysates and then the anion exchange resin was applied. Fermentation after this two-step detoxification showed no lag phase and the ABE production was satisfactory, which gave 11.11 g/L ABE production and a yield of 0.19 g/g sugar. The reason of the success of this two-step treatment is speculated to be the removal of mineral salts. We believe that $Ca(OH)_2$ could precipitate sulfate and therefore eliminate the lag phase. This speculation was proved by our investigation on the

effects of salts on ABE fermentation. The fermentation with sodium sulfate at the level of 1 to 5 g/L didn't show any lag. But when the sodium sulfate concentration reached 7 g/L, a lag of 36 hours occurred. The degradation compounds before and after two-step detoxification were determined by GC/MS. It was found the treatment by $Ca(OH)_2$ reduced the concentrations of some phenolic compounds and the resin could remove almost all the potent inhibitors.

Chapter 5

Acetone-Butanol-Ethanol Production from Organosolv Pretreated Loblolly Pine

5.1 Abstract

Acetone-butanol-ethanol production from ethanol organosolv pretreated loblolly pine was investigated both with separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) in the present study. In order to fully utilize the sugars present in feedstock, both hemicellulose dissolved in the prehydrolysates and cellulose left in remaining biomass solids were used for fermentation. It was found the ethanol organosolv was an effective pretreatment to remove 77.6% of original lignin in biomass leaving a cellulose rich solid which was suitable for ABE fermentation. The process of ethanol washing of pretreated solids played an important role in ABE fermentation and a good ABE fermentation yield (9.29 g/L butanol with a yield of 0.16 g/g and 15.74 g/L ABE with a yield of 0.27 g/g) was achieved with the SSF on ethanol-washed substrate. Therefore, the SSF is preferred process configuration compared to SHF. Moreover, the supplementation with detoxified prehydrolysates increased the butanol and ABE titer to 10.51 g/L and 18.29 g/L, respectively, which were 13% and 16% higher than that from solid only.

5.2 Introduction

Owing to the rapid growth of population and economic development, the conflict between increasing energy demand and limited fossil fuels has been rising since the past decades. In response to this situation, extensive research on biofuels production have been conducted on the most abundant organic resources, lignocellulosic biomass, such as corn stover [12, 235], wheat straw [236], switchgrass [17], paper sludge [237], and hardwood [238, 239]. Butanol, as a

potential alternative to gasoline, is superior to ethanol due to its high energy content, low vapor pressure, low volatility, low flammability and the property of being mixed with gasoline in any proportion [150, 152]. In addition, butanol has extremely similar characteristics to gasoline and thus it can be blended with gasoline or directly used in vehicle with no engine modification required [153]. However, fermentation- derived butanol suffers from high production cost and low productivity. Significant amount of research efforts have been made to improve the economics including using low cost substrates and increasing the butanol productivity by optimizing process variables, integration of product recovery to fermentation, and developing inhibitor-tolerant cultures through gene modification [152, 240, 241].

Bioconversion of pretreated substrates to butanol/ABE can be carried out in two main process configurations, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). SHF is to perform the saccharification/hydrolysis and fermentation sequentially, which allows each step to be conducted at the optimum conditions. But the need for more vessel tanks and longer operating time make this process less attractive. SSF is to carry out the enzymatic hydrolysis and fermentation in the same reactor simultaneously. The fermentable sugars are rapidly converted by microorganism once released from hydrolysis and thus the end product inhibition to enzyme is significantly reduced [239, 240, 242]. Additionally, the SSF offers low operating cost by reducing process step and low contamination risk resulting from lower glucose concentration [243]. However, the main drawback of SSF is the compromise on optimum conditions for enzyme activity and microbe. Particularly, the optimal temperature for enzymatic hydrolysis is 40-50 °C [242] while the traditional clostridia cannot have a good activity when the temperature is above 37 °C . It was reported the optimum growth of *Clostridium beijerinckii* BA101 was 35 °C [244]. Although extensive studies have

been conducted so far on evaluating the performance of SHF and SSF, to our best knowledge, few studies focused on ABE production with organosolv pretreated softwood.

Among the various pretreatment methods investigated, such as the acid pretreatment [245, 246], steam explosion[247], alkaline pretreatment [245, 246], and ionic liquid pretreatment [248], ethanol organosolv pretreatment has been considered to be one of the most promising strategies [48]. It was initially developed from pulping and paper industry and treated with a blend of ethanol and water using acid as a catalyst [224]. This process has been demonstrated to be effective on delignification of biomass [226]. Lignin is commonly believed to be a limiting factor to enzymatic digestibility [249] and has been proved to be adsorbed on enzyme and consequently impairs the enzymatic hydrolysis [250]. By comparing this method to the leading chemical pretreatments the unique advantage of this process is recovery of lignin as value-added product. The ethanol organosolv lignin (EOL) is of high purity and low molecular weight which can be used to synthesize polymeric materials [48]. As a result, the generation of high quality lignin from pretreatment is essential to improve the overall economic viability of biorefinery scheme.

To improve the overall bioconversion yield, complete utilization of sugars in biomass is desired and some research has been attempted to achieve a high butanol yield by using all the present sugar in feedstock but not only glucose. The solvent-producing Clostridia are capable of digesting a wide variety of sugars including glucose, xylose, galactose, arabinose and mannose although the efficiency was reported to be different [251-254]. Yang *et al*. [255] reported a significant elevated ABE yield from barley straw by increasing glucose and xylose yield from 53.2% and 36.2% to 86.9% and 70.2%, respectively, with adding xylanase and surfactants. A number of interests have been attracted on using the solubilized hemicellulose sugars in liquid

stream after pretreatment. However, the bottleneck of converting these sugars is the inhibitors forming through pretreatment, whereas the efficient detoxification is required in most cases [136, 203, 233].

In this chapter, the ABE fermentation was carried out on ethanol organosolv pretreated loblolly pine wood. Warm ethanol solution was normally involved in the washing process after pretreatment to prevent the lignin re-precipitated on the substrate. Therefore, the fermentation was performed on the substrate with and without ethanol washing to assess the necessity of this process. The objectives of the present study are to (1) evaluate the effect of ethanol washing post to pretreatment on ABE fermentation; (2) compare the two widely used process configurations, SHF and SSF; and (3) investigate the feasibility to supplement detoxified prehyrolysates into ABE fermentation.

5.3 Materials and Methods

Chemicals and Microorganisms

Glucose and NaOH were purchased from VWR (West Chester, PA). Ca $(OH)_2$, p-aminobenzoic acid and CH_3COONH_4 were purchased from Alfa Aesar (Heysham, England). Thiamine was purchased from Alfa Aesar (Ward Hill, MA). Dowex 1X4 resin (chloride form) and biotin were purchased from Sigma-Aldrich (St. Louis, MO). H_2SO_4 (98%) and NaCl were purchased from VWR (West Radnor, PA). K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ were purchased from Fisher Scientific (Fair Lawn, NJ). Citric acid was purchased from Mallinckrodt chemicals (Phillipsburg, NJ) $CaCO_3$ was purchased from EMD chemicals (Gibbstown, NJ). Reinforced Clostridial Broth medium (RCM) was purchased from HIMEDIA laboratories (Mumbai, India) with the composition of 10 g/L casein enzymic hydrolysate, 10 g/L

beef extract, 3 g/L yeast exact, 5 g/L glucose, 5 g/L sodium chloride, 3 g/L sodium acetate, 1 g/L soluble starch and 0.5 g/L L-cysteine hydrochloride. Cellic CTec 2 was obtained from Novozymes North America, Inc (Franklinton, UC). DI-water was produced by the Barnstead Nanopure UV Ultrapure Water System (Thermo Fisher Scientific, Marietta, OH).

Clostridium acetobutylicum ATCC 824 (obtained from Dr. Y. Y. Lee's lab at Auburn University) was used for butanol production. It was routinely stored as spores at 4°C and treated by heat shock at 75 °C for 10 min followed by cooling down in ice bath prior to cultivation. It was cultivated in Reinforced Clostridial Medium (RCM). The medium was bubbled through nitrogen for 15 min to remove oxygen and then autoclaved at 121 °C for 15 min. The heat-shocked cells were grown in RCM medium as seed inoculum until the optical density (OD) reached 1.30 determined by an UV-vis spectrometer (Thermo Scientific, Madison, WI) at 600nm.

Organosolv Pretreatment and mass balance

Loblolly pine wood chips were collected by Forest Products Laboratory at Auburn University and those with bark free and size of 1.0 × 1.0 cm (L × W) were selected for organosolv pretreatment. Wood chips (80 g, oven-dry weight) were soaked in 65% (*v/v*) ethanol solution with 1.1 % (*w/w*) sulfuric acid (on the basis of biomass dry weight) overnight (7:1 liquor/solid ratio) and then loaded into a 1 L Parr reactor (Parr Instrument Co., Moline, IL) to be treated at 170 °C for 60 min. Sample was taken from aqueous phase to determine the furfural and HMF and the spent liquor(aqueous phase) was separated from solid by vacuum filtration upon the completion of pretreatment. Afterwards, if ethanol washing was needed, the solid fraction was washed by 700 ml warm ethanol solution(65% (*v/v*), 50 °C) three times to dissolve the ethanol extractable lignin and followed by washing by 700 ml DI water four times to remove the

residue ethanol. The cellulose-rich solid fraction was homogenized in a blender for 15 s and then used to perform fermentation by *Clostridium acetobutylicum* ATCC 824 and the aqueous phase was subject to detoxification prior to supplementation to SHF and SSF. They were both stored at 4 °C until use.

In order to conduct the mass balance of organosolv pretreatment, the EOL was collected from the spent liquor and the ethanol washes. 3-fold DI water was added into the mixture of spent liquor and the ethanol washes to precipitate lignin and then the lignin fraction was prepared by filtration through Whatman NO.1 filter paper and then washed thoroughly with DI water, dried in air and then in oven (105 °C). Sample from the mixture of filtrate and water washes was taken to determine the water-solubles. The detailed process was shown in **Figure 5-1** [227].

The combined severity (CS) was used to determine the intensity of pretreatment, it was calculated as a function of reaction time (t, min), target temperature (T_H, ºC) and pH. The following equation was used for calculation [256],

$$CS = \log(t \cdot \exp[(T_H - T_R)/14.75]) - pH \qquad \text{Eq.5-1}$$

where T_R is reference temperature, 100 ºC

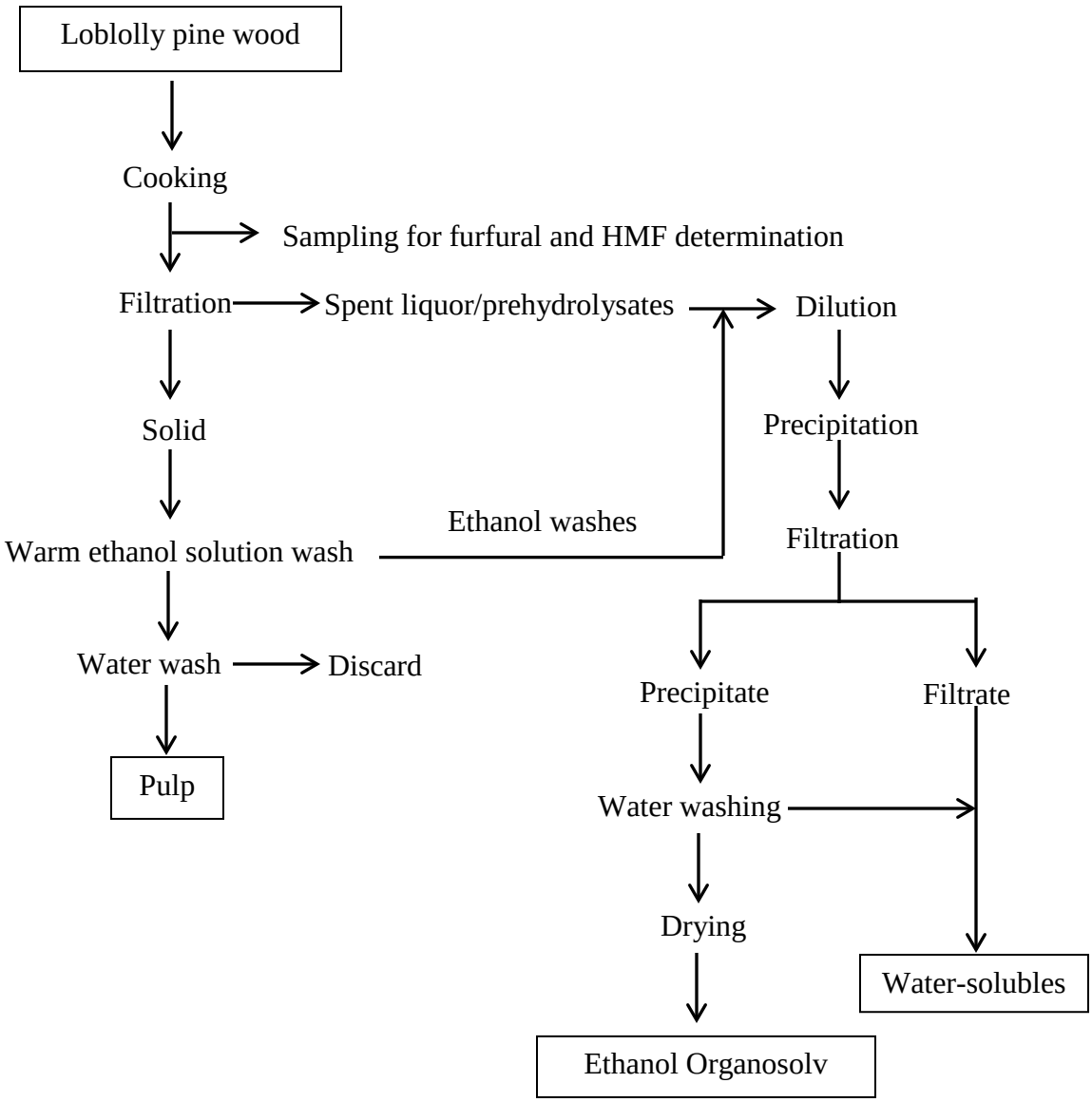

Figure 5-1 Flow chart of mass balance for ethanol organosolv pretreatment (Adapted from Pan *et al*. [227])

Chemical Analysis of Raw Biomass and Pretreated Substrates

The extractives content in raw biomass and organosolv pretreated substrate was determined according to NREL analytical procedure, NREL/TP-510-42619. The composition analysis of carbohydrate and lignin before and after ethanol organosolv pretreatment was carried

out using extractives-free sample according to NREL standard protocol, NREL/TP-510-42618. The sugar content of prehydrolysates was determined according to NREL standard method, NREL/TP-510-42623.

Detoxification

Ethanol in prehydrolysates was evaporated at 40 °C in a rotary evaporator (IKA RV10 basic) and the pH was adjusted to 4.0 before evaporation. The concentrated prehydrolysates was then diluted with DI water to make the total volume same as that before evaporation. Two-step detoxification was carried out as described in previous chapter. Briefly, the pH of prehydrolysates was adjusted to 10 by adding $Ca(OH)_2$ and incubated at 60 °C and 100 rpm for 30 min. The precipitate was removed by centrifuge at 4000 rpm for 15 min upon completion of incubation with $Ca(OH)_2$. In the second step, the Dowex 1X4 resin was activated by saturation NaCl followed by washing with NaOH and then DI water. 10 g activated resin was added into 100 mL prehydrolysates and the whole mixture was incubated at 25 °C and 100 rpm for 1 h. The liquid was separated from resin by centrifuge at 4000 rpm for 10 min and then the pH was adjusted back to 7 by NaOH.

Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)

Cellic CTec 2 was used in enzymatic hydrolysis of pretreated biomass and its filter paper enzyme activity was 126 FPU/ml. Enzymatic hydrolysis of ethanol washed and ethanol unwashed pulp (moisture content, ~70%) was carried out in 125mL serum bottle with working volume of 45 ml with glucan 5.8 % (*w/v*) corresponding to 60 g/L glucose in fermentation. The sterilization was conducted at 121 °C for 15 min prior to enzymatic hydrolysis to avoid sugar

degradation during autoclave. Enzyme loading of 25 FPU/glucan was used in hydrolysis with adding 50 mM citrate buffer to control pH at 4.8. The enzymatic hydrolysis was performed at 50 °C and 150 rpm for four days. The enzymatic hydrolysis yield was calculated with the released glucose content as a percentage of glucose available in substrate. After the completion of hydrolysis, the mixture was supplemented with previous filter-sterilized nutrients stock: 50μl vitamin (p-aminobenzoic acid, 1 g/L, thiamine, 1 g/L, biotin, 0.01 g/L), 0.25 mL mineral ($MgSO_4 \cdot 7H_2O$, 40 g/L, $MnSO_4 \cdot H_2O$, 2 g/L, $FeSO_4 \cdot 7H_2O$, 2 g/L, NaCl, 2 g/L) and 0.5 mL buffer (K_2HPO_4, 50 g/L, KH_2PO_4, 50 g/L, CH_3COONH_4, 220 g/L). $CaCO_3$ (0.25 g) was added into the medium to control the pH during fermentation. The medium in serum bottle was vacuumed and then flushed with nitrogen for 7 cycles to remove oxygen; schematic purge valve is shown in **Figure 3-1**. The inoculation volume was 10% (*v/v*) and the fermentation was carried out at 35 °C and 80 rpm.

EOL was added into ethanol-washed substrate prior to enzymatic hydrolysis to study the effect of lignin on separated hydrolysis and fermentation. 0.3 g EOL was supplemented to ensure the total lignin content was equivalent to that of ethanol-unwashed substrate.

SSF was conducted at the same conditions with separate hydrolysis and fermentation SHF described as above. But the enzyme and inoculation were induced at the same time.

In order to investigate the feasibility of integration prehydrolysates to solid fermentation, the detoxified prehydrolysates were supplemented into SSF to replace water previously used. The detoxified prehydrolysates were added into ethanol-washed substrate for doing SSF. The overall procedure is shown is **Figure 5-2**. All fermentations were performed in duplicates. Samples were taken periodically for analyzing sugar consumption and ABE production.

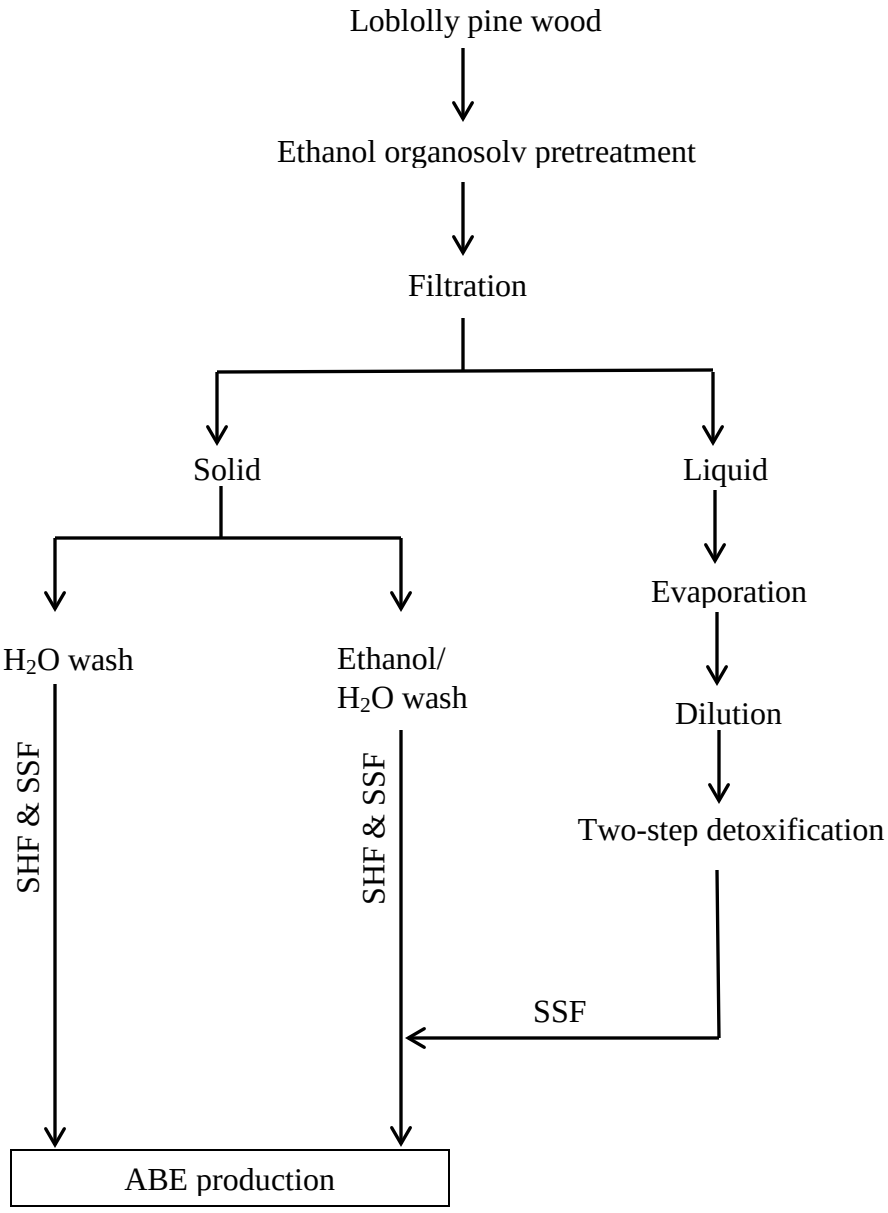

Figure 5-2 Flow chart of ABE production from loblolly pine

Butanol yield was calculated as butanol produced divided by glucose content in pretreated biomass and is expressed as g/g. ABE yield was calculated as the total ABE produced divided by glucose content in pretreated biomass and is expressed as g/g. In the case of prehydrolysates supplemented SSF, the sugars in liquid fraction was also considered, the glucose content was replaced by glucose in solid plus total sugars available in the prehydrolysates.

Sugar and Products Analysis

The sugar content was quantified by a Shimadzu (LC-20A) HPLC system consisting of a degasser, autosampler, LC-20AD pump, and RID-10A detector, equipped with a 300 mm × 7.8 mm i.d., 9 μm, Aminex HPX-87P column and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio-Rad, Hercules, CA). Nano-pure water was used as mobile phase running at 0.6 mL/min. The column temperature was maintained at 85 ºC. Acetic acid, butyric acid, ethanol, acetone, butanol, HMF and furfural were quantified by the same HPLC system (Shimadzu LC-20A) equipped with an Aminex HPX-87H column. The mobile phase was composed of 5 mM of sulfuric acid running isocratic at 0.6 mL/min. The column temperature was kept at 45 °C throughout the run.

5.4 Results and Discussion

5.4.1 Chemical Composition of Loblolly Pine Wood

The chemical composition of untreated and ethanol organosolv pretreated loblolly pine is shown in **Table 5-1.** The carbohydrates (cellulose and hemicellulose) accounted for approximately 64.92% of raw material and the total lignin (AIL and ASL) represented 30.01%. They were in good agreement with previous report on softwoods [257-259]. The glucan content increased after pretreatment was due to the fact that the loss of hemicellulose and lignin was

relative higher than glucan. The hemicellulose sugars were easier degraded than cellulose when exposed to heat [8, 229]. The ethanol extractives in pretreated substrate without ethanol washing (9.64%) was much higher than that in untreated biomass (1.18%). It was because the recalcitrant structure was broken during pretreatment and some of the lignin was extracted out by ethanol. While, the pretreated substrate with ethanol washing was observed an extremely low value of ethanol extractives, resulting from the lignin washed out by warm ethanol solution. The total lignin and ethanol extractives in substrate with ethanol washing was 9.17% lower than that in substrate without ethanol washing, suggesting the warm ethanol was effective to remove the lignin from cellulose-rich solid fraction and the lignin could be collected as a valued co-product.

Table 5-1 Chemical composition of untreated and ethanol organosolv pretreated loblolly pine

	Untreated (%)	**W/o ethanol washing (%)**	**W/ethanol washing (%)**
Glucan	41.50±0.38	72.74±0.20	82.14±0.03
Xylan	7.51±0.05	2.17±0.01	1.69±0.08
Galactan	2.96±0.05	0.36±0.03	0.40±0.02
Arabinan	1.78±0.03	0.63±.02	0.69±0.05
Mannan	11.17±0.08	1.36±0.00	0.99±0.02
Ethanol Extractives	1.18±0.05	9.64±0.12	0.79±0.04
Acid Insoluble Lignin(AIL)	29.45±0.27	12.11±0.15	11.72±0.03
Acid Soluble Lignin(ASL)	0.56±0.05	0.28±0.00	0.35±0.01
Ash	0.36±0.02	0.03±0.00	0.04±0.00
Total	96.47	99.31	98.81

5.4.2 Mass Balance of Organosolv Pretreatment

The mass balance of organosolv pretreatment was performed at the investigated condition, with a combined severity (CS) of 1.94, based on 80 g dry wood (**Fig. 5-3**). The solid recovery is 48.8% and 95.6% of glucan in untreated wood was retained in the solid fraction and only 1.30 g glucose was found in aqueous phase. In contrast, most of the hemicellulose released into liquid phase, approximately 83.7% of xylose, 90.3% of galactose, 55.3% of arabinose and 88.0% of

mannose was detected in water-solubles. The combination of hemicellulose sugars in solid fraction and in water-solubles was lower than the original content in untreated wood, indicating large portion of hemicellulose was degraded during pretreatment forming potential microbial inhibitors. It was found that all the sugars present in liquid are in the forms of monomers. It was known from the fact that the sugar concentrations in prehydrolysates after hydrolysis by 4% sulfuric acid were same as they were. Notably, the acid insoluble lignin decreased from 23.56 g to 5.27 g, corresponding to 77.6% lignin removal. Most of it was precipitated as ethanol organosolv lignin, representing 60.1% of original lignin. It holds great potential to isolate lignin as co-product with great value in industrial application. The mass loss was noticed after pretreatment and it resulted from undetermined components in water-solubles such as organic acids and sugar alcohols.

In general, increasing severity of pretreatment was helpful in increasing cellulose yield in solid fraction but as a result of enhancing the degradation of carbohydrates and delignification, the solid recovery ratio was reduced. Pan *et al*. [227] investigated the effect of process variables (temperature, acid dose, reaction time and ethanol concentration) on products yield and optimized the conditions. The condition in the present work is close to their center point condition (180 ºC, 60 min, 1.25% H_2SO_4 and 60% ethanol), resulting in the highest solid yield (52.72%), cellulose recovery (88% of glucose in raw material) and total xylose recovery (72% of xylose in raw material). It is apparent that the less server condition in our work resulted in a lower solid and sugar recovery. It was ascribed to the different response to organosolv pretreatment from hardwood and softwood. A consistent result was observed with lodgepole pine pretreated at same condition as the present study, 44.34% of solid yield and 63.18% of lignin recovery [260].

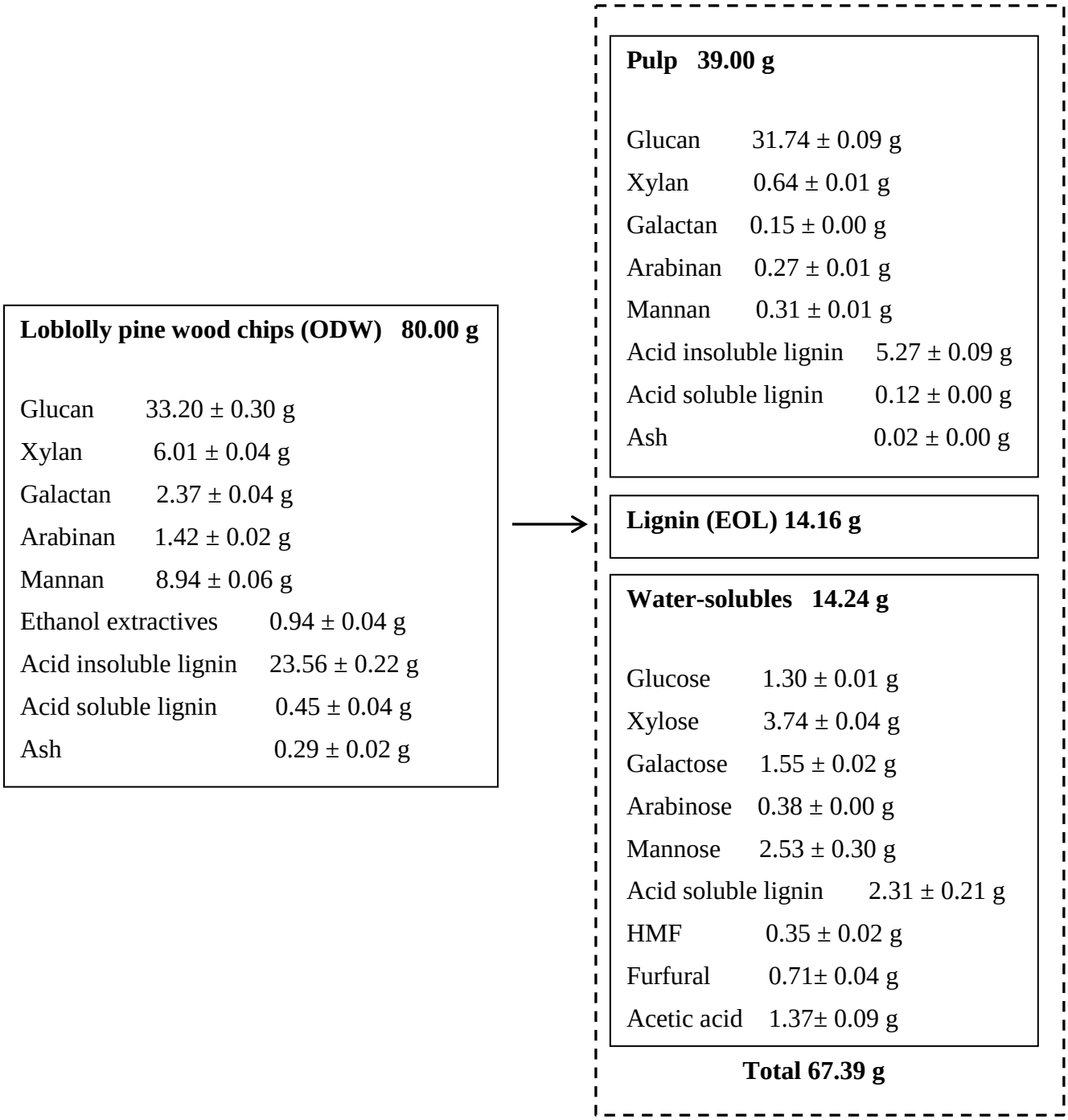

Figure 5-3 Mass balance of ethanol organosolv pretreatment of loblolly pine

5.4.3 Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)

One of the major benefits of organosolv pretreatment is that high quality of lignin can be isolated from cellulose rich solid, and thus significantly reduced the lignin content in pretreated substrate. Moreover, washing pretreated substrate by warm ethanol solution was shown to further remove the lignin by approximately 10% (**Table 5-1**). In order to investigate the effect of ethanol-wash process on butanol production, SHF and SSF were conducted on both ethanol-washed substrate and ethanol- unwashed substrate. The glucose released during enzymatic hydrolysis is shown in **Fig. 5-4**. The glucose yield could reach 90% on both ethanol- wash and ethanol- unwashed substrate, indicating the lignin content did not show any effect on glucan digestibility. By comparison SHF on ethanol- unwashed substrate with ethanol- washed substrate, it was found that the butanol production from ethanol- unwashed substrate was 8.16 g/L with a yield of 0.16 g/g, whereas the butanol production from ethanol-washed substrate was only 1.69 g/L with a yield of 0.03 g/g and the residual sugar was as much as 19.42 g (**Fig. 5-5, 5-6 and Table 5-2**). Notably, a remarkably high concentration of butyric acid was observed. It suggested the removal of lignin by ethanol washing was not helpful on butanol production in SHF process. Interestingly, it was surprised to find the addition of ethanol organosolv lignin (EOL) (equivalent amount to the lignin removal by ethanol washing) to ethanol- washed substrate substantially improved the ABE fermentation (**Fig. 5-7**). The butanol and ABE final concentration were increased to 7.60 g/L and 10.56 g/L respectively, which were comparable to that on ethanol- unwashed substrate. On the other hand, the lignin addition did not show any impact on enzymatic hydrolysis (**Fig.5-4**). The reason why supplement of lignin could enhance the ABE fermentation is not clear; it seems that in the case of the ethanol- washed substrate, a

transition from acidogenic phase to solventogenic phase was impaired considering the butyric acid was not re-assimilated and the fermentation stopped after 48 hours.

The effect of ethanol washing process on SSF strategy was also studied. With the substrate without ethanol washing, a poor ABE fermentation was observed (**Fig. 5-8 and Table 5-2**). The glucose kept increasing until reaching to a flat at 84 hours, indicating the hydrolysis was dominant all the time. The fermentation appeared to cease at approximately 60 hours, from which only 2.13 g/L butanol and 3.65 g/L ABE were produced, corresponding to a butanol yield of 0.04 g/g and ABE yield of 0.06 g/g. On the contrary, a far more satisfactory ABE fermentation was achieved by using ethanol-washed substrate (**Fig. 5-9 and Table 5-2**). Almost all the released glucose was utilized and converted to 9.29 g/L of butanol and 15.74 g/L ABE. The butanol and ABE yield were 0.16 g/g and 0.27 g/g respectively, which were 14% and 35% higher than that from SHF process (ethanol-unwashed substrate). It revealed the glucan digestibility was higher in SSF than that in SHF, due to the product inhibition in enzymatic hydrolysis through SHF. Hence, by comparing the ABE production and yield between SHF and SSF, the SSF process was a preferred configuration. Additionally, the ethanol- washed substrate associate in SSF demonstrated a better ABE fermentation and in turn, it could result in a predominant ethanol organosolv lignin (EOL) recovery.

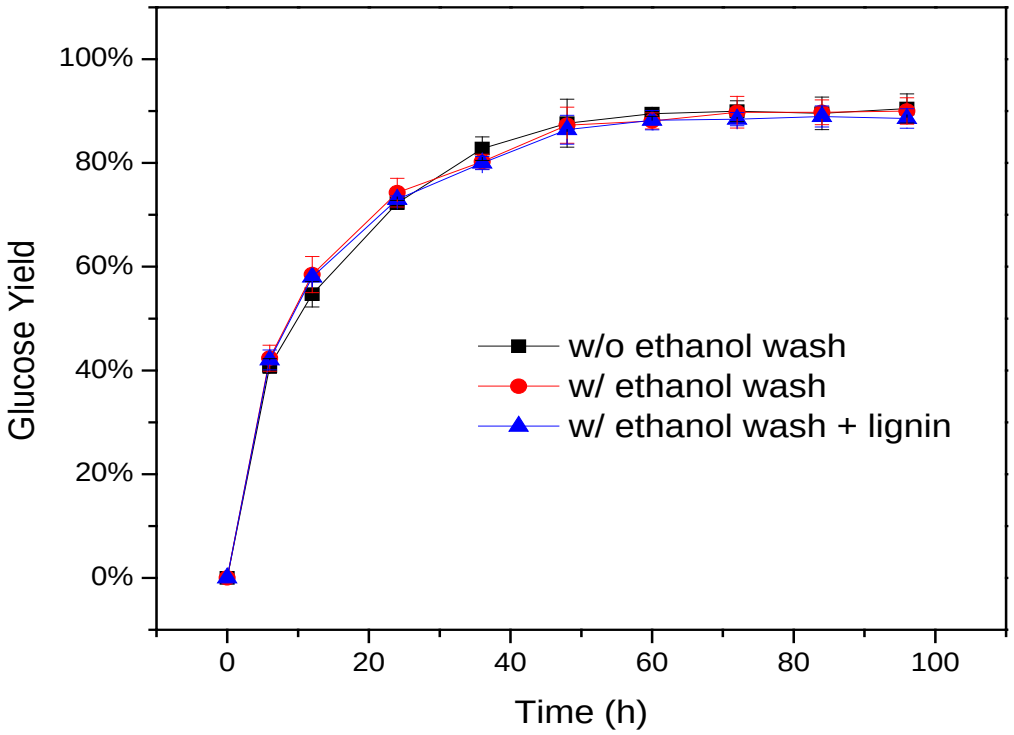

Figure 5-4 Effect of lignin on enzymatic hydrolysis

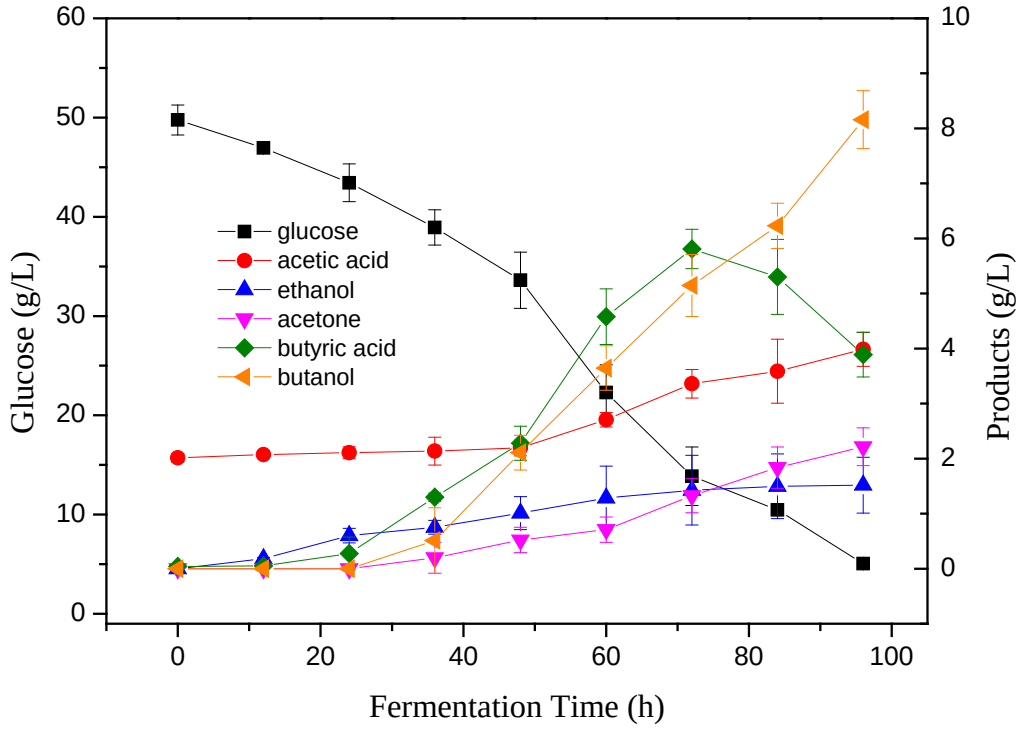

Figure 5-5 ABE Fermentation on enzyme hydrolyzed substrate (w/o ethanol washing)

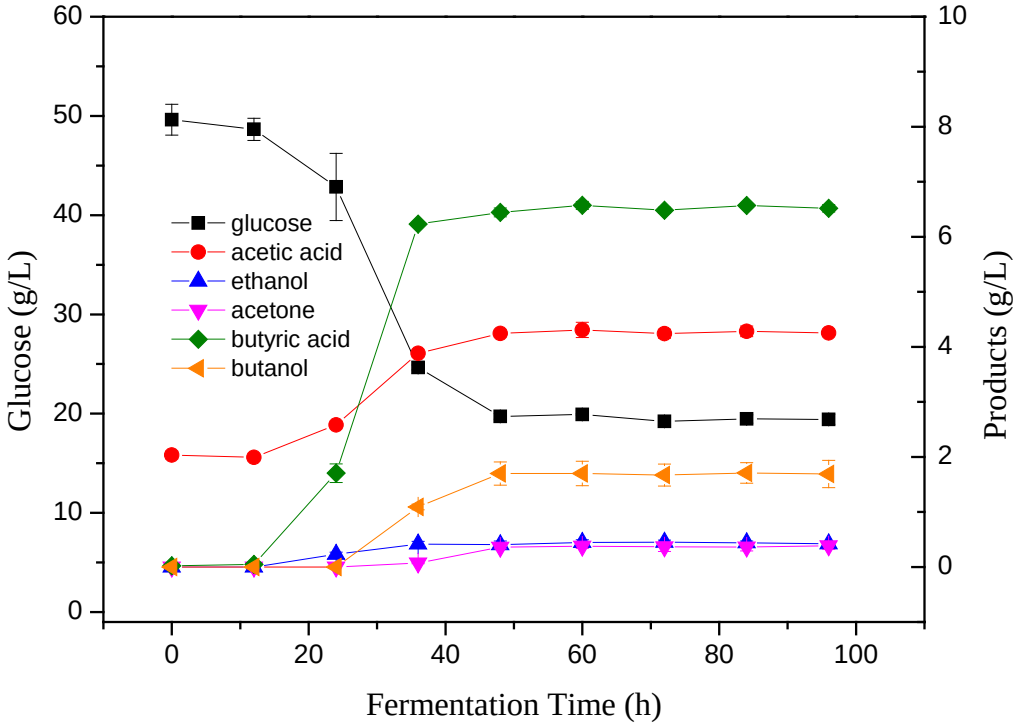

Figure 5-6 ABE Fermentation on enzyme hydrolyzed substrate (w/ ethanol washing)

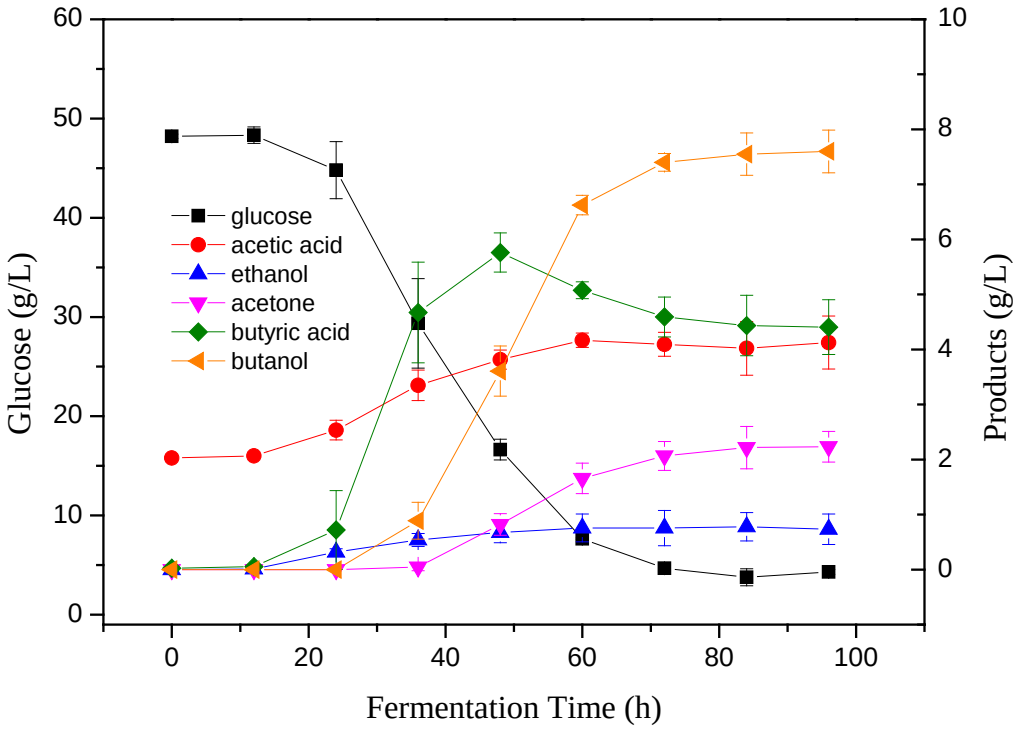

Figure 5-7 ABE Fermentation on enzyme hydrolyzed substrate (w/ ethanol washing) supplemented with lignin

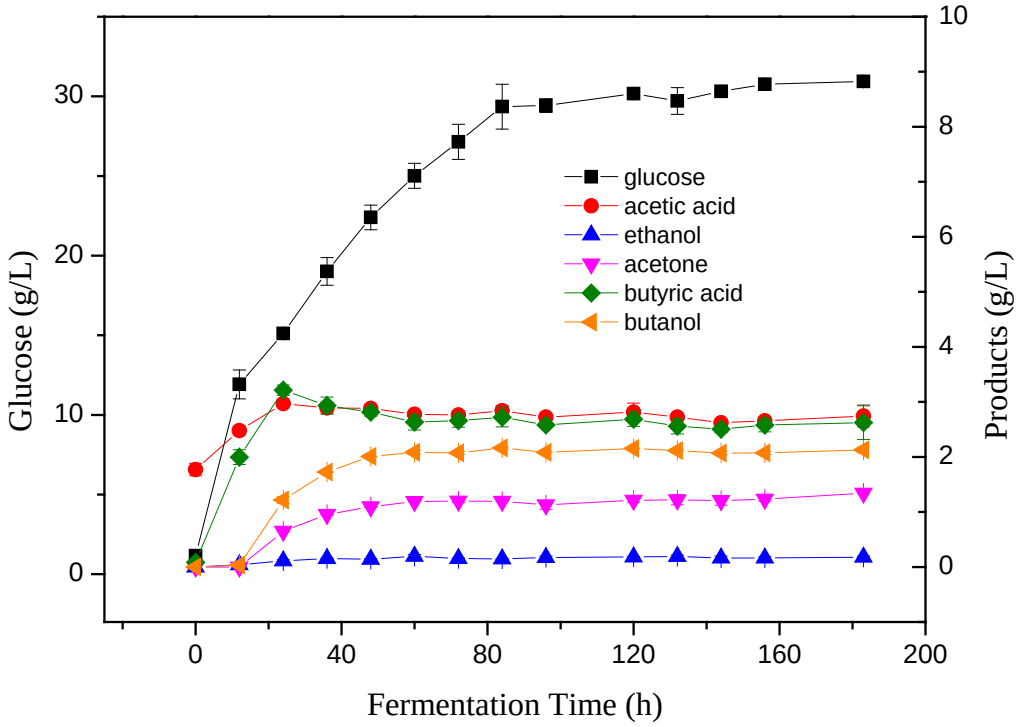

Figure 5-8 ABE production from substrate w/o ethanol washing in SSF process

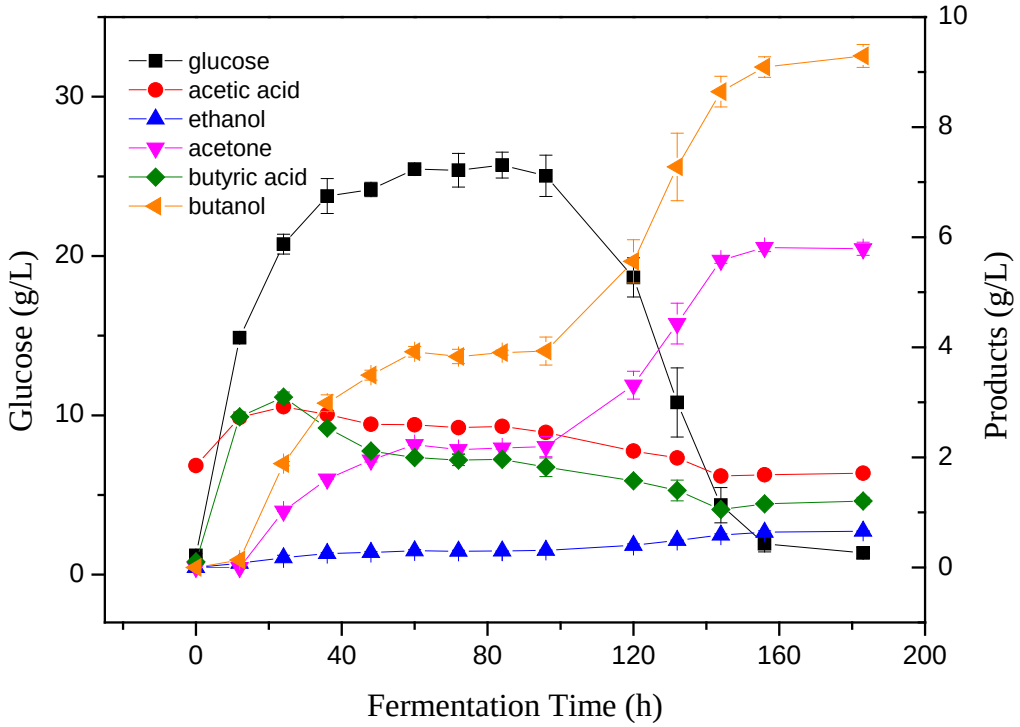

Figure 5-9 ABE production from substrate w/ ethanol washing in SSF process

Table 5-2 Acetone-butanol-ethanol fermentation with SHF and SSF

	SHF		SSF		
	Ethanol-unwashed substrates	**Ethanol-washed substrates**	**Ethanol-unwashed substrates**	**Ethanol-washed substrates**	**Ethanol-washed substrates + Prehydrolysates**
Residual glucose (g/L)	5.06±0.13	19.42±0.51	30.93±0.02	1.36±0.33	0.59±0.36
Butanol (g/L)	8.16±0.53	1.69±0.25	2.13±0.05	9.29±0.21	10.51±0.18
Butanol Yield (g/g)	0.14±0.01	0.03±0.00	0.04±0.00	0.16±0.00	0.15±0.00
ABE (g/L)	11.89±0.12	2.66±0.33	3.65±0.05	15.74±0.33	18.29±0.22
ABE Yield (g/g)	0.20±0.00	0.04±0.01	0.06±0.00	0.27±0.01	0.26±0.01
Butyric Acid (g/L)	3.89±0.41	6.52±0.07	2.62±0.31	1.21±0.07	1.68±0.04
Acetic Acid (g/L)	3.99±0.31	4.25±0.05	2.74±0.20	1.71±0.06	1.80±0.01

Note: the format was presented as mean value ± standard deviation

5.4.4 Supplement of Detoxified Prehydrolysates in SSF

In Chapter 4, the two-step detoxification strategy was proved to be effective on ABE fermentation with prehydrolysates obtained from ethanol organosolv pretreatment. It would be great benefits to integrate the aqueous phase into solid substrates fermentation to boost the solvent production. The feasibility of fermenting detoxified prehydrolysates and ethanol-washed substrates together in a SSF process was investigated in this study. The effect of detoxification on induvial sugars is shown in **Table 5-3**. The treatment by $Ca(OH)_2$ caused negligible sugar loss (less than 1%), and the second step resulted in larger amount of sugar loss, which was 9.65% reduction. It was lower than the sugar loss (17.3%) at the same condition reported in last chapter; the reason is the higher initial sugar concentration resulted in more sugar loss. Ethanol-washed substrates supplemented with prehydrolysates exhibited a satisfactory ABE fermentation (**Fig. 5-10**). Little amount of residual sugar was left and it produced 18.29 g/L ABE containing 10.51 g/L of butanol concentration with ABE and butanol yield of 0.26 g/g sugar and 0.15 g/g sugar, respectively (**Table 5-2**). It suggested one tonne of dry wood could produce 46.6 gallons of ABE and 26.5 gallons of butanol, respectively. As a result, the butanol and ABE production were 13% and 16% higher than that from solid only (**Table 5-2**), indicating the extra sugars in prehydrolysates benefited the solvent production.

Table 5-3 Effect of detoxification on sugar concentration (g/L) in prehydrolysates

	Prehydrolysates	**Treatment by $Ca(OH)_2$**	**Treatment by resin**
Glucose	2.36	2.36	2.19
Xylose	6.46	6.41	5.59
Galactose	3.09	3.05	2.71
Arabinose	0.62	0.60	0.59
Mannose	4.59	4.56	4.29
Total	17.12	16.99	15.35

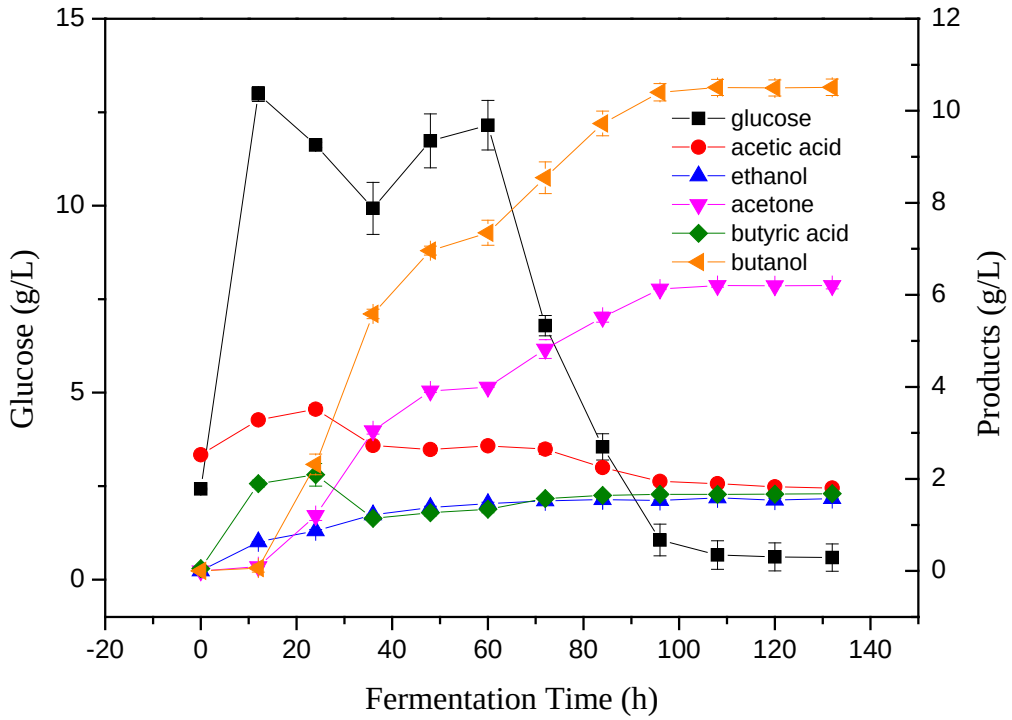

Figure 5-10 ABE fermentation on substrate (w/ ethanol washing) supplemented with detoxified prehydrolysates in SSF process

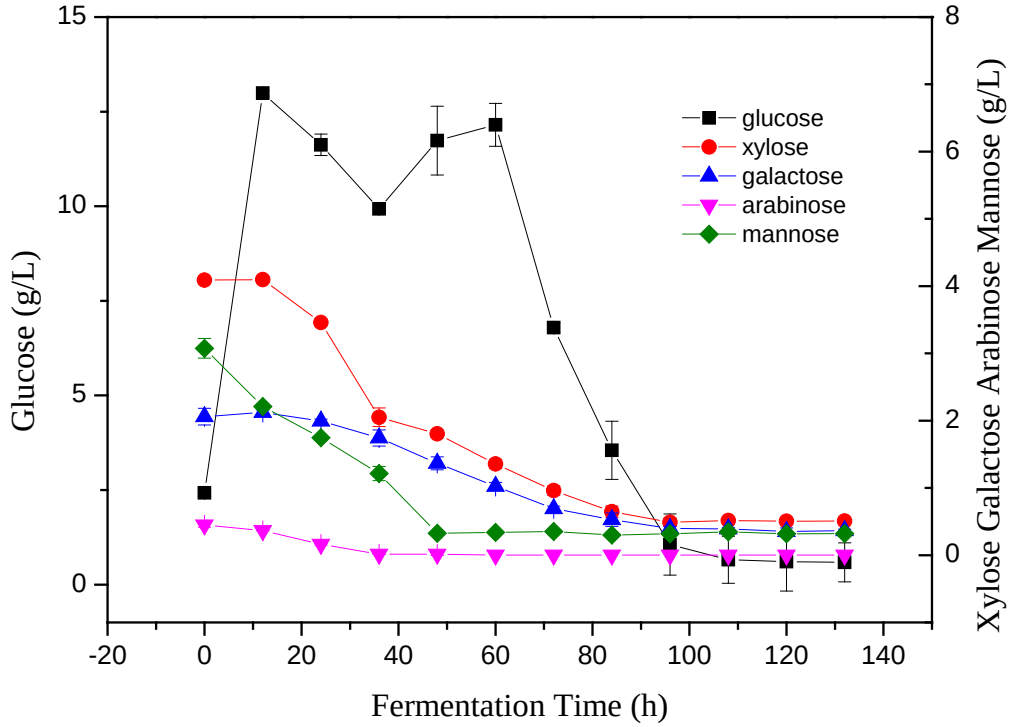

Figure 5-11 Sugar consumption in SSF process

In particular, the glucose concentration in this case started to decrease after 12 hours, indicating the glucose consumption rate was higher than hydrolysis rate; in contrast, it did not occur in the SSF process with only solid substrates. On the other hand, the prehydrolysates supplemented fermentation was faster comparing to that with solid substrate itself. These differences probably ascribed to the available sugars in prehydrolysates, which were ready for use by bacteria once the inoculum was induced and thus a comfortable environment was rapidly established for microbe growth. It is well known the solventogenic clostridia are capable of using both hexose and pentose as carbon source for ABE production. It was observed both of the sugars in prehydrolysates and released from solid substrates were utilized through the course of fermentation (**Fig. 5-11**). Except glucose, the mannose was used firstly and followed by xylose. All the available sugars were assimilated by *Clostridium* at the end of fermentation, leaving insignificant amount of residual sugars. Therefore, the integration of the prehydrolysates with pretreated substrates in SSF process has a good potential for industrial application. It can improve the overall carbohydrate utilization and increase the fermentation productivity.

5.5 Conclusions

Butanol production from organosolv pretreated loblolly pine was compared between SHF and SSF processes. The effect of ethanol washing of pretreated substrates on hydrolysis and fermentation was also examined. The ethanol-washed substrates with SHF showed a poor ABE fermentation. The corresponding butanol and ABE concentration was 1.69 g/L and 2.66 g/L, respectively. A large amount of butyric acid was produced. Surprisingly, the addition of lignin remarkable enhanced the solvent production, the butanol and total ABE was increased to 7.60 g/L and 10.56 g/L respectively, which was comparable to that with ethanol- unwashed substrate. The ethanol-unwashed substrate showed a good butanol and ABE yield (0.14 g/g and 0.20 g/g

respectively) in SHF process but not in the SSF process. The ethanol-washed substrates produced 9.29 g/L butanol with a yield of 0.16 g/g and 15.74 g/L ABE with a yield of 0.27 g/g in SSF process. Moreover, the detoxified prehydrolysates supplementation into SSF process was investigated. It improved the butanol and ABE titer to 10.51 g/L and 18.29 g/L, respectively, which were 13% and 16% higher than that from solid only. It suggested one tonne of dry wood could produce 46.6 gallons of ABE and 26.5 gallons of butanol, respectively. Therefore, we believe the prehydrolysates should be incorporated into the SSF process to improve the productivity of butanol production. In addition, the ethanol organosolv pretreatment resulted in a good glucan recovery (95.6%) in solid fraction and effective delignification (77.6%).

Chapter 6

Summary and Recommendations for Future Work

6.1 Summary

In this work, the inhibition effect of model carbonyl compounds (furans, phenolic compounds and carboxylic acids) on lactic acid and butanol fermentation was studied. It was found that the chemical structures played an important role in the inhibition of microbial fermentation. Aromatic aldehydes exhibited the strongest inhibition in lactic acid fermentation. The *ortho* hydroxyl group of phenolic compounds resulted in considerable inhibition in butanol fermentation. The structural properties such as hydrophobicity (Log *P*), energy of the lowest unoccupied molecular orbital (E_{LUMO}), energy of the highest occupied molecular orbital (E_{HOMO}), and dipole moment, were attempted to correlate with inhibition activity through quantitative structure-activity relationships (QSAR). Good linear relationships between the physicochemical descriptors and microbial inhibition were established. It serves as a useful tool to understand the inhibition mechanism of degradation compounds and predict their inhibition severity, as well as to guide an efficient detoxification method targeting specific inhibitors.

In the study of detoxification methods in butanol fermentation, the effectiveness of six typical detoxification strategies was compared. These methods have been extensively studied and proven to be effective for ethanol fermentation in the previous research. But they were found to be less efficient on butanol fermentation in this work. A direct comparison of the effectiveness of detoxification between our study and the previous is difficult because different microorganisms were used. Anion resin detoxification was the best one among the tested methods. Insignificant level of sugars was detected after the treatment by anion resin but a long lag phase was noticed

before the fermentation. To address this lag problem, a two-step detoxification ($Ca(OH)_2$ + anion resin) was developed and it significantly improved the fermentation efficiency. It produced 7.52 g/L of butanol and 11.11 g/L ABE, respectively, corresponding to a butanol and ABE yield of 0.13 g/g sugar and 0.19 g/g sugar, respectively. This study suggested the solventogenic *Clostridia* was more sensitive to the inhibitory compounds compared to yeast and ethanol producing bacteria.

Finally, the butanol production from organosolv pretreated softwood was studied in laboratory scale. The SHF and SSF process were compared and the effect of ethanol washing of pretreated substrates was investigated, as well as the feasibility of incorporation prehydrolysates into solid substrates was estimated. The ethanol washing step did affect the butanol fermentation, but the effect was found to be different in SHF and SSF process. The ethanol-unwashed substrates showed a better butanol yield in SHF process but it is not the case in SSF process, suggesting the lignin caused a positive effect in SHF. This is an interesting finding, and it was proved by adding ethanol organosolv lignin (EOL) back into ethanol-washed substrates for butanol fermentation. Moreover, the ethanol-washed solid substrates supplemented with detoxified prehydrolysates fermented through SSF proved to be suitable for butanol production. A satisfactory ABE fermentation yield was achieved with butanol and ABE yield of 0.15 g/g and 0.26 g/g sugar, respectively. Supplement of prehydrolysates improved the butanol and ABE production by 13% and 16%, respectively, compared to solid substrates only. The supplementation of prehydrolysates greatly utilized the sugars dissolved in liquid phase during pretreatment and improved the overall efficiency of bioconversion from biomass to biofuels.

6.2 Recommendations for Future Work

Although this work made good progress towards the inhibition and detoxification in the conversion to biofuels and biochemicals, a lot more effort is needed to make the second generation biofuels technical and economic viability. The following studies are recommended for future work to further advance the topic of this dissertation.

6.2.1 QSAR Analysis with More Inhibitory Compounds and Different Microbial Strains

Current study focused on the carbonyl inhibitors, but the ketones which are also important degradation compounds were not included. Hibbert's ketones are a group of phenolic ketones which have been mentioned in Chapter 1. It was found to take up to 72% of total phenolic compounds in prehydrolysates [63]. And it was observed that the phenolic ketones inhibition on ethanol fermentation was higher than that caused by phenolic acids but lower than phenolic aldehydes [217, 261]. Therefore, it is recommended to expand the study to the effect of aromatic ketones on butanol fermentation. Meanwhile, the methoxyl group is frequently present in prehydrolysates. The difference among p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin precursors is the various amount of methoxyl groups and consequently, one of the distinguishment in lignin derivatives is the amount of methoxyl groups. The introduction of methoxyl groups to aromatic ring in phenolic compounds significantly reduced the hydrophobicity [63] and it was reported the phenolic compounds with more methoxyl groups resulted in less inhibition on yeast fermentation [217]. Therefore, the study on the effect of methoxyl group on butanol fermentation is necessary.

The effect of carbonyl inhibition on lactic acid bacteria growth and fermentation has been examined in current work. A good correlation between inhibition and Log *P* has been found,

indicating the affinity to cell membrane probably be responsible for the inhibition. However, this is not the case in butanol fermentation by *C. acetobutylicum*. Their inhibition was closely related to E_{HOMO} rather than Log *P*. To examine more types of inhibitors mentioned above is able to fine tune these models and another way to train these models is done by investigation on different microorganisms. It is believed that different microorganisms have different tolerance and adaption to inhibitors. Thus, it is recommended to do the QSAR with various types of microorganisms such as yeast, lactic acid bacteria and butanol bacteria. It is needed to apply the same model compounds over different microbial strains. In addition, the influence of fermentation media could also be a reason resulted in different QSAR, it is better to avoid this issue as much as possible in the future work.

6.2.2 Further Investigation of Detoxification

6.2.2.1 Improvement on Economic Viability of Detoxification

The economic viability has been a great issue in bioconversion of biomass to biofuels and the cost of detoxification is one of the main barriers hampering the utilization of the sugars in prehydrolysates. Although, the detoxification by anion resin was found to be effective in removing inhibitors for butanol production in this work, the sugar loss was noticed and should be considered. Future study is needed on optimization of the reaction conditions to minimize the sugar loss and thus improve the biomass conversion efficiency. In addition, the cost of resin is relatively higher than common chemicals and therefore, reducing the amount of resin by optimization is required. On the other hand, comparing the effectiveness of detoxification by less expensive reagent with two-step detoxification method developed in this work is recommended.

Na_2SO_3 has been studied as detoxification reagent for ethanol fermentation of prehydrolysates and it might be a good candidate to benefit butanol fermentation as well.

6.2.2.2 Investigation of the Dominant Inhibition Factors in Prehydrolysates

Various detoxification methods including physical, chemical and biological strategies have been attempted to eliminate/reduce inhibition of softwood prehydrolysates, and two-step detoxification was found to be effective, however, why certain methods are superior to others? The reason behind these results has not been fully understood. We found the activated carbon treated prehydrolysates was colorless but it did not improve the fermentability. Overliming detoxification is a widely used and well established detoxification method; nevertheless, it just partially improved butanol fermentation in this work. We cannot explain these observations well with the knowledge gained in this dissertation. Further study is needed to explore the reason and the dominant inhibition factors could be found by the analytical tools such as GC/MS, LC/MS and NMR. First, the inhibitors in treated and untreated prehydrolysates should be quantified by GC/MS or LC/MS. By comparing the distinction of the chromatography, we might be able to find the clue of dominant inhibitors in butanol fermentation from the prehydrolysates. Second, we analyzed the prehydrolysates by GC/MS and found some potential inhibitors shown in Chapter 4. But, there are some unknown peaks left which are not able to be identified. In the future study, NMR is suggested to identify the inhibitors' structure or functional groups.

6.2.3 Study of the Effect of Lignin on Butanol Fermentation

We are surprised to find the positive effect of linin on butanol fermentation, but the effect only applied to butanol fermentation in SHF process not in SSF process. We have no clear explanations at this moment and the detailed examination is needed in the future. Various

amount of EOL is suggested to be added into pure glucose and cellulose fermentation to study how the dosages affect the butanol production. At the same time, the lignin prepared from different sources and different processes instead of EOL from softwood should be studied. Our group has found the lignin from softwood showed negative effect on enzymatic hydrolysis and that from hardwood exhibited positive effect [262]. The structures of lignin from different pretreatment conditions were found to be different by NMR and the lignin inhibitory effects on enzymatic hydrolysis were associated with their structures [263]. Similarly, the different types of lignin and lignin isolated from different pretreatment conditions may affect butanol fermentation differently. Furthermore, it would be helpful to examine how the lignin interacts with solid substrates and the *Clostridia* during the fermentation course.

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