Carbonyl inhibition and detoxification in microbial fermentation of biomass hydrolysates

by

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Abstract

Bioconversion of lignocellulosic biomass to biofuels has great potential to supplement petroleum-derived fuels. One of the major barriers to bioconversion is the release of considerable amount of carbonyl degradation compounds in the pretreatment of biomass, which because of their high reactivity toward biological nucleophiles inhibit the subsequent microbial fermentation. The development of a cost-effective detoxification approach and identification of the reaction mechanisms would alleviate the issue.

Overliming has been widely used to detoxify biomass hydrolysates. However, the chemical mechanisms were not very well understood. My initial work therefore was to explore possible detoxification mechanisms by using a carbonyl model compound *o*-phthalaldehyde. At 1 mM, *o*-phthalaldehyde completely inhibited ethanol production by *Saccharomyces cerevisiae*, but interestingly, the inhibition disappeared under alkaline conditions (pH~10) at 60°C for 2 h in the presence of a reducing sugar. Non-reducing sugar had no effect. LC/MS analysis of the detoxification mixture revealed an aldol condensation reaction between *o*-phthalaldehyde and a reducing sugar. The reducing sugar converted to its enolate ion under alkaline conditions, which then reacted with one of the aldehyde groups through nucleophilic addition. Loss of one aldehyde group could be the key for the detoxification.

In following work amino acids were used to detoxify the biomass hydrolysates for ethanol production by *S. cerevisiae*. I found cysteine was one of the amino acids that effectively detoxified loblolly pine hydrolysates. Ethanol production rate at 6 h increased from 0.18 in the

untreated hydrolysate to 1.77 g/L/h and the final yield from 0.02 to 0.42 g/g, significant increases in both production rate and yield. The extraordinary detoxification by cysteine was probably due to its reactive thiol group that, in addition to its amine group, reacted with aldehydes to form thiazolidine derivatives. Meanwhile, the amine group could attack the carbon of aldehydes/ketones via electrophilic substitution to form imines.

To understand the mechanism of aromatic aldehyde inhibition on yeast fermentation, I further investigated the structure-inhibition relationships using thirteen benzaldehydes. The results indicated that fermentation inhibition of benzaldehydes appeared related to their ortho-group (CHO > OH > OCH_3) and position of the OH group in the benzene ring (*o*-OH > *m*-OH > *p*-OH). Correlating the molecular descriptors to inhibition efficiency revealed a strong association between Log *P* and inhibitory activity.

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List of Abbreviations

μ	Dipole moment
ADH	Alcohol dehydrogenase
AFEX	Ammonia fibre explosion
ARP	Ammonia recycle percolation
$Ca(OH)_2$	Calcium hydroxide
C'_{carb}	Partial charge of the carbonyl carbon in the aromatic aldehydes
$CaSO_4$	Calcium sulfate
CFU	Colony forming units
E	Inhibition efficiency
E_{HOMO}	Energy of the highest occupied molecular orbital
E_{LUMO}	Energy of the lowest unoccupied molecular orbital
F	Fisher statistic
GC	Gas chromatography
HMF	5-Hydroxymethyl furfural
HPLC	High performance liquid chromatography
UPLC	Ultrahigh performance liquid chromatography
ILs	Ionic liquids
k	Capacity factor
LC-MS	Liquid chromatography-mass spectrometry
Log *P*	Partition coefficient

MLR	Multiple linear regression
MR	Molecular refractivity
n	Number of observations
NaOH	Sodium hydroxide
NAD^+	Nicotinamide adenine dinucleotide
NH_4OH	Ammonium hydroxide
NMR	Nuclear magnetic resonance
OPA	ortho-Phthalaldehyde
p-value	Significance
ppm	parts per million
QSARs	Quantitative structure-activity relationships
r^2	Coefficient of determination
R_s	Resolution
s	The standard error of the estimate
SEM	Scanning electron microscope
SHF	Separate hydrolysis and fermentation process
SSCF	Simultaneous saccharification and co-fermentation process
t_{acetic}	Retention time of acetic acid
$t_{levulinic}$	Retention time of levulinic acid
t_M	Retention time of the mobile phase
t_R	Retention time of an analyte peak

$W_{1/2,\ acetic}$	Peak width at half-height of acetic acid
$W_{1/2,\ levulinic}$	Peak width at half-height of levulinic acid

Chapter 1: Introduction

1.1 Background - the significance of lignocellulosic biofuel production and current issues

Biofuel production from plant biomass is of paramount significance to sustainable energy development due to the globally growing dependence on transportation fuels. During the past few decades, sugarcane and starch-based feedstocks have been successfully used for industrial ethanol production, while raising questions about the competition between food and energy (Sun and Cheng 2002). Moreover, starch-based ethanol is estimated to produce a maximum of around 15 billion gallons per year, not enough to cause a significant impact on petroleum usage in the United States, which consumes 140 billion gallons per year (Agbor et al. 2011). This has driven the exploration of the second generation bioenergy--lignocellulosic biofuels. Lignocellulosic biomass derived from grass, agricultural residues, forestry residues, and industrial solid waste is a cheap, abundant and environment-friendly source containing up to 70% carbohydrates (cellulose and hemicellulose) for biofuel production, the efficient use of which could bring sustainable and long-term benefits to our daily life.

Lignocellulosic biomass can be converted to biofuels and chemicals through a biochemical approach. The bioconversion process, although encouraging, is currently facing technical difficulties. This is mostly due to the strong recalcitrance of the lignocellulosic structure, the natural resistance of plant cells against bacterial and fungal attack (Sjöström 1993). Lignocellulosic biomass is mainly composed of three polymers: cellulose, hemicelluloses and lignin, which are tightly bound through chemical and non-chemical interactions to resist depolymerization. In order to overcome the lignocellulosic recalcitrance and make the carbohydrates accessible for further conversion, thermochemical pretreatment is used to fractionate the biomass into a solid portion rich in cellulose (hydrolysate) and a liquid portion

rich in hemicelluloses (prehydrolysate). After pretreatment, the biomass is hydrolyzed into monomeric sugars by cellulolytic enzymes and fermented into biofuels and chemicals by microorganisms (Galbe and Zacchi 2002; Kumar et al. 2008). However, rather than producing a tractable substrate, pretreatment produces a wide range of degradation compounds along with the sugars, potentially inhibiting the following microbial growth and fermentation (Ando et al. 1986; Klinke et al. 2004; Luo et al. 2002). These degradation compounds are generated from cellulose, hemicelluloses, lignin and extractives, with a variety of chemical structures including aldehydes, ketones, acids, alcohols and phenols, some of which are unsaturated compounds As a result, the inhibitory effect has become a major barrier to developing an industrially viable process for cellulosic biofuel production.

Numerous studies have focused on the chemical identification of potential inhibitors using high performance liquid chromatography (HPLC), gas and liquid chromatography mass spectrometry (GC-MS and LC-MS) and nuclear magnetic resonance (NMR) (Agblevor et al. 2004; Chen et al. 2006; Dizhbite et al. 2011). Potential inhibitors from various sources have been identified. For example, 5-hydroxymethyl furfural (HMF), formic acid and levulinic acid are produced from the degradation of cellulose; acetic acid and furfural are formed from hemicelluloses degradation; aromatic compounds are generated from lignin, extractives and sometimes sugars (Klinke et al. 2004; Nelson David et al. 1988; Palmqvist and Hahn-Hägerdal 2000). However, due to the large number of degradation compounds, and their extremely low concentration, identification of unknown microbial inhibitors has faced a mounting challenge in the past half century (Clark and Mackie 1984; Klinke et al. 2004; Palmqvist and Hahn-Hägerdal 2000). The knowledge of which compounds have the most pronounced inhibitory effect on fermentation is still lacking (Helm et al. 2010). Detoxification approaches from physical,

chemical and biological perspectives have been developed to remove and/or modify fermentation inhibitors and improve the fermentability of the biomass hydrolysates (Buchert et al. 1990; Hasmann et al. 2008; Jönsson et al. 1998; Larsson et al. 1999b). Among the detoxification methods investigated so far, alkaline treatment is the most widely used approach to improve hydrolysate fermentability (Larsson et al. 1999b; Wang and Feng 2010). However, its detoxification mechanism is still in question, mainly due to a lack of the right targets (Larsson et al. 1999b; Saha et al. 2005). Consequently, identification of the fermentation inhibitors and elucidation of the mechanisms by which that can be detoxified are recognized as major roadblocks for developing any effective detoxification approach and improving stress-tolerance yeast and bacterial ethanologens.

Linking the inhibitory activities of potential inhibitors to their structural features might be a key to revealing differences in levels of inhibition among different compounds. Structural properties are the fundamental basis for chemical reactivity of compounds, which in turn govern their inhibitory actions towards biological molecules (Chan and O'Brien 2008; Schwöbel et al. 2011). The correlation between structure and toxicity of the inhibitors could be established using quantitative structure-activity relationships (QSARs). QSARs link the biological activity of inhibitors/toxins to their structural features. It has proven to be a useful tool in studying drug potency and assessing contamination due to environmental toxicity. However, little work has been conducted to study and predict the inhibitory activity of inhibitors using QSARs in bioethanol production. Based on previous reports, most of the identified inhibitors associated with biomass hydrolysates are functional carbonyl compounds (R-C=O) (Ando et al. 1986; Clark and Mackie 1984; Klinke et al. 2004). These include carboxylic acids from carbohydrates, such as acetic acid and formic acid, and to some extent from lignin, such as ferulic acid and 4-

hydroxybenzoic acid (Klinke et al. 2002; Xie et al. 2011). Aldehydes, such as furfural and HMF, are dehydrated from monomeric sugars (Palmqvist et al. 1999); while aromatic aldehydes and ketones are mainly degradation products from lignin and extractives. These include a wide variety of compounds such as vanillin, syringaldehyde and Hibbert's ketones, as well as unknown compounds (Clark and Mackie 1984; Larsson et al. 2000). Carbonyl compounds are electrophilic, potentially forming covalent bonds with nucleophilic targets in the microorganisms. Reactions with proteins, nucleic acids, or related biological molecules, could lead to inhibition of important protein functions, DNA duplication, or even loss of cell activity. Therefore, linking the structural features/electrophilic reactivity of the carbonyl inhibitors to their antimicrobial activity might help to understand their inhibition mechanism in bioethanol production.

Loblolly pine (softwood) is an abundant source of lignocellulosic material in the southeastern United States. However, the use of woody biomass as feedstock is difficult because more intensive pretreatment conditions are required to disrupt the strong recalcitrance of the material, resulting in the generation of high levels of inhibition. In this dissertation, with the goal of improving the understanding of carbonyl inhibition and detoxification for biofuel production, I will focus on investigating the chemical mechanism of alkaline detoxification for biofuel production using carbonyl model compounds, developing a novel detoxification approach to remove the inhibition and improve the fermentability of wood biomass hydrolysates, and study the inhibitory activity of thirteen benzaldehydes on alcoholic fermentation and their QSARs.

1.2 Chemical composition of lignocellulosic biomass

Lignocellulosic biomass is mainly comprised of three types of polymers: cellulose, hemicelluloses and lignin. Cellulose is a homopolysaccharide consisting of D-glucopyranose

units linked by β-1, 4-glycosidic bonds, forming a completely linear structure. Hemicelluloses are a group of heterogeneous polysaccharides composed of D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, and small amounts of D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and D-galacturonic acid. Lignin is cross-linked co-polymers of phenylpropane units. These three polymers are tightly bound together through chemical and non-chemical forces. Apart from the structural components, extractives composed of a variety of compounds exist in the wood as minor fractions.

Cellulose, the most abundant biopolymer in the world, constitutes 40-50% of the dry weight of lignocellulosic biomass. The polymer chain is composed of (1→4) glycosidic bonds linked β-D-glucopyranose units, with a degree of polymerization of 10,000 (based on the wood cellulose) (Sjöström 1993). Existing as completely unbranched homopolymers, cellulose molecules have very high tendency to aggregate through intracellular and intercellular hydrogen bonds, forming microfibrils, which alternate between highly ordered crystalline regions and less ordered amorphous regions (Sjöström 1993). Microfibrils build up to form cellulose fibers. The stereochemical properties of cellulose give it high tensile strength, supporting plant cell walls. This is totally different from the chemical structure of starch, which consists of α-1, 4-glycosidic bonds, forming helical structures rather than linear chains.

Hemicelluloses are the second most abundant biopolymers on earth making up 20-30% of the dry wood and functioning as another supporting material in plant cells along with cellulose. However, unlike cellulose, hemicelluloses are branched heteropolysaccharides easily hydrolyzed by acids to their corresponding monosaccharides. In addition, most hemicelluloses have a degree of polymerization of only between 150 and 200. The hemicelluloses in softwoods and hardwoods differ from each other both qualitatively and quantitatively. Softwood hemicelluloses are rich in

mannose units while hardwood hemicelluloses are rich in xylose units. In softwood hemicelluloses, the principal backbone chains are galactoglucomannans (around 20% of dry wood), composed of 1→4 linked β-D-glucopyranose and β-D-mannopyranose units, with α-D-galactopyranose linked to the main chain by 1→6 bonds as a single unit side chain, and arabinoglucuronoxylan (5-10% of dry wood), built up by 1→4 linked β-D-xylopyranose, with 4-O-methyl-α-D-glucuronic acid partially linked to the main chain at C-2 (Sjöström 1993). In hardwood hemicelluloses, the major chains are typically glucuronoxylan (15-30% of the dry wood), made up of 1→4 linked β-D-xylopyranose units, with 4-O-methyl-α-D-glucuronic acid linked to the main chain about every ten xylose residues by 1→2 bonds (Sjöström 1993). In both softwood and hardwood, the hydroxyl groups at C-2 or C-3 positions on the backbone chains of hemicelluloses are often substituted by acetyl groups although softwoods are normally less acetylated than hardwoods.

Lignin is the third abundant biopolymer in nature. Softwood contains 26-32% lignin while hardwood normally contains 20-25% lignin (Sjöström 1993). Lignin is built up of phenylpropane units jointed by C-O-C and C-C linkages, with the former being dominant (over 2/3). The bond types are mainly including β-O-4, α-O-4, β-5, 5-5, 4-O-5, β-1 and β-β, among which β-O-4 makes up over 50% of wood lignin linkages. The monomer residues in lignin are ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, derived from the lignin precursors, ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively (Figure 1). Softwoods are typically rich in guaiacyl residues, with a H/G/S ratio of 2-18/82-98/trace (Klinke et al. 2004); while in hardwoods, lignin is a copolymer of sinapyl and guaiacyl units, with the H/G/S ratio around 0/22-66/44-86 (Klinke et al. 2004). It is generally accepted that lignin acts as the “glue” to hold cellulose and hemicelluloses together, resulting in high rigidity and limiting water

permeability, as well as acting as the natural defense of wood to microbial attack. Although direct evidence is still lacking, it is believed lignin is chemically binding to carbohydrates, possibly through ester and ether bonds (Sjöström 1993). Because of its close association with cellulose, lignin has been considered as the main obstacle to enzymatic hydrolysis of cellulose by physically blocking cellulose from enzymes and non-productively binding to the cellulosic enzymes. Moreover, the aromatic compounds produced from the degradation of lignin during pretreatment have strong inhibitory effect on the downstream microbial growth and biofuel production.

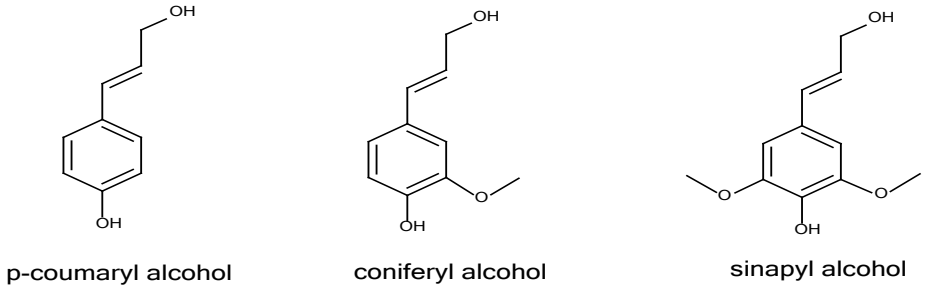


Figure 1: The three lignin precursors (building blocks) (Sjöström 1993)

Extractives are a large number of low-molecular-weight, aextracellular compounds that are considered as nonstructural constituents of wood (mostly <10% of dry wood). Some of the extractives are secreted by the wood as natural defense against microbiological attacks. Although there is distinct difference in the amount and composition of extractives between various species and even different parts of the wood, the extractives are typically composed of terpenoids, steroids, phenolic compounds, fats and waxes (Sjöström 1993). Terpenoids and steroids are derivatives of isoprene with hydroxyl, carbonyl and carboxyl functional groups, fats are composed of glycerol esters of fatty acids and waxes are esters of fatty alcohols or terpene alcohols (Sjöström 1993). Besides the insoluble constituents such as lignin and polysaccharides,

wood bark also contains high amount of extractives, from 20-40% of the dry weight (Sjöström 1993). During the bioconversion of woody biomass into biofuels and bioproducts, a large number of toxic compounds are derived from extractives and bark, with inhibitory effects on fermenting microorganisms.

1.3 Biochemical conversion of lignocellulosic biomass

The bioconversion of lignocellulosic biomass into fuels and chemicals involves three main steps. An initial pretreatment step is needed to disrupt the recalcitrant structure of lignocellulosic biomass and make cellulose accessible to hydrolytic enzymes, next hydrolysis is employed to degrade the carbohydrate polymers to fermentable monosaccharides, and finally a fermentation (anaerobic or aerobic) step is used to convert monomeric sugars into biofuels and chemicals (Figure 2). Pretreatment partially separates hemicelluloses and lignin from cellulose and thus significantly improving the enzyme accessibility of cellulose. After pretreatment, the biomass is fractionated into: a solid part rich in cellulose (hydrolysate) and a liquid part rich in solubilized hemicellulose sugars (prehydrolysate) (Figure 2). In a separate hydrolysis and fermentation process (SHF), the hydrolysate and prehydrolysate are fractionated prior to further conversion (Figure 2). In a simultaneous saccharification and co-fermentation process (SSCF), the hydrolysate and prehydrolysate are subjected to hydrolysis and fermentation simultaneously without fractionation. Although current pretreatment approaches can successfully disrupt the recalcitrant lignocellulosic biomass, a wide range of degradation compounds are produced, many of which tend to inhibit microbial growth and fermentation. As a consequence, a detoxification/conditioning step is needed in order to remove/modify these inhibitors and reach economically feasible biofuel productivities and yields (Figure 2).

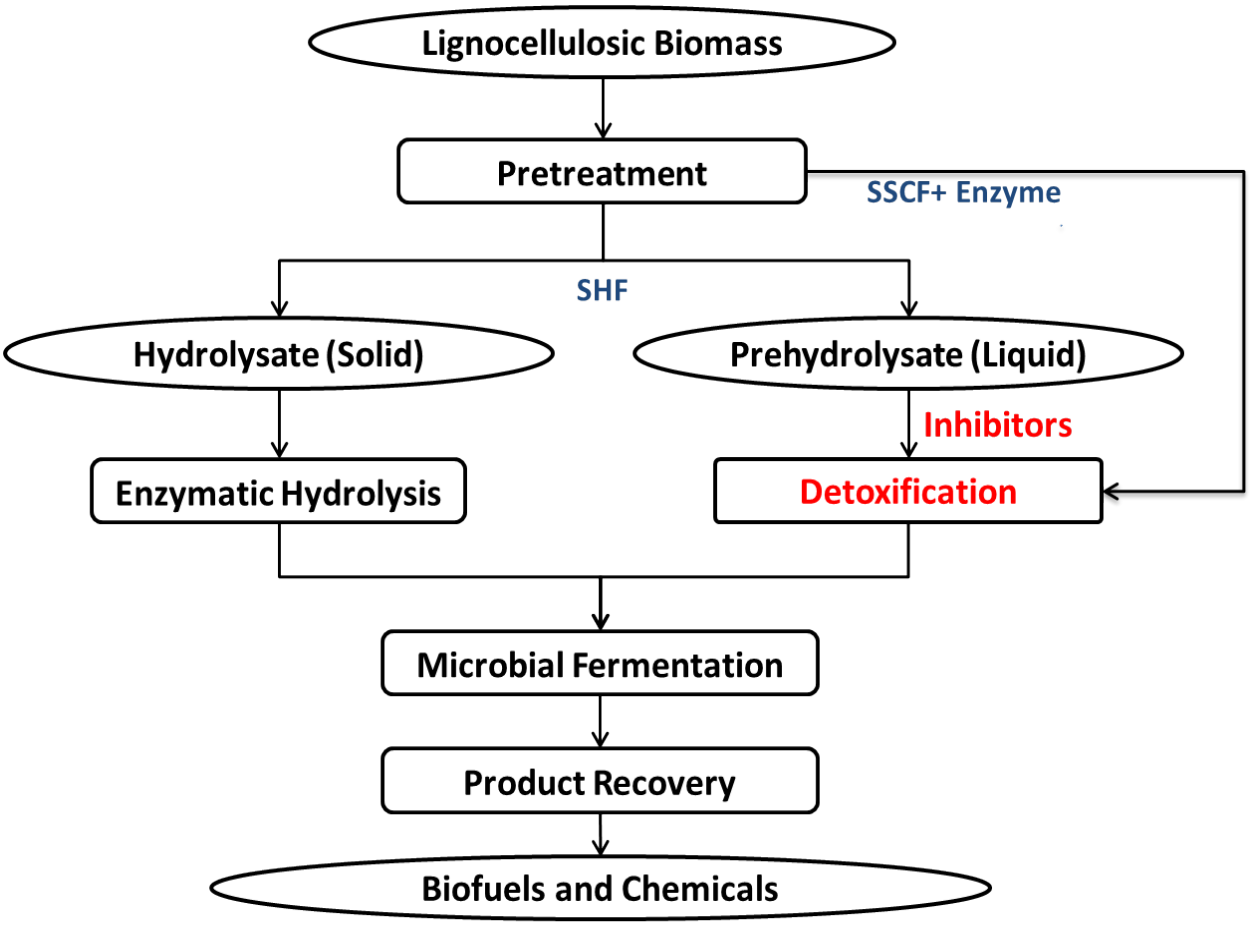


Figure 2: Biochemical conversion platform from lignocellulosic biomass to biofuels and chemicals

1.3.1 Pretreatment

Pretreatment plays an essential role in the production of biofuels and chemicals from lignocellulosic biomass. The main purpose of pretreatment is to disrupt the lignocellulosic matrix and increase the accessibility of celluloses to cellulosic enzymes (Wyman et al. 2005b; Wyman et al. 2009). Meanwhile, other factors might be considered (Agbor et al. 2011; Zhu and Pan 2010). It is desirable to recover hemicellulose sugars and high value lignin as biofuels and value-added co-products and reduce the production of degradation compounds that might inhibit microbial growth and fermentation. Only by taking all of these factors into consideration can a

pretreatment lead to a significant improvement on the downstream steps and achieve a cost-effective bioconversion process. Research has been focused on a variety of pretreatment options over the years in an effort to improve enzymatic hydrolysis and fermentation efficiency. Some of the popular pretreatment methods and their effects on the downstream steps and decomposition of the lignocellulosic biomass will be discussed as follows.

1.3.1.1 Chemical pretreatment

Chemical pretreatment is currently the most effective pretreatment approach, in which one or more chemicals, such as acids, alkali, organic solvents or ionic liquids are added to reduce or modify the recalcitrance of lignocellulosic biomass. Acid catalysts such as sulfuric acid, phosphoric acid, nitric acid and hydrochloric acid can achieve effective fractionation of cellulose, hemicelluloses and lignin at low concentration (typically 0.5-5%). Alkali salts such as sodium hydroxide, calcium hydroxide and potassium hydroxide, and ammonia are of promising base catalysts to disrupt the linkage between lignin and carbohydrates as well as decreasing the degree of polymerization of cellulose. Acetyl group and uronic acid derivatives on hemicelluloses are easily removed during alkaline pretreatment. Organosolv pretreatment occurs in an organic or water-organic solvent system at temperatures ranging from 100 to 250°C, often with the addition of acid to facilitate solubilization of hemicelluloses. Recently, ionic liquids (ILs) has been introduced as a new pretreatment system in which a new type of solvents, mostly imidazonium salts that have high polarities, low melting points, and high thermal stabilities, are used to disrupt the three dimension cellulose network.

Dilute acid pretreatment

Dilute acid pretreatment is the most studied and near-commercial process to fractionate agricultural biomass, with the pretreatment conditions conducted at acid charges on wood from 0.5-5%, temperatures between 120-215°C, and residence times from seconds to one hour (Saha et al. 2005; Schell et al. 2003; Wyman et al. 2005a). Normally, sulfuric acid is the acid of choice. This method can effectively solubilize and recover a large fraction of the hemicelluloses (80-90%) as oligomeric and monomeric sugars in the hydrolysate phase, at the same time disrupt the lignin structure and significantly increase the cellulose accessibility to enzymes. Although being proved to effectively improve the digestibility with agricultural residues, the efficiency of dilute acid pretreatment in digesting woody biomass, especially softwood, is not adequate and must be improved to achieve an industrially acceptable pretreatment process (Zhu and Pan 2010; Zhu et al. 2009). For example, the total glucose yield (% of maximum glucose) of a dilute acid pretreated poplar wood was 86.7% with a cellulase loading of 15FPU/G (Wyman et al. 2009), while the cellulose conversion of a pretreated spruce was only around 40% under similar pretreatment conditions (Zhu et al. 2009). A problem with the use of dilute acid pretreatment is the release of a large amount of degradation compounds, which potentially impede the subsequent processes, necessitating detoxification.

Steam explosion

Steam explosion is typically conducted at high pressure and temperature (between 160-240°C) with saturated steam, with the residence time ranging from a few seconds to minutes (Tucker et al. 2003; Wyman et al. 2009). This pretreatment typically solubilizes part of the hemicelluloses and somewhat modifies lignin structure, thereby increasing cellulose accessibility to enzymes. Because the acetyl group is easily released at high temperature and pressure during the pretreatment and acts as acid catalyst, this process is sometimes referred as autohydrolysis.

Due to low sugar yields, H_2SO_4, SO_2 or CO_2 is often added to increase sugar recovery. In this case, the acid-catalyst steam explosion turns into another form of dilute acid pretreatment, in which a vapor phase rather than aqueous phase is used for the pretreatment. Steam explosion has achieved high sugar recoveries with agricultural and hardwood biomass, but not with softwood (Wyman et al. 2005a; Wyman et al. 2009). Zhu et al. summarized the total monomer sugar recovery from steam explosion pretreated woody biomass based on recent studies, reporting values of 65% to 80% from poplar (hardwood), while the recovery was only about 65% for softwood under similar pretreatment conditions (Zhu and Pan 2010).

Organosolv pretreatment

Low boiling point alcohols such as methanol and ethanol, higher alcohols such as glycerol and ethylene glycol, as well as other organic solvents such as ketone, ethers or phenols have been used in organosolv pretreatment systems, with the operating temperature ranging from 100 to 250°C and residence time from 30 to 60 min (Pan et al. 2005; Sun and Chen 2008; Zhao et al. 2009b). Ethanol has been considered as the most promising solvent because of low cost, ease of recovery and low inhibition on fermenting microorganisms. The use of an organic solvent can effectively solubilize lignin so that a pure lignin can be recovered as a high-value by-product, which could be an alternative for epoxy resins and phenolic powder resins (Pan et al. 2005; Zhao et al. 2009b). Acid catalysts are also often added to increase the release of hemicellulose sugars and extraction of lignin. Unlike other pretreatment methods for woody biomass, organosolv pretreatments do not require significant size reduction to obtain high cellulose digestibility, thus reducing the energy consumption (Agbor et al. 2011). Although promising, the use of organic solvents and their recovery adds cost to the pretreatment system, and many inhibitors are produced in the hydrolysate phase after the pretreatment, most of which

are from the solubilization of lignin and extractives. These inhibitors will considerably hinder the microbial fermentation.

Ammonia fibre explosion and ammonia recycle percolation

Ammonia fibre explosion (AFEX) is typically conducted at high pressure (>3MPa), a variety of temperatures from 60 to 100°C, a residence time from 10 to 60 min, and a solid/ammonia ratio of 1:1-1:2 (Teymouri et al. 2005; Teymouri et al. 2004). AFEX can result in modification or partial removal of lignin as well as causing swelling of the cellulose structure enhancing digestibility to cellulases. When conducted at high temperatures (150-180°C), the aqueous ammonia flows through the biomass and is then recycled, in a process called ammonia recycle percolation (ARP). The ammonia-based pretreatments produce fewer inhibitors compared to the acid-based pretreatments and therefore detoxification is not needed. In addition, ammonia is a cheap chemical (1/4 of price of H_2SO_4), and is easy to recycle and recover (Agbor et al. 2011). On the other hand, this pretreatment method is inefficient in solubilizing hemicellulose sugars and subsequently the addition of hemicellulase is needed. Moreover, it is not effective on high-lignin containing materials such as woody biomass (Wyman et al. 2009).

Ionic liquid pretreatment

Ionic liquid pretreatment is a recent development for biomass pretreatment, in which a new class of non-derivatizing solvents with high polarities, high thermal stabilities and low vapor pressures are used to enhance digestibility of cellulose under low temperatures (Dadi et al. 2006; Zhao et al. 2009a). Imidazonium salts such as 1-allyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazonium are among the most commonly used ionic liquids (Xu et al. 2010; Zhang et al. 2012). The mechanism of the ionic liquid pretreatment is suggested to involve the

interruption of the hydrogen bonding networks of cellulose (Dadi et al. 2006). Nevertheless, the effect of ionic liquid pretreatment on lignocellulosic biomass is still not clear.

1.3.1.2 Biological pretreatment

Fungi that are able to produce lignin-degrading enzymes such as lignin peroxidases, laccases and manganese peroxidases have been used in attempt to remove lignin from lignocellulosic biomass. White rot fungi such as *Phlebia ochraceofulva* and *Phanerochaete chrysosporium* can achieve significant delignification (Hatakka 1994; Vares et al. 1993). However, industrial application of biological pretreatment is not practical because the fungi require strict growth conditions and long residence time for the production of lignin-degrading enzymes, some carbohydrates are consumed by the fungi, and finally large-scale equipment to conduct the biological pretreatment is difficult to obtain.

In summary, chemical pretreatments are more efficient than biological pretreatments to disrupt the recalcitrant structure of lignocellulosic biomass. Table 1 summarizes the effects of different chemical pretreatments on fractionation of biomass and the downstream steps. Dilute acid and organosolv pretreatment are promising approaches to fractionate woody biomass, in terms of recovering hemicellulose sugars.

Table 1: The effect of chemical pretreatments on biomass fractionation and downstream steps

Pretreatment	Hemicelluloses	Lignin	Detoxification required
Dilute acid	>80% Solubilized, mainly monomers	Little solubilization and extensive modification	Yes
Steam explosion	Partially solubilized to both monomers and oligomers	Little solubilization	Yes
Organosolv	Mostly solubilized	Solubilized in solvent	Yes
AFEX[a]	Remained, mostly to oligomers	Modification	No

a: Ammonia fibre explosion

1.3.2 Enzymatic hydrolysis and microbial fermentation

After being pretreated, lignocellulosic biomass becomes more accessible to cellulosic enzymes. Hydrolysis involves the transportation of enzymes from bulk solution to the surface of the pretreated substrates, adsorption of the enzymes to the substrates, hydrolysis of the polysaccharides into oligomeric and monomeric sugars, and release of the solubilized sugars into the bulk solution (Walker and Wilson 1991). For a complete hydrolysis, a combination of multiple enzymes is required. The cellulase enzymes work synergistically to hydrolyze cellulose into glucose monomers. The three cellulase enzymes that are mainly involved are: endoglucanases, exoglucanases and β-glucosidase (Lynd et al. 2002). Endoglucanases mainly bind to the internal amorphous sites in the cellulose polymer chains and cut them to shorter chains and oligosaccharides. Exoglucanases mainly catalyze the end sites of cellulose polymer chains and generate glucose and cellobiose. β-glucosidase acts as an accessory enzyme by hydrolyzing oligosaccharides and cellobiose into glucose. Over the last few decades, a great deal of research has been conducted to reduce the cost of enzymatic hydrolysis. Strategies include improving the production of the cellulosic enzymes, increasing the enzyme activity, adding

surfactants to improve the hydrolysis, and recovering the enzymes (Gadgil et al. 1995; Gruno et al. 2004; Lynd et al. 2002; Tu and Saddler 2010).

The monomeric sugars released after pretreatment and hydrolysis of lignocellulosic biomass include both hexose (glucose, mannose and galactose) and pentose sugars (xylose and arabinose). To achieve an efficient bioconversion process, the fermentation of all of the monomer sugars into bioethanol is desirable. Wild-type *S.cerevisiae* contains an elaborate uptake and consumption system for hexose sugars, but glucose is the preferred substrate (van Maris et al. 2006). Research from the perspectives of isolation and screening, as well as metabolic engineering has been conducted to improve mixed-substrate utilization (Keating et al. 2004; Ostergaard et al. 2001). *S. cerevisiae* is the most widely-used microorganism for hexose sugar fermentation. However, it is not able to ferment pentose sugars in that it lacks of two key enzymes for xylose metabolism, xylose reductase and xylitol dehydrogenase (Kötter and Ciriacy 1993). Some fungi (e.g. *Candida shehatae*; *Pichia stipitis*) and bacteria (e.g. *Clostridium acetobutylicum*) can utilize the pentose sugars (D-xylose and L-arabinose) in lignocellulosic biomass for biofuel production. These two type of microorganisms convert xylose and arabinose to a common intermediate, namely, D-xylulose-5-phosphate, through slightly different pathways (Chandel et al. 2011; Fernandes and Murray 2010).

Currently, although different fermenting microorganisms have been isolated to utilize all the monomeric sugars present in lignocellulosic biomass, an efficient and rapid fermentation process for the biomass hydrolysates is still in question because apart from the sugars, a wide range of inhibitory compounds are also produced during the pretreatment process, which seriously retard the microbial growth and biofuel production. The following section will focus on

discussing the strategies and problems involved in improving the fermentability of the biomass hydrolysates.

1.4 Strategies in improving lignocellulosic fermentability

1.4.1 Identification of inhibitors and their inhibitory mechanism

Current pretreatment approaches have been developed to disrupt the recalcitrance of the lignocellulosic biomass. However, they also produce a large number of inhibitors from lignin, extractives and carbohydrates which severely impede the utilization of the fermentable sugars. As a result, these inhibitors could become a limiting factor for the industrial success of biofuel production from lignocellulosic biomass. The formation of these inhibitors is dependent on biomass types and pretreatment conditions such as pH, temperature, residence time and addition of chemicals. For example, softwood mainly releases guaiacyl lignin compounds while hardwood releases both guaiacyl and syringyl lignin compounds (Klinke et al. 2004; Sjöström 1993). Acid-catalyzed pretreatment tends to produce ketone and aldehyde phenolic compounds while alkaline-based pretreatment tends to further oxidize these compounds into the corresponding acid forms (Sjöström 1993). Based on the functional groups, the previously-identified inhibitors can be classified into three groups: carboxylic acids, aldehydes/ketones and phenols. Aldehydes, ketones and carboxylic acids which contain R-C=O group are also called carbonyl compounds. Some of the carbonyl compounds are α,β-unsaturated compounds.

1.4.1.1 Carboxylic acids and their inhibition

Aliphatic acids are mainly degradation products from carbohydrates and to some extent from lignin due to pretreatment (Table 2). Acetic acid is a ubiquitous degradation product from

almost all biomass hemicelluloses. It is released in all pretreatments with a concentration ranging from 1 to 10 g/L (Klinke et al. 2004). Uronic acids such as glucuronic acid are also degraded from hemicelluloses but released at a higher temperature than acetic acid (Lu et al. 2009). Formic acid is generated from HMF and furfural, which are originally dehydrated from hexose and pentose sugars respectively in acidic and thermal conditions (Palmqvist and Hahn-Hägerdal 2000). Levulinic acid is generated from the degradation of HMF, with a much lower concentration than that of acetic acid (Xie et al. 2011). Other organic acids such as glycolic acid and lactic acid are also found in trace amounts from degradation of carbohydrates (Lu et al. 2009). Some saturated fatty acids derived from extractives have also been identified, such as caproic acid, caprylic acid and palmitic acid (Tran and Chambers 1985). Aromatic acids are generally generated in trace amounts from the degradation and oxidation of lignin (Klinke et al. 2002) (Table 2). The aliphatic substituents on the benzene ring depend mainly on the type of lignin precursors in the biomass feedstock (Sjöström 1993). Most of the aromatic acids are phenol derivatives.

Carboxylic acids released from the degradation of lignocellulosic biomass are weak acids, existing in both dissociated and undissociated forms (Piper et al. 2001; Taherzadeh et al. 1997). Only the undissociated forms are liposoluble and could diffuse into the cells through the plasma membrane (Piper et al. 2001). Because the pH value in the cytosol is neutral, the acids will re-dissociate and thus decreasing the intracellular pH. As an in situ detoxification defense, the microbial cells will pump out the protons to keep a neutral intracellular pH so as to maintain cell viability. This proton transportation is suggested to be conducted by the plasma membrane ATPase, the consumption of which, however, is traded off by decreasing cell biomass production and by-products (mostly glycerol) formation (Taherzadeh et al. 1997). Accordingly, researchers

often find a positive relationship between the presence of carboxylic acids and biofuel production in that extra carbon is used for promoting biofuel production when a low concentration of carboxylic acids is present. For instance, Taherzadeh et al. found that the presence of acetic acid (9 g/L, pH5) in anaerobic batch cultivation of *S. cerevisiae* increased the ethanol yield while it decreased the biomass and glycerol production compared to reference fermentation (Taherzadeh et al. 1997). Furthermore, since the effects of carboxylic acids on microbial activity are closely related to the concentration of their undissociated forms, the pH of the medium and the total acid concentration (dissociated+ undissociated) assume extra significance in this case. Taherzadeh et al. found that the minimum allowable pH at which the specific growth rate was equal or larger than 0.2/h increased with the increase of the concentration of acetic acid in the growth medium. The minimum allowable pH was 2.5 without the addition of acetic acid while the pH increased to 4.5 with the addition of 10 g/L acetic acid (Taherzadeh et al. 1997). However, the inhibition of carboxylic acid becomes obvious when a high concentration is present because the accumulation of protons in the cytosol could exhaust the proton pumping capacity of the cells (depletion of the ATP content), subsequently causing acidification of cytoplasm. In addition, the accumulation of anions was also suggested to assist the inhibition by creating abnormally high turgor pressure in the cytosol and affecting free radical production (Piper et al. 2001). Therefore, the effects of carboxylic acids on microbial growth are pH dependent.

The effects of carboxylic acids on microbial growth and fermentation are also related to their chemical structures. Aromatic acids were found to be more toxic than aliphatic acids due to the high liposolubility of benzene ring (Taherzadeh et al. 1997). Unsaturated aromatic acids were found to be more toxic than saturated acids. The unsaturated aromatic acids including ferulic

acid and 4-hydroxycinnamic acid severely inhibited fermentation even at low concentration (Ezeji et al. 2007). The unsaturated aromatic acids released during the biomass pretreatment are mostly α,β-unsaturated carbonyl compounds in which the carbonyl group is conjugated with an alkene. Due to the strong electron withdrawing ability of the carbonyl group, the alkene group (β-carbon) is deactivated towards an electrophile. Therefore, the β-carbon could attack the nucleophilic sites in the microbial cells. The α,β-unsaturated acids identified in previous studies are including fumaric acid, maleic acid, itaconic acid, cinnamic acid, ρ-coumaric acid, ferulic acid, 3-hydroxy-4-methoxycinnamic acid and sinapic acid (Table 2).

Based on previous studies (as discussed above) and research conducted in our lab, saturated carboxylic acids released in the biomass pretreatment are not potent inhibitors for retarding microbial activities. For example, in our study, a concentrated dilute-acid hydrolysate of loblolly pine containing 46.5 g/L hexose sugars, 1.9 g/L formic acid, 2.6 g/L acetic acid and 0.6 g/L levulinic acid was found to totally inhibit ethanol production with 2 g/L inoculation of Baker's yeast. After alkaline detoxification (NaOH, 2 h, 60°C), the hydrolysate did not lose any amount of carboxylic acids but the ethanol production became comparable to that in the reference control. On the contrary, the unsaturated carboxylic acids could be one of the groups of inhibitors contributing to the fermentation inhibition.

1.4.1.2 Aldehydes/ketones and their inhibition

Furfural and HMF are the most common aldehyde decomposition products from pentose and hexose sugars respectively, occurring in high concentrations after thermal and acidic pretreatment of lignocellulosic biomass (Klinke et al. 2004; Larsson et al. 1999a; Palmqvist et al. 1999). These furans have been used as model inhibitors in many previous studies on microbial

growth and biofuel production (Palmqvist et al. 1999; Taherzadeh et al. 1999). Recently, glycolaldehyde, produced from retro-aldol condensation of glucose after pressurized and thermal pretreatment of lignocellulosic biomass has been identified as a strong inhibitor for ethanol production (Jayakody et al. 2011). Similar to glycolaldehyde are numerous aliphatic aldehydes degraded from sugars, such as methylglyoxal, which may possess inhibitory activities towards fermenting microorganisms (Lu et al. 2009). Another major group of carbonyl compounds are aromatic aldehydes or ketones degraded from lignin and possibly extractives and sugars, most of which are phenol derivatives. Aromatic aldehydes such as 4-hydroxybenzaldehyde, vanillin and syringaldehyde have been used as model inhibitors for their effects on microbial growth and biofuel production in previous studies (Delgenes et al. 1996; Sakai et al. 2007). Among the aromatic carbonyl compounds are Hibbert's ketones derived from phenylpropane precursors of lignin with one or two keto group on the α or/and β position of the propyl groups (Klinke et al. 2004; Sjöström 1993). The formation of carbonyl groups on the benzene ring often depends on the pretreatment conditions. Usually, ketone and aldehyde compounds are formed under oxidative, acidic pretreatment conditions while alkaline pretreatment tends to further oxidize the aldehyde/ketone compounds into their corresponding acid forms. Moreover, the substituents on the benzene ring are often related to the type of biomass used for the pretreatment. Softwood mostly produces guaiacyl lignin derivatives while hardwood produces both guaiacyl and syringyl lignin compounds. Some compounds such as vanillic acid, syringic acids and their corresponding aldehyde forms are considered to be derived from the cleavage of ester and/or ether bonds in lignin, or degraded from labile compounds, such as Hibbert ketones. The previously identified aldehyde and ketone carbonyl compounds are summarized in Table 3.

Microbial cells were found to be capable of converting aldehyde compounds into their corresponding alcohols and trace amount of acids in both anaerobic and aerobic fermentation, most likely by multiple enzymes involved in reduction activities coupled with cofactors NADH or NADPH (Liu 2011; Palmqvist and Hahn-Hägerdal 2000). This in situ detoxification has been studied mostly in the conversion of furan derivatives (Gutiérrez et al. 2002; Palmqvist et al. 1999; Taherzadeh et al. 1999), as well as some aromatic aldehydes (Larsson et al. 2000). For example, furfural was converted to furfuryl alcohol and small amount of 2-furoic acid in an anaerobic fermentation by *S. cerevisiae*. This bio-reduction was suggested to act as an alternative redox sink competing for NADH oxidization which was regenerated (oxidized) through the formation of glycerol in microbial biosynthesis process without furfural. Therefore, the presence of furfural often results in decrease or absence in glycerol production (Palmqvist et al. 1999). In this case, the presence of furfural could enhance ethanol production since an extra carbon source was available due to the decrease in by-product formation. Similar results were also observed in our lab when using vanillin as a model phenolic aldehyde to study its effects on anaerobic fermentation by baker's yeast. Low concentrations of vanillin (≤10 mM) could increase the ethanol yield by 3 to 9% compared to the reference fermentation although they inhibited glycerol and cell-biomass production. In contrast, high concentration of vanillin (25 mM) considerably inhibited ethanol production (Cao et al. 2014). Moreover, we found that the enzyme activity of alcohol dehydrogenase, one of the main reductase enzymes that were considered to be responsible for aldehyde reduction, dropped from 3.85 to only 0.11 U/mg as the concentration of vanillin increased from 0 to 25 mM (Cao et al. 2014). 25 mM vanillin probably exceeded the reduction capacity of ADH enzyme and led to inhibition toward ethanol production.

The effects of aldehyde and ketone compounds on cellulosic fermentation are related to their chemical structures. After studying the effects of different aromatic model aldehydes (5 mM) on ethanol production by baker's yeast (2 g/L inoculum) in our lab, we found the fermentation inhibition followed the order: *o*-phthalaldehyde > 2-hydroxybenzaldehyde and 2,3-dihydroxybenzaldehyde > 2,4-dihydroxybenzaldehyde and 2,3,4-trihydroxybenzaldehyde > 4-hydroxy-3-methoxybenzaldehyde (vanillin) and 4-hydroxybenzaldehyde. The presence of OPA totally stopped glucose consumption, 2-hydroxybenzaldehyde inhibited the glucose consumption at 3 h by 94% compared to the control, 2,3-dihydroxybenzaldehyde and 2,4-dihydroxybenzaldehyde inhibited glucose consumption by 86 and 81 % respectively, 2,3,4-trihydroxybenzaldehyde inhibited the glucose consumption by 67%, while the last two compounds had little impact on glucose consumption (see chapter 5). The difference in toxicity of these aromatic aldehydes was believed to be related to the substituents on the benzene ring. In OPA, introduction of a second aldehyde group in the ortho position results in a strong resonance interaction which, therefore, leads to an increase in reactivity toward nucleophiles (Zuman 2004). The strong electrophilicity of OPA was also reflected by its low negative value of the energy of the lowest unoccupied molecular orbital (E_{LUMO}, -1.495 eV) (Cao et al. 2014). The strong inhibition of 2-hydroxybenzaldehyde was probably also related to its ortho hydroxyl group (-OH). -OH group is a strong electron withdrawing group that could enhance the electrophilicity of the CHO group in the α-carbon which in turn attacks the nucleophilic sites in the yeast cells. Similarly, when studying the effects of different aromatic compounds on oxygen-limited growth and ethanol fermentation by *S. cerevisiae*, Larsson et al. found that 0.2 g/L *o*-vanillin (with ortho-hydroxyl group) completely inhibited both cell growth and ethanol production while vanillin and isovanillin did not show any inhibition at the same concentration level (Larsson et al.

2000). Moreover, Friedman et al. found that the activities of different hydroxybenzaldehyes against *E. coli* were: 2-hydroxybenzaldehye > 3-hydroxybenzaldehye > 4-hydroxybenzaldehye (Friedman et al. 2003). Among the lignocellulose-derived aromatic carbonyls are a group of aldehyde compounds containing α,β carbon-carbon double bonds (C=C). Preliminary study in our group indicates these α,β-unsaturated carbonyl aldehydes have stronger fermentation inhibition compared to the conventional model compounds such as furfural and vanillin. Complicated chemistry towards biological nucleophiles was believed to contribute to their strong inhibition activity. In additional to the functional CHO group, α,β-unsaturated aldehydes have a second functional group, the C=C bond. Due to the proximity of CHO group, the C=C group is polarized and activated, resulting in enhanced electrophilicity on the β-carbon atom. As a result, both functional groups can participate individually or cooperatively a series of reactions with nucleophilic compounds such as amines, proteins and DNA (Chan et al. 2008b). Previous studies have identified α,β-unsaturated aromatic aldehydes in the biomass hydrolysate as potential inhibitors while their contribution to the fermentation inhibition has yet to be elucidated (Ando et al. 1986; Larsson et al. 2000).

Although studies have been conducted to investigate the inhibition mechanism of carbonyl (ketone and aldehyde) compounds, few conclusions have been reported. This is because most of the carbonyl inhibitors studied in model fermentation studies were at much higher concentrations compared to that in real biomass hydrolysate (Delgenes et al. 1996; Larsson et al. 2000), possibly leading to inconclusive results. Second, many carbonyl compounds have not been identified yet due to low concentration, which becomes an obstacle to studying their inhibition mechanism. Nevertheless, based on the discussion above, the fermentation inhibition of carbonyl inhibitors are closely related to their chemical structures. The electrophilicity of the

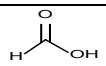
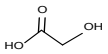
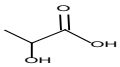
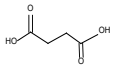
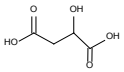
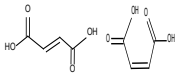
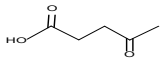
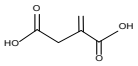
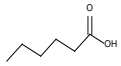
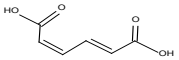
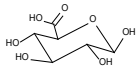
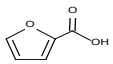
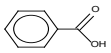
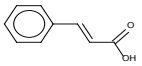
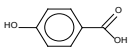
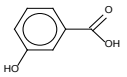
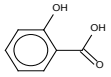
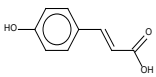
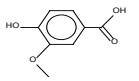
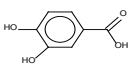
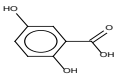
carbonyl groups (aldehyde or ketone) and the polarized α,β-unsaturated C=C bonds might initiate covalent binding with the nucleophilic sites in the microbial cells thus leading to adverse biological effects. Therefore, characterizing the electrophilic reactivity of the carbonyl inhibitors might help to understand their mechanism of inhibition.

1.4.1.3 Phenols and their inhibition

Phenols are generated mostly from lignin and to some extent extractives and carbohydrates (Table 4). It was reported that lignin hydrolysis occurred at a temperature as low as 160°C (Lu et al. 2009). Therefore, lignin degradation compounds are present in the biomass hydrolysate after most thermal pretreatments such as dilute acid, organosolv and steam explosion. Moreover, some phenol derivatives such as catechol, hydroquinone and 3-methylcatechol have been found to derive from carbohydrates under thermal and acidic conditions (Nelson David et al. 1988).

The toxicity of phenol derivatives was generally considered to be less than that of aldehydes and acids on a weight or molar basis (Zaldivar et al. 2000). Microbial growth was more sensitive to alcohol than ethanol production. The concentration required to completely inhibit ethanol production was 3-6 times higher than the concentration needed to inhibit cell growth (Zaldivar et al. 2000). The inhibition was believed to cause plasma membrane damage as measured by the leakage of magnesium from cells in PBS buffer (Zaldivar et al. 2000). Therefore, the inhibition mechanism was probably related to hydrophobicity.

Table 2: Carboxylic acids identified in lignocellulosic biomass after pretreatment

Compound	Structure	Formula	Exact mass	Origin	Reference
Formic acid		CH_2O_2	46.01	Furfural and HMF	(Chen et al. 2006)
Acetic acid		$C_2H_4O_2$	60.02	Hemicelluloses /lignin	(Klinke et al. 2002)
Glycolic acid		$C_2H_4O_3$	76.02	Sugars	(Lu et al. 2009)
Lactic acid		$C_3H_6O_3$	90.03	Sugars	(Chen et al. 2006)
Succinic acid		$C_4H_6O_4$	118.03	Not clear	(Klinke et al. 2002)
Malic acid		$C_4H_6O_5$	134.02	Not clear	(Klinke et al. 2002)
Fumaric acid/ Maleic acid		$C_4H_4O_4$	116.01	Not clear	(Luo et al. 2002)
Levulinic acid		$C_5H_8O_3$	116.05	Hexose	(Xie et al. 2011)
Itaconic acid		$C_5H_6O_4$	130.03	Not clear	(Chen et al. 2006)
Caproic acid		$C_6H_{12}O_2$	116.08	Extractives	(Tran and Chambers 1985)
2,4-Hexadienedioic acid		$C_6H_6O_4$	142.03	Extractives	(Luo et al. 2002)
Glucuronic acid		$C_6H_{10}O_7$	194.04	Sugars	(Lu et al. 2009)
2-Furoic acid		$C_5H_4O_3$	112.02	Furfural	(Luo et al. 2002)
Benzoic acid		$C_7H_6O_2$	122.04	ρ-Coumaryl unit	(Chen et al. 2006)
Cinnamic acid		$C_9H_8O_2$	148.05	ρ-Coumaryl unit	(Ando et al. 1986)
4-Hydroxybenzoic acid		$C_7H_6O_3$	138.03	ρ-Coumaryl unit	(Chen et al. 2006)
3-Hydroxybenzoic acid		$C_7H_6O_3$	138.03	ρ-Coumaryl unit	(Ando et al. 1986)
2-Hydroxybenzoic acid		$C_7H_6O_3$	138.03	Not clear	(Jönsson et al. 1998)
p-Coumaric acid		$C_9H_8O_3$	164.05	ρ-Coumaryl unit	(Klinke et al. 2002)
Vanillic acid		$C_8H_8O_4$	168.04	Guaiacyl unit	(Klinke et al. 2002)
3,4-Dihydroxybenzoic acid		$C_7H_6O_4$	154.03	Not clear	(Luo et al. 2002)
2,5-Dihydroxybenzoic acid		$C_7H_6O_4$	154.03	Not clear	(Luo et al. 2002)

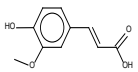
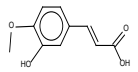
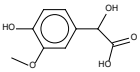
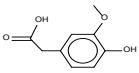
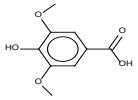
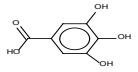
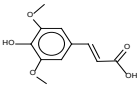
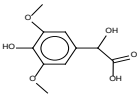
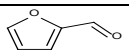
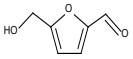
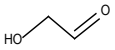
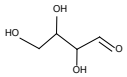
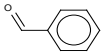
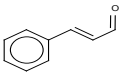
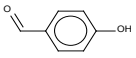
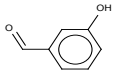
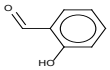
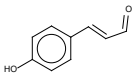
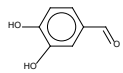
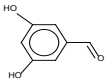
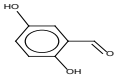
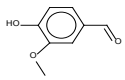
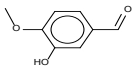
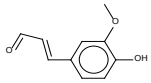
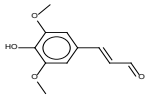
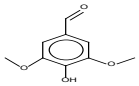
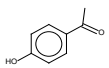
Ferulic acid		$C_{10}H_{10}O_4$	194.06	Guaiacyl unit	(Klinke et al. 2002)
3-Hydroxy-4-methoxycinnamic acid		$C_{10}H_{10}O_4$	194.06	Guaiacyl unit	(Chen et al. 2006)
Guaiacylglycolic acid		$C_9H_{10}O_5$	198.05	Guaiacyl unit	(Buchert et al. 1990)
Homovanillic acid		$C_9H_{10}O_4$	182.06	Guaiacyl unit	(Burtscher et al. 1987)
Syringic acid		$C_9H_{10}O_5$	198.05	Syringyl unit	(Chen et al. 2006)
Gallic acid		$C_7H_6O_5$	170.02	Extractives (tannin)	(Sjöström 1993)
Sinapic acid		$C_{11}H_{12}O_5$	224.07	Syringyl unit	(Klinke et al. 2004)
Syringylglycolic acid		$C_{10}H_{12}O_6$	228.06	Syringyl unit	(Buchert et al. 1990)

Table 3: Aldehydes and ketones identified in lignocellulosic biomass after pretreatment

Compound	Structure	Formula	Exact mass	Origin	Reference
Furfural		$C_5H_4O_2$	96.02	Pentose	(Klinke et al. 2002)
5-Hydroxymethylfurfural		$C_6H_6O_3$	126.03	Hexose	(Klinke et al. 2002)
Glycolaldehyde		$C_2H_4O_2$	60.02	Sugars	(Jayakody et al. 2011)
Methylglyoxal		$C_3H_4O_2$	72.02	Sugars	(Lu et al. 2009)
Erythrose		$C_4H_8O_4$	120.04	Hexose	(Lu et al. 2009)
Benzaldehyde		C_7H_6O	106.04	ρ-Coumaryl unit	(Ando et al. 1986)
Cinnamaldehyde		C_9H_8O	132.06	ρ-Coumaryl unit	(Ando et al. 1986)
4-Hydroxybenzaldehyde		$C_7H_6O_2$	122.04	ρ-Coumaryl unit	(Chen et al. 2006)
3-Hydroxybenzaldehyde		$C_7H_6O_2$	122.04	Not clear	Na
2-Hydroxybenzaldehyde		$C_7H_6O_2$	122.04	Not clear	Na
4-Hydroxycinnamaldehyde		$C_9H_8O_2$	148.05	ρ-Coumaryl unit	(Ando et al. 1986)
3,4-Dihydroxybenzaldehyde		$C_7H_6O_3$	138.03	ρ-Coumaryl unit	(Chen et al. 2006)
3,5-Dihydroxybenzaldehyde		$C_7H_6O_3$	138.03	Not clear	(Jönsson et al. 1998)
2,5-Dihydroxybenzaldehyde		$C_7H_6O_3$	138.03	Not clear	(Jönsson et al. 1998)
Vanillin		$C_8H_8O_3$	152.05	Guaiacyl unit	(Chen et al. 2006)
Isovanillin		$C_8H_8O_3$	152.05	Not clear	(Dizhbite et al. 2011)
Coniferyl aldehyde		$C_{10}H_{10}O_3$	178.06	Guaiacyl unit	(Lu et al. 2009)
Sinapaldehyde		$C_{11}H_{12}O_4$	208.07	Syringyl unit	(Lu et al. 2009)
Syringaldehyde		$C_9H_{10}O_4$	182.06	Syringyl unit	(Chen et al. 2006)
4-Hydroxyacetophenone		$C_8H_8O_2$	136.05	ρ-Coumaryl unit	(Burtscher et al. 1987)

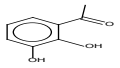
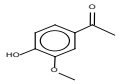
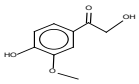
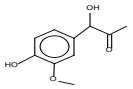
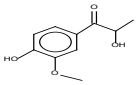
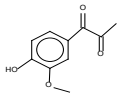
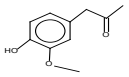
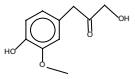
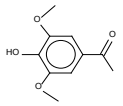
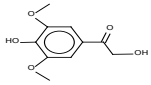
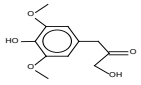
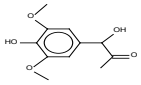
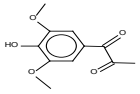
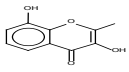
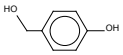
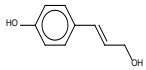
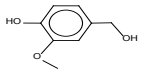
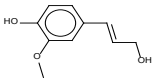
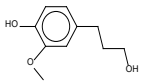
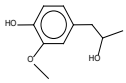
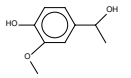
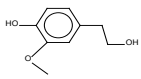
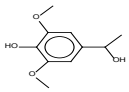
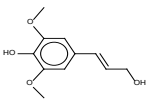
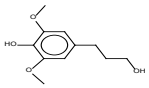
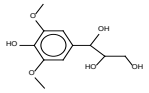
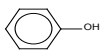
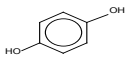
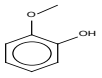
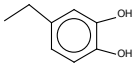
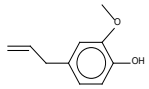
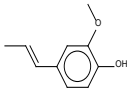
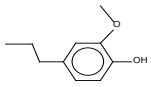
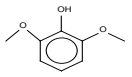
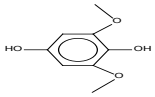
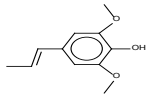
2,3-Dihydroxyacetophenone		$C_8H_8O_3$	152.05	Sugars	(Nelson David et al. 1988)
Acetovanillone		$C_9H_{10}O_3$	166.06	Guaiacyl unit	(Leonard and Hajny 1945)
2-Hydroxy-1-guaiacyl-ethanone		$C_9H_{10}O_4$	182.06	Guaiacyl unit	(Buchert et al. 1990)
1-Guaiacylacetol		$C_{10}H_{12}O_4$	196.07	Guaiacyl unit	(Buchert et al. 1990)
α-Hydroxypropiovanillone		$C_{10}H_{12}O_4$	196.07	Guaiacyl unit	(Clark and Mackie 1984)
NA		$C_{10}H_{10}O_4$	194.06	Guaiacyl unit	(Clark and Mackie 1984)
NA		$C_{10}H_{12}O_3$	180.08	Guaiacyl unit	(Clark and Mackie 1984)
β-Oxyconiferylalcohol		$C_{10}H_{12}O_4$	196.07	Guaiacyl unit	(Clark and Mackie 1984)
Acetosyringone		$C_{10}H_{12}O_4$	196.07	Syringyl unit	(Klinke et al. 2002)
2-Hydroxy-1-syringyl-ethanone		$C_{10}H_{12}O_5$	212.07	Syringyl unit	(Buchert et al. 1990)
β-Oxysinapylalcohol		$C_{11}H_{14}O_5$	226.08	Syringyl unit	(Fenske et al. 1998)
1-Syringyl acetol		$C_{11}H_{14}O_5$	226.08	Syringyl unit	(Buchert et al. 1990)
NA		$C_{11}H_{12}O_5$	224.07	Syringyl unit	(Fenske et al. 1998)
3,8-Dihydroxy-2-methylchromone		$C_{10}H_8O_4$	192.04	Sugars	(Nelson David et al. 1988)

Table 4: Phenols identified in lignocellulosic biomass after pretreatment

Compound	Structure	Formula	Exact mass	Origin	Reference
4-Hydroxybenzyl alcohol		$C_7H_8O_2$	124.05	ρ-Coumaryl unit	Na
ρ-Coumaryl alcohol		$C_9H_{10}O_2$	150.07	ρ-Coumaryl unit	(Agblevor et al. 2004)
Vanillyl alcohol		$C_8H_{10}O_3$	154.06	Guaiacyl unit	Na
Coniferyl alcohol		$C_{10}H_{12}O_3$	180.08	Guaiacyl unit	(Zaldivar et al. 2000)
Dihydroconiferyl alcohol		$C_{10}H_{14}O_3$	182. 09	Guaiacyl unit	(Buchert et al. 1990)
NA		$C_{10}H_{14}O_3$	182. 09	Guaiacyl unit	(Clark and Mackie 1984)
1-Guaiacylethanol		$C_9H_{12}O_3$	168.08	Guaiacyl unit	(Buchert et al. 1990)
Homovanillyl alcohol		$C_9H_{12}O_3$	168.08	Guaiacyl unit	(Buchert et al. 1990)
1-Syringylethanol		$C_{10}H_{14}O_4$	198.09	Syringyl unit	(Buchert et al. 1990)
Sinapyl alcohol		$C_{11}H_{14}O_4$	210.09	Syringyl unit	(Buchert et al. 1990)
Dihydrosinapyl alcohol		$C_{11}H_{16}O_4$	212.10	Syringyl unit	(Buchert et al. 1990)
Syringylglycerol		$C_{11}H_{16}O_6$	244.09	Syringyl unit	(Buchert et al. 1990)
Phenol		C_6H_6O	94.04	ρ-Coumaryl unit	(Jönsson et al. 1998)
Hydroquinone		$C_6H_6O_2$	110.04	Sugars	(Larsson et al. 1999b)
Catechol		$C_6H_6O_2$	110.04	Sugars	(Larsson et al. 1999b)
Guaiacol		$C_7H_8O_2$	124.05	Guaiacyl unit	(Klinke et al. 2002)
o-Cresol		C_7H_8O	108.06	ρ-Coumaryl unit	(Jönsson et al. 1998)
Ethylcatechol		$C_8H_{10}O_2$	138.07	ρ-Coumaryl unit	(Jönsson et al. 1998)
3-Methylcatechol		$C_7H_8O_2$	124.05	Sugars	(Jönsson et al. 1998)

Eugenol		$C_{10}H_{12}O_2$	164.08	Guaiacyl unit	(Larsson et al. 2000)
Isoeugenol		$C_{10}H_{12}O_2$	164.08	Guaiacyl unit	(Jönsson et al. 1998)
4-Propylguaiacol		$C_{10}H_{14}O_2$	166.10	Guaiacyl unit	(Jönsson et al. 1998)
Syringol		$C_8H_{10}O_3$	154.06	Syringyl unit	(Burtscher et al. 1987)
2,6-Dimethoxy-hydroquinone		$C_8H_{10}O_4$	170.06	Syringyl unit	(Buchert et al. 1990)
4-Propenylsyringol		$C_{11}H_{14}O_3$	194.09	Syringyl unit	(Buchert et al. 1990)

1.4.2 Development of analytical methods for identifying inhibitors

The inhibitors released during the biomass pretreatment are derived from all of the biomass components including cellulose, hemicelluloses, lignin and extractives. Frequently, more than 100 toxic compounds with extremely low concentration are introduced into the biomass hydrolysate inhibiting the subsequent microbial growth and ethanol productivity. Identification of these inhibitors has been conducted using analytical tools including HPLC, GC-MS and LC-MS, and NMR (Agblevor et al. 2004; Chen et al. 2006; Helm et al. 2010; Luo et al. 2002).

An analytical procedure using ion exchange resin has been developed by Luo et al. to identify fermentation inhibitors in the biomass hydrolysate (Luo et al. 2002). In this procedure, a concentrated wood hydrolysate was passed through an ion exchange resin column; the untreated and treated hydrolysates were extracted with ethyl acetate and derivatized with 95% BSTFA and 5% TMCS for GC-MS identification; the compositional difference and fermentabilities of the untreated and treated hydrolysates were then compared and evaluated. The compounds absorbed by the anion exchange resin were also regenerated using 3.6N NH_4OH and identified as potential

inhibitors. This procedure identified 35 potential inhibitors in dilute acid pretreated hybrid poplar. However, one problem of this procedure was that the chemical structure of the inhibitors, especially the potent and labile ones, probably changed in the ion exchange column and in the regeneration process since they were both at alkaline conditions.

HPLC has been extensively used in analyzing potential fermentation inhibitors in biomass hydrolysate. Reverse phase columns with gradient solvents are often used for the identification. Chen et al. developed a reversed-phase HPLC method for identification of degradation products in biomass hydrolysates using: aqueous 0.05% (v/v) phosphoric acid (pH 2.2–2.3) and water-acetonitrile (10:90) (Chen et al. 2006). 32 potential analytes were separated simultaneously with UV detection at 210 nm, including aliphatic acids, aromatic acids, aldehydes, and phenolic compounds. GC-MS is another effective analytical tool for the identification. Ando et al. used gas chromatography to identify the degradation products in a steam exploded poplar (Ando et al. 1986). The dried hydrolysate samples were silylated with TRI-Sil and then analyzed by GC, with which 12 aromatic monomers were identified. Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy has also been used to identify the functional groups of the lignocellulose-derived inhibitors. The ^{13}C-NMR spectra could detect aliphatic and aromatic acids, esters and other aromatic and aliphatic compounds, but the sensitivity towards ketone or aldehyde groups is very low (Agblevor et al. 2004).

Although efforts have been made to identify the hydrolysate inhibitors, there is still a lack of knowledge of which inhibitors have the most pronounced effects on microbial fermentation. This uncertainty in the fermentation inhibitors is widely recognized as one of the roadblocks for developing an effective detoxification approach and improving stress-tolerance yeast and

bacterial ethanologens. Therefore, it is important to develop a robust and reliable analysis procedure.

1.4.3 Detoxification strategies

Detoxification (conditioning) methods from physical, chemical and biological perspectives have been attempted to remove or modify inhibitors in biomass hydrolysates and improve cell-growth and fermentation. Physical treatments include evaporation, steam stripping, solvent extraction, aqueous two-phase extraction and supercritical liquid. Physical detoxification normally tends to remove the inhibitors rather than modify their chemical structures. Chemical treatments including alkaline detoxification, addition of reducing agents, ion exchange, activated charcoal and chemical extraction are mainly conducted to convert inhibitors into less toxic compounds. Biologically, many fermenting microorganisms are able to detoxify weak inhibitors such as aldehydes and carboxylic acids (see previous sections 1.4.1.1 & 1.4.1.2). This in situ detoxification is dependent on microorganism types, the inoculation size and the chemical structure of the inhibitors. Some fungi have been found to digest lignin degradation compounds. Laccase and peroxidase from the white-rot fungus *Trametes versicolor* are able to transform aromatic compounds. Some microorganisms can metabolize furan derivatives and other degradation compounds in biomass hydrolysate (bioabatement).

1.4.3.1 Physical detoxification

Stream stripping and evaporation

Steam stripping (steam distillation) introduces steam into the distillation system to remove temperature sensitive compounds. In the 1940s, this idea was used to improve the

fermentation performance of biomass hydrolysates by removing the volatile inhibitors (Leonard and Hajny 1945). Similarly, evaporation (mainly using a rotary evaporator) was a mild attempt to remove the volatile inhibitors at relatively low temperatures (between 40-55°C) to achieve a better fermentation (Larsson et al. 1999b). During the evaporation, volatile compounds such as furfural could be easily removed. On the other hand, the removal of carboxylic acids such as acetic acid and formic acid is pH dependent since it is the undissociated form of the acids that is being removed. When concentrating a biomass hydrolysate to 20% of the original volume, Palmqvist et al. detected 12 g/L acetic acid, indicating incomplete removal of acetic acid (Palmqvist et al. 1996). The major advantage of steam stripping and evaporation detoxification is to remove volatile inhibitors meanwhile concentrating biomass hydrolysate to achieve high fermentable sugar concentration. However, both methods could not remove non-volatile inhibitors, which indeed were considered as the major inhibitors in biomass hydrolysates (Palmqvist et al. 1996). Therefore, it is not practical to only use steam stripping/evaporation as a single detoxification approach to remove toxic inhibitors in biomass hydrolysate.

Physical extraction

Solvent extraction is a physical detoxification method that uses organic solvents such as ethyl acetate and diethyl ether to remove the inhibitory compounds mainly based on their solubility in two immiscible phases. This treatment could remove both volatile and non-volatile inhibitors such as phenolic compounds and furan derivatives (Clark and Mackie 1984). The main problem of this method is that the organic solvents used are often toxic to fermenting microorganisms. Thus, an extra separation step of the organic solvent is necessary. As a mild extraction method, aqueous two-phase extraction system has been used for removing inhibitors especially phenolic compounds from lignocellulosic hydrolysate while being benign to

fermenting microorganisms (Hasmann et al. 2008). In this method, one or two non-volatile polymers (copolymers) such as ethylene oxide were used as extractants which were soluble at lower temperatures but can be separated from water at higher temperatures due to their low critical solution temperature. The high cost of polymers and their recycle are the major obstacles for the industrial application of this method. Another alternative to organic solvent is the use of supercritical liquids, which are formed above their critical temperature and critical pressure. Supercritical CO_2 has been used to extract multiple degradation compounds including furans, phenolic compounds and carboxylic acids based on their polarities (Persson et al. 2002b). Since supercritical CO_2 has a low polarity, the removal of inhibitory compounds is limited. Moreover, the inhibition could only be partially removed because the ethanol productivity after the detoxification was only 33% of the reference control (Persson et al. 2002b). More importantly, the supercritical liquid is very costly for industrial applications.

1.4.3.2 Chemical detoxification

Alkaline detoxification

Alkali treatments, using calcium hydroxide ($Ca(OH)_2$, overliming) and sodium hydroxide (NaOH), or ammonium hydroxide (NH_4OH) added into biomass hydrolysate to maintain the solution at high pH (9-12) for a period of time prior to fermentation, have been used as one of the most effective approaches to detoxify lignocellulosic hydrolysates and improve their fermentability (Larsson et al. 1999b; Leonard and Hajny 1945). When compared to different detoxification methods for improving the fermentability (both final yield and productivity) of a dilute-acid pretreated spruce biomass, Larsson et al. found that alkaline detoxified hydrolysate had the best performance (Larsson et al. 1999b). Among the compounds being used, $Ca(OH)_2$

was believed to lead to better detoxification performance, but caused the formation of unfavorable gypsum precipitate ($CaSO_4$) (Alriksson et al. 2006). NaOH and NH_4OH also resulted in fermentations comparable with or even better than a reference control if the detoxification conditions were well controlled (Alriksson et al. 2006). An optimal combination of temperature and pH is crucial for achieving a high fermentability while avoiding sugar degradation. Alriksson et al. found the optimal combination of temperature and pH for a 3h-duration detoxification of dilute-acid hydrolysate was between pH9 / 80°C and pH12 / 30°C using NaOH and pH9 / 60°C using NH_4OH (Alriksson et al. 2006). Moreover, maintaining a high constant pH during the detoxification was equally important since the detoxification mechanism was believed to be the chemical conversion of inhibitors under alkaline conditions (Xie et al. 2012). Thus, NH_4OH is a better choice in that it has buffering effect that could maintain a high pH value without using pH-stat. The specific mechanism of alkaline detoxification has not been elucidated yet although alkali-assisted aldol condensation of furan and other aldehydes have been suggested (Alriksson et al. 2006; Horváth et al. 2005).

Addition of reducing agents

Addition of reducing agents to the biomass hydrolysate to improve fermentability was been proposed by Leonard et al. several decades ago (Leonard and Hajny 1945). In their research, Leonard et al. believed unfavorable oxidation-reduction potentials were the reason for poor fermentability of the biomass hydrolysate. Reducing substances such as Na_2SO_3, $NaHSO_3$, $Na_2S_2O_5$ and Na_2S were able to improve the hydrolysate fermentability. The effectiveness of reducing agent is concentration dependent. Larsson et al. found that detoxifying a dilute-acid treated spruce with 1% Na_2SO_3 at pH5.5 for 1 h led to a better fermentability than with 0.1% Na_2SO_3, probably because the latter was not sufficient to reduce furan derivatives (Larsson et al.

1999b). Nevertheless, using reducing agents as a single detoxification approach could not lead to a fermentability equal to the reference control (Olsson et al. 1995).

Chemical adsorption

Chemical adsorption has been examined as a detoxification approach of biomass hydrolysate for many years. The detoxification mechanism is involved in the extensive adsorption of solutes (inhibitors) from a liquid phase to an adsorbent solid phase, which results in the sharing of electrons between the solutes and the solid phase (weak chemical bonding). Chemical adsorption approaches used for removing inhibitors from biomass hydrolysate include ion-exchange resins, wood charcoal and activated charcoal.

Ion exchange resins have been proven to be an effective detoxification method for biomass hydrolysate. Nilvebrant et al. evaluated the effects of different ion exchangers (anion, uncharged and cation) on the fermentability of a toxic dilute-acid hydrolysate of spruce and found the detoxification efficiency in terms of improving ethanol productivity and yields was: anion > uncharged > cation (Nilvebrant et al. 2001). The desirable detoxification effect of the anion exchange resin was attributed to its positively charged functional groups. Anion exchanger contains quaternary ammonium groups with hydroxyl ions as the counterions on their surface, which exchange the ionized forms of carboxylic acids at pH 5.5. When pH is increased to 10, most of the phenolic compounds were ionized to phenolates and thus could also be retained on the cationic sites on the resin (Nilvebrant et al. 2001). Therefore, anion exchange at pH10 resulted in a better fermentability compared to that at pH5 because of exchange of toxic phenolic compounds (Larsson et al. 1999b; Nilvebrant et al. 2001). On the other hand, the uncharged and cation resins only removed part of furans and phenolic compounds by weak hydrophobic interactions (Nilvebrant et al. 2001). The negatively charged surface on the cation resin even led

to a repulsive effect on the anionic inhibitors in the hydrolysate and thus could not remove them, especially at high pH (Nilvebrant et al. 2001). The major problem associated with using anion exchange resin for detoxifying the hydrolysate is that a considerable amount of sugars are also ionized and retained on the cationic surface under alkaline conditions (Larsson et al. 1999b; Nilvebrant et al. 2001).

Activated charcoal and wood charcoal with high degrees of microporosity have been used to detoxify biomass hydrolysate by absorbing furans and phenolic compounds. The removal of phenolic compounds was dependent on hydrolysate/charcoal ratio and treatment time (Miyafuji et al. 2003; Mussatto et al. 2001). Moreover, increasing the treatment temperature for wood charcoal was found to increase the adsorption of phenolic compounds and furans due to increase in the hydrophobicity of the wood charcoal (Miyafuji et al. 2003). Major problems related to this approach are loss of fermenting sugars and high energy demand for preparing the charcoals. Furthermore, the improvement of fermentability in terms of productivity in the charcoal-treated hydrolysate was not comparable to that in other detoxification approaches. Miyafuji et al. found that the fermentability of biomass hydrolysate by baker's yeast was improved after wood charcoal treatment but ethanol production did not occur until 75 hours. However, a much faster productivity could be realized with overliming treatment (Miyafuji et al. 2003).

Chemical extraction

Complex extraction is conducted in ion-associated organic solvent systems in which the extraction is accomplished by both ion exchange and differential solubility. It has been extensively utilized in wastewater treatment as a separation and purification approach (Li et al. 2009; Li et al. 2007). A complex extraction system using trialkylamine as an alkali extractant was used recently for detoxifying a corn stover hydrolysate (Zhu et al. 2011). The optimal

condition for detoxifying the hydrolysate was found to be at a ratio of aqueous to organic phases of 1:2, at which the organic phase was composed of a mix of trialkylamine/n-octanol/kerosene (30/50/20%, v/v/v). The detoxification removed part of carboxylic acids and HMF, as well as all of furfural and improved the fermentability of the corn stove hydrolysate. One problem with this approach is that the organic solvents are toxic to the microorganisms if not removed completely. Moreover, the organic solvents are expensive and need to be cleaned up and recycled which will introduce another step in to the process.

1.4.3.3 Biological detoxification

Laccase and peroxidase from white-rot fungus are able to transform phenolic compounds. Some fungi are able to metabolize phenolic compounds and detoxify the biomass hydrolysate. Recently, several microorganisms are isolated to potentially abate inhibitors such as furan derivatives and aromatic acids in biomass hydrolysate.

Laccase and peroxidase

Laccase and peroxidase from white-rot fungus *Trametes versicolor* are able to transform aromatic compounds. Their effects on improving the fermentability of biomass hydrolysate by specifically removing aromatic compounds have been studied (Jönsson et al. 1998; Larsson et al. 1999b). After evaluating the effects of different detoxification on improving ethanol production, Larsson et al. found laccase treatment was one of the most efficient methods (Larsson et al. 1999b). Based on GPC analysis of laccase treated hydrolysate, an increase in large molecular weight substances and a decrease in small molecular weight substances were found. Using GC-MS analysis showed a decrease in the concentration of phenolic compounds, Jönsson et al. proposed an oxidative polymerization of phenolic compounds as the detoxification mechanism

(Jönsson et al. 1998). The main problem with this detoxification method is that the treatment time is very long which makes the industrial application infeasible. Moreover, the cost for preparing the enzyme is too high to compete with other detoxification approaches.

Trichoderma reesei and biological abatement

The use of cellulolytic fungus *Trichoderma reesei* for detoxifying biomass hydrolysate was proposed by Palmqvist et al. when they attempted to find an alternative way to utilize the inhibitory hydrolysate (Palmqvist et al. 1997). In order to recirculate the water stream without accumulating the nonvolatile inhibitors and utilize the solubilized hemicelluloses sugars, Palmqvist used this lignocelluloses-degrading fungus to pre-ferment the biomass hydrolysate, producing cellulolytic enzymes while detoxifying the hydrolysate. The pre-fermentation resulted in degradation of phenolic compounds, furan derivatives and acetic acid. However, the difficulty for enzyme extraction and complete consumption of hemicelluloses sugars during the pre-fermentation hindered the application of this method. Recently, the concept of bioabatement was introduced. Some microbes isolated from a screen of soil microorganisms were able to metabolize furan derivatives in a defined mineral medium and thus being chosen as candidates for biological abatement of biomass hydrolysate (López et al. 2004). *Coniochaeta ligniaria* NRRL30616, an ascomycete fungus, was identified because of its ability to metabolite furan derivatives in biomass hydrolysate (López et al. 2004). Subsequently, Nichols et al. used this fungus to successfully metabolize a wide range of inhibitors in a dilute-acid hydrolysate of corn stover, including furan derivatives, aromatic and aliphatic acids, aldehydes, and phenolic compounds (Nichols et al. 2008). The problems associated with this method were long treatment time and consumption of fermentable sugars.

The comparison of different detoxification approaches for biofuel production is presented in Table 5. Alkaline and ion-exchange are the most effective detoxification methods in terms of removal of potent inhibitors (aldehyde and phenols) and improvement of fermentation. However, given the considerable amount of sugar loss, it is not cost-effective to use ion exchange for industrial application. As a result, alkaline treatment is currently the mostly used approach for detoxifying biomass hydrolysate. It is therefore necessary to understand the chemical mechanism of alkaline detoxification in order to optimize the detoxification conditions, develop inhibitor-tolerant microbial strains and cost-effective detoxification methods. Moreover, based on the results obtained from different detoxification approaches, we could predict the inhibitors that contribute to the main inhibition are non-volatile, present at low concentration, containing both reduction and oxidization potential, and not stable in alkaline conditions.

Table 5: The effect of different detoxification on sugars, inhibitors and improvement of fermentability for biofuel production

	Detoxification	Sugars loss	Removal of inhibitors	Fermentation improvement	Reference
Physical	Steam stripping / Evaporation	Sugar degradation at low pH	Volatile inhibitors	Partially	(Larsson et al. 1999b; Leonard and Hajny 1945)
	Solvent extraction	No	Furans, aromatic compounds	Consumption of 80% sugar	(Clark and Mackie 1984)
Chemical	Alkaline	~ 5% (Isomerization)	Aldehydes, phenols	Equal to control	(Larsson et al. 1999b)
	Reducing agent	Small	Reducible compounds	Partially	(Leonard and Hajny 1945)
	Anion exchange resin	Over 20%	Acids, furan and aromatic compounds	Equal to control	(Nilvebrant et al. 2001)
	Wood/activated charcoal	Part	Acids, furan and aromatic compounds	Partially	(Miyafuji et al. 2003)
	Chemical extraction	No	Acids, furan and aromatic compounds	82.3% of theoretical yield	(Zhu et al. 2011)

Biological	*Trichoderma Reesei*	Over 20%	Furan and aromatic compounds	Partially	(Palmqvist et al. 1997)
	Bioabatement	Part	Some acids, furan and aromatic compounds	Partially	(Nichols et al. 2008)
	Laccase	No	Aromatic compounds	Largely	(Jönsson et al. 1998)

1.5 Research objectives

Over the past few decades, a great deal of research effort has been performed to identify the fermentation inhibitors in biomass hydrolysates and develop detoxification approaches to eliminate their inhibition. Although a wide range of potential inhibitors have been identified and evaluated, it is still unclear which compounds have the most pronounced inhibition on microbial fermentation because of the large number of the degradation compounds and their low concentration. Without the right inhibitor targets, development of cost-effective detoxification approaches for biomass hydrolysates and elucidation of their detoxification mechanism are difficult although physical, chemical and biological methods have been attempted. Consequently, identification of the potent inhibitors, understanding of their role in inhibition, and development of cost-effective detoxification have become major roadblocks to achieving an industrially feasible bioconversion process for lignocellulosic biomass and need to be addressed with urgency.

Linking antimicrobial activities of the potential inhibitors to their structural features could be an important clue to help overcome the above obstacles. Structural properties are the fundamental basis for chemical reactivity of compounds, which in turn governs their inhibitory actions towards biological molecules. As noted earlier, most of the identified potential inhibitors are carbonyl compounds, which are electrophilic. Due to the high electronegativity of oxygen

relative to carbon, the carbon-oxygen double bond is strongly polarized, creating a partial positive charge on the carbonyl carbon atom. The electron-poor (electrophilic) carbonyl carbon can form covalent bonds with nucleophilic targets in the microorganisms, such as proteins/enzymes, nucleic acids, or related biological molecules, leading to inhibition of important protein functions and DNA duplication, or even loss of cell activity. Among the carbonyls, aldehydes and ketones are commonly proposed as stronger inhibitors than their corresponding acids. Many fermenting microorganisms convert aldehydes/ketones into their corresponding less toxic alcohols and acids as in situ detoxification strategies (Larsson et al. 2000; Palmqvist et al. 1999). In addition, although furan derivatives are present in high concentration in the biomass hydrolysates, their inhibition is found to be much lower compared to aromatic aldehydes from lignin and extractives (Delgenes et al. 1996; Zaldivar et al. 1999). As a result, we hypothesize that aromatic aldehydes might be a group of compounds that contribute to the main fermentation inhibition in biomass hydrolysates.

The primary objective of this research is to investigate any possible mechanism of alkaline detoxification using appropriate model aromatic aldehydes. Alkaline detoxification is the most widely used approach to remove the inhibition from biomass hydrolysates for biofuel production, but its detoxification mechanism is not well understood. Elucidation of the detoxification mechanism is indispensable in order to optimize the detoxification conditions, enhance inhibitor-tolerant microbial strains, and develop efficient detoxification methods. Previously, model inhibitors such as furfural, HMF, acetic acid and syringaldehyde have been evaluated in model fermentation with regard to their effects on cell-growth and fermentation, yet these inhibitors did not show the same level of inhibition on yeast or bacterial fermentation compared to biomass hydrolysate, and in several cases they even increased the biofuel yields (He

et al. 2009; Larsson et al. 2000; Palmqvist et al. 1999; Taherzadeh et al. 1997). As a result, new model compounds with inhibition comparable to that in the biomass hydrolysates need to be used. Based on our hypothesis, potent inhibitors having aromatic and aldehyde functional groups could be the right targets to study the chemical mechanism of alkaline detoxification.

Giving that the carbonyls are electrophilic compounds, inactivation of the carbonyl groups might be a key for removing their inhibition. A combination of literature study and experimental experience leads us to the idea that using amino acids could be a cost-effective detoxification approach to remove carbonyl inhibition from biomass hydrolysates. Amino acids with nucleophilic properties in the biological cells often suffer electrophilic attacks from inhibitors or toxins (Chan et al. 2008a; Chan and O'Brien 2008). On the contrary, it is reasonable to presume free amino acids with reactive amino and/or thiol groups can also initiate nucleophilic reactions with the electrophilic groups of the carbonyl compounds, causing them to lose reactivity before contacting microbial cells. Therefore, proper utilization of free amino acids may prevent the carbonyl compounds from attacking microbial molecules prior to fermentation through covalent binding. In addition, the nutritional nature of amino acids renders them benign to both the fermenting microbes and environment.

The activity of aromatic aldehydes is often related to the types of the substituents (mostly hydroxyl or methoxyl group), the position of the substituents and the number of the substituents in the benzene ring (Larsson et al. 2000). Using the structural features of the compounds might help to investigate and predict their inhibitory activities on fermentation. Quantitative structure-activity relationships (QSARs) aim at linking the biological activity of inhibitors/toxins to their structural features. The structural properties of chemicals are often characterized by their physicochemical descriptors, such as hydrophobicity (Log *P*), dipole moment (μ), energy of the

lowest unoccupied molecular orbital (E_{LUMO}) and energy of the highest occupied molecular orbital (E_{HOMO}). The relationships between these molecular descriptors of the chemicals and their biological activities can help understand the inhibition mechanism of these compounds and predict which portion carries major inhibition. QSAR has been found to be a useful tool in studying drug potency and assessing environmental toxicity contamination (Chan et al. 2008a). It might also help to discover the unknown fermentation inhibitors in biomass hydrolysates.

With the goal of improving the understanding of inhibition of the aromatic aldehydes in lignocellulosic biomass and their detoxification, this research dissertation will be focused on the following objectives:

1. To investigate potential mechanism of alkaline detoxification in model fermentation using aromatic aldehyde inhibitors;
2. To develop a cost-effective detoxification approach specifically removing the carbonyl inhibitors from biomass hydrolysate for biofuel production;
3. To study the inhibitory activities of thirteen model aromatic aldehydes on alcoholic fermentation and their quantitative structure-activity relationships (QSARs).
4. To develop a simple analytical procedure for improvement of HPLC separation of lignocellulose-derived sugar degradation compounds.

Chapter 2: Improvement in HPLC separation of acetic acid and levulinic acid in the profiling of biomass hydrolysate

2.1 Background

The objective of this chapter is to develop a simple analytical procedure for improvement of HPLC separation of lignocellulose-derived sugar degradation compounds.

High performance liquid chromatography is a routine method in the analysis of degradation products in the hydrolysate or the hydrolysis liquor of lignocellulosic biomass (Chen et al. 2006). Cation-exchange column chromatography with a metal counter ion has been used for the quantitative analysis of carbohydrates with water as the eluent (Ruiz and Ehrman 1996). A cation exchange column with exchangeable hydrogen ion (H+) has been employed in quantification of organic acids and furan derivatives with acidic water as the mobile phase. In particular, the Aminex cation-exchange HPX-87H column has been developed for determining organic acids, furan derivatives and alcohols (Blake et al. 1987; Pecina et al. 1984; Scarlata and Hyman 2010). Although this column has been broadly used in biomass analysis, potential issues with co-elution of target analytes have been reported by several groups (Palm and Zacchi 2003; Scarlata and Hyman 2010). Scarlata and Hyman suggested that Aminex HPX-87H column was not suitable for biomass sugar analysis because xylose, galactose and fructose are co-eluted together, and glucose and mannose are co-eluted together (Scarlata and Hyman 2010). The co-elution of acetic acid and levulinic acid has not been addressed. Palm and Zacchi indicated the value of acetic acid in biomass hydrolysate was not reliable at high severity due to continuous degradation of furfural and HMF to levulinic acid (Palm and Zacchi 2003).

As one of the principle components in hemicelluloses, acetic acid has a noticeable amount in the acid hydrolysate of lignocellulosic biomass (Chen et al. 2006). Characterization of this organic acid becomes even more important during fermentation due to its inhibition to the microorganisms. Levulinic acid, one of the normal degradation compounds of HMF, is also present in the acid hydrolysate at low concentration. The separation of these two acids could be easily ignored due to their co-elution in the acid hydrolysate. Solutions to improve the separation of these organic acids included the use of a dual column system (Blake et al. 1987), employment of a capillary electrophoresis to separate and quantify the concentration of acetic acid and levulinic acid from softwood hydrolysate (Larsson et al. 1999a). However, no method has been developed to resolve co-eluting acetic acid and levulinic acid by optimizing the HPLC chromatographic conditions. In the present study, the improvement of HPLC separation of acetic acid and levulinic acid in acid hydrolysate on the Aminex HPX-87H column was investigated by varying column temperature, flow rate, and concentration of sulfuric acid in the mobile phase. Resolution and capacity factors were used as indicators for measuring the effectiveness of separation using pure standard compounds as well as real biomass hydrolysate from woody biomass.

2.2 Materials and methods

2.2.1 Reagents and biomass hydrolysate samples

Acetic acid was purchased from Fisher Scientific. Levulinic acid was purchased from Alfa Aesar. HMF was purchased from Acros Organics. Furfural was purchased from Aldrich. Freshly chopped hardwood sweetgum (*Liquidambar styraciflua*) from Forest Products Laboratory at Auburn University was used as the starting material in this study. Air dried

sweetgum chips (2.0 × 2.0cm) were milled by a centrifugal mill (Retsch ZM1) with a 0.75 mm screen and then passed through a 60 mesh sieve. The milled sweetgum was treated with a two-step acid hydrolysis (72% sulfuric acid for 2 h and 4% sulfuric acid for 1 h at 121°C). The resulting aliquot was defined as the hydrolysis liquor and was subsequently analyzed by HPLC for the degradation compounds.

2.2.2 High performance liquid chromatography

A HPLC system (Shimadzu) with a refractive index detector (RID-10A) was used in this study. The chromatographic separation uses a strong cation-exchange column (Aminex HPX-87H, 300×7.8 mm). The column temperature, flow rate and the concentration of H_2SO_4 mobile phase were tested for separation of acetic acid, levulinic acid, HMF and furfural.

The temperature conditioning of the HPX-87H column was tested at 60, 50, 40 and 30°C. The flow rate was tested at 0.6, 0.7 and 0.8 mL/min. Concentration of sulfuric acid in mobile phase was tested at 1.0, 2.5 and 5.0 mM. Optimum HPLC conditions were investigated using reference standards and then verified using the hydrolysis liquor from sweetgum. A 70min isocratic run was used for all samples. The resolution was calculated by measuring the peak width at half-height (Alasandro 1996; Calabrò et al. 2006)

$$R_s = \frac{1.18 \times (t_{levulinic} - t_{acetic})}{W_{1/2,\ levulinic} + W_{1/2,\ acetic}}$$

Where R_s is the resolution, $t_{levulinic}$ is the retention time of levulinic acid, t_{acetic} is the retention time of acetic acid, $W_{1/2,\ levulinic}$ is the peak width at half-height of levulinic acid and $W_{1/2,\ acetic}$ is the peak width at half-height of acetic acid.

The capacity factor was calculated based on the experimented retention times and void times

$$k = \frac{(t_R - t_M)}{t_M}$$

Where k is the capacity factor, t_R is the retention time of an analyte peak and t_M is the retention time of the mobile phase.

2.2.3 Linearity and recovery

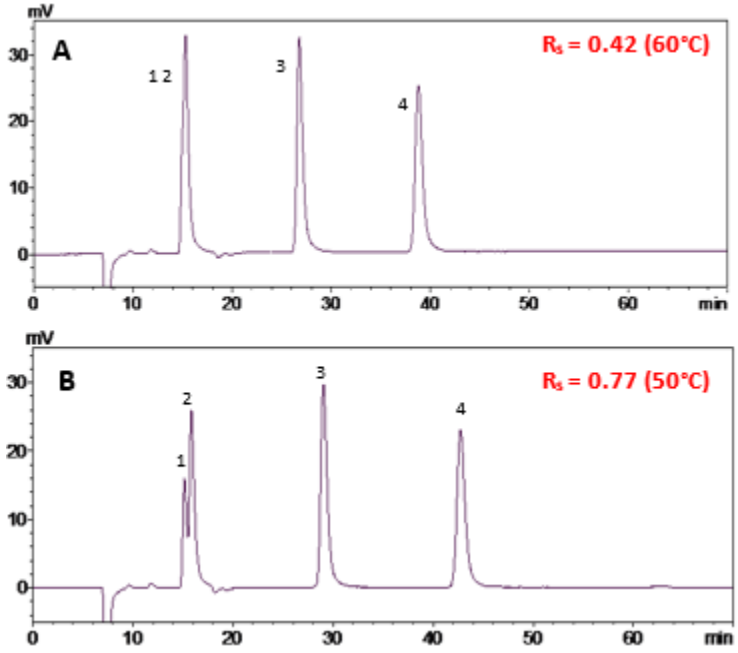
The linearity of calibration curve for acetic acid, levulinic acid, HMF and furfural was measured in the range of 0.02-1.0 mg/mL in the standard samples. The linear regression of the calibration curve was analyzed using excel spreadsheet software (Microsoft Excel 2007) and the results were compared their fitness by correlation coefficients (r^2). Recovery of acetic acid, levulinic acid, HMF and furfural was evaluated by addition of internal standard compounds at two concentrations to biomass hydrolysate prior to the two-stage hydrolysis of sweetgum. Spiked hydrolysate samples were prepared in triplicate, each in duplicate injection, and the results were averaged.

2.3 Results and discussion

2.3.1 Dropping column temperature leads to significant improvement in separation

To improve the separation of acetic acid and levulinic acid in the biomass hydrolysate, we first examined the change of column temperature on the resolution of acetic acid and levulinic acid with an Aminex HPX-87H column with 5 mM H_2SO_4 at 0.7 mL/min while other conditions remained fixed. The column temperature was dropped from 60°C to 50°C, 40°C, and 30°C (Figure 3). The resolution for peaks of acetic acid and levulinic acid under different temperature was calculated based on their retention time and peak width at half height. The

decrease of column temperature had a dramatic impact on the resolution of acetic acid and levulinic acid. Under the typical column temperature at 60°C, the separation of furfural and HMF was good, but the separation of acetic acid and levulinic acid was unsatisfactory and they often appeared as one combined peak in the chromatograph (Figure 3A). The resolution of these two acids was only 0.42. When the column temperature decreased to 50°C, the acetic acid and levulinic acid were partially separated with a resolution at 0.77 (Figure 3B). At 40°C column temperature the resolution was improved to 1.24 where peaks barely overlapped with each other (Figure 3C). Finally, when the column temperature reduced to 30°C, the two peaks from acetic acid and levulinic acid were baseline separated and their resolution reached 1.86 (Figure 3D).



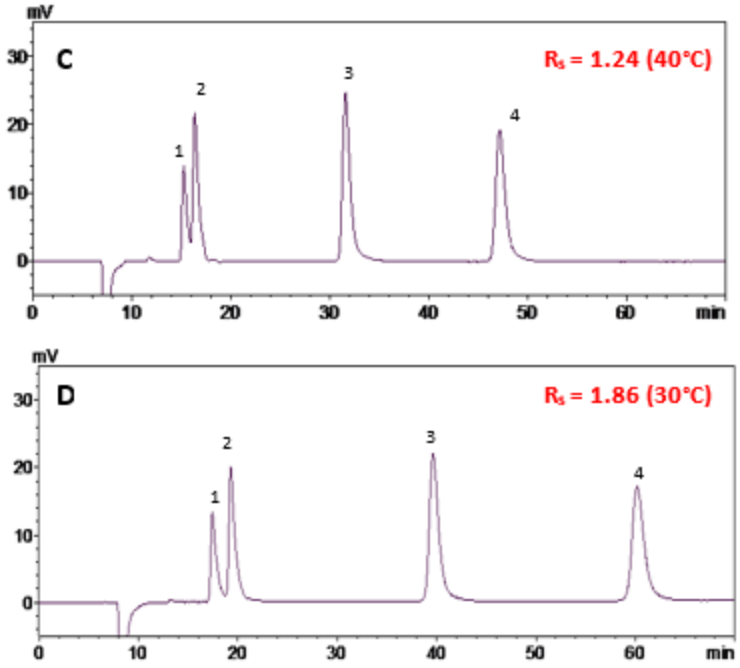


Figure 3: Effect of temperature on resolution of acetic acid and levulinic acid (peak 1, acetic acid; peak 2, levulinic acid; peak 3, HMF; peak 4, furfural). Column, Aminex HPX-87H; flow rate, 0.7m/min; mobile phase, 5.0 mM H_2SO_4

The temperature dependency of the capacity factors (k) was also determined (Figure 4). As the column temperature decreased from 60 to 30°C, the capacity factors of furfural and HMF increased considerably from 4.59 to 6.57 and 2.80 to 3.99, respectively. The capacity factor of levulinic acid also increased slightly from 1.20 to 1.44, the capacity factor of acetic acid at 1.20 did not change. Again, the capacity factor difference between levulinic acid and acetic acid increased due to the decreased column temperature.

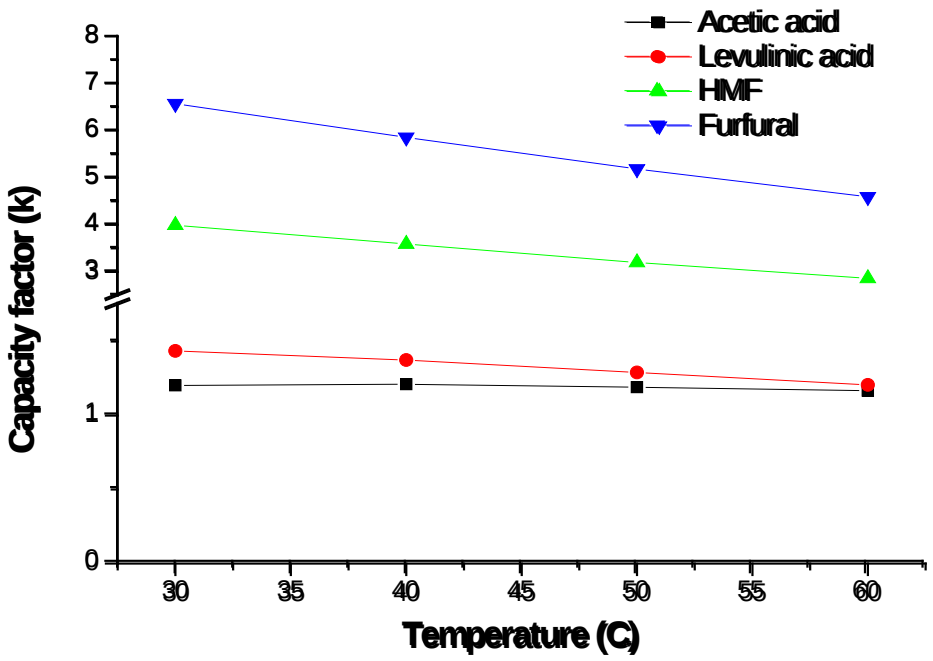


Figure 4: Effect of column temperature on the capacity factors (k) for acetic acid, levulinic acid, HMF and furfural. Column, Aminex HPX-87H, flow rate, 0.7m/min; mobile phase, 5.0 mM H_2SO_4

In the HPLC analysis of biomass hydrolysate with HPX-87H column, the acetic acid and levulinic acid were often eluted at the same time, which failed the quantization of these acids (Palm and Zacchi 2003; Scarlata and Hyman 2010). In the study, decreasing the column temperature resulted in a significant improvement in chromatography that resolved acetic acid and levulinic acid. In a typical condition, both acids fall into a narrow capacity factor range, expanding the difference in capacity factor between acetic acid and levulinic would indicate a good separation of two acids. As the temperature decreased, the capacity factor of levulinic acid increased and that of acetic acid stayed the same. Consequently, the capacity factor curve shows

separation between these two acids. This basically is consistent with previous finding that the capacity factor of levulinic acid was temperature dependent (Pecina et al. 1984).

It is believed that in order to separate two compounds their resolution (R_s) needs to be larger than 1.5 for baseline separation (Péter et al. 2005). According to the separation performance at different temperature, it is theoretically favored to utilize 30°C to separate acetic acid and levulinic acid (R_s >1.5). However, we observed the deterioration of column performance at this temperature after being used for a couple of weeks. The low temperature could shrink the polymer backbone in the resin and create higher backpressure in the column resulting in voids. Column deterioration would render inconsistency in retention time and resolution, or band tailing of peaks. To avoid potential shrinking of resins with HPX-87H column, we suggested a column temperature at 45°C to be used for separating acetic acid and levulinic acid (R_s = 0.89) and the resolution reached a practical sufficient limit (R_s>0.8, partial resolution) (Péter et al. 2005). Moreover, this temperature condition would reduce the analysis time for hydrolysate sample, especially for furan derivatives.

In addition to improvement on resolution, temperature also had an appreciable influence on the retention factor (capacity factor) of acetic acid, levulinic acid, HMF and furfural. The capacity factors of the four compounds decreased simultaneously as temperature increased. It was shown that acetic acid and levulinic acid had strong overlapping capacity factors at 60°C (Figure 4), which also results in an incomplete separation. The capacity factors of furan derivatives were strongly temperature dependent. In our observation, the capacity factors of HMF and furfural increased dramatically as temperature decreased. In this case, longer running time and more mobile solution would be needed for a complete separation of furan derivatives. The retention times of four peaks corresponding to acetic acid, levulinic acid, HMF and furfural

were increased gradually when column temperature was dropped (Figure 4). To compensate for increased retention time, the flow rate was increased which offset the delay of major compounds in the HPLC analysis of biomass hydrolysate.

2.3.2 Effect of flow rate and sulfuric acid concentration in mobile phase

To investigate the effect of flow rate on the resolution and capacity factor, we increased the flow rate from 0.6 mL/min, to 0.7 and 0.8 mL/min while kept the column temperature at 30°C with 5 mM H_2SO_4 mobile phase constant. As flow rate increased, the resolution of both acetic acid and levulinic acid did not change as much, they were 1.81, 1.86 and 1.63, corresponding to three flow rates tested. The capacity factors were similar to resolution in response to flow rate change (Figure 5). Increase in flow rate resulted in a decreased retention time for acetic acid, levulinic acid, HMF and furfural (Table 6). The retention time of the last peak (furfural) in the chromatography was reduced from 61.6 to 46.4 min. With this flow rate, the total HPLC running time were reduced usefully. There was no significant difference in resolution among three flow rates investigated. However, increased flow rate offered an advantage of considerably shortening the running time for acetic acid, levulinic acid, HMF and furfural. A running time of over 60 min was needed to elute the four compounds when the flow rate was 0.6 mL/min at 30°C while the running time can be reduced to within 50 min if flow rate increased to 0.8 mL/min at 30°C. Different flow rates had, however, little influence on capacity factors of the four compounds. When the flow rate increased, the retention time of the four compounds decreased. Meanwhile, the dead time of mobile phase was inversely proportional to the flow rate. The increase of flow rate reduced the column dead time. As a result, the capacity factor (k) did not change much as the flow rate changed.

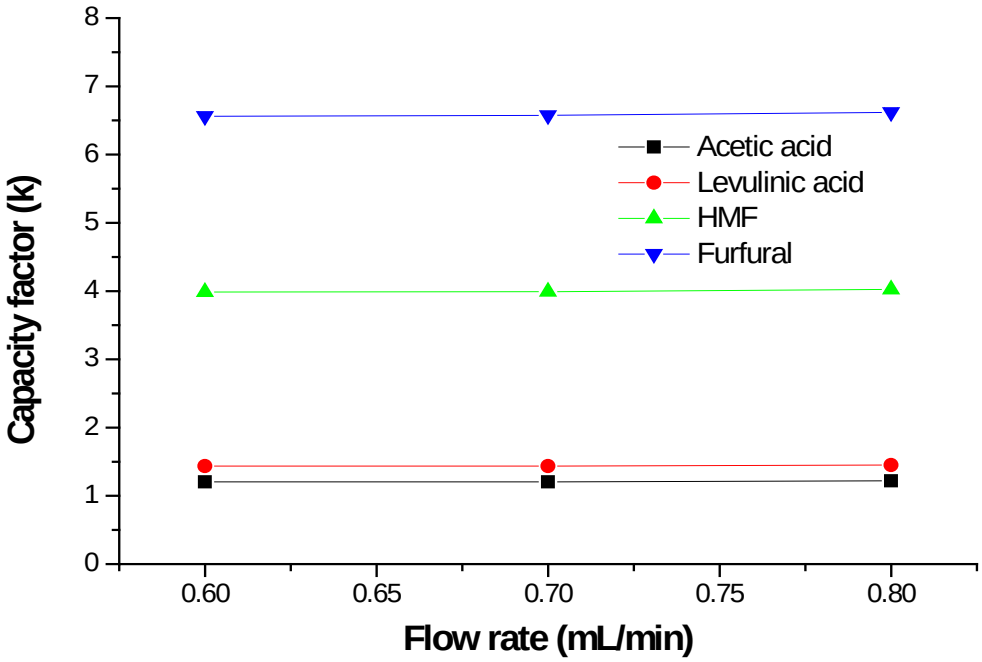


Figure 5: Effect of flow rate on the capacity factors of acetic acid, levulinic acid, HMF and furfural. Column, Aminex HPX-87H; column temperature 30°C; mobile phase, 5.0 mM H_2SO_4

Table 6: Effect of flow rate on retention times and capacity factors of acetic acid, levulinic acid, HMF and furfural

Flow rate (ml/min)	Analyte	Retention time (min)	Capacity factor
0.6	Acetic acid	17.9	1.2
	Levulinic acid	19.8	1.4
	HMF	40.6	4.0
	Furfural	61.5	6.6
0.7	Acetic acid	17.4	1.2
	Levulinic acid	19.3	1.4
	HMF	39.6	4.0
	Furfural	60.2	6.6
0.8	Acetic acid	13.5	1.2
	Levulinic acid	14.9	1.5
	HMF	30.6	4.0
	Furfural	46.4	6.6

Column, Aminex HPX-87H; column temperature, 30°C; mobile phase, 5.0 mM H_2SO_4.

Three concentration of sulfuric acid in mobile phase was tested on the separation of acetic acid and levulinic acid. The increase in sulfuric acid concentration from 1, 2.5 and 5 mM resulted in decrease of pH value to 2.98, 2.61 and 2.33, respectively in the mobile phase. The resolution of acetic acid and levulinic acid changed little to variation of sulfuric acid concentration (Table 7). Also, the capacity factors for acetic acid, levulinic acid, HMF and furfural did not change in tested sulfuric acid concentration (Figure 6). The effect of sulfuric acid concentration on the resolution of acetic acid and levulinic acid was negligible when compared to the effect of column temperature on resolution.

Table 7: Effect of sulfuric acid in mobile phase on resolution of acetic acid and levulinic acid

Concentration of sulfuric acid (mM)	pH of mobile phase	Resolution of acetic acid and levulinic acid
1.0	2.3	1.87±0.03
2.5	2.6	1.73±0.07
5.0	3.0	1.82±0.14

Column, Aminex HPX-87H; column temperature, 30°C; flow rate, 0.7 ml/min.

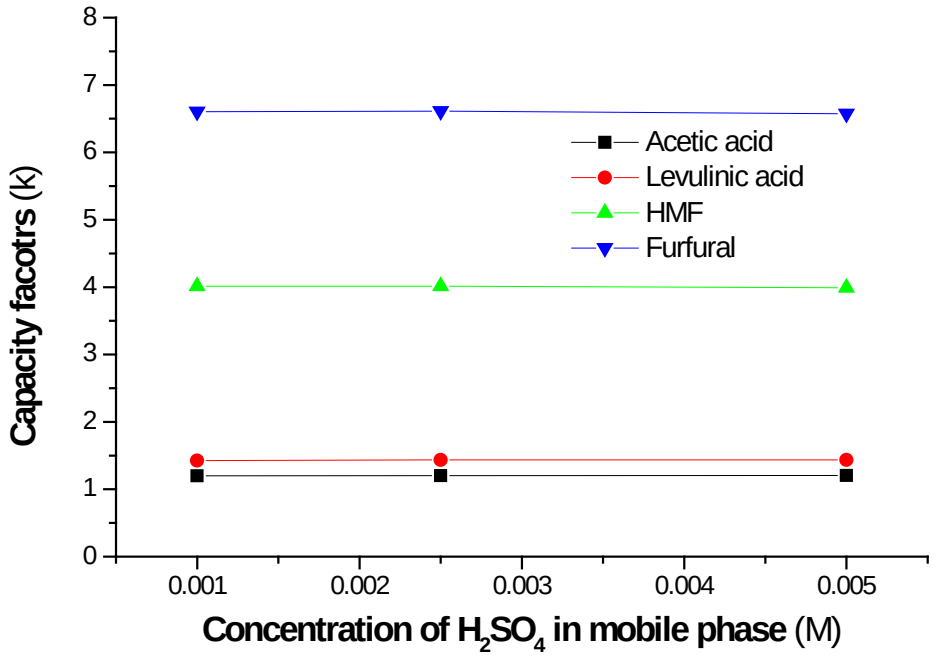


Figure 6: Effect of sulfuric acid concentration on capacity factors of acetic acid, levulinic acid, HMF and furfural. Column, Aminex HPX-87H; column temperature 30°C; flow rate 0.7 ml/min, 30°C

2.3.3 The separation of acetic acid, levulinic acid, HMF and furfural from biomass hydrolysate

As mentioned above, changes of flow rate and sulfuric acid concentration showed negligible effect on the separation of acetic acid and levulinic acid, but the temperature did. For biomass hydrolysate from sweetgum, we analyzed quantitatively the acetic acid and levulinic

acid concentration using Aminex HPX-87H column at 60 and 45°C. The resolution of two compounds was profoundly increased from 0.26 to 0.86 (Figure 7); once combined peaks at 60°C of acetic acid and levulinic acid were separated at 45°C. The optimized HPLC condition (0.6 mL/min of 5 mM H_2SO_4 at 45°C) was used for the analysis of hydrolysis liquor (Figure 7), showed the chromatograms of acetic acid, levulinic acid, HMF and furfural from sweetgum hydrolysis liquor. The chromatographic separations at 60 and 45°C were compared (Figures 7A and 7B); at 60°C, the resolution for acetic acid and levulinic acid was below 0.5 and failed to be resolved from each other. When the column temperature decreased to 45°C, the resolution of two acids was increased to 0.86, which was partially separated and could be quantified. This result demonstrated that HPX-87H column could be used for the quantification of levulinic acid and acetic acid in biomass hydrolysate with the column temperature at 45°C. The lifetime of a HPX-87H column typically depends on the amount of usage, and salt in the mobile phase. Neutralization of acid-pretreated biomass hydrolysate was often suggested before fermentation and HPLC analysis (Ruiz and Ehrman 1996), inevitably the HPX-87H column would be contaminated and gradually lose its resolution, especially when acetic acid and levulinic acid are analyzed. This makes a lower column temperature condition (45°C) more crucial in separating the organic acids in biomass hydrolysate. The performance of this column is consistent for at least 6 months at 45°C. The linearity and recovery tests have indicated that the optimized conditions determined in this study was satisfactory for biomass hydrolysate samples.

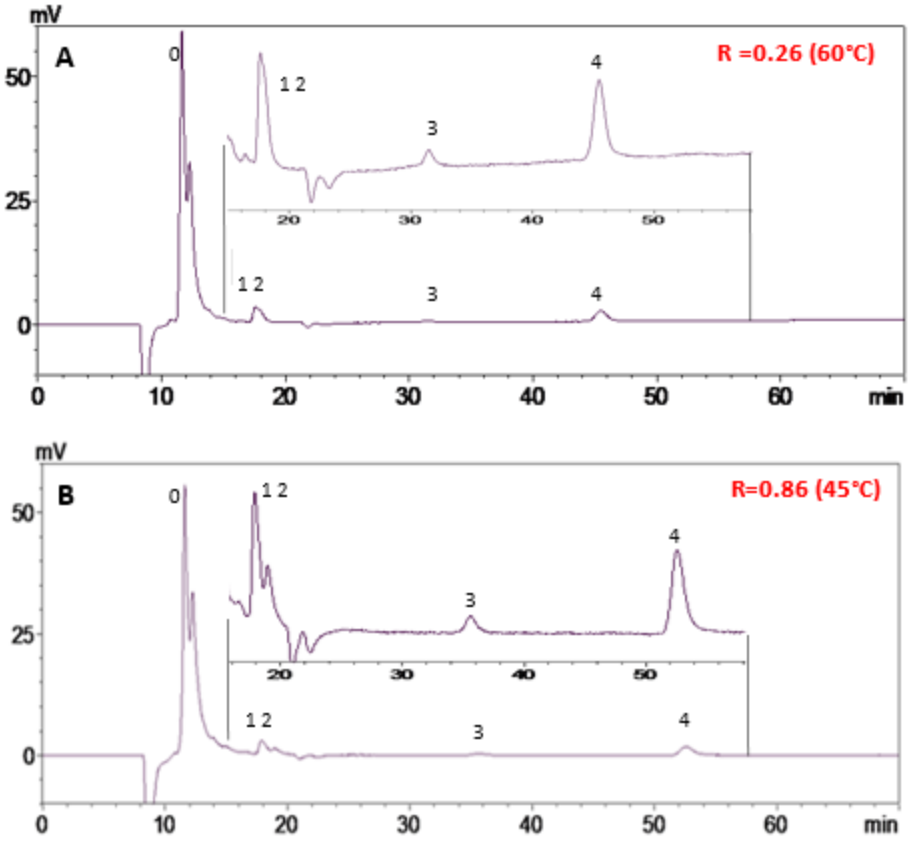


Figure 7: Acetic acid and levulinic acid separation in sweetgum hydrolysis liquor by Aminex HPX-87H column at the optimized conditions: column temperature, 45°C and 60°C; flow rate, 0.6 ml/min; mobile phase, 5.0 mM H_2SO_4. (peak 0, co-elution of sugars, peak 1, acetic acid; peak 2, levulinic acid; peak 3, HMF; peak 4, furfural)

2.3.4 Linearity and recovery

A five-point linear calibration curves was constructed at 45°C over the range of 0.02–1.0 mg/mL for acetic acid, levulinic acid, HMF and furfural (Table 8). The high degree of correlation coefficients ($r^2 > 0.99$) suggests the suitability of this HPLC method for acetic acid, levulinic acid, HMF and furfural analysis. Known amount of acetic acid, levulinic acid, HMF and furfural were spiked into the hydrolysis liquor of sweetgum at two levels as the internal standards for calculation of recovery. The results at two levels revealed a good recovery ranged from 101.6% to 108.8%, which is due to the intrinsic acids contributed to the recovery.

Table 8: Linearity and recovery tests of acetic acid, levulinic acid, HMF and furfural in biomass hydrolysate

Linearity and recovery		Acetic acid	Levulinic acid	HMF	Furfural
linearity	Range	0.02-1.0	0.02-1.0	0.02-1.0	0.02-1.0
	Regression	y = 9E-07x-0.0065	y = 4E-07x+0.003	y = 3E-07x-0.0005	y = 3E-07x-0.0014
	r^2	1	1	1	1
Recovery	Level 1	101.6±0.3	102.4±0.4	105.1±0.2	102.4±0.9
	Level 2	102.6±0.4	103.0±0.1	108.8±0.3	107.3±0.5

HPLC conditions: flow rate, 0.6ml/min; column temperature, 45°C; and mobile phase, 5.0 mM H_2SO_4

x=concentration (mg/mL), y=peak area.

Level 1: biomass hydrolysate was mixed with 10mg/ml of acetic acid, levulinic acid, HMF and furfural at a 19:1 (w/w).

Level 2: Biomass hydrolysate was mixed with 10 mg/ml of acetic acid, levulinic acid, HMF and furfural at a 9:1 (w/w).

2.4 Conclusion

A simple and effective method was developed to improve the separation of acetic acid and levulinic acid in biomass hydrolysate. Decreasing the column temperature from 60 to 45°C on the HPX-87H column should be used for the HPLC analysis of biomass degradation compounds. High column temperature (> 60°C) resulted in low resolution for acetic acid and levulinic acid. Low column temperature (< 40°C) improved the resolution of both acids considerably but in the expenses of tailing and longer retention time.

Chapter 3: Reducing sugars facilitated carbonyl condensation in detoxification of aromatic aldehyde model compounds for bioethanol fermentation

3.1 Background

The objective of this chapter is to investigate possible mechanisms of alkaline detoxification in a pure fermentation system using a carbonyl model inhibitor *o*-phthaldehyde.

Remarkably little is known about the mechanism of alkaline detoxification for biomass hydrolysate, despite it has been widely used in detoxifying the acid hydrolysate for biofuel and chemical production (Agblevor et al. 2004; Saha et al. 2005). Research has been concentrated largely on the chemical identification of potential inhibitors using HPLC, GC-MS, LC/MS and NMR (Agblevor et al. 2004; Ando et al. 1986; Chen et al. 2006). However, due to the large amount of degradation compounds from biomass and their extreme low concentration in the hydrolysate, there is still a lack of knowledge of which inhibitors have the most pronounced effects on yeast fermentation (Klinke et al. 2004; Palmqvist and Hahn-Hägerdal 2000). Consequently, the elucidation of overliming detoxification has not been achieved successfully although several model compounds have been evaluated. Identification of potent fermentation inhibitors and their detoxification mechanism is therefore widely recognized as one of the roadblocks for developing any effective detoxification approach and improving stress-tolerance yeast and bacterial ethanologens. Our objective of this research chapter is to elucidate the alkaline detoxification mechanism using special model compounds.

Based on previous study, Most of the identified inhibitors are carbonyl compounds. Moreover, aromatic compounds have been proposed as major fermentation inhibitors in hydrolysate (Palmqvist and Hahn-Hägerdal 2000). Most of the unknown aromatic compounds

were degradation compounds from lignin, or sometimes from extractives and sugars. Therefore, the compounds having aromatic and strong electrophilic carbonyl groups should be the right targets. As a result, we select ortho-phthaldehyde (OPA) as the appropriate carbonyl model inhibitor to study the alkaline detoxification mechanism. In OPA, a second aldehyde group is introduced at the ortho position of the first aldehyde group, resulting in a strong resonance interaction which, therefore, renders the first CHO group an increase in reactivity toward nucleophiles.

Here we report a new approach employing model carbonyl compounds to investigate the alkaline detoxification mechanism. The described work serves as an element in an overall scheme of unraveling the fermentation inhibitors and their detoxification mechanism. We first evaluated the effects of OPA, vanillin, furfural and HMF on the growth and fermentation of *S. cerevisiae*. Secondly, we assessed the influence of OPA concentration and inoculation size on yeast fermentation. Thirdly, we distinguished the distinct roles of reducing sugars and non-reducing sugars in detoxification of OPA. With the assistance of LC/MS, we finally identified the reaction products during the detoxification and proposed the new mechanism for alkaline detoxification.

3.2 Materials and methods

3.2.1 Microbial strain and medium

Baker's yeast (Fleischmann's), *S. cerevisiae*, was used in this study. The Yeast was activated in YDP liquid medium containing 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract at 30°C with 150rpm for 12-15 h and maintained at 4°C on YDP agar plate containing 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract and 15 g/L agar. Isolated colonies were

transferred and cultured in YDP liquid medium overnight and washed with sterile water for fermentation and growth inoculation. The yeast concentration was determined by UV-vis spectrophotometer at 600 nm based on a previous standard. Inoculum of 2.0 g/L was used for fermentation experiments.

3.2.2 Fermentation and growth curve

All fermentation broths were prepared in 125 mL serum bottles with 50 mL of 2% (w/w) sugar source (glucose, fructose or sucrose) using nanopure water. OPA, vanillin, furfural and HMF were added after the sugar solutions were prepared. Sugar solutions with no inhibitor were used as controls. All fermentation broths were sterilized using 0.2 µm sterile filter before inoculation. Fermentation was conducted at 30°C with 150 rpm for 48 h or 72 h. Samples were taken periodically for analysis. For testing the inhibitory potential of OPA, a series of concentration of OPA was added in the fermentation broths.

The growth curve was examined by measuring OD at 600 nm of 120 mL cultures grown at 30°C with 150 rpm in 250 mL flasks. M9 minimal medium containing 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.02% $MgSO_4$, 0.001% $CaCl_2$ and 2% (w/w) glucose was used as the growth media. OPA and vanillin were added before inoculation. A M9 medium was used as control. YDP solid medium was used for measurement of colony forming units (CFU). Colony forming units (CFU) were determined by the following equation:

CFUs (number/mL) = Number of colonies × dilution/Volume of culture on plate (mL)

3.2.3 Alkaline detoxification of OPA

Fermentation broths containing OPA and sugar sources were used to conduct alkaline

detoxification. The alkaline detoxification was held at 60°C in a temperature-controlled water bath for 2 h. pH was controlled at 10 by adding NaOH with vigorous vortex mixing. Reducing sugars including glucose and fructose and non-reducing sugar sucrose were used as sugar sources respectively to test their effects on alkaline treatment of OPA. Same amount of NaOH was added into each sugar source fermentation broth to avoid the effect of salt on yeast (Carvalheiro et al. 1999; Watson 1970). pH value of each broth was adjusted above 10. After 2 h of alkaline treatments, the solution was cooled to ambient temperature in an ice water bath and then adjusted pH to 6.0 with H_2SO_4. The treated broths were used immediately for analysis and fermentation without further storage. All the alkaline-treated broths were sterilized through 0.2 μm filters before fermentation.

3.2.4 HPLC and LC/MS analysis

The fermentation products including sugars and ethanol were analyzed by HPLC. The HPLC system (Shimadzu) was equipped with a strong cation-exchange column (Aminex HPX-87H, 300×7.8mm) and a refractive index detector (RID-10A). The conditions are 45 °C, 0.6 mL/min, and 5.0 mM H_2SO_4 as mobile phase. A 35 min isocratic run was used for all fermentation samples. OPA was analyzed with a C30 column (Waters Carotenoid 5μm, 4.6×250 mm) and Photo Diode Array (PDA) detector (SPD-M20A). The conditions are 30 °C, 0.6 mL/min and 45 min. Solvent was 100% CH_3CN. The alkaline detoxification products were analyzed by an Ultra Performance LC system (ACQUITY, Waters Corp.) coupled with a quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI) in negative ESI-MS operated by the Masslynx software (V4.1).

3.3 Results and discussion

3.3.1 Effects of OPA, vanillin, furfural and HMF on fermentation and growth of *S. cerevisiae*

The effect of OPA on fermentation of *S. cerevisiae* was firstly investigated and compared to vanillin, furfural and HMF in batch fermentation. We examined ethanol production of *S. cerevisiae* in the presence of 5.0 mM of OPA, vanillin, furfural or HMF respectively. Without adding any inhibitors, pure glucose (2%) fermentation to ethanol was used as a control. The results showed that OPA inhibited ethanol production completely during 48 h fermentation period at the concentration tested, while vanillin, furfural and HMF showed no inhibition on ethanol production compared to the control (Figure 8). This indicated that OPA had much higher toxicity on ethanol production than vanillin, furfural and HMF. During the biochemical conversion of lignocellulosic materials to ethanol, vanillin was identified as a lignin degradation compound which decreased ethanol productivity of microorganisms such as *S. cerevisiae* (Delgenes et al. 1996; Lee et al. 1999). Furfural and HMF were degradation compounds of lignocellulosic sugars that are also known for their inhibition on ethanol production and microorganism growth (Delgenes et al. 1996; Taherzadeh et al. 1999). Vanillin has also been recommended in food industry as an antimicrobial agent (Cerrutti and Alzamora 1996; Fitzgerald et al. 2003). A wide range of research has been conducted to test their inhibition on ethanol production and growth of *S.cerevisiae*. Lee et al. reported that vanillin, furfural and HMF had complete inhibition on ethanol fermentation of baker's yeast in the range of 23-110 mM with 0.57 g/L yeast inoculation (Lee et al. 1999). Delgenes et al. reported that ethanol production was 49% and 66% of the control at a culture time of 32 h when 39.6 mM of HMF and 13.1 mM of vanillin were added respectively (Delgenes et al. 1996). In real hydrolysates, however the concentration of these degradation products especially vanillin produced in lignocellulosic

hydrolysate was much lower than the amount used in the tested fermentation (Larsson et al. 1999b; Nigam 2001). This indicated that the inhibition in the lignocellulosic hydrolysate on fermentation microorganisms could not be fully represented by those model inhibitors. The fact that OPA as a dialdehyde could completely inhibit the ethanol production of *S. cerevisiae* at low concentration (5 mM or 0.67g/L) gives us a new perspective of the real type of inhibitors that exist in the lignocellulosic hydrolysate.

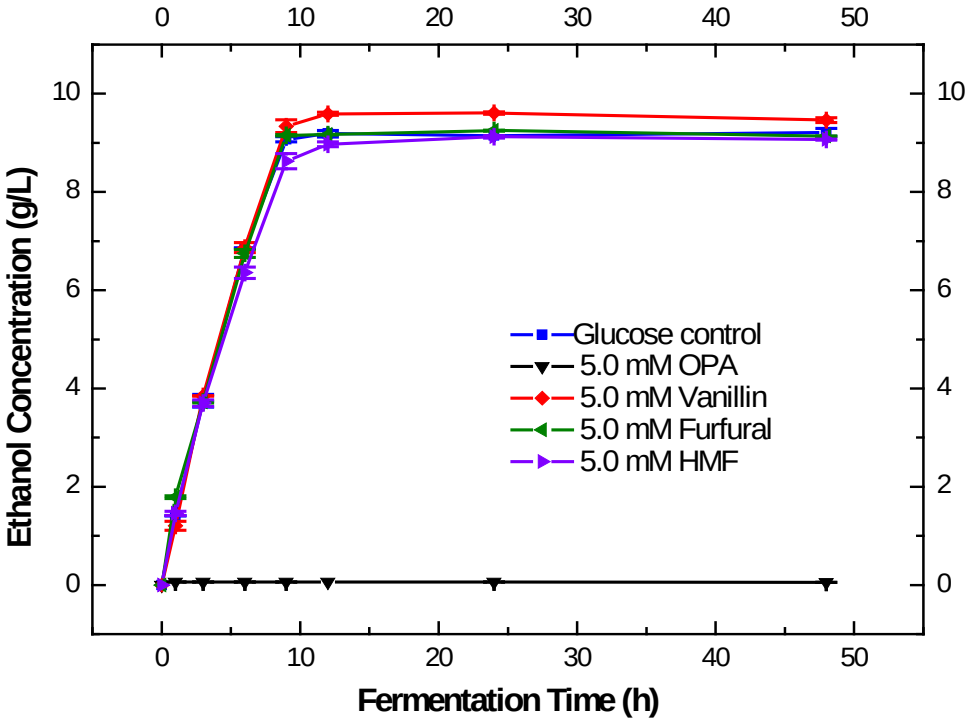


Figure 8: Effects of OPA, vanillin, furfural and HMF on fermentation of *S. cerevisiae* (5.0 mM of OPA, vanillin, furfural or HMF with 2% glucose at 30°C with 150 rpm)

The effects of OPA and vanillin on cell growth of *S. cerevisiae* were also investigated. Compared to the growth of the control, an addition of 5.0 mM OPA executed complete inhibition on cell growth, while vanillin reduced cell concentration by 10% of the control and did not affect

the lag phase (Figure 9). This indicated that the toxicity of OPA on yeast growth was also much higher than vanillin. Fitzgerald at al reported that an addition of 5.0 mM of vanillin increased the lag time by 53% and reduced the final cell density by 46% of the control (Fitzgerald et al. 2003). The difference of our results might attribute to the different yeast strain we used.

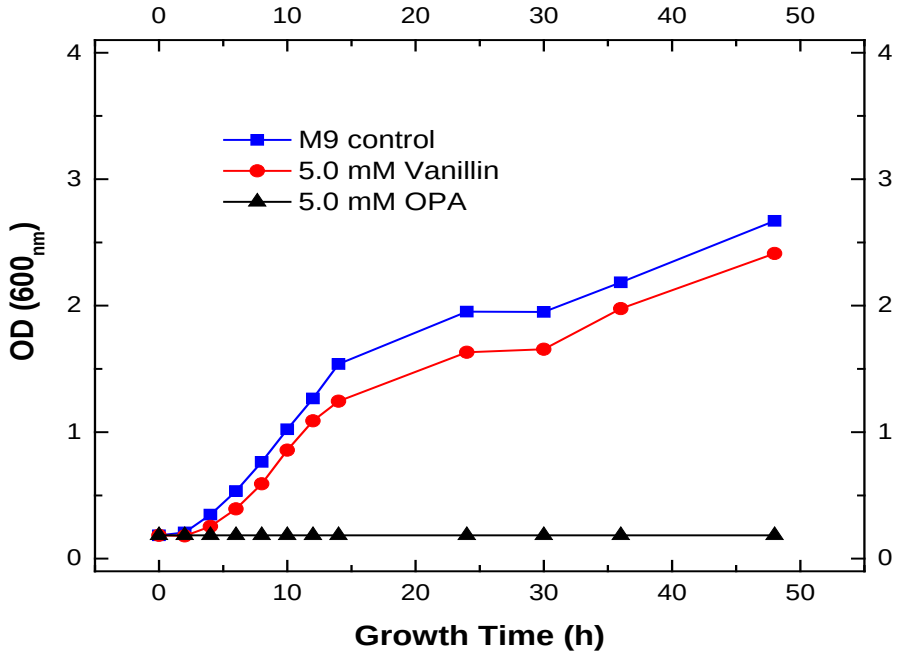


Figure 9: Effects of OPA and vanillin on growth of *S. cerevisiae* (5.0 mM of OPA and vanillin with 2% glucose, inoculation size 0.14 g/L)

Interestingly, we also found that the addition of 5.0 mM of vanillin increased the ethanol final yield by 3.8% of the control (Figure 8). However, the same level of vanillin inhibited the cell growth by 10% of the control (Figure 9). In our fermentation process, we had detected that the production of glycerol was reduced by 25% in the presence of 5.0 mM vanillin. These results indicated that the increase in ethanol production in the presence of 5.0 mM vanillin might result from the compromise of yeast biomass yield. More sugars have been converted to ethanol, rather than yeast biomass. Similar results have been reported previously when the tested inhibitors

increased the ethanol or butanol yields (Ezeji et al. 2007; He et al. 2009; Taherzadeh et al. 1997). The addition of acetic acid (3.3 g/L) was reported to increase the ethanol yield by 20% with *S. cerevisiae*, and decrease the yeast biomass and glycerol yields by 45% and 33% (Taherzadeh et al. 1997). Glycerol formation has been suggested to be essential for maintaining cytosolic redox balance and providing important intermediates (glycerophospholilids) for yeast biosynthesis (Albers et al. 1996; Rigoulet et al. 2004). Thus, the decrease of glycerol production probably resulted in low yeast biomass yield. Other inhibitors (such as furfural and HMF) in certain range of concentration have also been reported to increase the alcohol yields (Ezeji et al. 2007; Wahlbom and Hahn–Hägerdal 2002). In our case, we did not see the increase of ethanol yields with the addition of furfural and HMF. This probably is due to the very low concentration of furfural and HMF (5.0 mM) that we added, which could not interfere with the biosynthesis of yeast.

3.3.2 Effects of OPA concentration on fermentation inhibition and growth of *S. cerevisiae*

Effects of different concentration of OPA (between 0-1.0 mM) on fermentation inhibition of *S. cerevisiae* were further determined (Figure 10). The higher concentration of OPA (1.0 mM) resulted in complete inhibition of ethanol production within 48 h fermentation. Low concentration of OPA (0.02 mM) did not show any inhibition on ethanol production of *S. cerevisiae*, even slightly increased the final ethanol yield (Figure 10). The addition of OPA between 0.1-0.5 mM decreased the fermentation rate significantly, but the final ethanol yields were dependent on fermentation time (Figure 10). The volumetric ethanol productivity decreased dramatically from 1.16 to 0.28 and 0 g/(L·h) respectively as the OPA concentration increased from 0.02 to 0.1 and 0.5 mM (Table 9). This indicated the yeast cells could adapt themselves to

OPA inhibition and the adaptation probably was time dependent. This could be further demonstrated by the ethanol production curve (Figure 10). Both 0.02 mM OPA and the control had their ethanol production leveled off within 10 h. The slight increase in ethanol final yield in the presence of 0.02 mM OPA might have the same explanation as the presence of 5.0 mM vanillin. During the fermentation, we also detected that glycerol production was decreased in the presence of 0.02 mM OPA. Increase the concentration of OPA to 0.1 mM considerable decreased the ethanol productivity but not the final yield. The ethanol production was not leveled off until 36 h, suggesting lower ethanol productivity at higher OPA concentration. The final ethanol yield was 0.45 g/g, similar to the yield in the control. The addition of 0.5 mM OPA severely inhibited both ethanol productivity and the final yield. Ethanol production was significantly inhibited within first 24 h. The final yield was 0.29 g/g, 60% of the control. Interestingly, the ethanol production was not completely inhibited in the first hour of fermentation. This suggested that a short time was required for OPA to be adsorbed by yeast and enable the subsequent inhibition probably due to the low solubility of OPA in water. Increasing OPA concentration to 1.0 mM has lead to a complete inhibition on ethanol production. This result indicated that OPA as a carbonyl model compound is a very potent inhibitor on ethanol production of *S. cerevisiae* that it totally stopped ethanol production at the concentration as low as 1.0 mM (0.13 g/L).

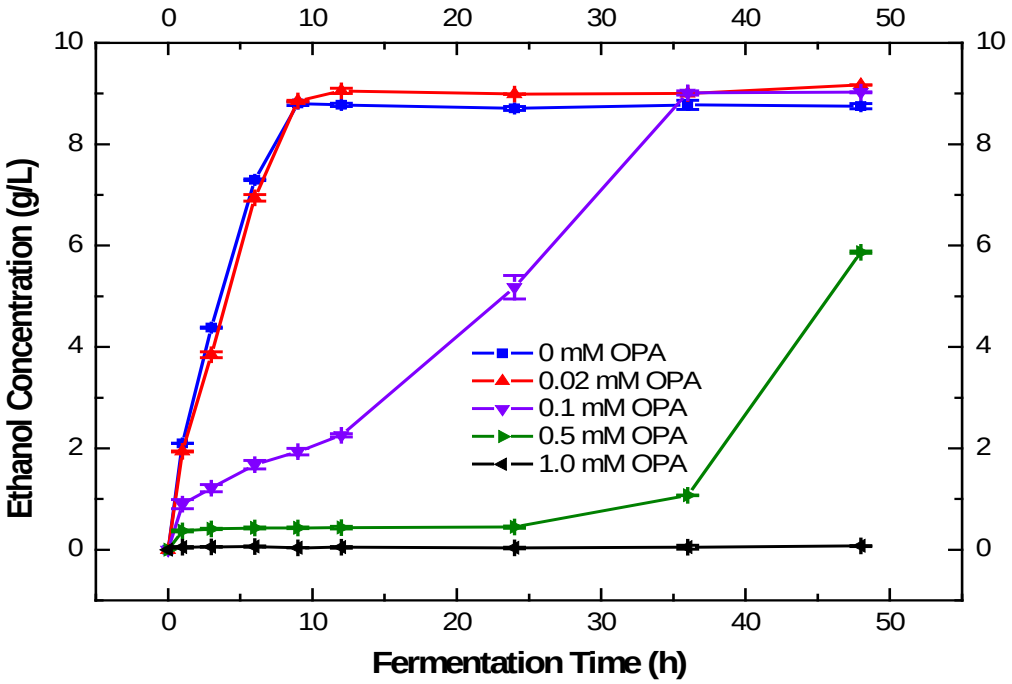


Figure 10: Effects of OPA concentration on fermentation inhibition of *S. cerevisiae*

Table 9: Effects of OPA concentration on ethanol productivity and yields

OPA(mM)	C_{EtOH}[a](g/L)	Y_{EtOH}[b](g/g)	Q_{EtOH}[c](g/L·h)
0	8.75±0.05	0.44±0.00	1.22±0.00
0.02	9.17±0.00	0.46±0.00	1.16±0.01
0.10	9.03±0.01	0.45±0.00	0.28±0.01
0.50	5.87±0.02	0.29±0.00	0.07±0.00
1.00	0	0	0

[a]C_{EtOH} Ethanol concentration at 48 h. [b]Y_{EtOH} Ethanol yield from total glucose at 48 h. [c]Q_{EtOH} Volumetric ethanol productivity after 6 h.

Effects of different concentration of OPA (between 0-1.0 mM) on growth curve of *S. cerevisiae* were also investigated (Figure 11). The higher concentration of OPA resulted in lower

the growth yield of yeast biomass. An addition of 0.02 mM OPA showed a negligible effect on the yeast growth by only 4% reducing of OD. As mentioned above, 0.02 mM of OPA increased the ethanol production by 4.5%. The fact that the same amount of OPA slightly decreased the growth rate gave us more evidence that the increase of ethanol with 0.02 mM of OPA was compromised by the decrease of biomass yield. Increasing OPA concentration to 0.1mM significantly decreased the yeast growth rate and the lag phase of *S. cerevisiae*. The growth of yeast was reduced to 25% as compared to the control and the lag phase was observed to increase to 34 h. Further increasing OPA concentration to 0.5 and 1.0 mM completely stopped the yeast growth. No cell growth was observed during the 48 h growth period. These results indicated that the inhibition of OPA on yeast growth was much higher than that on ethanol production.

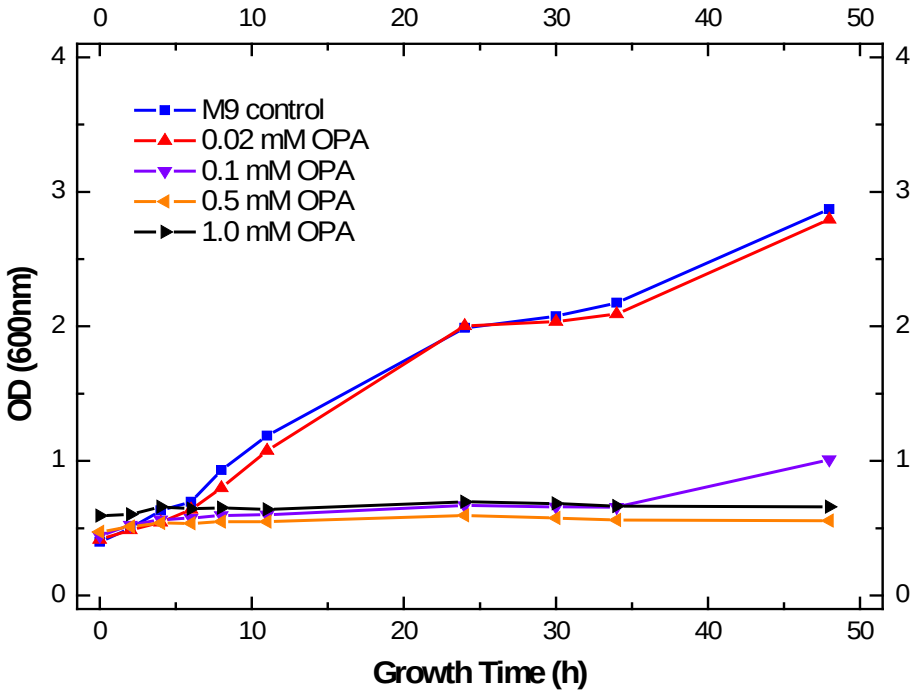


Figure 11: Effect of OPA concentration on growth of *S. cerevisiae*

Effects of different concentration of OPA (between 0-1.0 mM) on yeast growth were also investigated by counting the colony forming units (CFU) on YPD solid media after incubating yeast cells with OPA at different time (Table 10). Similar trend as growth curve was observed. 0.02 mM OPA addition showed slightly lower CFU compared to control. Increasing OPA concentration to 0.1 mM considerably decreased the CFU. An addition of 0.5 mM OPA concentration stopped the cell growth till 48 h, at which 0.17×10^5 CFU was observed. This result indicated that OPA might suppress the growth of yeast cells but didn't kill all of them. The yeast cells need a long lag phase to be adapted to the high toxicity of OPA. With an OPA concentration of 1.0 mM, no colony was observed on the YDP plates after 48 h of incubation.

Table 10: Colony Forming Units (CFU) of *S. cerevisiae* on YDP agar plates in the presence of 0 mM (M9 control), 0.02 mM, 0.1 mM, 0.5mM and 1.0 mM OPA at 30°C

Incubation time	M9 ($\times10^5$)	0.02mM ($\times10^5$)	0.1mM ($\times10^5$)	0.5mM ($\times10^5$)	1mM ($\times10^5$)
0 h	157	151	19	0	0
12 h	730	510	4.0	0	0
24 h	1070	1470	8.1	0	0
48 h	4000	1400	206	0.2	0

3.3.3 Effect of inoculation size of *S. cerevisiae* on OPA fermentation inhibition

It was reported that inoculation size of *S. cerevisiae* affected the ethanol production in the presence of inhibitors (Huang et al. 2011; Leonard and Hajny 1945). In order to test if the inoculation size of *S. cerevisiae* affected OPA inhibition on fermentation, two different levels of inoculation size (0.5 and 2.0 g/l) of *S. cerevisiae* were used in the presence of 0.02 mM OPA.

Starting with 2.0 g/L of yeast (inoculation size), the volumetric ethanol productivity and final ethanol yields were similar in *S. cerevisiae* fermentation with and without the addition of 0.02 mM OPA (Figure 12). When the inoculation size was decreased to 0.5 g/L, the volumetric

ethanol productivity dropped significantly to 0.07 and 0.12 g/L ·h with and without 0.02 mM of OPA; and the final ethanol yields was 3.4 g/L and 8.9 g/L with and without 0.02 mM of OPA. This indicated that higher inoculation size could overcome the fermentation inhibition when the inhibitors concentration was low. It also suggested the ethanologenic yeasts could adapt and tolerate the low concentration of inhibitors (Keating et al. 2006; Liu et al. 2004).

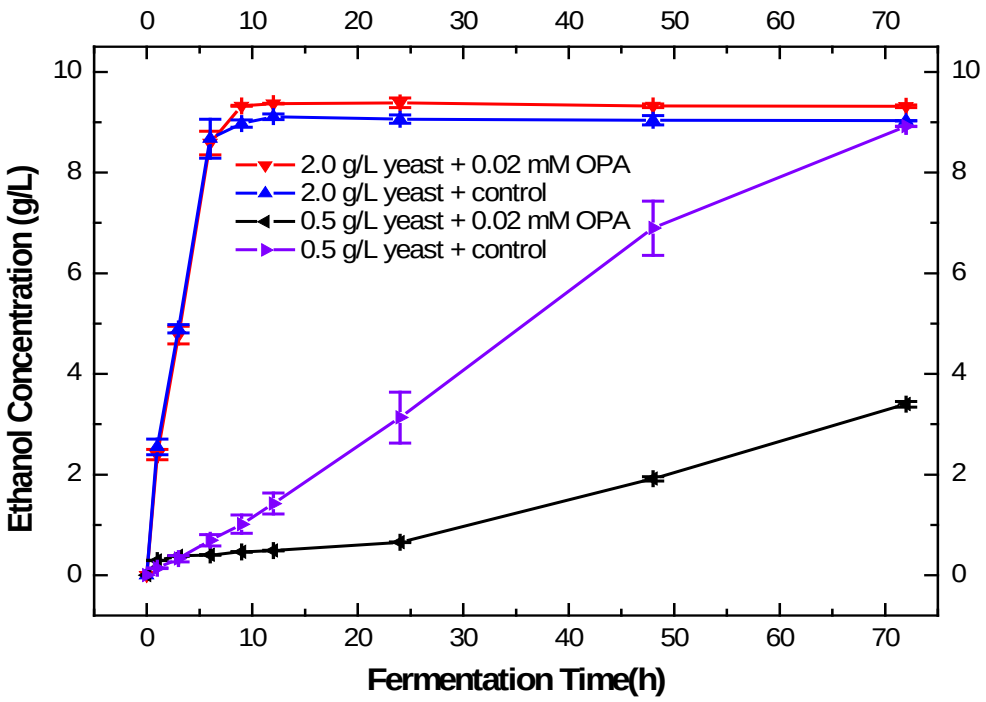


Figure 12: Effect of inoculation size of *S. cerevisiae* on OPA fermentation inhibition (with and without addition of 0.02 mM OPA)

The results showed that, compared to the control, 0.02 mM OPA showed no inhibition on ethanol fermentation when the inoculation size was 2.0 g/L, while the same amount of OPA showed strong inhibition on ethanol productivity when the inoculation size was dropped to 0.5 g/L (Figure 12). When the inoculation size was 2.0 g/L, the volumetric ehtanol productivity and

final ethanol yields were similar between the fermentation with and without addition of OPA (0.02 mM). Overall, the ethanol production curves showed little difference between addition of 0.02 mM OPA and the control. When the inoculation size was decreased to 0.5 g/L, an addition of 0.02 mM OPA decreased ethanol productivity to 58.3% and final yield to 39.3% as compared to the control. The explanation might be that at high inoculation size, the toxicity of OPA to each yeast cell was relatively low, because the toxicity is dose dependent. Therefore, ethanol production was compromised at low inoculation loading.

3.3.4 Effects of reducing sugars on alkaline detoxification of OPA

It has been shown that alkaline treatment could be used to remove the inhibition of aldehydes and ketones on microorganisms during fermentation (Alriksson et al. 2006; Persson et al. 2002a). Alkaline treatment was reported to decrease furaldehydes concentration in the spruce hydrolysate by up to 40% (Persson et al. 2002a). Alkaline treatment could also effectively remove phenolic compounds with $Ca(OH)_2$, NaOH and NH_4OH under the pH of 9-11 (Alriksson et al. 2006).

Based on our preliminary research, interestingly it was the first time that we found that the OPA inhibition would not be detoxified if glucose was not added before alkaline treatment of OPA. Therefore, we hypothesized that reducing sugars played a very important role in the alkaline detoxification of OPA. In order to test this hypothesis, we investigated that the alkaline detoxification (pH 10 at 60 °C for 2 h) of OPA (1.0 mM) with the addition of reducing and non-reducing sugars. Glucose and fructose were chosen to represent aldose amd ketose reducing sugars respectively; Sucrose was chosen as non-reducing sugar. Fermentations were conducted to examine the removal of OPA inhibition in the presence of reducing and non-reducing sugars.

Fermentations with the addition of only sugar sources were conducted as controls.

After alkaline treatments, OPA inhibition was removed almost completely in fermentation when glucose and fructose were presented in the detoxification process. Ethanol production achieved the plateau within 9 h (Figure 13). On the other hand, OPA inhibition was evident and no changed when sucrose was presented in the detoxification process (Figure 13 and Table 11). This indicated that the detoxification of OPA could not be conducted effectively unless reducing sugars (either aldose or ketose) were present. This probably is the first time that suggested reducing sugars played a significant role the alkaline detoxification of carbonyl compounds. When compared to their corresponding controls, the treated reducing sugar broths had faster fermentation rate (Table 11). The improved fermentation rate might be attributed to the addition of salts. The alkaline treatments and the following neutralization brought a considerable amount of sodium ions and sulfate ions into the fermentation broth, these salts could be helpful to the yeast. The final concentration of ethanol, on the other hand, was similar in the treated broths compared to corresponding controls (Table 11).

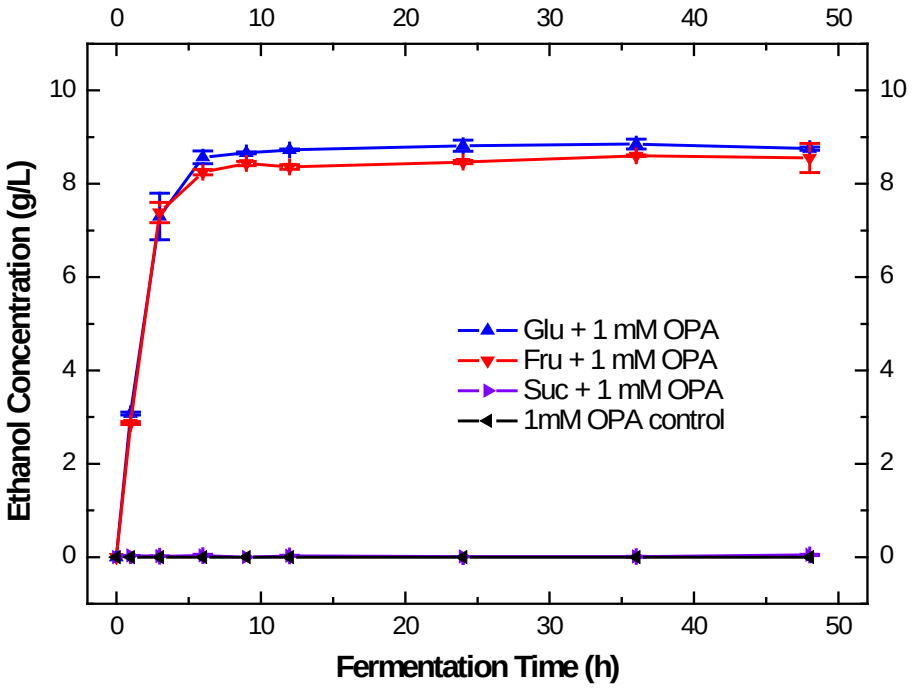


Figure 13: Effects of reducing and non-reducing sugars on alkaline detoxification of OPA

Table 11: Effects of reducing sugars and non-reducing sugar on alkaline detoxification of OPA

Samples	R_s[a] (g/L·h)	C_{EtOH}[b] (g/L)	Y_{EtOH}[c] (g/g)	Q_{EtOH}[d] (g/L·h)
Glu/OPA	5.67±0.13	8.75±0.04	0.45±0.02	2.43±0.50
Glucose	4.19±0.01	8.95±0.04	0.44±0.01	1.77±0.09
Fru/OPA	5.93±0.01	8.55±0.31	0.44±0.01	2.46±0.22
Fructose	4.08±0.10	8.88±0.03	0.44±0.00	1.71±0.09
Suc/OPA	0	0	0	0
Sucrose	3.53±0.12	9.35±0.02	0.46±0.00	1.65±0.09

[a]R_s Sugar consumption rate after 3 h. [b]C_{EtOH} Ethanol concentration at 48 h. [c]Y_{EtOH} Ethanol yield from total glucose at 48 h. [d]Q_{EtOH} Volumetric ethanol productivity after 6 h.

As mentioned above, the treated sucrose fermentation showed no ethanol production within the 48 h fermentation period (Figure 13). In the corresponding control, however, the

sucrose was fermented successfully (Figure 13 and Table 11). This implied OPA was not detoxified during alkaline treatment with sucrose as the sugar source. Interestingly, we also observed that in the alkaline treated broth, sucrose was hydrolyzed to glucose and fructose quickly after inoculation of yeast (Figure 14), which indicated the yeast released enzymes (invertase) for sucrose hydrolysis (Koschwanez et al. 2011). It suggested OPA was not detoxified in the presence of sucrose, but the toxic OPA appeared not to affect the invertase for sucrose hydrolysis.

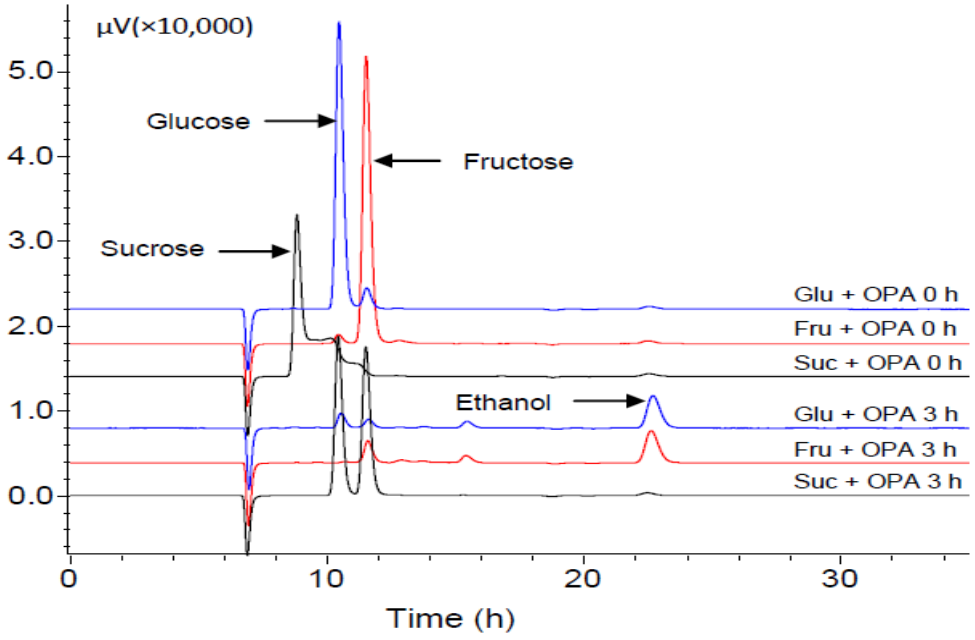


Figure 14: HPLC Chromatogram of fermentation samples after alkaline detoxification of OPA in the presence of glucose, fructose and sucrose at 0 and 3 h

We evaluated the role of pH in this reducing-sugar facilitated OPA detoxification. In the control without pH adjustment (to 10), the reducing sugar (glucose) and OPA was heated for two hours before the inoculation of yeast, glucose could not be fermented. This indicated that alkaline condition (pH~10) is required for this detoxification. Actually, we found that pH was

changed differently during alkaline treatments with reducing and non-reducing sugars (Table 12). We adjusted the pH of solution (sugars and OPA) to 10 with NaOH initially, then heated and mixed at 60°C. The pH in solution with glucose and fructose dropped from 10 to around 9 and 8 respectively every 30 min (additional NaOH was added into solution to keep the pH at 10). However, pH didn't change in the sucrose treatment. With the addition of same amount of NaOH into sucrose, pH was actually increased to 11 at the end of treatment. Before the fermentation, we readjust the pH of solution back to 6 with H_2SO_4. It suggested reducing sugars were reacted with OPA and brought the pH down, and the non-reducing sugars did not react with OPA and did not decrease the pH of solution. Subsequently, it will be very interesting to identify the potential reaction mechanism between OPA and reducing sugars. This probably will shed great insight on the detoxification reaction mechanism for biomass hydrolysate.

Table 12: The change of pH during the alkaline treatment of 1.0 mM OPA with different sugars (2h, 60°C)

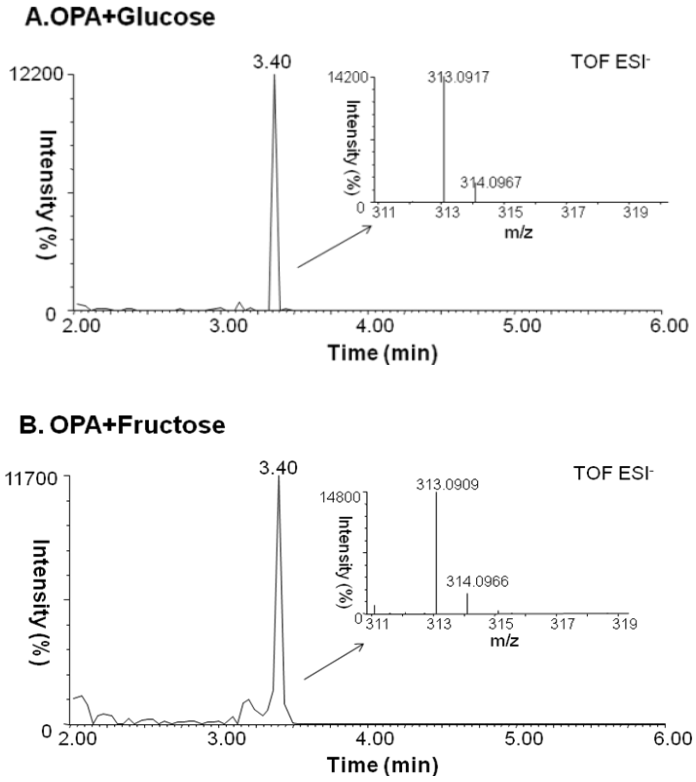
Samples	Initial	pH adjusting[a]	30 min	pH adjusting	60 min	pH adjusting	90 min	pH adjusting	120 min
Glu/OPA	6.3	10.0	9.0	10.1	9.5	10.2	9.5	10.2	9.7
Fru/OPA	6.4	9.9	8.2	9.9	8.2	9.9	8.1	10	8.7
Suc/OPA	6.3	10.5	10.4	10.9	10.8	11.1	10.9	11.1	11

a: NaOH was added at the same level in each treatment to avoid the effect of salts on fermentation

3.3.5 Identification of potential mechanism for alkaline detoxification of OPA

The potential mechanism of reducing sugars facilitated alkaline detoxification of OPA was explored further based on the analyzing reaction products of OPA and glucose by TOF LC/MS. As mentioned above, the alkaline detoxification could not be achieved unless a reducing sugar is present. It implied that the OPA might be converted into a non-toxic compound by a

reducing sugar. LC/MS was used to analyze of the potential products in alkaline treated OPA solution in the presence of 2% (w/w) glucose, fructose and sucrose respectively. The results showed that a new compound with a mass of 313.09 m/z was produced in the alkaline treated OPA solution of reducing sugars (Figure 15). This compound existed at a very high intensity in OPA solution treated with glucose or fructose (Figures 15A and 15B), while existed at a negligible intensity in OPA solution treated with sucrose (Figure 15C).



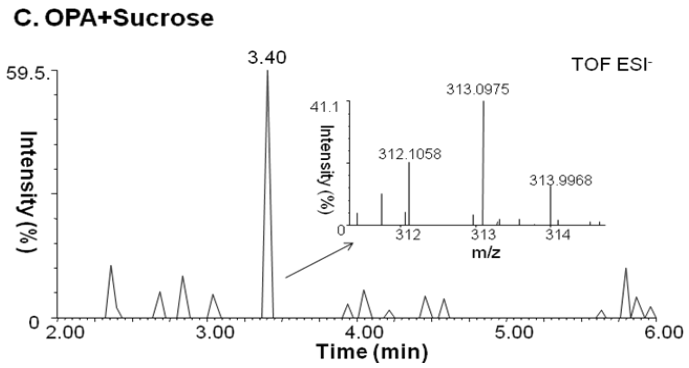


Figure 15: Mass spectra of alkaline treatment of OPA in the presence of glucose (A), fructose (B) and sucrose (C)

A composition analysis of this compound in the Masslynx software gave a formula of $C_{14}H_{18}O_8$ (ppm-1.9), which was exactly the addition of chemical formula of OPA $C_8H_6O_2$ and a reducing sugar $C_6H_{12}O_6$. A quick search of literature leads us to the prediction of this compound as aldol reaction product (glucosyl (β-hydroxyl) benzene-carboxaldehyde) (Horváth et al. 2005; Persson et al. 2002a). Generally, aldol reaction takes place under base condition when two carbonyl partners are present (McMurry 2008). Specifically, one of the carbonyl partners with α-hydrogen atom is converted into its corresponding enolate ion under base condition. Then the enolate ion acting as nucleophile adds to the carbonyl group of the second partner. The resultant intermediate is then protonated to give an alcohol product. Therefore, the presence of a carbonyl compound with α-hydrogen atom was the key point for the reaction to start. This carbonyl condensation reaction occurs frequently in biosysthesis pathways as one of the most important method forming C-C bonds (De Bruijn et al. 1986; Sultana et al. 2004). Normally, the aldolization product contains the molecular weight of the addition of the two carbonyl reactants (McMurry 2008). In our case, glucose and fructose as reducing sugars both have carbonyl group and α-hydrogen atom, which could be converted by hydroxide ion into their corresponding enolate ion. Then the enolate ion attacks one of the -CHO group of OPA to form the aldolization

product (Figures 16 and 17). Addition of another enolate ion to the second CHO group on OPA become less favored since the newly formed alcohol product on the first CHO group had much less electron withdrawing effect on the second CHO group on OPA, which makes the nucleophilic reaction less favorable (Zuman 2004). Steric effect could also be another factor. Therefore, the aldolization product between reducing sugar and OPA could be mainly the reaction of one reducing sugar with one CHO group on OPA. The prediction of aldol reaction between reducing sugar and OPA could well explain our experimental result. LC/MS results showed that sucrose had no aldol reaction product with OPA under alkaline condition (Figure 15C). In addition, the chemical formula of $C_{20}H_{28}O_{13}$ as aldol reaction product of OPA and sucrose was not found. Sucrose as a non-reducing sugar has glucose and fructose bonded through α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside. In such case, the reducing end of both glucose and fructose is linked together and could not be open. Therefore, sucrose is not carbonyl compound anymore. The aldol reaction could not take place with chemical compound with no carbonyl group. Similarly, OPA contains zero α-hydrogen, which makes the aldol reaction impossible between OPA molecules. Consequently, OPA inhibition was not able to be removed after alkaline treatment with OPA and sucrose.

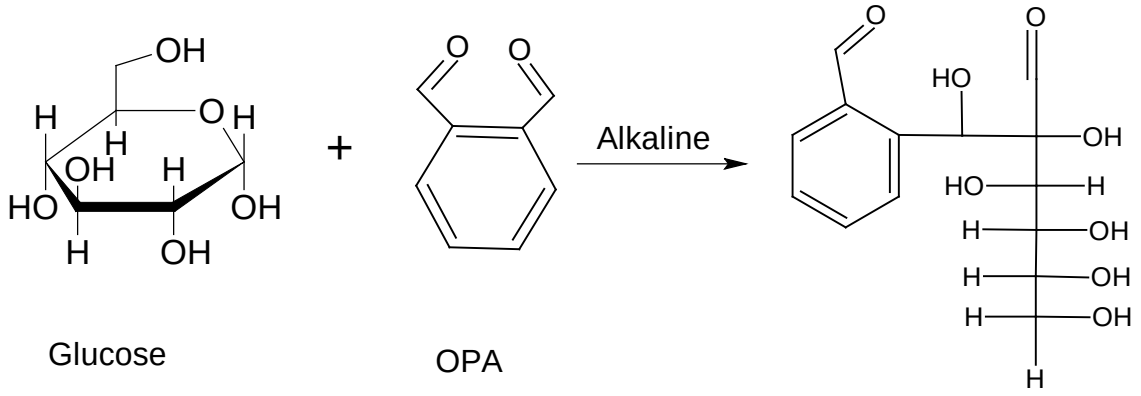


Figure 16: Potential Carbonyl condensation for reducing sugar and OPA under alkaline condition

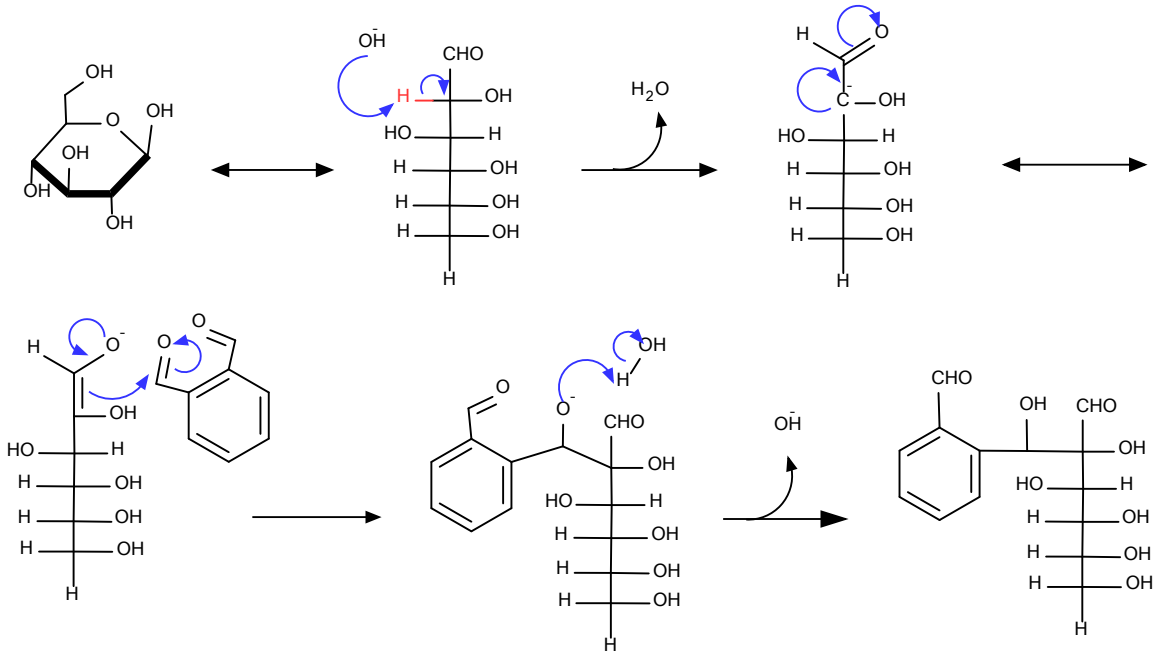


Figure 17: potential reaction mechanism for reducing sugar and OPA under alkaline condition

Aldol reactions between reducing sugars and non-aromatic aldehyde compounds have also been reported by other researchers. De Bruijn and coworkers reported that formaldehyde and fructose could react through aldolization in aqueous alkaline solution (De Bruijn et al. 1986). The author also reported that formaldehydes oligomerized to form monosaccharides through aldolization in alkaline condition with the requirement of addition of >C2 aldehydes (De Bruijn et al. 1986). This indirectly emphasized the importance of α-hydrogen on the carbonyl compounds for the aldol reaction to take place. Moreover, condensation reactions between OPA and aliphatic ketones have been utilized to produce benztropones (Davey and Gottfried 1961). This also indicated OPA could possibly undergo aldol condensation if appropriate carbonyl compounds with α-hydrogen are present.

The detoxification product (aldol reaction product) of OPA in the presence of reducing sugars also gives us the suggestion that the potent inhibition of OPA indeed comes from the two aldehyde groups. As our fermentation results showed (Figure 8), OPA was a considerably strong

inhibitor on both fermentation and growth of *S. cerevisiae* compared to the monoaldehyde compounds such as vanillin, furfural and HMF. On the other hand, OPA lost one of its CHO group and thus its inhibition after reacting with reducing sugars in base condition.

In fact, the alkaline treatment of OPA in the presence of reducing sugars or non-reducing sugar is a very complicated process. It was also reported that OPA could be oxidized to o-(hydroxymethyl) benzoic acid under alkaline condition (Zhu et al. 2003; Zuman 2004). During LC/MS analysis, the oxidization product of OPA was detected after alkaline treatment in the presence of glucose, fructose and sucrose. It was found that the oxidization products of OPA, including o-(hydroxymethyl) benzoic acid and phthalic acid, had considerably higher intensity in alkaline treatment with glucose and fructose than with sucrose. The difference oxidization rates of OPA in base condition with different sugars are still unknown and need further investigation. Moreover, compared to the aldol reaction products, the oxidization products of OPA under alkaline condition had relatively low intensity. This further indicated that reducing sugars are the key factors for removal of OPA. In addition, saccharides such as fructose and glucose were reported to undergo retro-aldol reaction under base conditions (De Bruijn et al. 1986). Some of the degradation products of saccharides are also aldehyde or ketone compounds with α-hydrogen. These retro-aldol reaction products from sugars could also react with OPA through aldol reaction to convert OPA into other compounds. Further research is necessary for a full understanding of chemical conversion of OPA under alkaline condition with reducing and non-reducing sugars.

3.4 Conclusion

Ortho-phthalaldehyde as a carbonyl model compound showed strong inhibitory effects on

fermentation and growth of *S. cerevisiae.* We found its alkaline detoxification potentially was a reducing sugars facilitated aldol condensation reaction. It was the first time we proposed the detoxification mechanism was a carbonyl aldol condensation reaction between sugars and potent carbonyl inhibitors. It was the first time that we suggested reducing sugars played a significant role the alkaline detoxification of carbonyl compounds. OPA could completely inhibit fermentation and growth of *S. cerevisiae* compared to vanillin, furfural and HMF at 5mM. The inhibition of OPA on fermentation and growth of *S. cerevisiae* was found to dose dependent. The OPA inhibition on fermentation and growth of the yeast decreased with the decrease of the OPA concentration. OPA showed no inhibition on both fermentation and growth of yeast at low concentration (0.02mM). The inhibition of OPA could be overcome by increasing inoculation size of yeast. OPA inhibition could be removed under alkaline condition (pH~10) at 60°C for 2 h in the presence of a reducing sugar (ketone or aldose). Non-reducing sugar could not result in the removal of OPA inhibition. LC/MS analysis of reaction products after detoxification in negative ion mode revealed a potential product, glucosyl (β-hydroxyl) benzene-carboxaldehyde with molecular weigh at 313.09. This compound was predicted to be the aldol reaction product of reducing sugar and OPA under base condition. One of the CHO groups on OPA was converted into hydroxyl group by nucleophilic addition of enolate ion of reducing sugar. Loss of one CHO group of OPA might be the key factor for detoxification.

Chapter 4: Effect of Amino acids on detoxification of biomass hydrolysate and carbonyl inhibitor cinnamaldehyde for bioethanol fermentation

4.1 Background

The objective of this chapter is to develop a cost-effective detoxification method by using amino acids to remove carbonyl inhibition from biomass hydrolysates and model inhibitor for bioethanol production.

Previously, physical, biological and chemical attempts have been employed to detoxify biomass hydrolysates. These methods are not without drawbacks in terms of time-efficiency, cost-effectiveness and follow-up processing issues. Evaporation/steam stripping was found to remove the volatile compounds but not the non-volatile yet more toxic compounds (Larsson et al. 1999b; Leonard and Hajny 1945). Ligninolytic enzymes such as laccase and peroxidase were able to transform phenolic compounds through putative oxidative polymerization but requiring long treatment time (12 h) and high cost for preparing the enzymes (Jönsson et al. 1998). Bioabatement was a recent biological attempt using certain microbes isolated from screens of soil microorganisms, for example, *Coniochaeta ligniaria* NRRL30616, to metabolize a wide range of inhibitors in dilute-acid biomass hydrolysates, which also required long treatment time (24 h) (Nichols et al. 2008). Activated charcoal and anion exchange resins were effective detoxification methods to adsorb inhibitors through weak chemical interaction and increase fermentabilities of the hydrolysates to desirable levels (Canilha et al. 2004; Larsson et al. 1999b; Mussatto et al. 2001; Nilvebrant et al. 2001). However, both methods caused substantial loss of fermentable sugars with high costs for the detoxifying materials. Alkaline/overliming detoxification has been the most commonly-used method to extensively remove the inhibitors

from biomass hydrolysates while the gypsum salts and precipitates produced during the detoxification could be significant issues for the subsequent processes (Alriksson et al. 2006; Horváth et al. 2005). Moreover, failing to target the detoxification of the compounds providing the most profound fermentation inhibition instead of randomly removing the degradation compounds was a disadvantage among the detoxification attempts.

Linking antimicrobial activity of the potential inhibitors to their structural features could be an important clue to design an industrially practical detoxification approach for biomass hydrolysates (Chan et al. 2008a; Schwöbel et al. 2011). Most of the identified inhibitors associated with biomass hydrolysates are functional carbonyl compounds (R-C=O) (Ando et al. 1986; Chen et al. 2006; Klinke et al. 2004). Carbonyl compounds possess electrophilic reactivity. The electron-poor carbonyl carbon can therefore form covalent bonds with nucleophilic targets in the microorganisms, leading to adverse biological effects. Consequently, inactivation of the electrophilic carbonyl groups (CHO) might be the key to remove the fermentation inhibiting carbonyl compounds. Amino acids with nucleophilic properties in the microbial cells often suffer electrophilic attacks from inhibitors or toxins (Friedman et al. 1965; Labenski et al. 2009). On the contrary, free amino acids with reactive amino and/or thiol groups can also initiate nucleophilic reactions with the electrophilic groups on carbonyl compounds, causing them to lose reactivity before inhibiting microbial cells. Therefore, we hypothesize that proper use of free amino acids could prevent the lignocelluloses-derived carbonyl compounds from attacking biological molecules prior to microbial fermentation through covalent binding with the reactive electrophilic functional groups. Additionally, the nutritional nature of amino acids renders them benign towards the microbe.

The objective of this study is to investigate the detoxification effectiveness of amino acids on biomass hydrolysates and their detoxification mechanisms. We first examined the effects of the 20 amino acids on detoxifying a non-fermentable dilute-acid biomass hydrolysate of loblolly pine (*Pinus taeda*) by evaluating the fermentation performance of the detoxified hydrolysates by *Saccharomyces cerevisiae*. Second, we investigated the effects of pH and temperature on the detoxification. Third, the detoxification effectiveness of amino acid was further evaluated by comparing SEM images of the fermenting cells in the untreated and detoxified hydrolysates. Finally, we studied the detoxification mechanisms of amino acids using a potent model carbonyl inhibitor from the hydrolysate.

4.2 Material and methods

4.2.1 Chemical reagents and stock preparation

Glucose, mannose, galactose, xylose and arabinose were obtained from Fluka (Milwaukee, WI) and Alfa Aesar (Ward Hill, MA). Glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, serine, threonine, cysteine, methionine, asparagine, glutamine, lysine, histidine, arginine, aspartate and glutamate were purchased from Alfa Aesar, Sigma-Aldrich (St. Louis, MO) and Acros Organics (Morris Plains, NJ). Acetic acid, levulinic acid, formic acid, furfural, hydroxymethylfurfural (HMF), vanillin and cinnamaldehyde were purchased from Alfa Aesar, Fisher Scientific (Fair Lawn, NJ), Aldrich (Milwaukee, WI) and Pickering laboratories (Mountain View, CA). All chemical reagents were of chromatographic grade. Stock solutions (1.0 M) of furfural, HMF, vanillin and cinnamaldehyde were prepared in ethanol (HPLC grade, Sigma-Aldrich) separately before further use. Stock solutions (1.0 M) of acetic acid, levulinic acid and formic acid were prepared in nanopure water (Barnstead)

separately. All stocks were protected from light and kept at 4°C. The stocks were used within 1 month.

4.2.2 Preparation of biomass hydrolysate

Fresh loblolly pine (*Pinus taeda*) from the research forest adjacent to the Forest Products Laboratory at Auburn University was used as the starting material in this study. The air-dried wood (without debarking) was chipped using a mechanical chipper and passed through a Chip Class machine (TMI) with a tray size of 7.0 mm. The wood chips (≤7 mm) were then stored in a walk-in freezer (2°C) until later use. The moisture content of the wood chips was around 8.0 wt%.

Dilute acid pretreatment was conducted in this study to produce biomass hydrolysate. Briefly, 400 g wood chips were saturated in 1% sulfuric acid (w/w biomass) overnight prior to pretreatment. The S/L ratio was 1:7. The saturated wood chips were then loaded in a 4 L Parr reactor (4842) and pretreated at 170°C for 1 h. After 1 h, the pretreatment was stopped immediately by running cold water through the reactor. After pretreatment, the biomass hydrolysate (liquid portion) was separated from the wood pulp (solid portion) through filtration. The initial pH of the hydrolysate was 1.8.

In order to achieve higher ethanol production yields, the loblolly pine hydrolysate was concentrated to approximately one-third of its initial volume before fermentation using a rotary evaporator (IKA®RV10 basic) at 40°C and 60 rpm. The hydrolysate was adjusted to pH 4 before evaporation to avoid sugar degradation. After the evaporation, the concentration of the sugars including glucose, mannose, xylose and arabinose was 16.8, 38.3, 29.2 and 6.0 g/L respectively, which gave a 55.1 g/L of total fermentable sugars. With regard to the sugar degradation

compounds, the concentration of formic acid, acetic acid, levulinic acid and HMF was 2.1, 5.5, 0.4 and 3.7 g/L respectively. Furfural was completely removed during the evaporation due to its high volatility. The concentrated hydrolysate (called hydrolysate hereafter) was considered not fermentable (Y_{EtOH}: 0.02 g/g total glucose) and used for all the detoxification and fermentation processes in this study. The hydrolysate was stored in a walk-in freezer at 2°C and used within 1 month.

4.2.3 Detoxification with amino acids

The loblolly pine hydrolysate and sugar solution added with model inhibitor (called inhibitor solution hereafter) were treated with amino acids prior to fermentation. All the detoxification treatments were conducted in a temperature-controlled water bath. The pH was adjusted with a pH meter (AR20, Fisher Scientific) by adding NaOH or H_2SO_4. After detoxification, the detoxified hydrolysates / inhibitor solution were cooled to ambient temperature using an ice water bath and then adjusted to pH 6. The detoxified hydrolysates /inhibition solution were used for fermentation immediately without further storage to test the detoxification effectiveness.

4.2.4 Microbial strain and fermentation

Baker yeast (Fleischmann's), *S. cerevisiae*, was used for all the fermentation experiments in this study. The yeast was isolated and maintained on YDP agar medium containing (g/L): 20 glucose (J.T. Baker), 20 peptone (BD), 10 yeast extract (Amresco) and 20 agar (BD). Isolated colony was grown in YDP liquid medium (without adding agar) overnight and harvested as fermentation inoculum. The yeast concentration was measured using an UV-vis

spectrophotometer based on a previous standard. Inoculum of 2.0 g/L was used for all fermentation experiments.

Batch fermentation was carried out in 125 mL serum bottles containing 50 ml of fermentation broth (untreated or detoxified hydrolysates, control solution or inhibitor solution) without any additional nutrient supplement. In the control and inhibitor solutions, the sugar concentration was prepared comparable to that in the biomass hydrolysate. All the fermentation broths were adjusted to pH 6 with NaOH or H_2SO_4 and sterilized by passing 0.2 μm sterile filters (VWR, Suwanee, GA). After inoculation, the serum bottle was sealed with rubber stopper and aluminum seal, and equipped with cannulas for CO_2 release. All fermentation experiments were incubated at 30°C, spun at 150 rpm in a shaker (E24, New Brunswick Scientific). Aliquots of samples were withdrawn at 0, 1, 3, 6, 9, 12, 24, 36 and 48 h for the time course analysis of both starting material and product. Each fermentation treatment was run in duplicate.

4.2.5 HPLC and LC/MS analysis

The biomass sugars, including glucose, mannose, galactose, xylose and arabinose, were quantified by integrating the peak area of the compound eluted from a HPLC system equipped with a strong cation-exchange column (Aminex HPX-87P, 300 × 7.8 mm), a refractive index detector (RID-10A), with column temperature of 85°C, and nanopure water as the mobile phase at a flow rate of 0.6ml/min for a 35 min isocratic run. Ethanol, acetic acid, levulinic acid, formic acid, furfural and HMF were analyzed using an Aminex HPX-87H column (300 × 7.8 mm) with a refractive index detector. The elution conditions were column temperature of 45°C and flow rate of 0.6 ml/min with 5.0 mM H_2SO_4 as the mobile phase in a 60 min isocratic run. The detoxification products were analyzed by an Ultra Performance LC system (ACQUITY UPLC,

Waters) coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer with electrospray ionization (ESI) in positive ion mode, with or without C18 column chromatography operated by the Masslynx software (V4.1).

In a loop injection without a column, each sample, in H_2O, was injected directly into ion source and acquired spectrum. With column, each sample was injected onto a C18 column (ACQUITY UPLC® BEH C18, 1.7 µm, 2.1 x 50 mm, Waters) with a 150 µL/min flow rate of mobile phase of solution A (95% H_2O, 5% acetonitrile, 0.1% formic acid) and solution B (95% acetonitrile, 5% H_2O, 0.1% formic acid) in a 10 min gradient starting at 95% A to 5% A in 6 min and back to 95% in 8 min. The ion source voltages were set at 3 KV, sampling cone at 37 V and the extraction cone at 3 V. In both modes the source and desolvation temperature were maintained at 120°C and 225°C, respectively, with the desolvation gas flow at 200 L/h. The TOF MS scan was from 200 to 800 m/z at 1 s with 0.1 s inter-scan delay using extended dynamic range acquisition with centriod data format. For real time mass calibration, direct infusion of sodium formate solution (10% formic acid/0.1M NaOH/isopropanol at a ratio of 1:1:8) at 1 sec/10 sec to ion source at 1 µL/min was used.

The instrument was calibrated at the time of data acquisition in addition to real time calibration by the lockmass. Mass accuracy at 5 ppm or less was the key for assuring the presence of target molecules. Ion source parameters such as the source temperature (gas and sample cone), mobile phase flow rate, and cone voltage were fixed throughout the study. Ions of interest were analyzed for mass accuracy, elemental composition (using accurate mass measurement of less than 5 ppm error) and isotope modeling to identify the formula. Quantification of unknowns was performed by computing intensity of the chromatogram using either the ion count in the spectrum or the peak area displayed target ion mass in the

chromatogram, referencing to known amount of standard peptides acquired under the same conditions in the same time period.

4.2.6 SEM of yeast cell

The morphology of the yeast cells at 12 h fermentation of untreated and amino acid-treated loblolly pine hydrolysates, as well as pure sugar solution, were observed using a field emission scanning electron microscopy (SEM, JEOL 7000F), with accelerating voltage at 20.0 KV. 0.2 mL of the fermentation broths were withdrawn at 12 h and centrifuged at 13.2×10^4 rpm for 10 min. The yeast cell pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min twice at ambient temperature. The cells were subsequently fixed in 4% glutaraldehyde for 1 h, washed and equilibrated in 0.1 mM sodium phosphate buffer for 10 min twice. After fixation, the cells were dehydrated for 15 min in each of the following levels of ethanol solutions sequentially: 50, 60, 70, 80, 90 and 100%. The dehydrated cells were then dried with hexamethyldisilazane (HMDS) for 30 min. The dried samples were immediately coated with a thin layer of gold (50 nm) by PELCO SC-6 Sputter Coater and imaged under SEM.

4.3 Results and discussion

4.3.1 Detoxification of loblolly pine hydrolysate by amino acids

The presence of carbonyl compounds in the biomass hydrolysates can initiate covalent binding with important biological nucleophiles such as proteins/enzymes and reduce biofuel yield by the fermenting microorganisms. We hypothesize that amino acids could remove the inhibition by neutralizing the electrophilic carbonyl compounds prior to the fermentation. In order to examine this hypothesis, all 20 regular amino acids were examined for detoxification of

a non-fermentable loblolly pine hydrolysate containing 55.1 g/L of fermentable sugars (glucose, 16.8 g/L; mannose, 38.31 g/L). The detoxification assay, based on our experience, was performed at 60°C and pH 6 for 2 h, with addition of 0.2% (w/v) of individual amino acids. Batch fermentation by *S. cerevisiae* was conducted to evaluate the detoxification effectiveness of each amino acid. Pure sugar fermentation in which 17.7 g/L of glucose and 35.1 g/L of mannose were added but without inhibitor was used as a positive control for the fermentation. The results showed amino acids improved the fermentability of the barely fermentable loblolly pine hydrolysate to various degrees, but the results were quite variable among different amino acids (Table 13). Fermentation of the untreated hydrolysate had an ethanol productivity of 0.18 g/L/h at 6 h and final ethanol concentration of 1.16 g/L at 48 h (Table 13 and Figure 18). However, the fermentability was improved, at least partially, after the hydrolysates were detoxified with amino acids. Among the 20 amino acids, cysteine had the highest detoxification activity, it increased the ethanol productivity to 1.77 g/L/h (or 27% higher than the control), the final ethanol concentration to 23.14 g/L, and the final ethanol yield to 0.42 g/g (similar to the control, Table 13 and Figure 18). Histidine detoxification also resulted in the final ethanol concentration and yield of 23.07 g/L and 0.42 g/g respectively, but the ethanol productivity at 6 h (0.78 g/L/h) was lower than that of cysteine detoxified hydrolysate and the control (Table 13 and Figure 18). Ethanol production in fermentation of the cysteine-detoxified hydrolysate leveled off within 24 h, while it extended to 48 h in the histidine-detoxified hydrolysate and the control (Figure 18). The rest of 18 amino acids had less detoxification activities, as indicated by their reduced final ethanol production than that of the control (Table 13). Among these, lysine, tryptophan, asparagine and glycine showed some advantageous detoxification activities, with the final ethanol production at 14.40, 11.59, 11.18 and 8.58 g/L respectively, considerably higher than the untreated hydrolysate

(1.16 g/L) (Table 13 and Figure 18). The remaining amino acids had negligible detoxification effects (Table 13). Consumption of fermentable sugars followed a similar trend to that of ethanol production. In the cysteine-detoxified hydrolysate, sugar consumption rate at 6 h was 4.26 g/L/h, 18% faster than that in the control (3.61 g/L/h). The sugars were almost completely consumed within 24h, while in the control 48 h was required to consume with 98% of the sugars (Figure 19). In the histidine-detoxified hydrolysate, the sugar consumption rate at 6 h (1.95 g/L/h) was lower than that in the control, but the total sugar consumption at 48 h reached 96%, similar to that of control (Figure 19). In contrast, the sugar consumptions at 48 h were incomplete among the rest of the amino acids. In particular, lysine, tryptophan, asparagine and glycine detoxification led to 59.46, 47.48%, 45.16% and 36.06% sugar consumption respectively, and the remaining amino acids led to less than 25% sugar consumption.

The fact that various levels of detoxification occurred between the amino acids was probably due to their difference in nucleophilicity towards the carbonyls in the hydrolysates. Brotzel and Mayr compared the nucleophilicities of 16 amino acids based on their reaction with electrophilic benzhydrylium terafluoroborates and found that cysteine had a much higher nucleophilicity parameter (N=23.43) than all other amino acids (Brotzel and Mayr 2007). The high nucleophilicity of cysteine was attributed to its thiol group, which exceeded the reactivities of the primary amino groups by a factor of 10^4 (Brotzel and Mayr 2007). Similar results were also reported when nucleophilic reactivities of amino group and thiol group were reacted with α,β-unsaturated compounds (Friedman et al. 1965), in which the thiol group was around 280 times more reactive than amino group. These agreed with our finding that cysteine completely detoxified a non-fermentable loblolly pine hydrolysate, resulting in better fermentation than the control without inhibitors. This indicated the sulfhydryl side chain in cysteine played a

detrimental role in the neutralization of reactive carbonyl compounds in the hydrolysate. Interestingly, we found that histidine also exhibited promising detoxification activity by consuming 96% of the fermentable sugars at 48 h. The secondary amine in the imidazole side chain of histidine makes it one of the strongest bases at neutral pH due to the low pK_a (6.1). It was reported that histidine-containing dipetides conjugated and detoxified aldehyde compounds in biological cells, as an aldehyde scavenger (Xie et al. 2013). Therefore, the favorable detoxification effect of histidine was probably also attributed to its high nucleophilic side chain. Indeed, the side chains of cysteine, histidine and lysine often serve as important biological nucleophilic sites that are attacked by reactive aldehydes or other electrophilic toxins, forming a complex of stable products (Casini et al. 2002; Labenski et al. 2009; Xie et al. 2013). From our study, cysteine, histidine and lysine were the top three candidates for biomass hydrolysate detoxification, probably because they contain a secondary nucleophilic functional group, apart from the primary amino group.

It is not clear why asparagine, tryptophan and glycine had higher detoxification activity than the rest of amino acids. It seems that the α-amino group was the only available detoxifying group towards carbonyl inhibitors. It was unlikely that basicity of the primary amino groups among different amino acids governed the detoxification because a correlation between pK_a of the primary amino groups and their detoxification activity was not found ($r^2<0.01$). Similarly, when comparing the nucleophilicity parameters of the primary amino groups in amino acids, Brotzel and Mayr found the difference were not significant ($12.7 < N < 14.1$) (Brotzel and Mayr 2007).

Interestingly, although cysteine and histidine successfully detoxified the hydrolysates, they did not remove any of the sugar degradation compounds such as acetic acid (5.5 g/L),

formic acid (2.1 g/L), levulinic acid (0.4 g/L) and HMF (3.7 g/L), revealing these compounds were not the major inhibitors. This was not surprising because a number of previous studies have reported, that rather than inhibiting ethanol production, these sugar degradation compounds indeed increased the ethanol yields. For example, Taherzadeh et al reported that 9 g/L acetic acid at pH 5 increased the final ethanol yield in *S.cerevisiae* fermentation by 16% (Taherzadeh et al. 1997). In our preliminary study, we also found that addition of 25 mM (3.16 g/L) HMF did not affect the ethanol production by *S.cerevisiae*. Therefore, nucleophilic reactions with unknown reactive carbonyl inhibitors might be the main reason for the detoxification. Although amino acids did not remove HMF in the hydrolysate, they promoted HMF consumption in the fermentation of the detoxified hydrolysates (Table 13). HMF consumption at 48 h of the fermentation was 6.37% in the untreated hydrolysate, but increased from 11.9, 39.2, 42.12, 50.20 and 81.94 to 96.40% in the glycine, asparagine, tryptophan, lysine, histidine and cysteine detoxified hydrolysates, respectively (Figure 20). Overall, the increase in HMF consumption followed the same pattern as the ethanol production after the hydrolysates were detoxified with different amino acids (Table 13). It is well known that yeast cells are able to convert furans to their corresponding alcohols as the major products and acids as the minor products using multiple enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase (Palmqvist et al. 1999; Uchida 2000). The detoxification seems affected the ethanol-producing enzyme activities in the yeast cells.

Table 13: Detoxification by 20 amino acids (pH6, 60°C and 2 h) on improving the fermentability of concentrated loblolly pine hydrolysate by *S. cerevisiae*

Treatment	R_S [a] (g/L/h)	Q_{EtOH} [b] (g/L/h)	C_{EtOH} [c] (g/L)	Y_{EtOH} [d] (g/g)	C_{HMF} [e] (g/g)
Control	3.61±0.04	1.39±0.00	22.68±0.12	0.43±0.00	NA
Untreated	0.45±0.08	0.18±0.00	1.16±0.01	0.02±0.00	6.37±0.67
Glycine	0.89±0.03	0.42±0.01	8.58±0.27	0.16±0.01	11.19±0.20
Alanine	0.65±0.08	0.28±0.00	2.14±0.01	0.04±0.00	11.03±1.64
Valine	0.74±0.03	0.32±0.00	2.69±0.18	0.05±0.00	12.66±1.50
Leucine	0.91±0.16	0.34±0.01	2.83±0.19	0.05±0.00	12.84±1.32
Isoleucine	1.03±0.08	0.44±0.08	5.03±1.46	0.09±0.03	17.36±3.50
Proline	0.93±0.23	0.33±0.00	3.12±0.13	0.06±0.00	13.35±1.05
Phenylalanine	0.79±0.01	0.28±0.00	1.87±0.02	0.03±0.00	12.69±1.12
Tyrosine	0.83±0.03	0.30±0.02	2.16±0.25	0.04±0.00	12.17±0.92
Tryptophan	1.58±0.04	0.63±0.01	11.59±0.25	0.21±0.00	42.12±0.81
Serine	1.03±0.12	0.45±0.00	3.87±0.08	0.07±0.00	15.66±0.11
Threonine	1.03±0.05	0.41±0.01	3.96±0.13	0.07±0.00	16.19±0.87
Cysteine	4.26±0.16	1.77±0.03	23.14±0.10	0.42±0.00	96.40±0.03
Methionine	0.89±0.03	0.31±0.00	3.11±0.02	0.06±0.00	12.78±0.10
Asparagine	1.52±0.11	0.58±0.01	11.18±0.15	0.20±0.01	39.20±0.50
Glutamine	1.11±0.03	0.40±0.00	3.52±0.06	0.060.01	13.38±0.99
Lysine	1.89±0.19	0.72±0.01	14.40±0.57	0.26±0.01	50.20±4.97
Histidine	1.95±0.01	0.78±0.00	23.07±0.35	0.42±0.01	81.94±1.57
Arginine	0.88±0.09	0.34±0.01	2.62±0.03	0.05±0.00	12.69±0.27
Aspartate	1.32±0.02	0.47±0.00	3.86±0.18	0.07±0.00	16.27±0.30
Glutamate	0.90±0.04	0.41±0.01	3.63±0.23	0.07±0.00	15.40±0.72

[a]R_s, sugar consumption rate at 6 h. [b]Q_{EtOH}, volumetric ethanol productivity at 6 h. [c]C_{EtOH}, final ethanol concentration at 48 h. [d]Y_{EtOH}, ethanol yield from total glucose at 48 h. [e]$C_{HMF,}$ HMF consumption from initial HMF at 48 h.

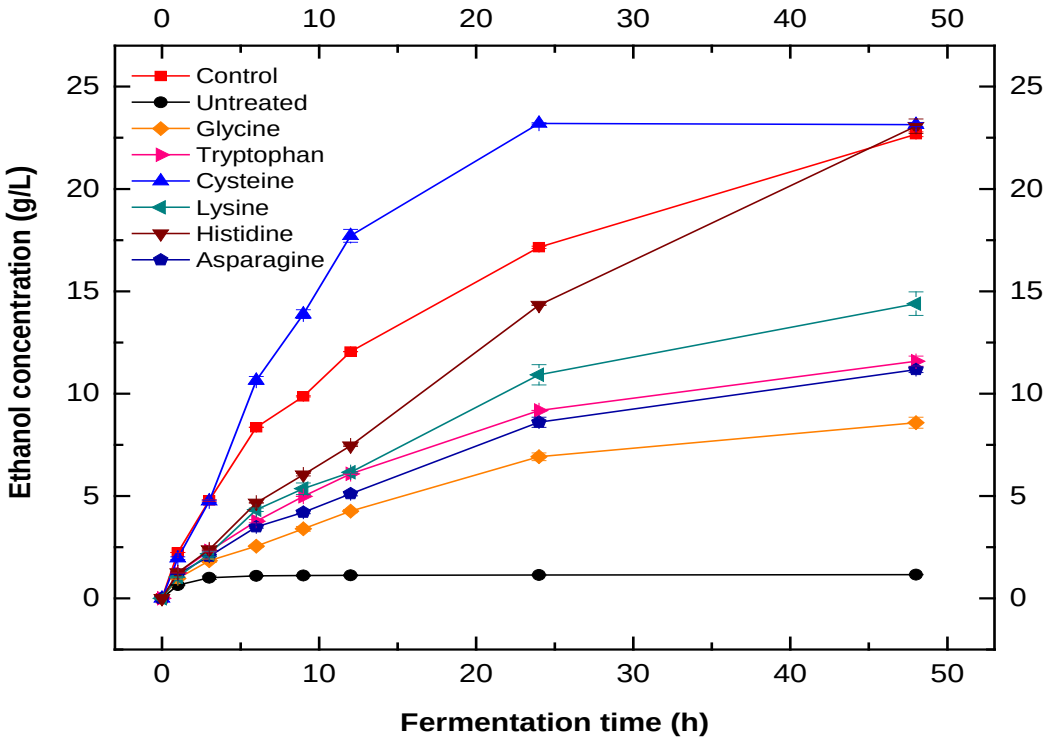


Figure 18: Ethanol production by *S. cerevisiae* in the concentrated loblolly pine hydrolysate detoxified by different amino acids (detoxification condition: pH 6, 60°C, 2 h, 0.2% (w/v) of amino acid)
Untreated: untreated loblolly pine hydrolysate;

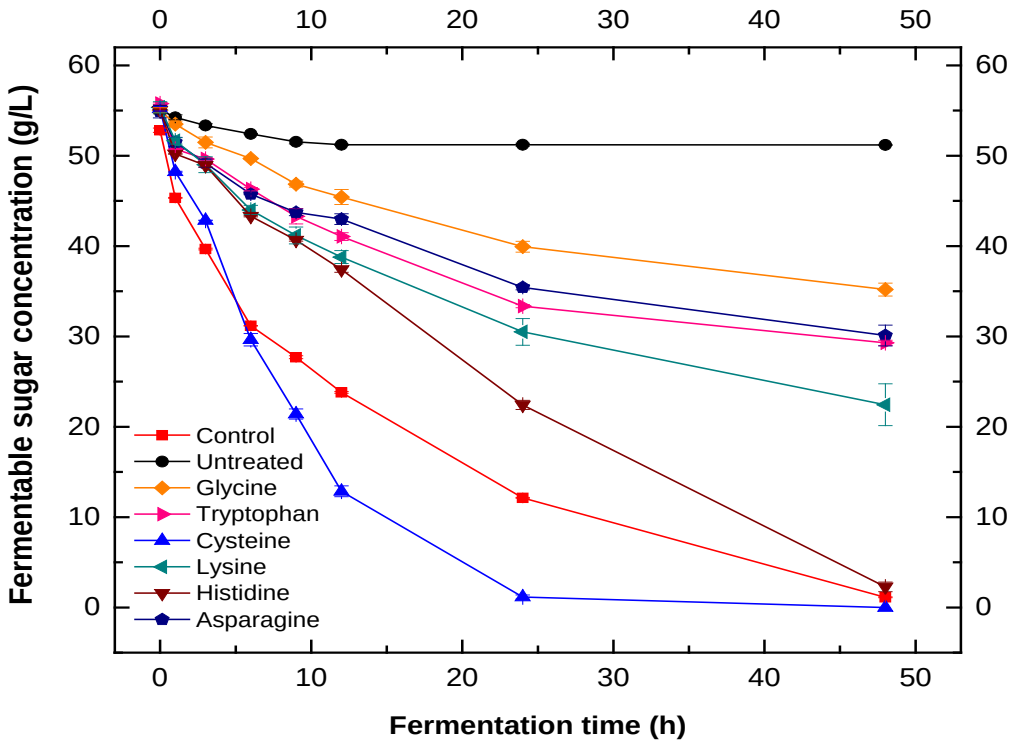


Figure 19: Sugar consumption by *S. cerevisiae* in the concentrated loblolly pine hydrolysate detoxified by different amino acids (detoxification condition: pH 6, 60°C, 2 h, 0.2% (w/v) of amino acid)
Untreated: untreated loblolly pine hydrolysate;

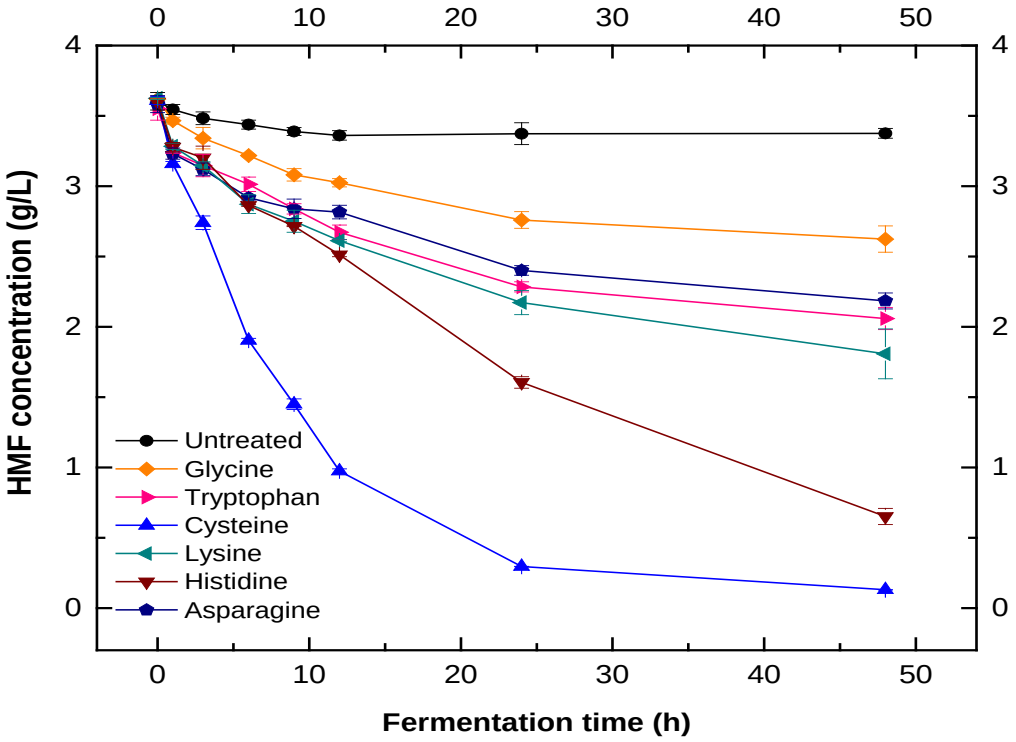


Figure 20: HMF consumption by *S. cerevisiae* in the concentrated loblolly pine hydrolysate detoxified with different amino acids (detoxification condition: pH 6, 60°C, 2 h, 0.2% (w/v) of amino acid)
Untreated: untreated loblolly pine hydrolysate;

4.3.2 Effect of temperature and pH on cysteine and glycine detoxification

It was interesting to find that the 20 amino acids had different degree of detoxification on the biomass hydrolysate. Cysteine and histidine with reactive nucleophilic side chains showed promising detoxification activity. On the contrary, the amino acids containing only primary amine showed less detoxification activity. This led us to the assumption that the primary amine group was probably less reactive towards the inhibitors and dependent more on the detoxification conditions. To obtain a better understanding, we further investigated the effects of temperature

and pH on cysteine and glycine detoxification, one represents each situation. For cysteine, the temperature was increased from 20, 40 and 60 to 80°C while keeping the pH at 6 for 2 h. The pH levels of 2, 4 and 6 were used while keeping the temperature at 60°C for 2 h. For glycine, the temperature was varied from 60 to 80°C while keeping the pH at 6 for 2 h. The pH was increased from 2 and 4 to 6 while keeping the temperature at 60°C. The results from cysteine showed that a change in the detoxification temperature or pH affected only the fermentation rates but not the final ethanol yields in the hydrolysates, whereas for glycine temperature or pH affected both the fermentation rates and final yields (Figures 21, 22 and 23). As shown in Figure 21, as the temperature was increased from 20, 40 and 60 to 80°C, the volumetric ethanol productivities at 6 h in cysteine-detoxified hydrolysates increased from 0.83, 1.19 and 1.77 to 1.90 g/L/h respectively, concurrently the sugar consumption rates increased from 2.03, 2.88 and 4.26 to 4.63 g/L/h. However, the final ethanol production and yield at these four detoxification temperatures were similar, with ethanol final concentrations of 23.42, 23.66, 23.14 and 23.12 g/L, and yields of 0.43, 0.43, 0.42 and 0.42 g/g, for 20, 40, 60 and 80°C, respectively. From the production curve, the ethanol production reached a plateau at 24 h when the detoxification temperature was at 40, 60 or 80°C, but it extended to 48 h when detoxified at 20°C. These data indicated that increasing the detoxification temperature for cysteine from 20 to 80°C increased the fermentation rates, but did not affect the final yields. Furthermore, when the cysteine detoxification was conducted at pH 2, 4 and 6, the resulting volumetric ethanol productivities at 6 h were 0.92, 0.92 and 1.77 g/L/h, while the ethanol final production reached 23.65, 23.9 and 23.14 g/L respectively (Figure 22), indicating the detoxification pH for cysteine also affected only the fermentation rates but again not the final yields.

As shown in Figure 23 from glycine detoxification, both temperature and pH affected the fermentation rate and final yield. Glycine detoxification of the hydrolysate at 60°C resulted in volumetric ethanol productivity at 6 h of 0.42 g/L/h and a final ethanol concentration of 8.58 g/L. However, when the detoxification temperature was increased to 80°C, the volumetric productivity improved to 1.68 g/L/h, 34.8% higher than that in the control, and the final ethanol production increased to 23.14 g/L, 2.7% higher than that of the control. This indicated temperature played an important role in the detoxification of inhibitors by amino acids with primary amine groups. Moreover, when holding the temperature at 60°C but dropping the pH to 4 and 2, glycine barely showed any detoxification effect, as shown in the final ethanol production of 2.70 and 2.11 g/L. These results indicated a neutral pH was important for the detoxification reaction to initiate.

In order to examine if high temperature helped to alleviate the inhibition of hydrolysate, we also treated the hydrolysate at 80°C and pH 6 for 2 h without addition of cysteine or glycine. It was found that a high temperature slightly improved the final production of ethanol from 1.16 in the untreated hydrolysate to 2.93 g/L, but the improvement was negligible compared to that in the cysteine or glycine detoxification at the same conditions (Figures 21 and 23). This indicated temperature probably accelerated the reactivity of cysteine and glycine towards the carbonyl inhibitors in the biomass hydrolysates, while temperature itself did not lead to the detoxification.

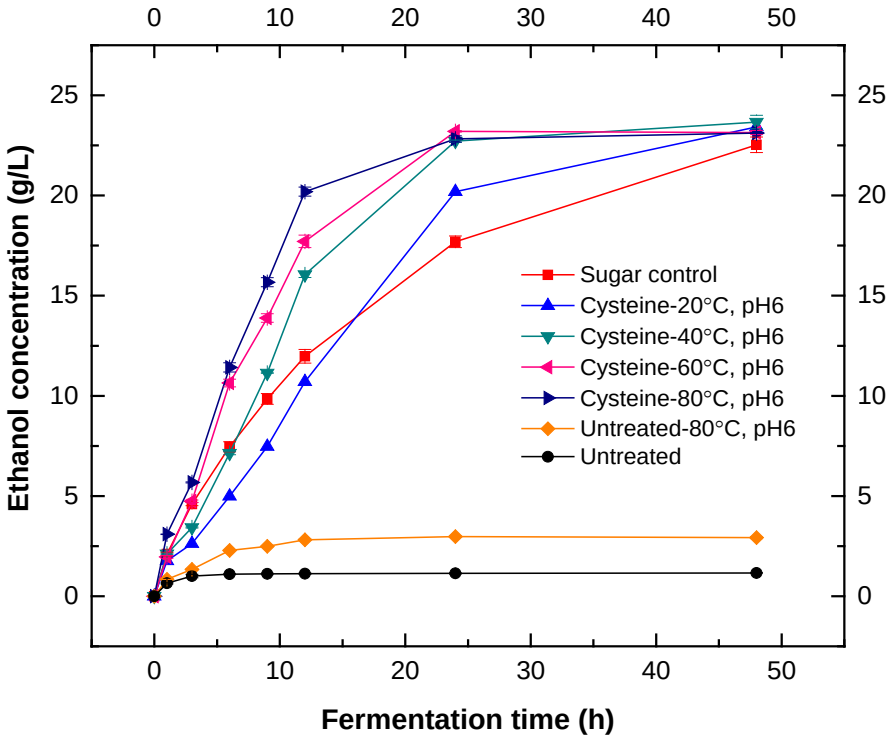


Figure 21: Ethanol production by *S. cerevisiae* of concentrated loblolly pine hydrolysate detoxified with cysteine at different temperature (cysteine: 0.2% (w/v), pH 6, 2 h) Untreated-80°C: loblolly pine hydrolysate detoxified at 80°C and pH6 without cysteine or other amino acids; Untreated: untreated loblolly pine hydrolysate;

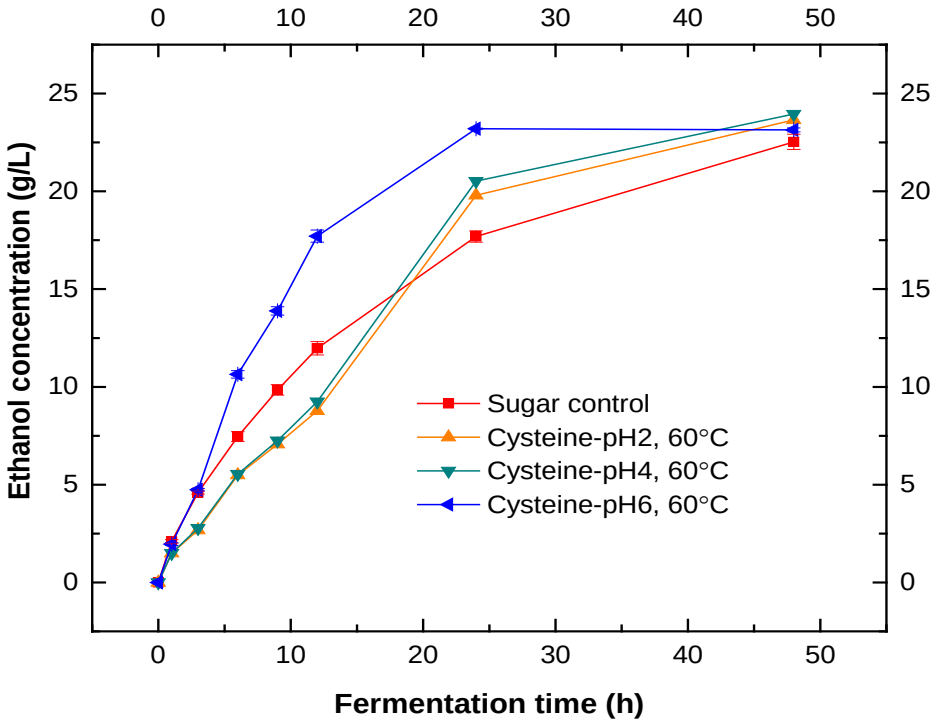


Figure 22: Ethanol production by *S. cerevisiae* of concentrated loblolly pine hydrolysate detoxified with cysteine at different pH (cysteine: 0.2% (w/v), 60°C, 2 h)

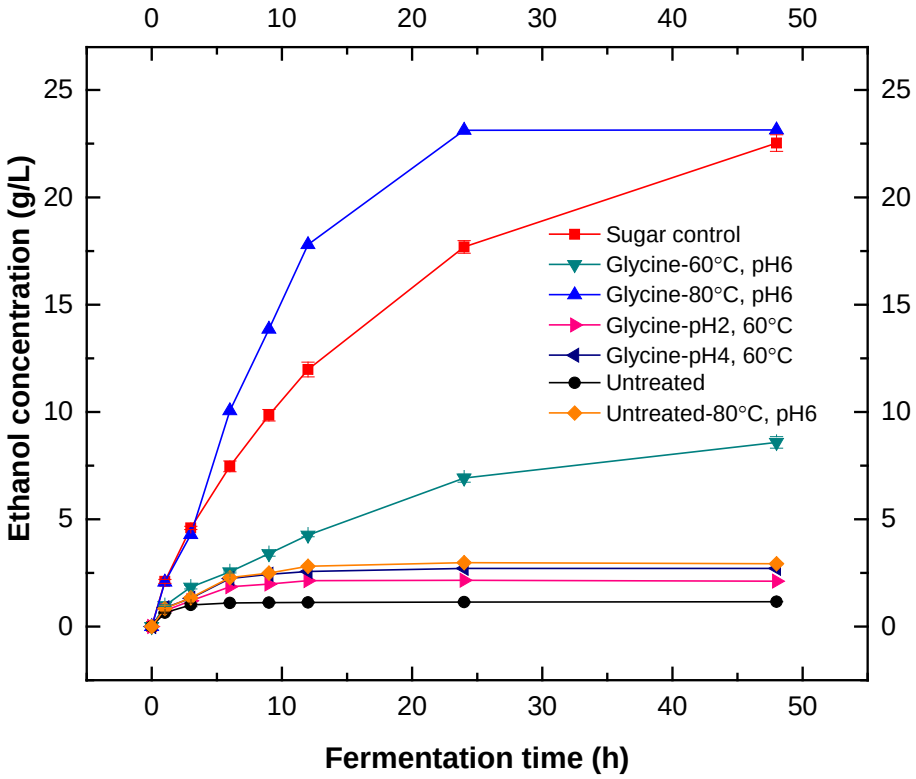


Figure 23: Ethanol production by *S. cerevisiae* of concentrated loblolly pine hydrolysate detoxified with glycine at different temperature and pH (cysteine: 0.2% (w/v), 2 h) Untreated-80°C: loblolly pine hydrolysate detoxified at 80°C and pH6 without glycine or other amino acids;

4.3.3 SEM image of yeast cells

SEM was further used to visually estimate the effects of untreated and cysteine-detoxified loblolly pine biomass hydrolysate on the healthiness of the yeast cells. Figure 24 shows the SEM images of *S. cerevisiae* cells 12 h after inoculation in the fermentation of sugar control solution (A), untreated loblolly pine hydrolysate (B) and cysteine-detoxified hydrolysate (C). Fermenting yeast cells were more round and plump, with smooth surfaces in image A, reflecting their healthy growth. In image B, where the yeast was inoculated in the untreated biomass hydrolysate medium, the cells showed different morphology. Cells appear to have less volume, many

wrinkles and compromised cell wall integrity, reflecting a sign of stressful growth condition, likely from general carbonyl toxicity targeting all aspects of cellular metabolism. Interestingly, healthy cells were observed in the hydrolysate detoxified with cysteine in image C, supporting the notion that the inhibitory compounds were removed and cells were less intoxicated. It was speculated the lignocellulose-derived phenolic inhibitors could cause loss of microbial cell wall integrity, thereby disabling their function as selective barriers and enzyme matrices (Palmqvist and Hahn-Hägerdal 2000). Many phenolic inhibitors contain carbonyl functional groups (Ando et al. 1986; Chen et al. 2006; Klinke et al. 2004). The fact that cysteine alone could effectively reverse the inhibition suggests that carbonyl functional groups of the phenolic compounds might be the major player in disrupting the cell membrane integrity and cell growth through electrophilic interaction, and their reactivity were depleted by reacting with cysteine. To view this from an opposite perspective, the antimicrobial effects of carbonyl compounds are often implied in the study of antibiotic drugs, in which the antimicrobial agents having carbonyl functional groups are referred to have irreversibly inactivated the biological nucleophilic sites, among which the cell wall polypeptides and membrane-bound enzymes are potential targets (Cowan 1999).

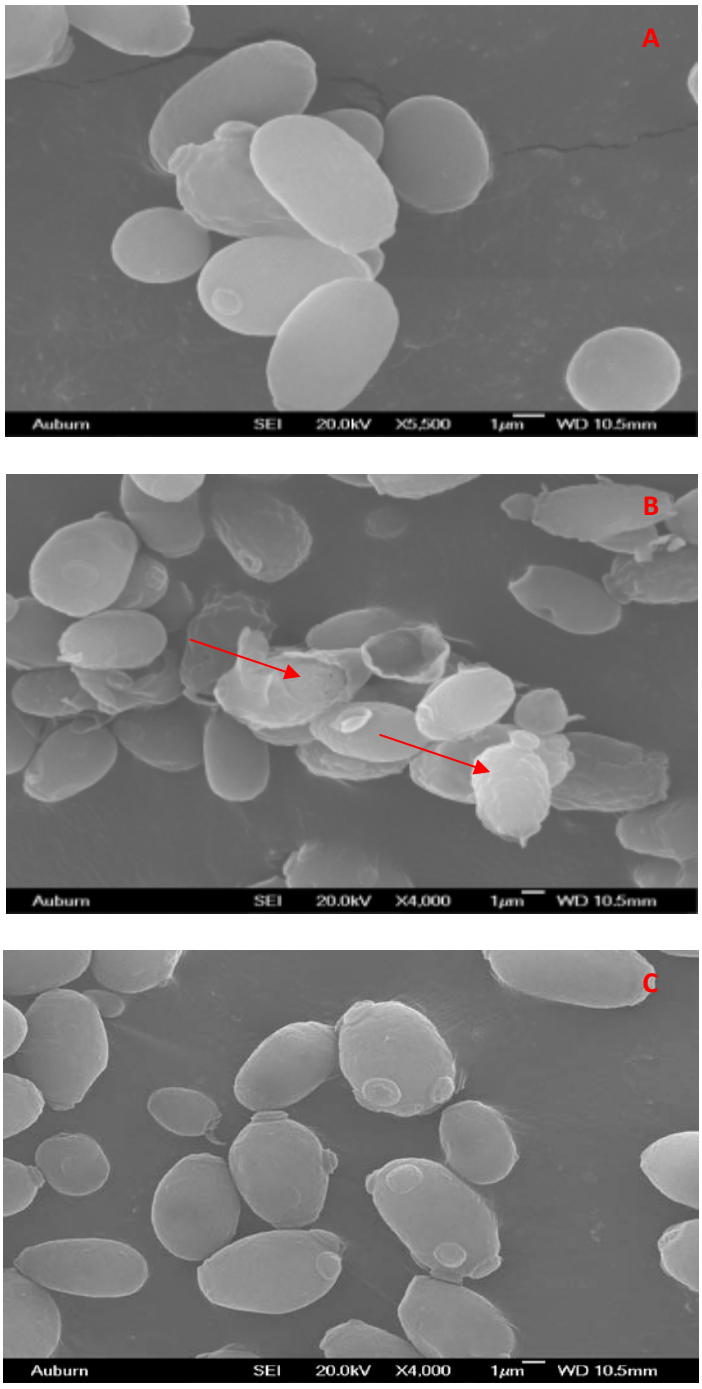


Figure 24: SEM images of *S. cerevisiae* cells at 12 h fermentation: A. control fermentation with sugar alone; B. untreated concentrated loblolly pine hydrolysate; C. concentrated loblolly pine hydrolysate detoxified with 0.2% (w/v) cysteine at 60°C and pH5 for 2 h.

4.3.4 Identification of potential detoxification products using a lignocellulose-derived carbonyl inhibitor cinnamaldehyde

As discussed earlier, amino acids detoxified the biomass hydrolysate through presumable nucleophilic reaction to the carbonyl inhibitors in the hydrolysate. To better understand the detoxification process, we could use a model inhibitor present in the biomass hydrolysate to identify the detoxification products. After screening a bunch of aromatic carbonyl inhibitors from the biomass hydrolysate, we selected cinnamaldehyde as a representative potent inhibitor for identifying any potential detoxification products. The effect of cinnamaldehyde on fermentation by *S.cerevisiae* containing 20 g/L of glucose was examined using vanillin as a comparison. Vanillin as a lignin degradation compound has been used as a model phenolic inhibitor in many previous studies (Larsson et al. 2000; Xie et al. 2012). Our results showed cinnamaldehyde was a considerably stronger inhibitor to the yeast fermentation than vanillin (Figure 25). In both the control and the solution containing 10 mM vanillin, ethanol production reached their plateau at 12 h, indicating vanillin had no inhibitory activity. On the contrary, cinnamaldehyde at 2.5 mM already showed complete inhibition to the ethanol production (Figure 25), indicating this compound was a stronger inhibitor than vanillin.

The detoxification effects of cysteine and glycine on cinnmaldehyde were investigated further using amino acid to inhibitor at 2.5:1. It was shown when detoxifying at 60°C, pH 6 for 2 h, cysteine showed a complete removal of cinammaldehyde inhibition. Both ethanol productivity and final yield reached a level higher than that in the control (Figure 26). However, glycine at 60°C, pH 6 for 2 h had barely any detoxification effect on cinnamaldehyde inhibition. Increasing the detoxification temperature to 80°C, it showed partial removal of the inhibition. The ethanol production had a lag phase for the first 24 h, after which the ethanol production was increased

and reached plateau at 48 h. These results were similar to the hydrolysate detoxification with cysteine and glycine, in which cysteine has completely removed the inhibition from the hydrolysate at 60°C while glycine detoxification required a higher temperature.

The possible mechanism for cysteine detoxification reaction with cinnamaldehyde was explored further using LC/MS tools where reaction products could be identified and quantified by their relative amount. As noted before, cysteine detoxification must have thiol group involved, any compound that contains sulfur could be the reaction products from cysteine. After analyzing the detoxified solution containing only cysteine and cinnamaldehyde to start with, two major peaks were present at high intensities with 236.07 and 357.09 ions ($[M+1]^+$). An elemental composition analysis of these two compounds revealed the formula of $C_{12}H_{13}NO_2S$ and $C_{15}H_{20}N_2O_4S_2$, with mass error at less than 5 ppm and matched isotope pattern. After a search of the literature and study of the reaction mechanism, we predicted these two compounds were the nucleophilic addition products of cysteine to cinnamaldehyde, with the mechanism depictured in Figure 27. We believed the thiol group in addition to the primary amino group has reacted with the carbonyl group on the cinnamaldehyde to form thiazolidine derivatives.

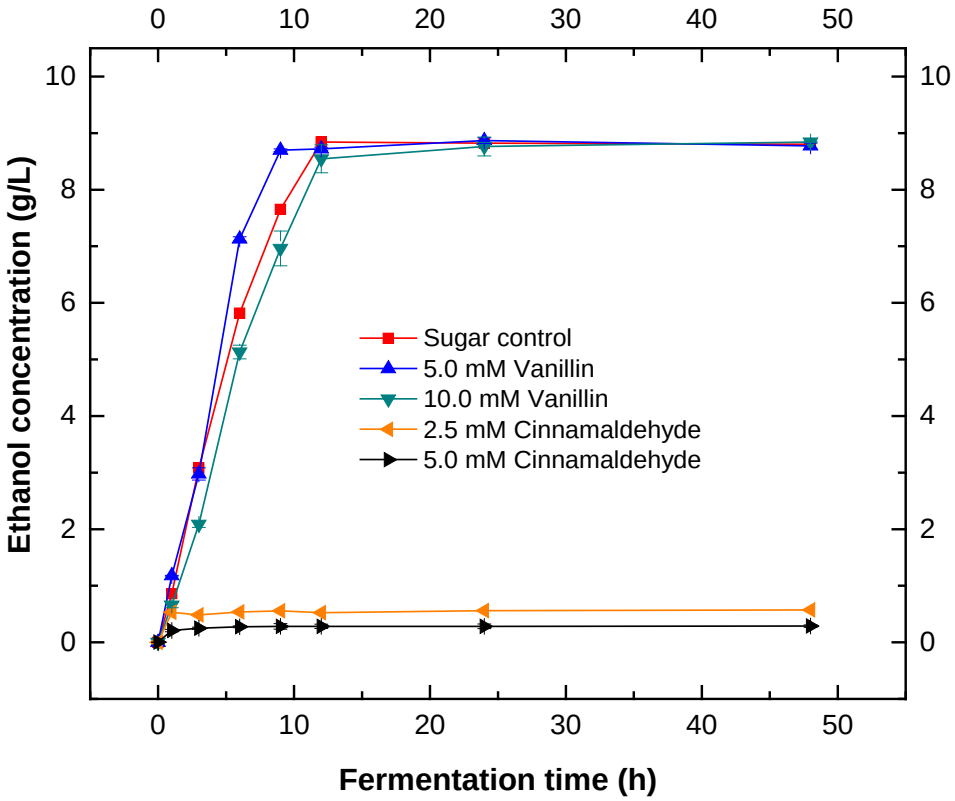


Figure 25: Effect of vanillin and cinnamaldehyde on ethanol production by *S. cerevisiae*

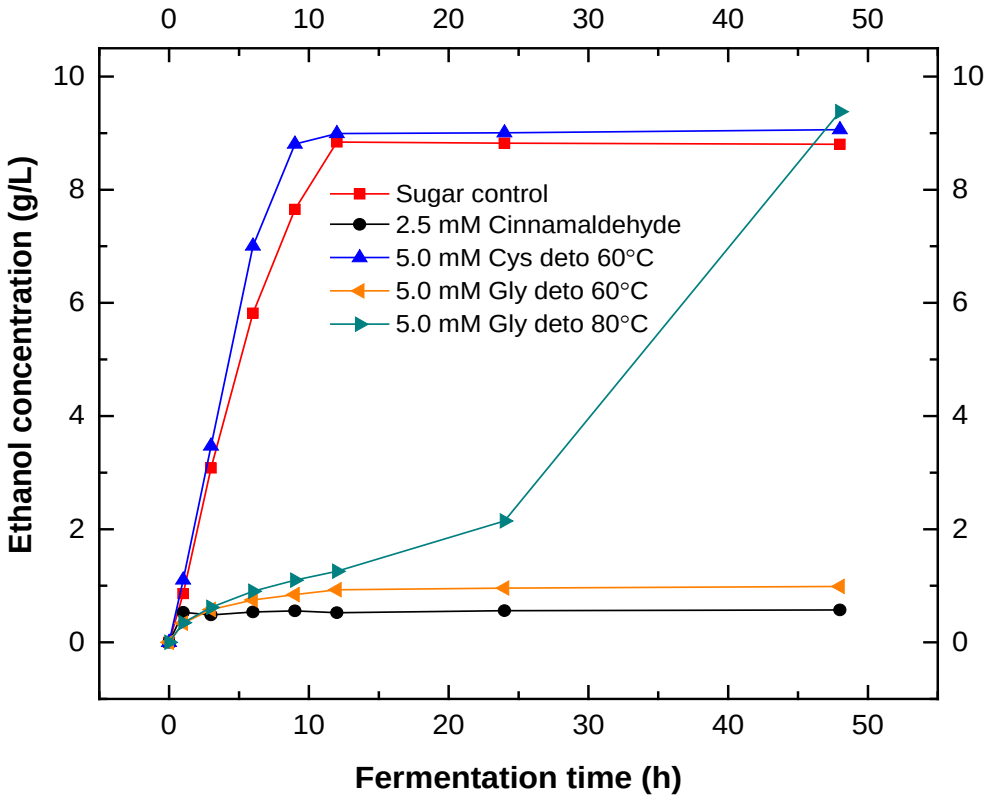


Figure 26: Effect of cysteine and glycine on cinnamaldehyde detoxification

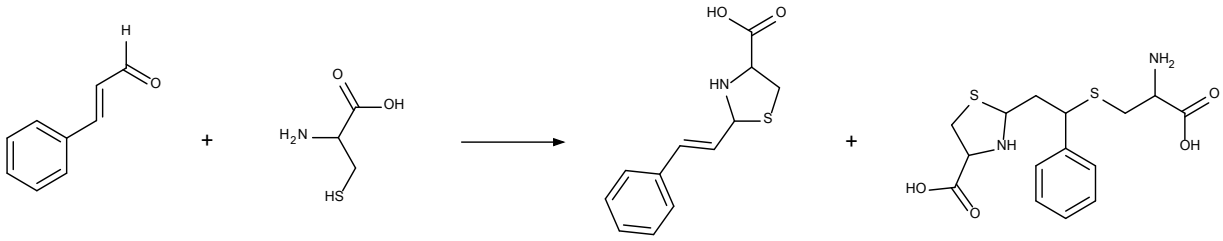


Figure 27: Possible detoxification products from cysteine reaction with cinnamaldehyde

4.4 Conclusion

A novel approach was presented to effectively detoxify a non-fermentable loblolly pine biomass hydrolysate and a carbonyl model inhibitor cinnamaldehyde in the hydrolysate for

ethanol production by *S.cerevisiae*, namely, amino acid detoxification. Among the 20 amino acids, cysteine had the highest detoxification activity. It increased the ethanol productivity at 6 h from 0.18 g/L/h in the untreated hydrolysate to 1.77 g/L/h (or 27% higher than the control without inhibitor), and increased the final yield from 0.02 to 0.42 g/g (the same as the control). Detoxification mechanism study using cinnamaldehyde as a representative inhibitor indicated the extraordinary detoxification of cysteine was probably attributed to its thiol side-chain group, which in addition to its amino group reacted with the aldehyde group to form thiazolidine derivatives.

Chapter 5: Substituent-related inhibition by benzaldehydes on fermentation and their quantitative structure-activity relationships

5.1 Background

The objective of this chapter is to study the inhibitory activities of benzaldehydes on yeast fermentation and their quantitative structure-activity relationships (QSARs).

The lignocellulose-related carboxylic acids and furans have been studied in great detail with regard to their effects on microbial growth and fermentation in the past decades. Although present in high concentration, these compounds were found to have minor inhibition on the microbial fermentation of the biomass hydrolysates (Palmqvist et al. 1999; Taherzadeh et al. 1997). On the contrary, lignin-derived aromatic aldehydes/ketones are believed to be more toxic than either their corresponding acid equivalents and the sugar-derived aliphatic acids, or the furan derivatives (Larsson et al. 2000; Zaldivar et al. 1999). The aromatic compounds are formed through oxidative cleavages of the ether bonds from lignin in thermochemical pretreatments. Due to the high polydispersity and rigidity of lignin, a wide range of low-molecular-weight aromatic compounds with a variety of substituents are formed at extremely low concentration after the pretreatments, including those of aromatic aldehydes/ketones. The fermentation inhibition of the aromatic aldehydes was often related to the type (mostly hydroxyl or methoxyl), the position and the number of the substituents (Friedman et al. 2003; Larsson et al. 1999b). However, the large number of the aromatics and their low concentration make the understanding of their contribution to inhibition difficult. Whether due to an individual compound or a group of compounds, the inhibition in biofuel production is not clearly understood.

Quantitative structure-activity relationships (QSARs) are concerned with linking the biological activity of inhibitors/toxins to their structural features. Structural properties are the

fundamental basis for the chemical reactivity of the compounds, which in turn controls their inhibitory actions towards biological molecules (Cao et al. 2014; Chan et al. 2008a; Chan et al. 2008b). The structural properties of chemicals are often characterized by their physicochemical descriptors, such as hydrophobicity (Log *P*), dipole moment (μ), energy of the lowest unoccupied molecular orbital (E_{LUMO}) and energy of the highest occupied molecular orbital (E_{HOMO}). The relationships between these molecular descriptors of the chemicals and their biological activities can help understand the inhibition mechanism of these compounds and predict their level of inhibition. The QSARs have been reported to be a useful tool in studying, and in assessing environmental toxicity contamination (Hansch 1971; Könemann 1981; Schwöbel et al. 2011)

In this section, we selected 13 representative aromatic aldehydes (benzaldehydes) and studied their fermentation inhibition by *S. cerevisiae* to address the questions: (1) how the substituents affected the inhibition of these benzaldehydes? (2) is there any correlation between physicochemical descriptors of the structural features of the benzaldehydes and their inhibitory activity. The QSARs attempted in this study could serve as a useful tool to predict the component of aromatic aldehydes that contribute significantly to the fermentation inhibition in biofuel production, and to guide an effective detoxification method targeting specific inhibitors, as well as in improving the stress-tolerance of yeasts.

5.2 Materials and methods

5.2.1 Chemical reagents and stock preparation

2-Hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 2-methoxybenzaldehyde, 2,3-dihydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde, ortho-phthaldehyde (OPA) and glucose (anhydrous) were obtained from Alfa Aesar (Ward Hill, MA). 4-Hydroxybenzaldehyde, 2,3,4,-

trihydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin) and glycerol were obtained from Acros Organics (Morris Plains, NJ). 3,4,5-Trihydroxybenzaldehyde and 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin) were obtained from TCI (Tokyo, Japan). 3,5-Dihydroxybenzaldehyde was obtained from Matrix Scientific (Columbia, SC). Benzaldehyde was obtained from Aldrich (Milwaukee, WI). Ethanol was obtained from Sigma-Aldrich (St. Louis, MO). All chemical reagents were purchased as chromatographic grades. 2-Hydroxybenzaldehyde, 2,3-dihydroxybenzaldehyde, *o*-vanillin and OPA were prepared in ethanol individually as 1M stock solutions and stocked at 4°C before use. The other benzaldehydes were used in fermentation directly.

5.2.2 Microbial fermentation

Batch fermentation was conducted in 125 ml sterile serum bottles containing 50 ml of 2% (w/w) glucose solution. No additional nutrients were added to the glucose solution. The fermentation was conducted at 30°C in a shaking incubator at 150 rpm. The glucose solutions were sterilized by an autoclave (Sanyo, MLS-3781L) at 115°C for 20 min. Benzaldehydes were added to sterilized glucose solution from their stock solutions or from the powders directly. After addition of the benzaldehydes, the glucose solution was incubated in a temperature-controlled water bath at 60°C for half an hour to dissolve the benzaldehyde powders. Each benzaldehyde was added at four concentration levels to examine their level of inhibition. Aliquots of 0.25 ml were withdrawn from the fermentation at times of 0, 1, 3, 6, 9, 12, 36 and 48 h for the time course analysis of metabolic flux by HPLC. All fermentations were run in duplicate batches.

5.2.3 Inhibitory activity

The inhibitory activities among the tested benzaldehydes on fermentation were evaluated by inhibition efficiency [*E*, (%)] and inhibition of yield [IY_{50}, (mM)]. *E* (%) was used to indicate the inhibition of tested benzaldehydes on fermentation rates. It was calculated as a decrease in the initial glucose consumption in the presence of 5 mM of tested benzaldehydes. The equation is shown below:

$$E(\%) = \frac{R_{SC} - R_S}{R_{SC}} \times 100$$

where R_S is the glucose consumption rate in the first 3 h of fermentation in the presence of 5 mM of selected benzaldehydes $R_s = (C_0 - C_3)/t$, C_0 and C_3 are the glucose concentration at 0 and 3 h respectively, and R_{SC} is the consumption rate in the first 3 h without inhibitor. The higher the E value, the higher the inhibitory activity.

IY_{50} was defined as the concentration of tested benzaldehydes that resulted in an ethanol final yield of 50% of the control (without inhibitor) at 48 h. In particular, the ethanol final yields in the presence of four concentration of each benzaldehyde were calculated as percentage of the control. These calculated yield percentages covered a range in which 50% was approximately the mid-point. The dose-response profiles of each benzaldehyde were fitted to linear relationships (yield percentages vs. concentration). The IY_{50} value was then estimated based on the linear regression relationships. The lower the IY_{50} value, the higher the inhibitory activity.

5.2.4 HPLC analysis

Glucose, ethanol and glycerol were analyzed by using a strong cation-exchange column (Aminex HPX-87H, 300×7.8 mm) of a HPLC system (Shimadzu) with a refractive index detector (RID-10A). The conditions were column temperature of 45°C, mobile phase of 5.0 mM

H_2SO_4 at 0.6 ml/min flow rate. All compounds were quantified by integrating their peak areas under the curve when they were eluted off the column in the chromatographic traces.

5.2.5 Calculation of physicochemical descriptors

E_{LUMO}, E_{HOMO}, μ and C'_{carb} (partial charge of the carbonyl carbon in the aromatic aldehydes) were calculated using the PM6 semi-empirical method (GaussView 5.0). Marvin sketch (6.1) was used to prepare the molecular structures of the examined aromatic aldehydes and to calculate the Log *P* and MR (Molecular refractivity). Multiple linear regressions (MLR, Origin 9.0) were used to calculate the correlations between the physicochemical descriptors and the inhibition efficiency. The coefficient of determination (r^2), the number of observations (n), the standard error of the estimate (s), the Fisher statistics (*F*) and the significance (*p*) were used in establishing the model.

5.3 Results and discussion

5.3.1 Effects of benzaldehydes on the fermentation by *S. cerevisiae*

In order to test the inhibition magnitude, four concentrations of each benzaldehyde (structures shown in Figure 28) were used. From the results, all benzaldehydes investigated showed a dose-dependent inhibition on the fermentation, while the inhibition severities of different benzaldehydes were quite different (Table 14). Without inhibitor, the initial ethanol productivity and glucose consumption rate (at 3 h) in the control were 1.27 and 3.71 g/L/h respectively; the final ethanol yield and glycerol production were 0.41 g/g and 0.76 g/L respectively. Overall, glucose was completely consumed and the ethanol production curve leveled off within 12 h. When the benzaldehyde (without substituent) was added to the fermentation medium, it slightly slowed the initial productivity but not the final yields at

concentrations of 5 mM and 10 mM. The ethanol production curves were comparable to that of the control, indicating benzaldehyde had no inhibition at these two concentrations. Increasing the concentration to 20 mM partially decreased both the productivity and final yield to 0.41 g/g and 0.36 g/g respectively. Further increasing the concentration to 40 mM resulted in almost complete inhibition to the ethanol production, with an ethanol yield of 0.03 g/g. Similar to this compound were 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde and vanillin, which at low concentration (5 and 10 mM) only slightly delayed the initial fermentation rates but not the final yields; while at higher concentration (20 and 40 mM) largely inhibited both the rates and yields. In the case of 3,4,5-trihydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde, both the fermentation rates and yields were barely inhibited at concentration ranges from 5 mM to 20 mM. When increased to 40 mM, 3,5-dihydroxybenzaldehyde extended the ethanol production curve to 24 h but did not affect the final yield; 3,4,5-trihydroxybenzaldehyde still showed no inhibition to both the rate and yield. These results indicated that these two benzaldehydes were not inhibitors even at high concentration. On the other hand, 2-methoxybenzaldehyde showed no inhibition at the concentration of 5 mM or less; while it led to significant inhibition at the concentration of 10 mM, suggesting it was a stronger inhibitor. Moreover, the inhibition increased considerably in the case of 2,4-dihydroxybenzaldehyde and 2,3,4-trihydroxybenzaldehyde, both of which showed strong inhibition at the concentration of 5 mM and 10 mM, and the inhibition did not disappear until the concentration was reduced to 1 mM. The inhibition further increased in the case of 2-hydroxybenzaldehyde, 2,3-dihydroxybenzaldehyde and *o*-vanillin, in which ethanol production was substantially inhibited at the concentration of 2.5 mM and 5 mM, and the inhibition did not disappear until the concentration was decreased to 0.5 mM. When it came to OPA, the inhibition was further increased a great deal, in which 1 mM completely inhibited

ethanol production and the inhibition did not disappear until the concentration was dropped to 0.02 mM.

To understand how the substituents affect the inhibition, benzaldehydes with different ortho-substituents, different positions of hydroxyl (OH) groups and different amount of OH groups were compared in parallel all at 5 mM. If two benzaldehydes led to the same ethanol production curve at 5mM, the concentration was further increased or decreased for comparison.

Table 14: Effect of different types of benzaldehydes on fermentation by *S. cerevisiae*

Compound	Conc. (mM)	R_S [a] (g/L/h)	Q_{EtOH} [b] (g/L/h)	Y_{EtOH} [c] (g/g)	C_{Gly} [d] (g/L)
Control		3.71±0.14	1.27±0.00	0.41±0.00	0.76±0.00
Benzaldehyde	40.0	0.52±0.14	0.16±0.00	0.03±0.00	0.03±0.00
	20.0	1.18±0.10	0.41±0.01	0.36±0.07	0.07±0.00
	10.0	2.29±0.04	0.89±0.01	0.46±0.00	0.12±0.00
	5.0	3.15±0.10	1.28±0.01	0.46±0.00	0.20±0.00
2-Hydroxybenzaldehyde	5.0	0.24±0.03	0.04±0.01	0.01±0.00	0.07±0.00
	2.5	0.33±0.03	0.11±0.00	0.02±0.00	0.08±0.01
	1.0	1.39±0.09	0.57±0.00	0.22±0.03	0.35±0.04
	0.5	2.99±0.09	1.24±0.03	0.42±0.00	0.64±0.00
3-Hydroxybenzaldehyde	40.0	0.32±0.06	0.08±0.00	0.03±0.00	0.17±0.00
	20.0	0.53±0.05	0.20±0.00	0.10±0.00	0.31±0.00
	10.0	1.60±0.11	0.49±0.14	0.42±0.01	0.41±0.01
	5.0	2.74±0.13	0.97±0.03	0.41±0.01	0.53±0.00
4-Hydroxybenzaldehyde	40.0	0.50±0.01	0.18±0.00	0.05±0.00	0.09±0.00
	20.0	0.97±0.12	0.37±0.00	0.18±0.00	0.2±0.00
	10.0	2.88±0.02	1.12±0.01	0.42±0.00	0.43±0.02
	5.0	3.20±0.00	1.02±0.06	0.40±0.00	0.47±0.02
2,3-Dihydroxybenzaldehyde	5.0	0.52±0.03	0.13±0.00	0.05±0.00	0.2±0.01
	2.5	0.61±0.08	0.22±0.01	0.06±0.00	0.26±0.00
	1.0	1.35±0.02	0.58±0.00	0.11±0.00	0.31±0.01
	0.5	2.49±0.02	1.10±0.01	0.45±0.00	0.81±0.01
2,4-Dihydroxybenzaldehyde	10.0	0.51±0.00	0.24±0.00	0.06±0.00	0.07±0.00
	5.0	0.71±0.16	0.30±0.00	0.07±0.00	0.15±0.00
	2.5	1.66±0.08	0.69±0.01	0.18±0.01	0.33±0.00
	1.0	3.16±0.07	1.24±0.01	0.44±0.00	0.67±0.00
2,3,4-Trihydroxybenzaldehyde	10.0	0.70±0.11	0.27±0.01	0.08±0.00	0.10±0.00
	5.0	1.20±0.09	0.57±0.01	0.17±0.00	0.21±0.00
	2.5	2.09±0.08	0.86±0.04	0.44±0.00	0.72±0.00
	1.0	3.02±0.13	1.24±0.02	0.44±0.01	0.82±0.00
3,5-Dihydroxybenzaldehyde	40.0	1.53±0.06	0.51±0.01	0.40±0.00	0.67±0.00
	20.0	3.43±0.04	1.29±0.00	0.41±0.00	0.76±0.01
	10.0	3.56±0.03	1.37±0.01	0.40±0.00	0.82±0.00
	5.0	3.96±0.07	1.52±0.02	0.41±0.00	0.82±0.00
3,4,5-Trihydroxybenzaldehyde	40.0	3.57±0.05	1.32±0.01	0.40±0.00	0.93±0.00
	20.0	3.84±0.09	1.42±0.01	0.40±0.00	0.95±0.00
	10.0	4.15±0.08	1.56±0.01	0.40±0.00	0.96±0.02
	5.0	4.12±0.04	1.41±0.02	0.38±0.00	0.80±0.06
2-Methoxybenzaldehyde	10.0	0.79±0.03	0.19±0.00	0.06±0.00	0.14±0.00
	5.0	2.19±0.05	0.81±0.03	0.43±0.00	0.43±0.00
	2.5	3.40±0.00	1.31±0.03	0.42±0.00	0.61±0.01

Vanillin	40.0	0.92±0.11	0.31±0.01	0.13±0.00	0.10±0.00
	20.0	1.16±0.07	0.46±0.00	0.22±0.00	0.14±0.00
	10.0	2.37±0.04	0.70±0.02	0.43±0.00	0.24±0.01
	5.0	3.11±0.00	0.99±0.04	0.42±0.00	0.41±0.02
o-Vanillin	5.0	0.46±0.06	0.08±0.03	0.05±0.00	0.15±0.01
	2.5	0.73±0.17	0.18±0.05	0.07±0.01	0.21±0.03
	1.0	1.49±0.12	0.57±0.04	0.43±0.00	0.48±0.06
	0.5	3.05±0.06	1.21±0.00	0.43±0.00	0.60±0.00
OPA	1.0	0.11±0.01	0.02±0.00	0.00±0.00	
	0.5	0.12±0.01	0.14±0.00	0.29±0.00	
	0.1	1.11±0.03	0.40±0.02	0.45±0.00	
	0.02	2.94±0.04	1.28±0.02	0.46±0.00	

[a]R_S, sugar consumption rate at 3 h. [b]Q_{EtOH}, volumetric ethanol productivity at 3 h. [c]Y_{EtOH}, final ethanol yield from total glucose at 48 h. [d]C_{Gly}, glycerol final concentration at 48 h..

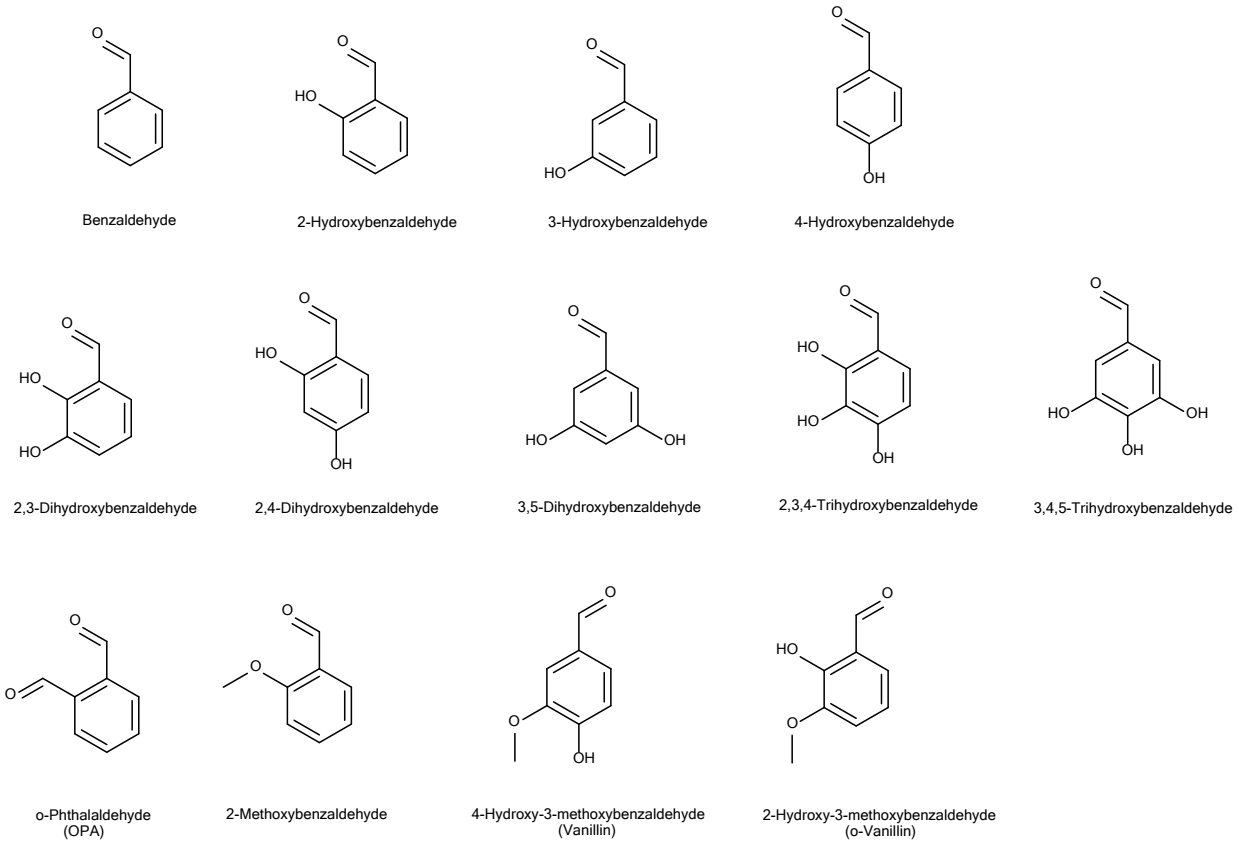


Figure 28: Structure of benzaldehydes tested

5.3.1.1 Fermentation inhibition by benzaldehydes with different ortho substituents

The substituents, including aldehyde (CHO), OH and methoxyl (OCH_3) groups, were selected because of their frequent appearance in lignin-derived aromatic compounds. The results showed that at the concentration of 5 mM, benzaldehyde without substituents showed no inhibition to the ethanol production (Figure 29). In fact, it increased the final yield by 12% as compared to the control. With an ortho-OCH_3 group, 2-methoxybenzaldehyde at 5 mM partially decreased the ethanol productivity and the ethanol production curve was extended to 24 h. However, the final yield of ethanol was not affected (0.43 g/g) (Figure 29). Interestingly, the ethanol production was almost completely inhibited when the ortho substituent was an OH or CHO group, in which the ethanol final yields were 0.01 and 0.00 g/g respectively (Figure 29). To further compare the inhibition levels of OPA and 2-hydroxybenzaldehyde, the concentration was decreased to 1 mM. It was shown that OPA at 1 mM still completely inhibited ethanol production (ethanol yield: 0.00 g/g). However, the inhibition of 2-hydroxybenzaldehyde was decreased at this concentration, in which the ethanol productivity and final yield were increased to 0.57 g/L/h and 0.22 g/g respectively (Figure 29). These results indicated the fermentation inhibitions of these benzaldehydes were: OPA > 2-hydroxybenzaldehyde > 2-methoxybenzaldehyde > benzaldehyde (no substituent).

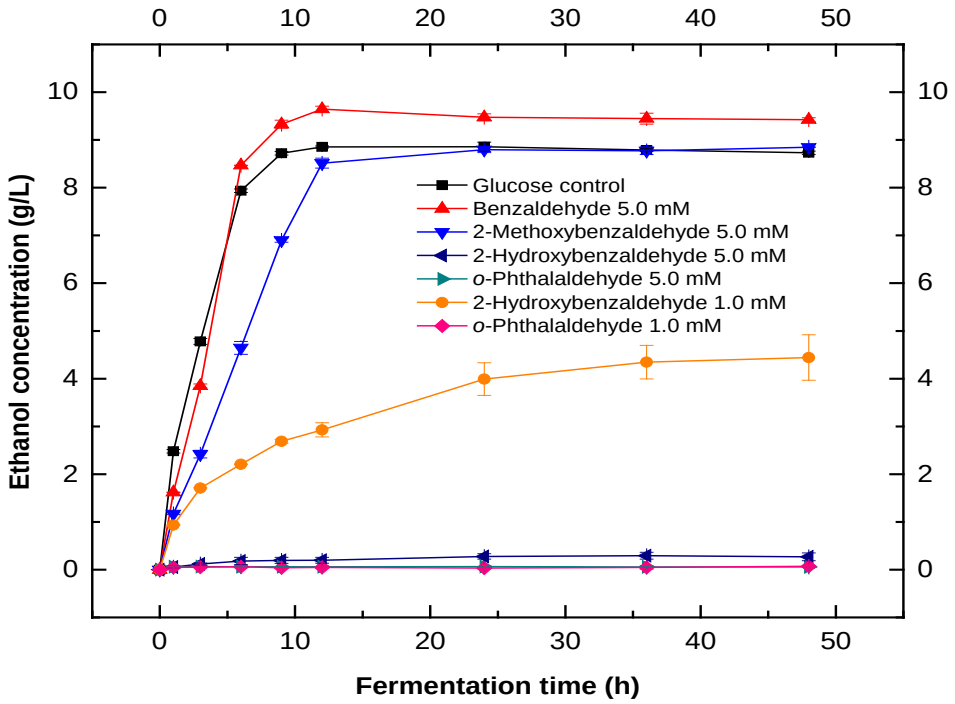


Figure 29: Effect of ortho substituents on the fermentation inhibition of benzaldehydes

5.3.1.2 Fermentation inhibition by benzaldehydes substituted with hydroxyl group at different positions

The OH group is the most common substituent in the benzene ring of lignin derived aromatic compounds. We have shown that addition of an OH group in the ortho position of the benzaldehyde led to a completely inhibition of ethanol production at 5 mM (Figures 29 and 30). However, when the OH group was shifted to the meta or para positions (3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde), they only slightly decreased the initial ethanol productivities but not the final yields. Overall, the ethanol production curves in the presence of 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde reached the plateau within 12 h, the same as the control (Figure 30). When the concentration was increased to 10 mM, 4-hydroxybenzaldehyde still showed no inhibition, with regard to both the ethanol productivity and

final yield (1.12 g/L/h and 0.42 g/g) (Figure 30). On the other hand, the ethanol production curve was extended to 24 h when 10 mM of 3-hydroxybenzaldehyde was used. The ethanol productivity was decreased to 0.49 g/L/h but the final yield was not affected (0.42 g/g) (Figure 30). This indicated that 3-hydroxybenzaldehyde was slightly more inhibitory than 4-hydroxybenzaldehyde. These results rank the inhibition of these mono-hydroxybenzaldehydes as: 2-hydroxybenzaldehyde > 3-hydroxybenzaldehyde > 4-hydroxybenzaldehyde. The effect of OH position was also observed in the case of vanillin isomers. With the OH group in the para position of the benzene ring, vanillin showed no inhibition at 5 mM. The ethanol production reached the plateau within 9 h, with a final ethanol yield of 0.42 g/g, 2.4% higher than that in the control (Figure 31). In the case of *o*-vanillin, where the OH group was in the meta position, the ethanol production was largely inhibited. The final yield was 0.05 g/g, only 12% of the control (Figure 31). Taken together, these results led to the conclusion that the ortho-OH group played an important role in increasing the inhibition of the benzaldehydes on yeast fermentation.

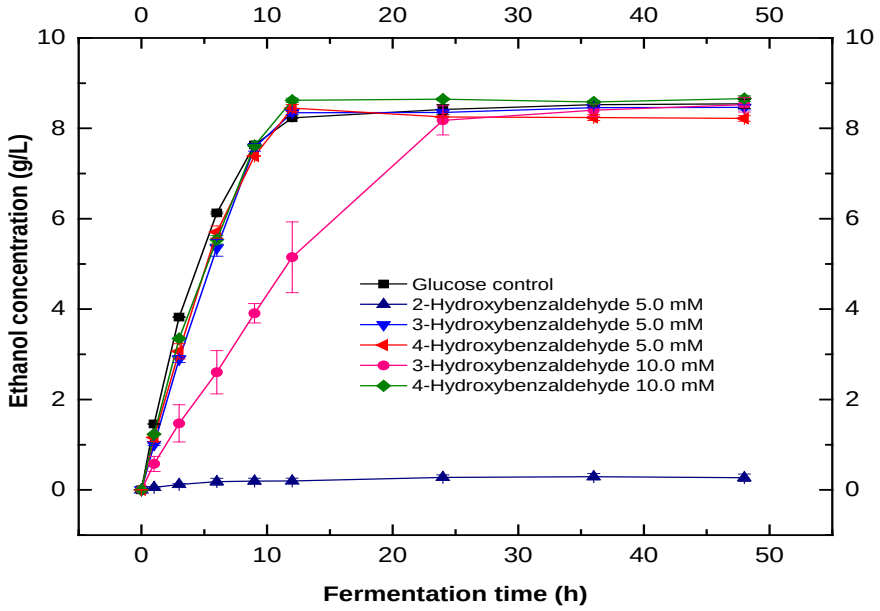


Figure 30: Effect of hydroxyl position of the benzaldehydes on the fermentation inhibition

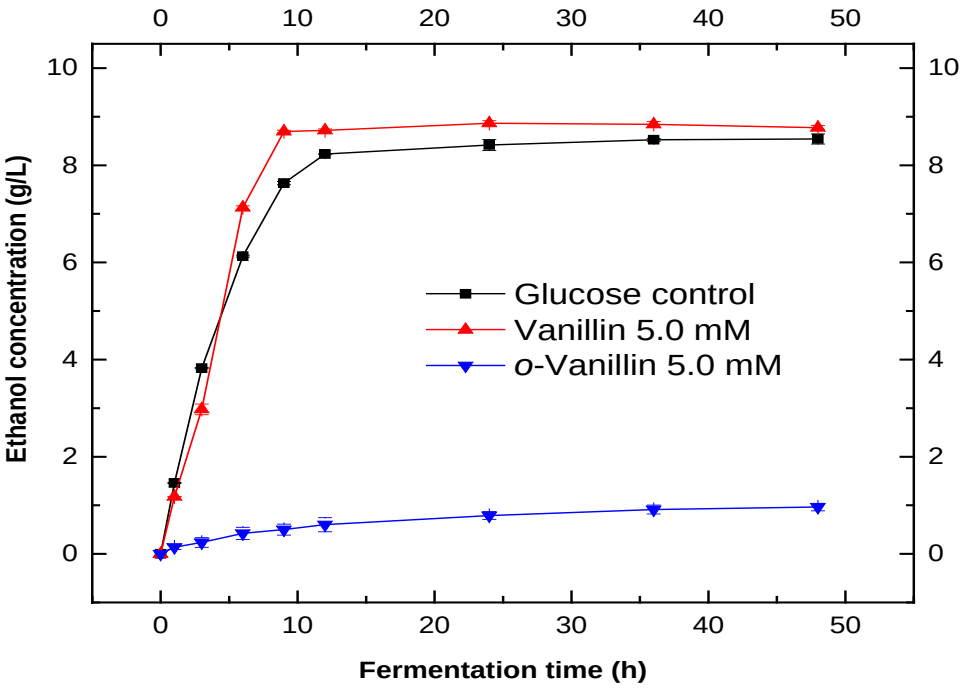


Figure 31: Effect of vanillin and *o*-vanillin on the fermentation inhibition

5.3.1.3 Fermentation inhibition by benzaldehydes substituted with different number of hydroxyl groups

In the previous section, we found that in the case of mono-hydroxybenzaldehydes, ortho-OH was more toxic than meta or para-OH with regard to their inhibition on the fermentation. In this section, we further examined the impacts of the number of OH groups on inhibition. Specifically, dihydroxybenzaldehydes including 2,3-dihydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde and 3,5-dihydroxylbenzaldehyde and trihydroxybenzaldehydes including 2,3,4-trihydroxybenzaldehyde and 3,4,5-trihydroxybenzaldehyde (Figure 28) were examined. Similar to 2-hydroxybenzaldehyde, 2,3-dihydroxybenzaldehyde and 2,4-dihydroxybenzaldehyde at 5 mM showed strong inhibition on ethanol production, both with

reduced ethanol final yields of 0.05 and 0.07 g/g respectively (Figure 32). On the contrary, although containing two OH groups, 3,5-dihydroxybenzaldehyde was not inhibitory to ethanol production at the same concentration (Figure 32). In the case of trihydroxybenzaldehydes, 2,3,4-trihydroxybenzaldehyde at 5 mM showed a considerable inhibition, in which an initial productivity of 0.57 g/L/h and a final ethanol yield of 0.17 g/g were obtained (Figure 32). In contrast, 3,4,5-trihydroxybenzaldehyde at the same concentration had no inhibition (Figure 32). Based on these results, it was suggested that an increase in the number of OH groups in the benzaldehydes did not increase the inhibition. It was ortho-OH group that increased the inhibition by hydroxybenzaldehydes.

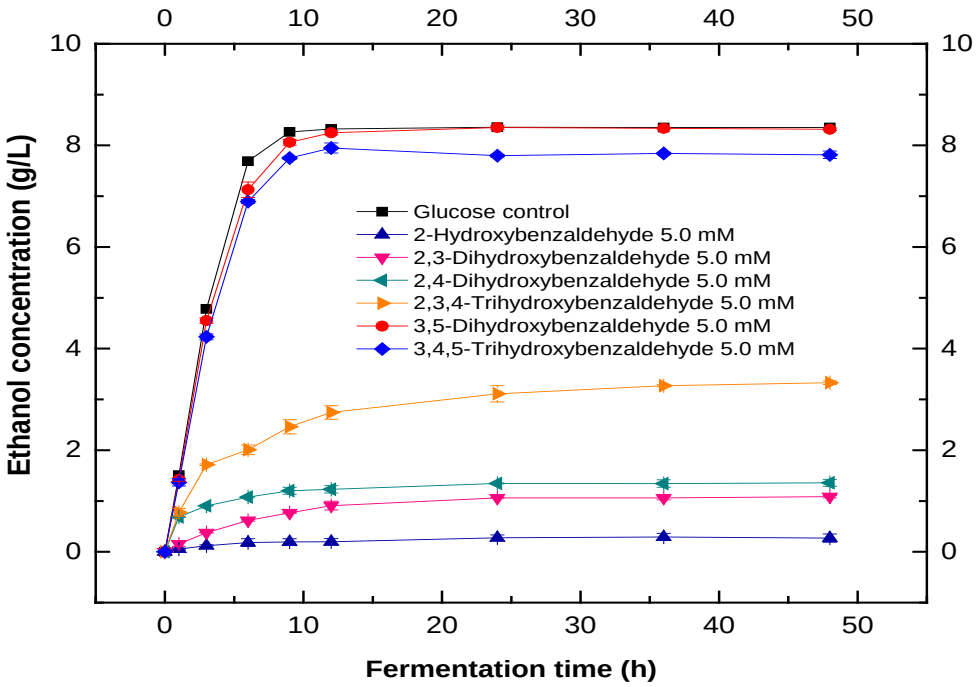


Figure 32: Effect of amount of hydroxyl groups of benzaldehydes on the fermentation inhibition

In summary, it was suggested that the inhibition of the benzaldehydes on yeast fermentation was related to their ortho-group (CHO > OH > OCH_3) and position of the OH

group in the benzene ring (*o*-OH > *m*-OH > *p*-OH). The inhibitory activities of the benzaldehydes were shown in Figure 33 and Figure 34. Interestingly, an ortho CHO or OH group could enhance the inhibition to a large extent while a meta or para OH group did not increase the inhibition. Similar results were also reported in previous studies. When studying the effects of different aromatic compounds on oxygen-limited growth and ethanol fermentation by *S. cerevisiae,* Larsson et al. found that 1.3 mM *o*-vanillin completely inhibited both cell growth and ethanol production while vanillin and isovanillin at the same concentration did not show any inhibition (Larsson et al. 2000). Also similarly, after evaluating the bactericidal activities of 35 benzaldehydes, 34 benzoic acids, and 1 benzoic acid methyl ester against *Campylobacter jejuni, Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella enterica,* Friedman et al found that the compounds that showed the most inhibitory activities were benzaldehdyes with ortho-OH groups, including 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2,3,4-trihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, 2,3-dihydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde, and 2-hydroxybenzaldehyde (Friedman et al. 2003). These were similar to our results, which all indicated the ortho-OH group could considerably enhance the microbial inhibition of benzaldehydes. More interestingly, we found that increase in the amount of OH groups decreases the inhibition. For example, the estimated IY_{50} of 2,4-dihydroxybenzaldehyde and 2,3,4-trihydroxybenzaldehyde were estimated to be 2.1 mM and 5.2 mM, much higher than that of 2-hydroxybenzaldehyde (0.9 mM). In addition, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde had estimated IY_{50} value at 14.8 and 18.6 mM respectively. However, the IY_{50} value of 3,5- dihydroxybenzaldehyde and 3,4,5-trihydroxybenzaldehyde could not be calculated because they both showed no inhibition on the fermentation even at 40 mM.

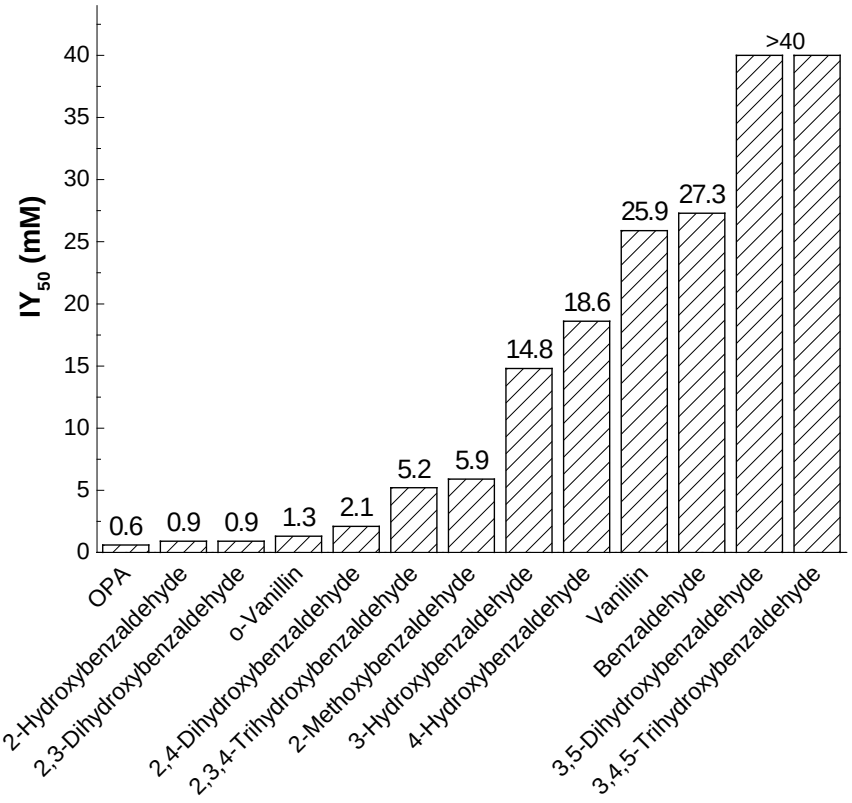


Figure 33: Estimated IY_{50} of the examined benzaldehydes

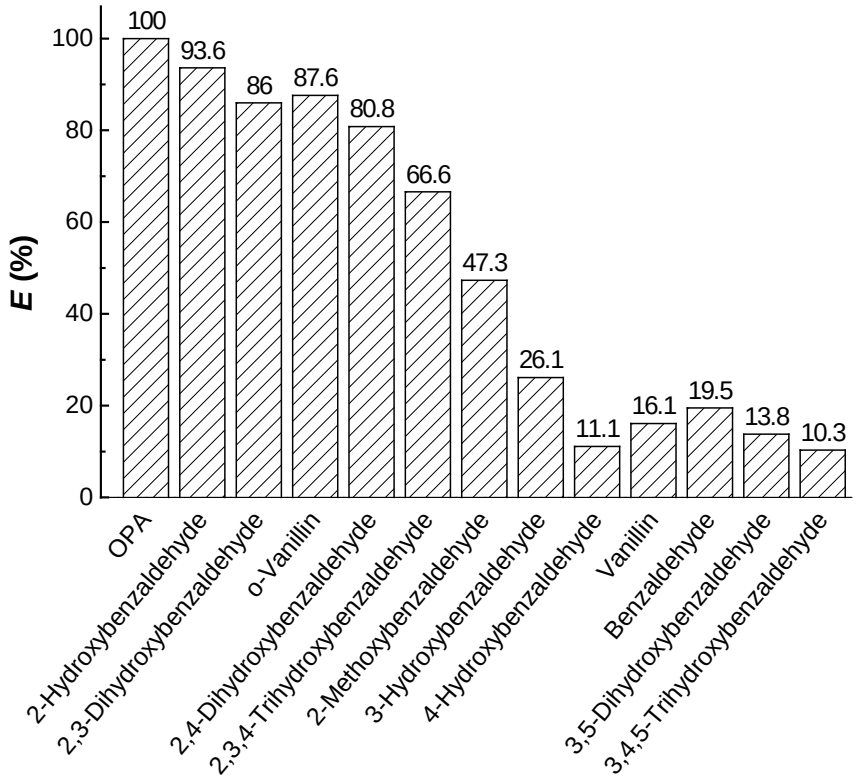


Figure 34: Inhibition efficiency (*E*) of the examined benzaldehydes

5.3.2 QSARs analysis

The inhibition efficiency of the 13 benzaldehydes in this study was correlated with their physicochemical parameters (Table 15). The correlations with good prediction were reported in Table 16. Among the six physicochemical parameters investigated, a linear regression was found between log *P* and fermentation efficiency (r^2=0.641, *p*=0.002). Log *P* is a measure of the hydrophobicity of the molecule, which is related to the availability of the compound to its targeting molecule. The correlation between Log *P* and inhibition efficiency indicated the compound with high hydrophobicity, or less soluble in aqueous environment, probably had a high diffusion rate through the cell membrane. OPA has a different story, due to its dialdehyde nature, OPA was more reactive than other benzaldehydes tested and made it a poor fit with the

model. A good correlation was also found between C'_{carb} and the fermentation efficiency (r^2=0.595, p=0.003) (Table 16). The partial charge is an atomic parameter that indicates the asymmetricity in the chemical bonds. In the benzaldehydes, the electronegativity of the oxygen draws electrons away from the carbonyl carbon, giving the carbon atom a partial positive charge and nucleophilic character. As a result, the correlation between C'_{carb} and the inhibition efficiency indicated the carbonyl carbon was probably the reactive site that initiated covalent bonding with nucleophilic targets in the yeast cells. On the other hand, we did not find good correlation between E_{LUMO} and the inhibition efficiency in a simple linear regression. E_{LUMO} is a global parameter representing the tendency of a molecule to accept electrons, in other words, the electrophilicity of the molecule. The poor correlation indicated electrophilic reactivity is not involved in inhibition by benzaldehydes. Interestingly, the relationship between E_{LUMO} and the fermentation inhibition of the benzaldehydes was established when E_{LUMO} and log P were used in a multiple linear regression (Table 16 and Figure 35). Benzene ring brings hydrophobicity which may control the diffusion of the compounds into plasma membrane of the microbial cell to reach its nucleophilic target.

Table 15: Molecular descriptors and inhibition efficiency of 13 benzaldehydes

	C'_{carb}	Log *P*	E_{LUMO} (eV)	E_{HOMO} (eV)	Dipole (Debye)	MR	Inhibition efficiency E (%)
Benzaldehyde	0.431	1.69	-0.88	-10.09	4.53	32.64	19.51
2-Hydroxybenzaldehyde	0.471	2.03	-0.91	-9.61	6.53	34.62	93.60
3-Hydroxybenzaldehyde	0.414	1.38	-1.06	-9.47	4.65	34.62	*26.10*
4-Hydroxybenzaldehyde	0.452	1.38	-0.85	-9.62	5.99	34.62	11.05
2,3-Hihydroxybenzaldehyde	0.458	1.73	-1.09	-9.23	6.75	36.60	86.02
2,4-Hihydroxybenzaldehyde	0.485	1.73	-0.83	-9.70	5.91	36.60	80.79
3,5-Hihydroxybenzaldehyde	0.404	1.08	-0.92	-9.44	6.04	36.60	13.79
2,3,4-Trihydroxybenzaldehyde	0.469	1.43	-1.11	-9.25	0.79	38.58	66.57
3,4,5-Trihydroxybenzaldehyde	0.410	0.78	-1.17	-9.56	5.05	38.58	10.31
2-Methoxybenzaldehyde	0.471	1.53	-0.87	-9.45	7.09	39.11	47.25
Vanillin	0.435	1.22	-1.01	-9.14	6.41	41.09	16.05
o-Vanillin	0.458	1.87	-1.05	-9.10	7.76	41.09	87.58
OPA	0.419	1.40	-1.40	-10.31	7.11	39.23	100.00

Table 16: Linear regression analysis between benzaldehydes' physicochemical descriptors and their inhibition efficiency

No.	Equation	n	r^2	r^2_{adj}	*s*	*F*	*p*	Outlier
1	E=0.774Log *P*-0.685	12	0.641	0.605	0.214	17.848	0.002	OPA
2	E=9.711C'_{carb}-3.871	12	0.595	0.555	0.227	14.709	0.003	OPA
3	E=0.919Log *P*-1.302E_{LUMO}-2.171	13	0.798	0.758	0.176	19.773	0.0003	
4	E=11.174C'carb-1.522E_{LUMO}-5.999	13	0.695	0.634	0.217	11.391	0.003	

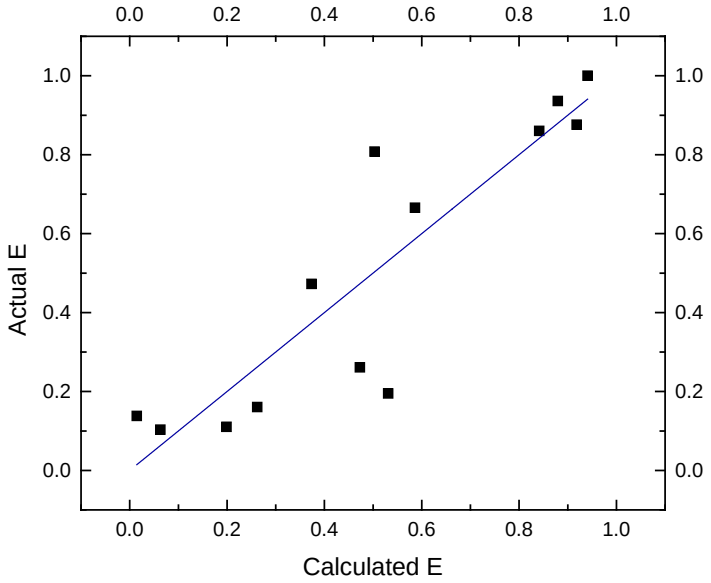


Figure 35: Scatter plot of experimental inhibition efficiency (*E*) vs calculated E using: E=0.919Log *P*-1.302E_{LUMO}-2.171 (r^2=0.798, p<0.05)

5.4 Conclusion

In this section, we found the fermentation inhibitory activity of thirteen benzaldehydes were closely related to their ortho-group (CHO > OH > OCH_3) and position of the OH group (*o*-OH > *m*-OH > *p*-OH) in the benzene ring. QSARs analysis indicated a strong correlation the between Log *P* of the benzaldehydes and their inhibition efficiency (r^2=0.641, p=0.002). This correlation indicated the compound with high hydrophobicity (less soluble in an aqueous environment) probably had high diffusion rate through the cell membrane to reach the biological targets.

Chapter 6: Future work

Targeting the right compounds is the key for developing a reliable procedure for identifying the inhibitors associated with biomass hydrolysate and a cost-effective detoxification approach to removing these inhibitors. As a result of literature summary and experimental screening, we hypothesized that aromatic aldehydes were a group of compounds that contributed to the main inhibition in biomass hydrolysates. Accordingly, we used *o*-phthalaldehyde as a potent model inhibitor to study the mechanism of alkaline detoxification. We found the detoxification might be an aldol condensation reaction between *o*-phthalaldehyde and a reducing sugar. This suggested we could use a reducing sugar as derivatization reagent to pull out carbonyl inhibitors in biomass hydrolysates by aldol condensation reaction. Using reducing sugar for derivatization has the following advantages: Firstly, the reaction product is easy to identify in LC-MS in the negative ion mode by the accurate mass measurement, so the unknown molecule will have a mass plus-sugar conjugate. Secondly, since the derivatization process is also a detoxification process, the information obtained from LC-MS analysis could be compared with the fermentation result of those with and without derivatization. Therefore, using reducing sugar as a derivatization reagent to specifically identify any potent carbonyl inhibition in biomass hydrolysate can be one of our future efforts.

We have successfully utilized amino acids as nucleophiles to detoxify a non-fermentable biomass hydrolysate and a lignocellulose-derived inhibitor cinnamaldehyde. Among the 20 regular amino acids, cysteine and histidine at 60°C, pH6 for 2 h completely detoxified the hydrolysate. Glycine representing amino acids with only primary amine group did not have the same detoxification activity at the same condition; it could not detoxify the hydrolysate until the

detoxification temperature was increased to 80°C. Similarly, cysteine at 60°C, pH6 for 2 h also completely detoxified cinnamaldehyde, while glycine at the same condition could not detoxify cinnamaldehyde. By using LC-MS analysis, we found outstanding detoxification by cysteine was probably due to its reactive thiol group, which in addition to its primary amine group, reacted with the aldehyde group in cinnamaldehyde to form thiazolidine derivatives. Further work in our group could continue on identifying the detoxification products of histidine and glycine with cinnamaldehyde. Histidine contains a secondary amine group, which probably also contributes to its outstanding detoxification activity. Also, it is interesting to investigate the temperature dependence of detoxification reaction between glycine and cinnamaldehyde.

We also studied the quantitative structure-activity relationships of thirteen benzaldehydes. We found the inhibition efficiency of the benzaldehydes on yeast fermentation was strongly correlated with Log *P*. We hope more aromatic aldehydes will be studied in the future to fine-tune the model.

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