**Detoxification of biomass hydrolysates with nucleophilic amino acids
enhances alcoholic fermentation**

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Abstract

Carbonyl compounds generated in biomass pretreatment hinder the biochemical conversion of biomass hydrolysates to biofuels. A novel approach of detoxifying hydrolysates with amino acids for ethanol production was developed. Among the 20 amino acids assessed for their detoxification efficiency and nucleophilicity, cysteine was the most effective one. It increased both ethanol productivity and final yield of biomass hydrolysates from 0.18 (untreated) to 1.77 g/L/h and from 0.02 to 0.42 g/g, respectively. Detoxification efficiency was followed by histidine and it increased the final yield to 0.42 g/g, then by lysine, tryptophan and asparagine. It was observed all five effective amino acids contained reactive side-chain functional groups, which played important roles in the amino acid detoxification reaction. The study further showed cysteine and glycine detoxifications were temperature and pH dependent. The mechanistic study using mass spectrometry revealed thiazolidine carboxylic acid, a Schiff base, was formed by condensation of aldehyde and cysteine.

Keywords: fermentation; detoxification; amino acids; hydrolysates; *Saccharomyces cerevisiae*.

1. Introduction

Producing biofuels from lignocellulosic biomass via biochemical conversion shows great promise for reducing the dependence on petroleum based fuels (Department of Energy, 2012; Soudham et al., 2014). Biomass typically needs to be pretreated prior to subsequent enzymatic hydrolysis to monomeric sugars and fermentation to biofuels by microorganisms such as *Saccharomyces cerevisiae* (Sun & Cheng, 2002). Pretreatment, however, generates a wide range of toxic compounds from the degradation of carbohydrates, lignin and extractives, which significantly inhibit microbial fermentation (Klinke et al., 2004; Palmqvist & Hahn-Hägerdal, 2000). Numerous studies have concentrated on identifying potential inhibitors and developing detoxification methods to remove their inhibition (Chen et al., 2006; Franden et al., 2013). However, due to the large number of degradation compounds and low concentrations, identification of the compounds those are the most potent to fermentation remains elusive. Consequently in the absence of correct targets, the detoxification or conditioning methods developed have not been cost effective or chemically selective.

In previous research, various physical, biological and chemical approaches have been developed to detoxify biomass hydrolysates (Nichols et al., 2010; Pienkos & Zhang, 2009; Soudham et al., 2014). Evaporation and steam stripping were used to remove the volatile compounds but not the non-volatile toxic compounds (Larsson et al., 1999; Leonard & Hajny, 1945). Ferrous sulfate and hydrogen peroxide recently have been used to detoxify spruce hydrolysates and improve fermentation yield (Soudham et al., 2014). Ammonium hydroxide has also been used to detoxify woody biomass hydrolysates, which resulted in better yield than overliming (Alriksson et al., 2005). Ligninolytic enzymes such as laccase and

peroxidase have been found to transform phenolic compounds presumably through
presumable oxidative polymerization but require long treatment times (12 h) and high costs
of enzymes. Bioabatement has been used to metabolize inhibitors from the dilute-acid
hydrolysis of biomass, but required long treatment times (24 h) (Nichols et al., 2008).
Activated charcoal and anion exchange resins can adsorb inhibitors through physical
interactions and have increased fermentability of hydrolysates, but both methods caused
considerable loss of sugars and increased the cost of the bioconversion process. Alkaline
treatment or overliming has been a widely-used method to detoxify biomass hydrolysates, but
the gypsum salts and precipitates produced during the detoxification step could be significant
issues for subsequent processes. Although alkaline treatment has been one of the simple and
effective methods to remove the toxic compounds in hydrolysates, it was also reported the
total sugar loss was up to 11% by overliming treatment (Hodge et al., 2009). This would
make the alkaline treatment less economic for inhibitors removal. Moreover, failing to target
the most potent inhibitors instead of randomly removing the degradation compounds is not a
cost-effective approach to improve the hydrolysates fermentability.

Correlating the inhibitory activity of toxic degradation compounds to their structural
features could help to design a cost-effective detoxification approach for biomass
hydrolysates. Structural properties are the fundamental basis for chemical reactivity of
compounds, which in turn governs their inhibitory actions towards biological molecules
(Chan et al., 2008; Schwöbel et al., 2011). Previous studies indicated most of the identified
inhibitors associated with biomass hydrolysates are carbonyl compounds (Chen et al., 2006;
Klinke et al., 2004), such as carboxylic acids-related acetic acid, ferulic acid and syringic acid,

sugar-derived furfural and hydroxymethyl furfural (HMF) and lignin-derived aromatic aldehydes and ketones (syringaldehyde and coniferyl aldehyde) (Larsson et al., 2000). Among them, the aromatic aldehydes with a carbon-carbon double bond (C=C) next to a carbonyl group are α,β -unsaturated compounds such as cinnamaldehyde and coniferyl aldehyde (Klinke et al., 2004). Carbonyl compounds are electrophilic with the high electronegativity of oxygen relative to carbon, polarizing the carbon-oxygen double bond and creating a partially positive charge on the carbonyl carbon atom. The electron-poor carbonyl carbon could form covalent bonds with biological nucleophiles such as proteins and nucleic acids, leading to inhibition of protein functions, DNA duplication, or even loss of cell activity (Schwöbel et al., 2011). In the case of α,β -unsaturated aldehydes, the electronegative oxygen atom in the carbonyl group can also withdraw electrons from the β carbon, polarizing it and making it more electrophilic than a regular alkene carbon. Therefore, α,β -unsaturated aldehydes have two reactive functional groups that can participate individually or cooperatively in a series of reactions with nucleophiles in microbial cells (Chan et al., 2008; Chan & O'Brien, 2008). Consequently, inactivation of these electrophilic groups may be the key for removing the fermentation inhibition of carbonyl compounds. Based on literature reports and the preliminary study, it can be hypothesized that the inhibitory effects of carbonyl compounds are controlled by their electrophilic functional groups, which can be detoxified by reacting with nucleophiles such as amino acids. Most amino acids contain a primary amine group and important side chain functional groups such as the thiol in cysteine. These functional groups can react readily with electrophilic carbonyl groups, thus detoxifying the carbonyl compounds. Therefore, it is believed highly nucleophilic amino acids (such as

cysteine) are prospective candidates for the selective and environmentally benign detoxification of biomass hydrolysates.

The objective and originality of this study was to design carbonyl-based selective chemical reactions for detoxifying biomass hydrolysates to improve ethanol or butanol fermentation.

The detoxification efficiency of amino acids for biomass hydrolysates was investigated and their potential detoxification mechanisms were determined. The fermentability of biomass hydrolysates detoxified with 20 individual amino acids was compared along with the untreated hydrolysates based on their volumetric ethanol productivity in the first 6 h and final ethanol yield from glucose and mannose at 48 h. The effects of temperature and pH on the detoxification of biomass hydrolysates were also examined. Cinnamaldehyde, a model inhibitory compound, was used to elucidate the potential reaction mechanisms between a carbonyl aldehyde and cysteine. Mass spectrometry was used to identify all major detoxification products and a possible detoxification mechanism was proposed.

2. Materials and methods

2.1. Pretreatment of lignocellulosic biomass

Loblolly pine (*Pinus taeda*) wood chips (1.0×1.0 cm, L $\times$ W) were collected at the Forest Products Laboratory at Auburn University. The chemical composition of untreated biomass is: 41.3% glucan, 12.2% mannan, 6.3% xylan, 2.2% galactan, 1.3% arabinan, 28.7% lignin and 1.6% extractives. Dilute acid pretreatment was used to produce biomass hydrolysates in a 4.0 L Parr batch reactor (Parr Instrument Co., Moline, IL) as previously described (Lai et al., 2014). Briefly, wood chips (400 g, dry weight) were soaked in 1% (w/w) sulfuric acid overnight (7:1 liquor/solid ratio) prior to the pretreatment, and then loaded into the reactor and treated at 170 °C for 60 min. After pretreatment, the resulting slurry was separated into a solid fraction and a liquid fraction by filtration, and the biomass hydrolysates (liquid fraction with an initial pH of 1.8) was collected for further study.

To achieve higher ethanol titer in fermentation, loblolly pine hydrolysates were concentrated to approximately one-third of the original volume using a rotary evaporator (IKA RV10 basic) at 40 °C and 60 rpm, with the pH adjusted to 4.0 before evaporation. After the concentration, the sugar contents of the hydrolysates were analyzed by HPLC with a refractive index detector. The hydrolysates contained 90.3 g/L of total sugars with 16.8 g/L glucose, 38.3 g/L mannose, 29.2 g/L xylose and 6.0 g/L arabinose. The total fermentable glucose and mannose are 55.1 g/L. With regard to the sugar degradation compounds, the concentrations of formic acid, acetic acid, levulinic acid and HMF were 2.1, 5.5, 0.4 and 3.7 g/L respectively. Furfural was completely removed during the evaporation. The concentrated hydrolysates (called hydrolysates hereafter) were not fermentable and used for all the

detoxification and fermentation processes in this study.

2.2. Chemicals and amino acids

Glucose, mannose, galactose, xylose and arabinose were obtained from Fluka (Milwaukee, WI) and Alfa Aesar (Ward Hill, MA). Amino acids 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 (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 stock solutions were protected from light and kept at 4°C and used within 1 month.

2.3. Detoxification of biomass hydrolysates with amino acids

The biomass hydrolysates (50 mL) were treated with the 20 amino acids (0.2% w/v) individually at 60 °C and pH 6.0 for 2 h. The detoxification experiments were performed in a 250 mL flask, placed in a temperature-controlled water bath. To investigate the effect of temperature on detoxification efficiency, the biomass hydrolysates were treated at pH 6.0 with cysteine or glycine (0.2% w/v) under various temperatures (20, 40, 50, 60, 70, and 80 °C). To investigate the effect of pH, the biomass hydrolysates were treated with cysteine or glycine at 60 °C and various pHs (2.0, 4.0, 6.0 and 8.0). Detoxified hydrolysates were cooled to room temperature and then readjusted to pH 6.0. Similarly for the model compound

(cinnamaldehyde) detoxification, glucose (~20 g/L) was mixed with 2.5 mM cinnamaldehyde, and then treated with 5 mM cysteine or glycine at 60 °C for 2 h prior to fermentation. A mixture of glucose (17.7 g/L) and mannose (35.1 g/L) without inhibitors was used as the positive sugar control for the fermentation.

2.4. Microbial fermentation

Baker yeast (Fleischmann's), *S. cerevisiae*, was used for the fermentation experiments. The yeast was maintained on YDP medium containing (g/L): 20 glucose, 20 peptone, 10 yeast extract and 20 agar. **Single** colony was cultured in YDP liquid medium overnight and harvested as the fermentation inoculum. The yeast concentration was measured using an UV-vis spectrophotometer. The initial yeast inoculation was 2.0 g/L in fermentation. Batch fermentation was carried out in 125 mL serum bottles containing 50 mL broths (untreated or detoxified hydrolysates, untreated or detoxified cinnamaldehyde sugar solution) without any supplemental nutrients. All the fermentation broths were adjusted to pH 6 with NaOH or H₂SO₄ and filtered through 0.2 µm sterile filters. After inoculation, the serum bottle was sealed with a rubber stopper and an aluminum seal, and cannulas **were attached** for CO₂ 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 products. **Fermentation and detoxification** were carried out in duplicate. The volumetric ethanol productivity and initial consumption rate of glucose (̑G) and mannose (̑M) were calculated based on the sugar consumed in the first 6 h of fermentation as described previously (Cao et al., 2014).

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 strong cation-exchange column (Aminex HPX-87P, 300 × 7.8 mm) of a HPLC system with a refractive index detector (RID-10A). The column temperature was maintained at 85°C, and nanopure water was the mobile phase at 0.6 ml/min for a 35 min isocratic run. Ethanol, acetic acid, levulinic acid, formic acid, furfural and HMF were determined using an Aminex HPX-87H column (300 × 7.8 mm) chromatography with a refractive index detector. The column was kept at 45°C, mobile phase with 5.0 mM H₂SO₄ was at 0.6 mL/min 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 the positive and negative ion mode operated by the Masslynx software (V4.1).

In a loop injection without a column, each sample, in H₂O, was injected directly into the ion source and spectra were acquired. With column, each sample was injected onto a C18 column (ACQUITY UPLC® BEH C18, 1.7 μm, 2.1 × 50 mm, Waters) with a 150 μL/min flow rate of mobile phase of solution A (95% H₂O, 5% acetonitrile, 0.1% formic acid) and solution B (95% acetonitrile, 5% H₂O, 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 ion 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 centroid 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 μ L/min was used.

MS/MS of product ions of interest, or the parent ions, was acquired M+1 or M-1 of its accurate mass, and entered as the Set Mass for the MS/MS fragmentation. Collision energy, the kinetic energy of argon gas, was adjusted so the daughter ions produced have similar intensity, with mass range up, to the parent ion.

3. Results and discussion

3.1. Detoxification of biomass hydrolysates with 20 amino acids

It is hypothesized that strong nucleophilic amino acids (such as lysine, cysteine and histidine) can react readily with carbonyl aldehydes and ketones through nucleophilic addition and thus detoxify the hydrolysates. To examine this hypothesis, the biomass hydrolysates were detoxified with 20 amino acids (Table 1) individually at 60°C and pH 6 for 2 h initially and the glucose and mannose consumption rates, ethanol productivity, final concentration and ethanol yields were determined. The results showed five amino acids have improved the fermentability of biomass hydrolysates significantly (Table 1). Detoxification with cysteine and histidine have produced 23.14 and 23.07 g/L ethanol respectively at 48h, and detoxification with tryptophan, asparagine and lysine generated 11.59, 11.18 and 14.40 g/L ethanol respectively, as compared to 1.16 g/L ethanol in the untreated biomass hydrolysates.

Detoxification with these five amino acids has also improved the glucose and mannose consumption rate significantly (Table 1). Cysteine, histidine, lysine, tryptophan and

asparagine have increased the glucose consumption rate from 0.11 g/L/h (untreated) to 2.10, 0.94, 0.80, 0.69, and 0.65 g/L/h, respectively, and increased the mannose consumption rate from 0.34g/L/h (untreated) to 2.16, 1.02, 1.09, 0.89 and 0.87 g/L/h respectively. Compared with the positive sugar control, cysteine detoxification has enabled a faster sugar consumption rate, especially for mannose, which was improved by 43%. This agreed with the previous findings about effect of sodium acetate salts on yeast fermentation (Cao et al., 2014). Because the hydrolysates were neutralized by NaOH, considerable amount of salts (sodium acetate and sodium sulfate) were presented in the hydrolysates with amino acids; thus sodium acetate and sodium sulfate activated cell membrane permeability and increased glucose consumption (Kirk & Doelle, 1992). Consequently, the volumetric ethanol productivity was increased from 0.18 to 1.77 g/L/h by cysteine treatment, which was 27% higher than the positive sugar control (1.39 g/L/h). This indicated that cysteine was the most effective detoxifying amino acid. This was further confirmed by the highest final ethanol yield (0.42 g/g) after 48 h fermentation (Fig.1a). The second most effective amino acid was histidine, which resulted in the same final ethanol yield, but the sugar consumption rates (0.94 and 1.02 g/L/h for glucose and mannose) were 50% lower than those from cysteine detoxification (Table 1 and Fig. 1b). In addition, detoxification with these five amino acids (cysteine, histidine, lysine, tryptophan and asparagine) resulted in a significant increase of HMF consumption (39.20-96.40%) with cysteine the most effective (Fig.1c).

The remaining 15 amino acids had minor improvement in ethanol yield (<0.10 g/g) except for glycine, which had a higher ethanol yield at 0.16 g/g. Based on the increase in ethanol yields, amino acids could be characterized into four groups. The most effective amino

acids were cysteine and histidine (0.42 g/g), followed by tryptophan, asparagine and lysine (0.20-0.26 g/g). The third group was glycine (0.16g/g). All the other amino acids showed marginal improvement in fermentation of biomass hydrolysates.

Detoxification efficiency of effective amino acids can be related to the side chain functional group. They are the thiol group of cysteine, the imidazolyl group of histidine, the ϵ -amino group of lysine, the indolyl group of tryptophan and the carboxamide group of asparagine (Tischer & Wedekind, 1999). The thiol group of cysteine has been reported as the most potent nucleophile among amino acids. Previously, Brotzel and Mayr found cysteine had a much higher nucleophilicity parameter ($N = 23.43$) than all the other amino acids when they compared with the nucleophilicities of 16 amino acids based on their reactions with electrophilic benzhydrylium tetrafluoroborates (Brotzel & Mayr, 2007). The high nucleophilicity of cysteine was attributed to its thiol group, which exceeded the reactivity of the primary amine groups by a factor of 10^4 (Brotzel & Mayr, 2007). Similar results were also reported where nucleophilic reactivity of amine and thiol groups were assessed by reacting with α,β -unsaturated compounds individually (Friedman et al., 1965), in which the thiol group was around 280 times more reactive than amine group. These agreed with the finding that cysteine completely detoxified hydrolysates, resulting in a higher fermentation rate and yield than all the other amino acids. This indicated the thiol group side chain in cysteine played a critical role in detoxifying reactive carbonyl compounds in the hydrolysates. Interestingly, it was observed that histidine also exhibited strong detoxification efficiency with the same ethanol yield as cysteine detoxification. 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 dipeptides detoxified aldehyde compounds in biological cells, as aldehyde scavengers (Xie et al., 2013). Therefore, the favorable detoxification effect of histidine can probably be attributed to its high nucleophilicity of imidazolyl group. 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 (Xie et al., 2013). The study suggested that cysteine, histidine, and lysine were the top three amino acids for biomass hydrolysates detoxification, because they contain a secondary nucleophilic functional group in addition to the primary amine group.

Tryptophan has been reported to react readily with phenolic aldehydes in biological systems with its indole group. Asparagine with a carboxamide group can react with carbonyl compounds to form acrylamide in the maillard reaction (Mottram et al., 2002). It was believed the side chain functional groups played more important role than the primary amine groups in detoxification, because a correlation between pK_a of the primary amine group and their detoxification activity was not observed ($r^2 < 0.01$). Similarly, when comparing the nucleophilicity parameters of the primary amine groups in amino acids, Brotzel and Mayr suggested the difference were not significant ($12.7 < N < 14.1$) (Brotzel & Mayr, 2007).

Although cysteine and histidine have successfully detoxified hydrolysates, they did not react with or remove sugar degradation compounds in the detoxification process. The concentration of acetic acid (5.5 g/L), formic acid (2.1 g/L), levulinic acid (0.4 g/L) and HMF (3.7 g/L) did not changed much ($< 10\%$) after detoxification, suggesting that these degradation compounds were not the major inhibitors in the biomass hydrolysates. Similar

observations have been reported previously about the effects of sugar degradation compounds on ethanol fermentation. Taherzadeh et al. reported that acetic acid (9 g/L) at pH 5 increased the final ethanol yield in *S.cerevisiae* fermentation by 16% (Taherzadeh et al., 1997). In the preliminary study, it was also found that addition of 25 mM (3.16 g/L) HMF did not affect the ethanol production. Therefore, nucleophilic reactions with unknown highly reactive lignin-derived carbonyl inhibitors could be the main reason for the detoxification. Although amino acids did not remove HMF from the hydrolysates during the detoxification, HMF consumption at 48 h was increased significantly in the fermentation of the detoxified hydrolysates (Table 1). HMF consumption was 6.37% in the untreated hydrolysate, but was 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 (Fig. 1c). The improvement of HMF consumption was likely related to the cell viability. Detoxification increased the yeast cell viability, and thus more viable cells converted more HMF to HMF alcohol. Overall, the increase in HMF consumption followed the same pattern as the ethanol yield (Table 1). This agreed with a previous report that yeast cells were 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 (Liu et al., 2004).

3.2 Effects of temperature on both cysteine and glycine detoxification

To examine whether temperature affects the detoxification, the biomass hydrolysates were detoxified with cysteine and glycine at various temperature (20, 40, 50, 60, 70 and 80

°C) for 2 h at pH 6.0 (Fig. 2a and 2b). The results showed higher temperature resulted in higher fermentation rates in detoxified hydrolysates. For cysteine detoxification, the volumetric ethanol productivity was increased by 43-130% from 0.83 to 1.19, 1.77 and 1.90 g/L/h when temperature increased from 20 to 40, 60 and 80 °C respectively (Fig.2a). However, their final ethanol concentration and yield were similar (23.34 g/L and 0.42 g/g). Undetoxified hydrolysates treated at 80 °C have resulted in negligible fermentation, indicating cysteine is an excellent detoxification reagent and the detoxification process is temperature-dependent.

For glycine detoxification, the temperature showed dramatic improvement on the fermentation of detoxified hydrolysates (Fig.2b). At 50 and 60 °C, the ethanol productivity was improved slightly from 0.18 (untreated) to 0.24 and 0.42 g/L/h respectively, with the final ethanol concentration at 1.87 and 8.58 g/L respectively. However at 70 and 80 °C, the ethanol productivity was increased significantly to 1.08 and 1.33 g/L/h and the final ethanol concentration have reached 23.68 and 23.00 g/L respectively, which was comparable to the cysteine detoxification. These results indicated glycine without side chain functional group could also detoxify the hydrolysates, and the primary amine group in amino acids reacted with carbonyl groups at relatively high temperature (80 °C).

3.3. Effects of pH on both cysteine and glycine detoxification

To examine the effect of pH, the biomass hydrolysates were detoxified with cysteine and glycine for 2 h at various pH (2.0, 4.0, 6.0, and 8.0) while keeping the temperature at 60 °C (Fig.3a-c). The results showed increase in pH enhanced the fermentation rate and yield considerably. For cysteine detoxification, the resulting volumetric ethanol productivity was

increased by 92% (from 0.92 to 1.77 g/L/h) when pH was increased from 2 and 4 to 6 (Fig.3a), but, there was no change in ethanol productivity when the pH was increased from 2.0 to 4.0. Under these three pH conditions, it was observed that the final ethanol concentrations were similar (23.65, 23.9 and 23.14 g/L). This indicated cysteine detoxification was effective between pH 2.0-6.0 and higher pH facilitated more effective detoxification.

For glycine detoxification, higher pH not only improved the fermentation rate significantly but also enhanced the final ethanol concentration substantially (Fig.3b). At pH 2.0 and 4.0, the detoxified biomass hydrolysates were not fermentable, while at pH 6.0 and 8.0; the resulting ethanol productivity increased from 0.18 (untreated) to 0.42 and 0.87 g/L/h respectively. Furthermore, their final ethanol concentrations have reached 8.58 and 23.77 g/L. The undetoxified biomass hydrolysates (incubated at pH 8.0) were not fermentable. This indicated that glycine detoxification required higher pH conditions (>6.0) and the detoxification process was pH dependent.

To further examine whether the pH dependency of glycine detoxification could be compromised by higher temperature, the biomass hydrolysates were detoxified at pH 2.0, 4.0 and 6.0 at 80 °C (Fig.3c). The results showed the detoxified hydrolysates at pH 2.0 and 4.0 were still not fermentable, although higher temperature improved the fermentation at pH 6.0. The temperature and pH dependence of detoxification was probably related to the chemical reaction between amino acids and carbonyl inhibitors (aldehydes) in the hydrolysates. It has been reported that the reaction of amino acids with aldehydes to form Schiff bases was temperature dependent and the reaction was favored at higher temperatures (Chobpattana et

al., 2000). Compared with glycine, cysteine contains thiol group, which could react rapidly with carbonyl compounds. Consequently, it reacted fast with carbonyl inhibitors under milder conditions (Pripis-Nicolau et al., 2000).

This study indicated that amino acid detoxification consumes little acid or base as comparing to the alkaline detoxification. It suggested that amino acid detoxification at pH 6.0-8.0 and 80 °C could potentially be an environmentally friendly detoxification method, because no considerable amount of salts are produced in the detoxification process and excessive amino acids can be easily consumed by microorganisms. Therefore, it was believed amino acid detoxification could be an alternative approach in detoxifying biomass hydrolysates, although the cost of addition amino acids into hydrolysates could be a potential issue. In the future work, the amino acids detoxification process will be optimized and it is expected to reduce the cost significantly.

3.4. Detoxification of model inhibiting compound with cysteine and glycine and identification of potential detoxification products

To ascertain the potential reaction mechanism of amino acids in the detoxification of biomass hydrolysates, cinnamaldehyde was detoxified in a glucose solution with cysteine and glycine. Cinnamaldehyde was chosen as a model inhibitor, because it has been identified in wood hydrolysates and had strong inhibition on fermentation. Although vanillin has been evaluated as a model inhibitor previously (Cao et al., 2014), the preliminary results showed 2.5 mM cinnamaldehyde inhibited glucose fermentation significantly while 10 mM vanillin did not inhibit the final ethanol concentration and yield (Fig. 4a) under the same conditions. This probably was due to the higher electrophilic reactivity in cinnamaldehyde than that in

vanillin.

Comparing the detoxification efficiency of cysteine and glycine on cinnamaldehyde inhibition (Fig. 4b), it was observed that 5.0 mM cysteine detoxified cinnamaldehyde very effectively and enabled a fast fermentation that was comparable to that of glucose control. Glycine did not detoxify the cinnamaldehyde at 60 °C, but did at 80 °C. This confirmed the previous findings that cysteine had higher detoxification activity than glycine.

The possible reaction mechanism between cysteine and cinnamaldehyde was investigated using Q-TOF LC/MS of detoxified products. Two major ions were present at high intensities, 236.07 and 357.09 [M+1]⁺/z. An elemental composition analysis of the two ions revealed the formula of C₁₂H₁₃NO₂S and C₁₅H₂₀N₂O₄S₂, with relative mass error less than 5 ppm. The first compound was probably a Schiff base (thiazolidine carboxylic acid), which can be formed by the condensation of cinnamaldehyde and cysteine (Kallen, 1971).

Previously, the Michael addition has been suggested as the reaction between thiol group of cysteine and α,β-unsaturated bond of cinnamaldehyde. However, the Michael addition product was not observed in the detoxification process. The second peak (357.09, [M+1]⁺) was probably a diadduct, formed between thiol group of cysteine and α,β-unsaturated bond of thiazolidine-4-carboxylic acid. MS/MS of precursor ion (236.07) showed four major fragments (115.06, 132.08, 147.03 and 190.07). The fragmentation pattern can be explained as the precursor ion lost a COOH group to form ion 190.07, which could further lost C₂H₅N to form ion 147.03 or lost CH₂S to form ion 132.08. The ion 115.05 was produced from the ion 147.03 by losing S or from the ion 132.08 by losing NH₃. For the second product (357.09, [M+1]⁺), it appeared the precursor ion lost one cysteine (C₃H₇NSO₂) to form ion 236.07. The

confirmed products further indicated that primary amine and thiol groups present in cysteine played an important role in detoxifying cinnamaldehyde. The reaction of the thiol group with carbonyl aldehyde to form hemithioacetal has been reported previously (Kallen, 1971). The amine group further reacts with hemithioacetal to form a five-membered thiazolidine ring. For glycine detoxification, the reaction of aldehydes and ketones with primary amine group in amino acids to form imine derivatives could be the main mechanism of detoxification. Therefore, Schiff base formation was likely the main reaction mechanism in detoxifying carbonyl aldehydes with nucleophilic amino acids.

4. Conclusions

A selective detoxification of biomass hydrolysates with nucleophilic amino acids was evaluated in the study. Five amino acids (cysteine, histidine, lysine, tryptophan and asparagine) showed very good detoxification efficiency with cysteine the most effective. This study indicated both cysteine and glycine detoxification were temperature and pH dependent. Mass spectra of detoxified hydrolysates with cysteine revealed that the detoxification mechanism was probably related to the Schiff base formation by a condensation between carbonyl aldehyde (cinnamaldehyde) and nucleophilic amino acid (cysteine). Future work will focus on the cost evaluation of amino acid detoxification for real biomass hydrolysates.

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510 **Table 1**

511 Fermentability of biomass hydrolysates detoxified by 20 amino acids (pH 6.0, 60°C and 2 h).

Treatment	R_G^a (g/L/h)	R_M^b (g/L/h)	Q_{EtOH}^c (g/L/h)	C_{EtOH}^d (g/L)	Y_{EtOH}^e (g/g)	C_{HMF}^f (%)
Glucose control	2.10±0.01	1.51±0.03	1.39±0.00	22.68±0.12	0.43±0.00	NA
Untreated	0.11±0.01	0.34±0.07	0.18±0.00	1.16±0.01	0.02±0.00	6.37±0.67
Glycine	0.39±0.01	0.50±0.05	0.42±0.01	8.58±0.27	0.16±0.01	11.19±0.20
Alanine	0.21±0.02	0.44±0.07	0.28±0.00	2.14±0.01	0.04±0.00	11.03±1.64
Valine	0.25±0.00	0.49±0.03	0.32±0.00	2.69±0.18	0.05±0.00	12.66±1.50
Leucine	0.27±0.04	0.64±0.12	0.34±0.01	2.83±0.19	0.05±0.00	12.84±1.32
Isoleucine	0.37±0.02	0.66±0.06	0.44±0.08	5.03±1.46	0.09±0.03	17.36±3.50
Proline	0.27±0.05	0.67±0.18	0.33±0.00	3.12±0.13	0.06±0.00	13.35±1.05
Phenylalanine	0.24±0.01	0.56±0.01	0.28±0.00	1.87±0.02	0.03±0.00	12.69±1.12
Tyrosine	0.25±0.02	0.59±0.05	0.30±0.02	2.16±0.25	0.04±0.00	12.17±0.92
Tryptophan	0.69±0.00	0.89±0.04	0.63±0.01	11.59±0.25	0.21±0.00	42.12±0.81
Serine	0.38±0.00	0.65±0.13	0.45±0.00	3.87±0.08	0.07±0.00	15.66±0.11
Threonine	0.36±0.02	0.67±0.03	0.41±0.01	3.96±0.13	0.07±0.00	16.19±0.87
Cysteine	2.10±0.04	2.16±0.12	1.77±0.03	23.14±0.10	0.42±0.00	96.40±0.03
Methionine	0.27±0.01	0.63±0.02	0.31±0.00	3.11±0.02	0.06±0.00	12.78±0.10
Asparagine	0.65±0.01	0.87±0.10	0.58±0.01	11.18±0.15	0.20±0.01	39.20±0.50
Glutamine	0.38±0.02	0.73±0.01	0.40±0.00	3.52±0.06	0.06±0.01	13.38±0.99
Lysine	0.80±0.06	1.09±0.13	0.72±0.01	14.40±0.57	0.26±0.01	50.20±4.97
Histidine	0.94±0.00	1.02±0.01	0.78±0.00	23.07±0.35	0.42±0.01	81.94±1.57
Arginine	0.29±0.02	0.59±0.07	0.34±0.01	2.62±0.03	0.05±0.00	12.69±0.27
Aspartate	0.43±0.02	0.90±0.01	0.47±0.00	3.86±0.18	0.07±0.00	16.27±0.30
Glutamate	0.34±0.02	0.56±0.03	0.41±0.01	3.63±0.23	0.07±0.00	15.40±0.72

^a R_G , glucose consumption rate in the first 6 h.^b R_M , mannose consumption rate in the first 6 h.^c Q_{EtOH} , volumetric ethanol productivity in the first 6 h.^d C_{EtOH} , ethanol final concentration at 48 h.^e Y_{EtOH} , ethanol yield from total glucose and mannose at 48 h.^f C_{HMF} , percentage of HMF consumed at 48 h.

Table 2

MS/MS data of products 1 and 2 from cysteine reaction with cinnamaldehyde.

MS peaks	Elemental composition	Exact mass	$[M+1]^+$	Calculated mass $[M+1]^+$	<i>ppm</i>
Product 1	C ₁₂ H ₁₃ NO ₂ S	236.0745		236.0733	5.0
Fragment 1-1	C ₉ H ₇	115.0548		115.0553	4.3
Fragment 1-2	C ₉ H ₁₀ N	132.0813		132.0816	2.3
Fragment 1-3	C ₉ H ₇ S	147.0268		147.0270	1.4
Fragment 1-4	C ₁₁ H ₁₂ NS	190.0690		190.0692	1.1
Product 2	C ₁₅ H ₂₀ N ₂ O ₄ S ₂	357.0943		357.0938	1.4
Fragment 2-1	C ₁₂ H ₁₃ NO ₂ S	236.0745		236.0743	0.9
Fragment 2-2	C ₉ H ₇	115.0548		115.0546	1.7
Fragment 2-3	C ₉ H ₁₀ N	132.0813		132.0812	0.8
Fragment 2-4	C ₁₁ H ₁₂ NS	190.0690		190.0695	2.6
Fragment 2-5	C ₉ H ₇ S	147.0268		147.0269	0.7

Figure Legends

Fig.1. Fermentation of biomass hydrolysates detoxified by various amino acids (0.2% w/v) at pH 6 and 60°C for 2 h (a: ethanol production in the fermentation of hydrolysates; b: sugar consumption in the fermentation of hydrolysates; c: HMF consumption in the fermentation of hydrolysates).

Fig. 2. Effects of temperature on cysteine and glycine detoxification (pH 6.0 for 2 h) of hydrolysates (a: ethanol production from the hydrolysates detoxified by cysteine; b: ethanol production from the hydrolysates detoxified by glycine).

Fig. 3. Effect of pH and temperature on cysteine and glycine detoxification of hydrolysates (a: effect of pH on cysteine detoxification of hydrolysates at 60 °C; b: effect of pH on glycine detoxification of hydrolysates at 60 °C; c: effect of pH on glycine detoxification of hydrolysates at 80 °C).

Fig. 4. Detoxification of model inhibitors with cysteine and glycine (a: effects of vanillin and cinnamaldehyde on ethanol production; b: ethanol production from detoxified sugar solution with cinnamaldehyde (2.5 mM)).

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Fig.1.

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Fig. 2.

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Fig. 3.

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(b)

Fig. 4.