Inhibitory Effects of Biomass Degradation Products on Ethanol Fermentation and a Strategy to Overcome Them

Shiyu Fu,* Jinfeng Hu, and Hao Liu

The influence of buffers, as well as inhibitors such as formic acid, furfural, HMF, guaiacol, and vanillin, on ethanol formation was investigated. Compared to phosphoric buffer, the acetic and citric buffers were less inhibitory on ethanol fermentation. The addition of formic acid (2.5 g/L) to the buffer reduced the ethanol yield by 8%. Guaiacol (3 g/L) and vanillin (2.5 g/L) decreased ethanol production by 50% and 20%, respectively. Furfural and HMF delayed the yeast fermentation without reducing the total yield. The fermentation was seriously inhibited by the mixture of furfural (1 g/L), HMF (1 g/L), formic acid (1 g/L), vanillin (1 g/L), and guaiacol (1 g/L). The ethanol yield of the fermentation based on enzymatic hydrolyzate from treated biomass was 82%. The addition of 1 g/L $MgSO_4$ as a shielding protector rehabilitated nearly 100% of the total yield.

Keywords: Ethanol; Fermentation; Inhibitors; Shielding agents; Rehabilitation

*Contact information: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640; *Corresponding author: shyfu@scut.edu.cn*

INTRODUCTION

The shortage of fossil fuel is one of the greatest global issues. To address this problem, many researchers have focused on biomass, especially lignocellulose, which is the most abundant organic resource worldwide (Nichols *et al.* 2010). The major components of lignocellulose include cellulose and hemicelluloses, both of which could be depolymerized to monosaccharides. Microorganisms can subsequently ferment these sugars to ethanol. Utilization of ethanol from biomass could reduce both the consumption of fossil energy and environmental pollution (Balat *et al.* 2008). The natural structures of lignocellulosic make it difficult for microorganisms to convert it to ethanol. Pretreatment, such as dilute acid, hot water, steam explosion, and so on, is necessary to make it possible that the subsequent enzymatic hydrolysis takes place efficiently (Jorgensen *et al.* 2007).

Among biomass pretreatment methods, dilute acid pretreatment has been found to be highly effective for releasing hemicelluloses and enhancing the accessibility of cellulose to cellulolytic enzymes (Hahn-Hägerdal *et al.* 2006; Lin and Tanaka 2006; Mosier *et al.* 2005; Yang and Wyman 2008). Harsh pretreatment conditions are usually required to deconstruct the plant cell walls, but such treatments have resulted in many by-products from the degradation of cellulose, hemicellulose, and lignin. These by-products, some of which may inhibit the subsequent fermentation, are divided into three groups: weak acids, furan derivatives, and aromatic compounds (Klinke *et al.* 2004). The degradation of hemicelluloses liberates xylose, mannose, acetic acid, and galactose. Xylose is further converted to furfural with high temperature and pressure (Dunlop 1948). Similarly, 5-hydroxymethyl furfural (HMF) is formed from the degradation of hexoses (Ulbricht *et al.* 1984).

Formic acid is produced when furfural and HMF are broken down (Dunlop 1948). Hydrolysis or oxidation of lignin gives rise to solubilized aromatic compounds (acids, aldehydes, phenols, and alcohols) (Lapierre *et al.* 1983).

The hydrolysate of spruce (softwood) by dilute sulfuric acid (Larsson *et al.* 1999) contains significant amounts of formic acid, acetic acid, furfural, and HMF. Both the enzymatic hydrolysate of sugarcane bagasse pretreated with steam explosion and the enzymatic hydrolysate of chipped tobacco stalks pretreated with steam contain low levels of inhibitory compounds (Martín *et al.* 2002).

When the biomass hydrolyzates are fermented, the presence of inhibitory compounds have the potential to retard or inhibit cell growth and fermentation. Furfural and HMF could cause a lag-phase during ethanol fermentation and reduce the growth rate of yeasts. However, they do not impact the final ethanol yield (Chung and Lee 1985) because yeast can grow in the medium containing furfural, but slowly (Palmqvist *et al.* 1999). During fermentation, furfural can be metabolized by *S. cerevisiae* to furfuryl alcohol (Villa *et al.* 1992; Taherzadeh *et al.* 1997). Furthermore, furfural is metabolized more rapidly than 5-HMF (Larsson *et al.* 1999).

A high concentration of weak acids could decrease ethanol yield from biomass hydrolysates. Low concentrations, however, can have a positive effect (Pampulha and Loureiro-Dias 1989). Larsson *et al.* (1999) reported that low concentrations of acetic or formic acid (<100 mmol/L) in medium could increase the yield of ethanol, while ethanol yield decreases at high concentrations.

Phenolic compounds are considered to have a significant inhibitory effect during fermentation. The lower the phenol's molecular weight is, the stronger its toxicity is to the yeast (Clark and Mackie 1984). Vanillin has the potential to completely inhibit the fermentation and cell growth at 6 g/L (Lin *et al.* 2007) and cause an increased lag-phase in cell growth at 2 g/L. No obvious lag-phase was seen under concentrations below 2 g/L. The mechanism by which phenols produce this inhibition is not fully understood due to the lack of accurate qualitative and quantitative analysis methods (Palmqvist and Hahn-Hägerdal, 2000).

In this study, the fermentation with various inhibitors including acid, furfural, and phenols was carried out. A cheap but efficient detoxification method was proposed. The enzymatic hydrolyzate from pretreated bagasse was fermented with the proposed method for detoxification.

EXPERIMENTAL

Material and Methods

Yeast strain and culture methods

The yeast strain Angel High-temperature Resistant & Highly-active Dry Yeast (AHD yeast) from Angel Yeast Company, Yichang, Hubei, China was used. The characteristics of AHD yeast include high-speed fermentation, ethanol, and temperature resistance, and excellent stability in storage. 2 g AHD yeast was activated in 20 mL of 2.5% glucose solution for 20 min in 38 °C and then kept at 34 °C for 2 h. The activated yeast solution was centrifuged at 10000 rpm, and washed three times with distilled water. Then 20 mL distilled water was added to the precipitate, producing the final solution of rehydrated yeast for fermentation.

Fermentation

200 g/L glucose solution was used as the fermentation medium. The fermentation was conducted in 25 mL triangular flasks containing 9 mL medium and 1 mL rehydrated yeast solution for 48 h with silica gel stoppers. The precipitate was separated by centrifugation, and the supernatant was collected for ethanol measurement as described below.

Ethanol fermentation with inhibitors

At the initiation of fermentation, formic acid, furfural, HMF, guaiacol, vanillin, and lignin were added to the medium at varying concentrations (0 to 3 g/L). Bagasse from the sugarcane mill in Guangxi, China, was treated with a $FeCl_3$ solution according to Chen's method (Chen and Fu 2013). The pretreated bagasse was hydrolyzed with cellulase from Youtell Biotechnology, Hunan, China, and the hydrolyzate was used in the ethanol fermentation.

Ethanol determination

The ethanol concentration in the fermentation supernatant was measured using full evaporation headspace gas chromatography (Li *et al.* 2009) with a DANI HSS86.50 Automatic Headspace Sampler and an Agilent 7890A gas chromatographer (GC, Agilent Corporation). The GC operating conditions consisted of a HP-5 capillary column at 40°C and nitrogen as the carrier gas (25 mL/min). A flame ionization detector was employed at 250°C and the flow rates of hydrogen and air were 30 and 400 mL/min respectively. The headspace operating conditions were as follows: 10 μL supernatant was injected into a closed 22 mL vial, and the vial was placed in an Automatic Headspace Sampler, and incubated at 105 °C for 5 min to get equilibrium. 20 μL gas was drawn from the equilibrium vial for GC measurement. A minimum of three replicates was performed for all analyses.

RESULTS AND DISCUSSION

Influence of Buffer Solution and pH on Ethanol Yield

Two buffer solutions, a citric acid-citrate sodium buffer solution (CSB) and a KH_2PO_4-Na_2HPO_4 buffer solution (KPB) at different pH levels were used for ethanol formation with AHD yeast (Table 1). Values of pH and buffer types were shown to have a significant effect on fermentation. The fermentation ethanol yield with CSB or KPB buffer was significantly higher than that without buffer (50.93%). The ethanol yield with KPB was 10 to 15% lower than the CSB yield, indicating a more productive fermentation with the use of CSB. The optimal pH for fermentation with ADH yeast in either buffer solution was 5.5. Generally, the optimal pH range for *S. cerevisiae* growth was 5.0 to 5.5 (Verduyn *et al.* 1990).

In commercial production, the acetic acid-sodium buffer solution (SAB) is commonly employed due to its lower price. Because acetate at concentrations below 0.1 mol/L could have a positive effect on ethanol production, while higher concentrations could decrease the ethanol production (Larsson *et al.* 1999), a 0.05 mol/L SAB buffer solution (pH 5.5) was used in our study. Figure 1 shows that the SAB and CSB buffer used as fermentation medium could improve the ethanol yield during the fermentation process. The highest ethanol yield (95%) was obtained using CSB medium after a 24 h

incubation, while the ethanol yield when using SAB or KPB medium was only 86% and 78%, respectively. In contrast, the ethanol yields with a water medium (with HCl to adjust pH to 5.5), were 51%, 75%, and 78%, at 24 h, 36 h, and 48 h, respectively. In SAB and CSB buffer medium, the ethanol yields only changed slightly from 36 h to 48 h. The SAB buffer was selected as the fermentation medium in the study below to explore the effects of inhibitors. The fermentation of glucose (200 g/L) with SAB buffer (pH 5.5) was set as reference fermentation (control).

Table 1. Influence of Buffer Solution on Ethanol Yield

Buffer solution	pH	Y^{a}_{24h}/%
None	-	50.9±3.1
Citric acid-citrate sodium (0.05 mol/L)	4.8	88.5±1.1
	5.5	95.2±0.8
	6	89.7±1.0
KH_2PO_4-Na_2HPO_4 (0.033 mol/L)	4.9	73.4±2.4
	5.5	78.3±0.8
	5.9	77.9±0.4

[a] Yield of ethanol fermentation for 24 h at 38 °C.

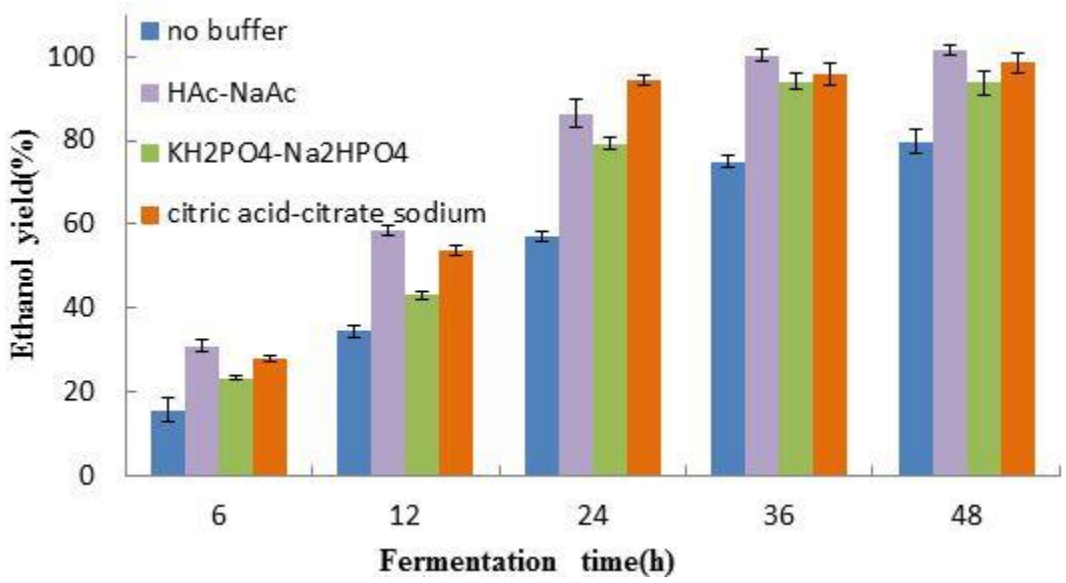

Fig. 1. Influence of different buffer solutions (pH=5.5) on ethanol yield. Note: the concentration of glucose was 200 g/L.

Influence of Temperature on Ethanol Yield

Temperature is an important parameter affecting microorganism activities. The AHD yeast, with high temperature resistance, was investigated within the range 30 °C to 42 °C. As shown in Fig. 2, temperature had a strong impact on fermentation. During the initial 12 h, the ethanol yield improved along with the increase of fermentation temperature. However, after 24 h, this increasing trend vanished when the fermentation temperature reached 42 °C. The optimal fermentation temperature was 38 °C, at which the yeast exhibited the highest activity and growth rate. Fermentations at lower

temperatures had a longer lag phase and a lower ethanol production rate, particularly at 30 °C. Fermentation at 42 °C had no lag phase, resulting in higher ethanol yield because it had a quick exponential phase. The fermentation proceeded with a very short stationary phase, likely leading to a decrease in yeast cell viability (Blieck *et al.* 2007), suggesting 42 °C might exceed the tolerable temperature of AHD yeast, causing the yeast cells to be no longer viable after 24 h of fermentation.

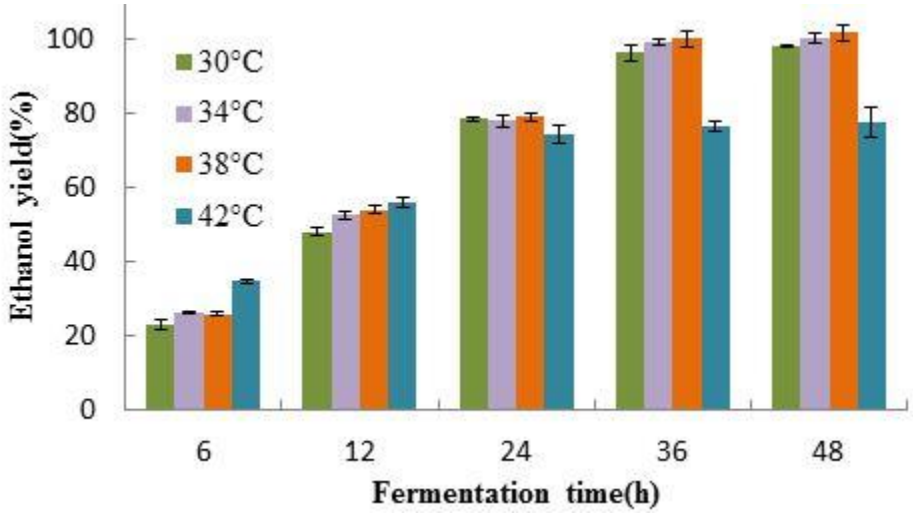

Fig. 2. Influence of temperature on ethanol yield. Note: 1) The concentration of glucose in medium: 200 g/L; 2) The concentration of pH 5.5 SAB buffer solution: 0.05 mol/L

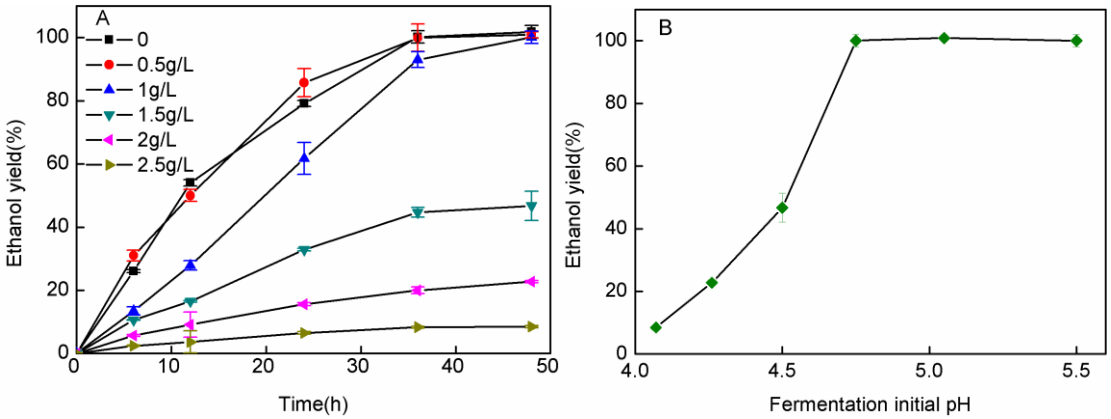

Fig. 3. Influence of formic acid concentration (A) and fermentation initial pH (B) on ethanol yield. Note: 1) The concentration of glucose in medium: 200 g/L, 2) Fermentation at 38 °C for 48 h

Influence of Inhibitors on Ethanol Fermentation

In general, organic acids and aldehydes are formed during biomass pretreatment, and these could potentially inhibit ethanol fermentation. In this study, formic acid was added to the fermentation medium, and its effect on ethanol production is displayed in Fig. 3(A). Formic acid at 0.5 g/L did not affect the ethanol fermentation, while 1 g/L formic acid delayed the time reaching the highest ethanol conversion, but it did not decrease the final ethanol yield compared to the reference fermentation and the 0.5 g/L formic acid fermentation. The ethanol yield with 0.5 g/L formic acid was almost the same (100%) as with 1 g/L after 48 h fermentation. The ethanol production rate and yield were

both significantly decreased in the presence of 1.5 g/L formic acid and the yield was decreased by about 53%. The ethanol yield was heavily restrained to 8% by addition of 2.5 g/L formic acid.

When formic acid was added to the medium, the ethanol fermentation was possibly inhibited. Figure 3(B) shows that the final ethanol yields decreased with the addition of formic acid because the medium pH dropped below 5.5. When the pH was 4.07, the ethanol yield only reached 8%; when the medium was adjusted to pH 5.5, the yield of fermentation was 100%. These pH effects may be explained by the intracellular pH and ATPase activity (Pampulha and Loureiro-Dias 1990). A suitable range for the external pH for AHD yeast fermenting was 5.0 to 5.5, which is consistent with other reports (Verduyn *et al.* 1990). Maintaining neutral intracellular pH is crucial for cell activity, because the undissociated weak acid in the medium can diffuse across the plasma membrane into the cytosol and lower the intracellular pH (Pampulha and Loureiro-Dias 1990). In order to maintain the neutral intracellular pH, membrane ATPase pumps protons out of the cell by ATP hydrolysis (Stouthamer 1979; Verduyn *et al.* 1992), which leads to intracellular ATP scarcity, resulting in a shortage of enzymes, coenzymes and nutrients and slower cell metabolism. This can ultimately result in cell death when the weak acid concentration is too high.

The influences of furfural and HMF on ethanol fermentation are shown in Fig. 4(A) and (B). The results show the ADH yeast was well tolerant to the furfural, and there was no inhibition for ethanol fermentation when the furfural was below 2.5 g/L because it can be metabolized by yeast (Taherzadeh *et al.* 1997). When the concentration was increased, furfural metabolized more slowly, causing a reduced ethanol production rate and introducing a lag-phase in ethanol fermentation. When the furfural concentration was added to 5 g/L, the ethanol yield was reduced to 87% at 32 h, but the final ethanol yield was not decreased if the fermentation time was extended to 53 h as shown in Fig. 4(A). These results are consistent with Chung's report (1985). When the medium contained furfural from 7.5 g/L to 10 g/L, the yeast fermentation could proceed, but the ethanol production was saliently inhibited in the initial stage. The fermentation with 10 g/L furfural was slower about 25 h than 0 g/L furfural, and the ethanol yield was 90% of that with furfural (control).

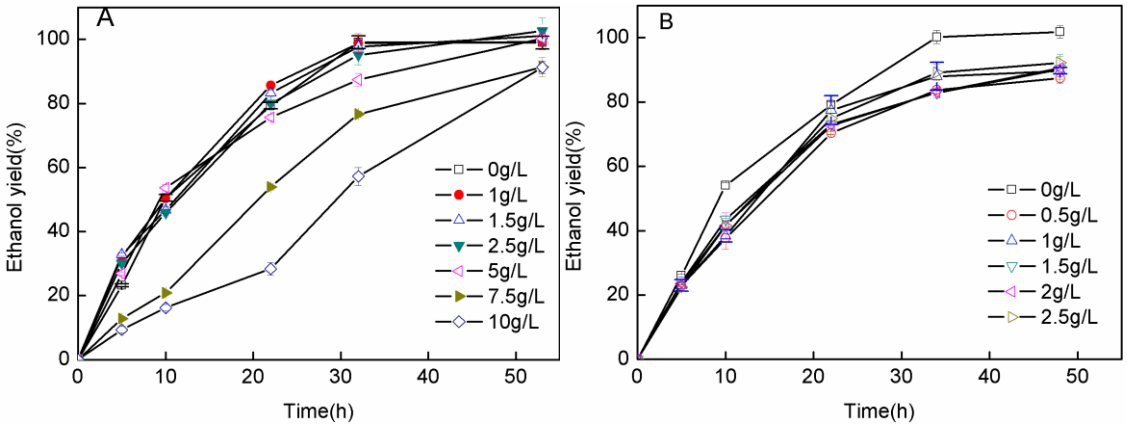

Fig. 4. Influence of furfural (A) and HMF (B) on ethanol yield. Note: 1) The concentration of glucose in medium: 200 g/L; 2) Fermentation at 38 °C for 53 h (A), 48 h (B)

Figure 4(B) shows that 0.5 to 2.5 g/L HMF exhibited similar effects on ethanol production, and the final ethanol yield in the presence of HMF was about 90% of that without HMF. The fermentation rate in the presence of HMF was slower than that of the reference fermentation, suggesting that the furfural is metabolized faster than the HMF. Additionally, it suggests that HMF is more toxic than the furfural at the same concentrations.

Phenolic compounds destroy yeast membranes, causing loss of integrity and reducing the membranes ability to function as a selective barrier and enzyme matrix (Heipieper *et al.* 1994). Phenolic compounds, especially the phenols with lower molecular weight, have been shown to inhibit yeast fermentation (Clark and Mackie 1984). The effects of guaiacol and vanillin, two byproducts of lignin degradation, on fermentation are shown in Fig. 5. Results indicate that both guaiacol and vanillin significantly inhibited fermentation. The guaiacol at dosages of 1 g/L, 2 g/L, and 3 g/L caused 12%, 29%, and 50% decreases in ethanol yield compared to the reference fermentation, respectively. Vanillin at 1 g/L, 2 g/L, and 2.5 g/L led to 5%, 17%, and 20% decreases in ethanol yield, respectively. These data suggest that vanillin is less toxic than guaiacol, most likely due to the differences in structures between guaiacol and vanillin. This structure-activity relationship is complex and relies on the strain caused by distinct metabolic and membranous features of an individual microorganism (Mikulášová *et al.* 1990). It was reported that the hydrophobic parts of enzymes, proteins, or membrane transport systems are possible sites of inhibition. There is evidence that the more hydrophobic a phenolic compound is, the stronger the inhibition activity is (Klinke *et al.* 2004). Therefore, the introduction of a hydrophilic group such as an aldehyde in the aromatic ring of guaiacol, forming the vanillin, drastically reduces the hydrophobicity compared with guaiacol, reducing the inhibition on ethanol yields.

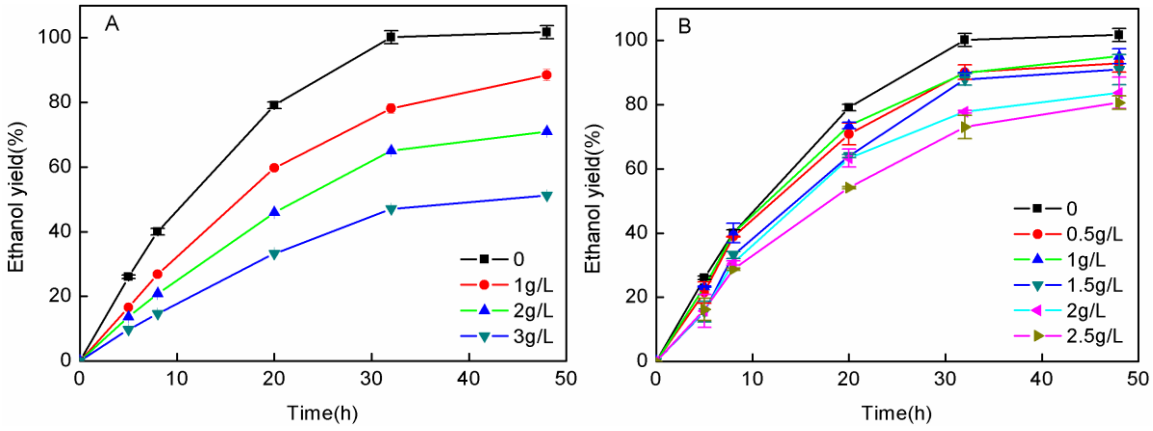

Fig. 5. Influence of guaiacol (A) and vanillin (B) on ethanol yield. Note: 1) The concentration of glucose in medium: 200 g/L; 2) Fermentation at 38 °C for 48 h

Synergic Effect of Inhibitors

Inhibition of fermentation by certain compounds can be enhanced by other compounds. When the inhibition obtained with combined compounds is significantly higher than the sum with each individual compound, it is a synergistic effect. The effects of combination between furfural (1 g/L), HMF (1 g/L), formic acid (1 g/L), vanillin (1 g/L), and guaiacol (1 g/L) on ethanol yields were measured at 48 h fermentation, and the results are shown in Table 2. The ethanol yield obtained in the presence of 1 g/L HMF

and 1 g/L furfural was only slightly lower compared to 1 g/L HMF alone. The inhibition obtained from the combination of vanillin (1 g/L) and guaiacol (1 g/L) was significantly higher than each alone. These data suggest the phenolic compounds are the most toxic. The ethanol yield was decreased to 78% in the presence of formic acid (1 g/L) and furan derivatives (1 g/L). The synergistic inhibitory effects of vanillin (1 g/L) and furan derivatives (1 g/L) was stronger than that of guaiacol (1 g/L) and furan derivatives (1 g/L), despite the stronger inhibition of individual guaiacol (1 g/L) than vanillin (1 g/L).

Table 2. Interaction Effects on Ethanol Yield

Furfural (g/L)	HMF (g/L)	Formic acid (g/L)	Vanillin (g/L)	Guaiacol (g/L)	Ethanol yield (%)
1					99.3±1.1
	1				92.9±1.0
		1			100.1±2.0
			1		95.1±1.8
				1	88.5±2.3
	1	1			77.8±0.6
1	1				90.2±1.8
1		1			78.5±1.6
			1	1	39.7±0.9
1				1	80.2±2.0
	1		1		66.6±1.3
		1		1	61.2±0.6
1	1	1			75.6±2.1
	1	1	1		60.1±1.5
1	1		1		64.1±2.4
1		1		1	54.7±0.7
1	1	1	1	1	12.4±2.0

The ethanol production rate and the yield were significantly decreased in the presence of formic acid (1 g/L) and guaiacol (1 g/L), and the inhibition was 39%. This result differed from other reports (Larsson *et al.* 1999) in that the ethanol yield in the presence of acetic acid (5 g/L), formic acid (10 g/L), levulinic acid (23 g/L), furfural (1.2 g/L), and HMF (1.3 g/L) decreased only slightly compared to a reference fermentation when an initial cell mass of 10 g/L was used. These poisonous compounds inhibited cell growth rather than ethanol production. The yield was decrease by 88% in the presence of furfural (1 g/L), HMF (1 g/L), formic acid (1 g/L), vanillin (1 g/L), and guaiacol (1 g/L). Lignin degradation products largely contributed to the inhibition effect in these cases. The toxicity of the inhibitors in descending order is: phenolic compounds > weak acids > furan derivatives. Therefore, it was necessary to remove or shield the phenolic compounds to increase the ethanol production.

Inhibiting of Lignin on Ethanol Fermentation and Retrieving with Shielding Agent

Lignin exists naturally within biomass hydroyzate, particularly when the substrate is pretreated. The majority of lignin in biomass is not removed during pretreatment, so it is necessary to consider the effects of lignin on fermentation. The lignin-derived compounds, black liquid extracted from bagasse with formic acid (Tu 2008), was added in the medium for ethanol fermentation with AHD yeast. The results showed that lignin-derived compounds, in the medium decreased ethanol yield (Fig. 6). When the concentration of lignin-derived compounds in the medium was increased from 0 to 1 g/L, the ethanol yield was reduced sharply from 100% to 86%. However, the ethanol yield decreased only slightly from 85% to 81% when the lignin-derived compounds concentration was increased from 2 g/L to 5 g/L. Hence, it is proposed that there are some sites in yeast that bind tightly to lignin, inhibiting ethanol fermentation.

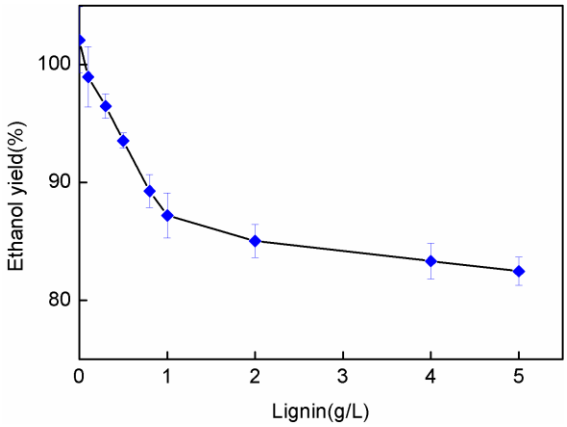

Fig. 6. Influences of lignin on ethanol yield. Notes: 1) The concentration of glucose in medium: 200 g/L; 2) Fermentation at 38 °C for 48h.

Table 3. Effects of $MgSO_4$ on the Ethanol Fermentation in the Presence of Lignin[a]

Lignin (g/L)	$MgSO_4$ (mol/L)	Ethanol yield (%)
0.5	0	92.9±0.6
1	0	85.1±1.9
0.5	0.005	99.7±3.3
0.5	0.01	102.0±2.7
0.5	0.05	99.5±3.0
0.5	0.1	99.3±2.3
0.5	0.5	100.7±1.8
1	0.005	95.6±3.0
1	0.01	102.3±2.8
1	0.05	100.0±1.4
1	0.1	99.7±1.0
1	0.5	100.1±1.4

[a]: Lignin from the black liquid of bagasse-organosolv pulping with formic acid

Bioethanol is a staple product of the chemical and energy industries. Needless to say, the yield of ethanol from starting substrates is of paramount importance for commercial production. Frequently, the conversion from pure sugar to ethanol via yeast is close to the theoretical yield. Due to the presence of inhibitors in biomass hydrolyzate, strategies are needed to remove inhibitors or reduce their effects (Van Maris *et al.* 2006). Because the magnesium ion is able to chelate with carboxyl groups or phenolic hydroxyl groups in lignin, the addition of magnesium ions in pretreated wood was shown to improve the enzymatic hydrolysis of wood by lessening the inhibition of lignin on enzymes (Liu *et al*. 2010). In order to shield the inhibiting effects of lignin, $MgSO_4$, called a shielding agent, was added to the medium of ethanol fermentation in the presence of lignin (in Table 3). It is interesting to find that the ethanol yield increased significantly with $MgSO_4$ in medium compared to the fermentation without $MgSO_4$, and the ethanol yield almost reached up to 100% with addition of higher levels of $MgSO_4$. When the medium contained 0.5 to 1 g/L lignin in the ethanol fermentation, 0.01 mol/L $MgSO_4$ was shown to be efficient to reduce the inhibition of lignin and promote the ethanol production.

A test of fermentation of biomass hydrolysate was conducted with pretreated bagasse. The treated bagasse was hydrolyzed, and the resulting sugars were used for fermentation with yeast. The consistency of pretreated bagasse (2%, 15%, and 20%) for enzymatic hydrolysis can cause the concentration of lignin degradation products and low molecular weight lignin to differ in the enzymatic hydrolyzates. After enzymatic hydrolysis of bagasse, the enzymatic hydrolyzates were adjusted to pH 5.5 by NaOH solution, and the concentration of glucose was measured. If the glucose in the enzymatic hydrolyzates was less than 90 g/L, additional glucose was added to raise total glucose to over 90 g/L. The fermentation with $MgSO_4$ was compared with that without $MgSO_4$.

The ethanol yield from fermentation of bagasse-hydrolyzate increased up to nearly 100% in the presence of $MgSO_4$, demonstrating the positive effects of $MgSO_4$ on ethanol fermentation (Table 4).

Table 4. Effect of Shielding Agent on Fermentation of Bagasse Hydrolyzate

Substrate concentration for enzymatic hydrolysis	Glucose (g/L)	Ethanol yield 1[c] (%)	Ethanol yield 2[d] (%)
2%	108.9[a]	95.8±1.4	101.6±2.1
2%	90.1[a]	92.7±0.9	98.7±1.8
15%	116.1[a]	88.2±1.6	100.7±1.3
20%	92.1[b]	84.6±2.5	101.2±2.9

[a] Glucose including two parts, one came from the enzymatic hydrolysis and the other came from the additional glucose; [b] Glucose came from the enzymatic hydrolysis only; [c] Fermentation without additional $MgSO_4$; [d] The fermentation with additional $MgSO_4$ (0.01mol/L)

When the substrate (pretreated bagasse) solid content was as low as 2%, the concentration of lignin in hydrolyzate was so low that it was possible to obtain an ethanol yield of over 92% from the fermentation. For the substrate in 20% solid content, the enzymatic hydrolyzate contained glucose (92 g/L), which was not accounting for the whole cellulose in the used biomass. This is an important issue that needs to be solved in our future work. The ethanol yield was only 84.6% when the above hydrolyzate was used

for fermentation. The lignin in the hydrolyzate could be shielded by $MgSO_4$ so that the negative impact of lignin on fermentation could be reduced, and the yeast would not bind to lignin. When 0.01 mol/L $MgSO_4$ was added in the hydrolyzate of bagasse (20% concentration at hydrolysis stage), the ethanol fermentation was rehabilitated completely (as shown in Table 4).

CONCLUSIONS

Ethanol fermentation with yeast preferred HAc-NaAc buffer over phosphoric or citric buffer. Acids, aldehydes, phenolic compounds, and lignin were found to be yeast inhibitors. The toxicity from highest to lowest are: phenolic compounds, weak acids, lignin, and furan derivatives. The furfural caused a lag-phase in ethanol fermentation, but did not influence the final ethanol yield. 1 g/L of lignin caused a 15% decrease in ethanol yield. The fermentation of hydrolyzate from pretreated bagasse was suppressed due to the presence of degraded lignin and phenolic compounds. The addition of 0.01 mol/L $MgSO_4$ was able to shield these compounds and completely rehabilitate the ethanol fermentation.

ACKNOWLEDGEMENTS

The financial supports of the National Natural Science Foundation of China (No. 31170549).

REFERENCES CITED

Balat, M., Balat, H., and Öz, C. (2008). "Progress in bioethanol processing," *Progress in Energy and Combustion Science* 34, 551-573.

Blieck, L., Toye, G., Dumurtier, F., Vertstrepen, K., Delvaux, F., Thevelein, J., and Van Dijck, P. (2007). "Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions," *Applied and Environmental Microbiology* 73, 815-824.

Chen, L., and Fu, S. (2013). "Enhanced cellulase hydrolysis of eucalyptus waste fibers from pulp mill by Tween 80-assisted ferric chloride pretreatment," *Journal Agriculture Food Chemistry* 61, 3293-3300.

Clark, T., and Mackie, K. (1984). "Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus radiata*," *Journal Chemistry Biotechnology* 34, 101-110.

Chung, I., and Lee, Y. (1985). "Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inoculation," *Biotechnology and Bioengineering* 27, 308-315.

Dunlop, A. (1948). "Furfural formation and behavior," *Industry Engineering and Chemistry* 40, 204-209.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M., Lidén, G., and Zacchi, G. (2006). "Bioethanol: the fuel of tomorrow from the residues of today," *Trends in Biotechnology* 24, 549-556.

Heipieper, H., Weber, F., Sikkema, J., Kewelo, H., and de Bont, J. (1994). "Mechanism of resistance of whole cells to toxic organic solvents," *Trends in Biotechnology* 12, 409-415.

Jorgensen, H., Kristensen, J., and Felby, C. (2007). "Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities," *Biofuels Bioproducts Biorefining* 1, 119-134.

Klinke, H., Thomsen, A., and Ahring, B. (2004). "Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass," *Applied Microbiology Biotechnology* 66, 10-26.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., and Nilvebrant, N. (1999). "The generation of fermentation inhibitors during dilute acid hydrolysis of softwood," *Enzyme and Microbial Technology* 24, 151-159.

Lapierre, C., Rolando, C., and Monties, B. (1983). "Characterization of poplar lignins acidolysis products: Capillary gas-liquid and liquid-liquid chromatography of monomeric compounds," *Holzforschung* 37, 189-198.

Li, H., Chai, X., Deng, Y., Zhan, H., and Fu, S. (2009). "Rapid determination of ethanol in fermentation liquor by full evaporation headspace gas chromatography," *Journal of Chromatography A* 1216, 169-172.

Lin, B., Zhao, X., and Ge, X. (2007). "The effects of dilute acid hydrolysate by-products of corn stover on ethanol fermentation of xylose-utilising *Saccharomyces cerevisiae* 6508-127," *Chinese Biotechnology* 27, 61-67.

Lin, Y., and Tanaka, S. (2006). "Ethanol fermentation from biomass resources: Current state and prospects," *Applied Microbiology Biotechnology* 69, 627-642.

Liu, H., Zhu, J., and Fu, S. (2010). "Effects of lignin−metal complexation on enzymatic hydrolysis of cellulose," *Journal of Agriculture and Food Chemistry* 58, 7233-7238.

Martín, C., Galbe, M., Wahlbom, C., Hahn-Hägerdal, B., and Jönsson, L. (2002). "Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*," *Enzyme and Microbial Technology* 31, 274-282.

Martín, C., Fernández, T., García, R., Carrillo, E., Marcet, M., Galbe, M., and Jönsson, L. (2002). "Preparation of hydrolysates from tobacco stalks and ethanolic fermentation by *Saccharomyces cerevisiae*," *World Journal of Microbiology and Biotechnology* 18, 857-862.

Mikulášová, M., Vodný, Š., and Pekarovičová, A. (1990). "Influence of phenolics on biomass production by *Candida utilis* and *Candida albicans*," *Biomass* 23, 149-154.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresource Technology* 96, 673-686.

Nichols, N., Dien, B., and Cotta, M. (2010). "Fermentation of bioenergy crops into ethanol using biological abatement for removal of inhibitors," *Bioresource Technology* 101, 7545-7550.

Palmqvist, E., Almeida, J., and Hahn-Hägerdal, B. (1999). "Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture," *Biotechnology Bioengineering* 62, 447-454.

Palmqvist, E., and Hahn-Hägerdal, B. (2000). "Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition," *Bioresource Technology* 74, 25-33.

Pampulha, M. E., and Loureiro-Dias, M. C. (1989). "Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast," *Applied Microbiology Biotechnology* 31, 547-550.

Pampulha, M., and Loureiro-Dias, M. (1990). "Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in the presence of acetic acid," *Applied Microbiology Biotechnology* 34, 375-380.

Stouthamer, A. (1979). "The search for a correlation between theoretical and experimental growth yields," *Int. Rev. Biochem. Microb. Biochem.* 21, 28.

Taherzadeh, M., Niklasson, C., and Lidén, G. (1997). "Acetic acid—Friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*?," *Chemical Engineering Science* 52, 2653-2659.

Tu, Q., Fu, S., Zhan, H., Chai, X., and Lucia, L. (2008). "Kinetic modeling of formic acid pulping of bagasse," *Journal of Agriculture and Food Chemistry* 56, 3097-3101.

Ulbricht, R. J., Sharon, J., and Thomas, J. (1984). "A review of 5-hydroxymethylfurfural in parental solutions," *Fundam. Appl. Toxicol*. 4, 843-853.

Van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., Wisselink, W., Scheffers, W., van Dijken, J., and Pronk, J. (2006). "Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: Current status," *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 90, 391-418.

Verduyn, C., Postma, E., Scheers, W., and van Dijken, J. (1990). "Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures," *Journal General Microbiology* 136, 405-412.

Verduyn, C., Postma, E., Scheers, W., and Dijken, J. (1992). "Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation," *Yeast* 8, 501-517.

Villa, G., Bartroli, R., López, R., Guerra, M., Enrique, M., Peñas, M., Rodríquez, E., Redondo, D., Jglesias, I., and Díaz, M. (1992). "Microbial transformation of furfural to furfuryl alcohol by *Saccharomyces cerevisiae*," *Acta Biotechnologica* 12, 509-512.

Yang, B., and Wyman, C. (2008). "Pretreatment: The key to unlocking low-cost cellulosic ethanol," *Biofuels Bioproduct Biorefinery* 2, 26-40.

Article submitted: April 2, 2014; Peer review completed: May 14, 2014; Revised version received and accepted: May 30, 2014; Published: June 4, 2014.