Evaluation of the Main Inhibitors from Lignocellulose Pretreatment for Enzymatic Hydrolysis and Yeast Fermentation

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To produce cellulosic ethanol more economically, utilization of whole slurry of pretreated lignocellulose without separating liquid and solid fractions after thermal and/or chemical pretreatment of lignocellulose may be advantageous in terms of process economics. To carry out such processing on mixtures, which contain pretreatment byproducts, quantitative evaluation of the degree of inhibition of enzymatic hydrolysis and yeast fermentation by pretreatment byproducts are important. Therefore, in this study, the inhibitory effect of byproducts, focusing on sugar degradation products including furfural, hydroxymethylfurfural (HMF), acetic acid (AA), formic acid (FA), and levulinic acid (LA), on enzyme and microbial performance was investigated. The experimental conditions for SSF media containing the inhibitors were optimized by response-surface methodology-ridge analysis. The saccharification using commercial cellulase was most remarkably inhibited (approximately 28%) by HMF. The ethanol production by *Saccharomyces cerevisiae* was nearly completely inhibited (approximately 80%) by furfural. The toxicity was noted as HMF > FA > furfural > AA ≈ LA for enzymatic hydrolysis, and furfural > HMF > FA > AA > LA for yeast ethanol production. The results indicated that the inhibitor accumulation during pretreatment should be controlled for subsequent effective saccharification and fermentation.

Keywords: Lignocellulose; Pretreatment; Inhibitors; Enzymatic hydrolysis; Fermentation

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INTRODUCTION

The efficient bioconversion of lignocellulosic biomass to ethanol requires pretreatment because of the recalcitrance of the source material (Yang and Wyman 2008; Zhu and Pan 2010). In particular, with the development of integrated processes, such as whole slurry fermentation and one-pot processing, the economic feasibility of cellulosic fuel and chemical production are improved (Jung *et al*. 2013, 2014). Thus, the utilization of the hydrolysates from pretreatment has become increasingly important. However, different byproducts such as furans and organic acids are formed from sugar in hydrolysates of lignocellulose during pretreatment, depending on the solution conditions employed (Jönsson and Martín 2016). In addition, the performance of enzymes and microorganisms during the subsequent hydrolysis and fermentation processes has been found to be lowered due to those pretreatment byproducts (Palmqvist *et al*. 1999; Zaldivar and Ingram 1999; Klinke *et al*. 2004; Jönsson *et al*. 2013). To minimize these inhibitory effects, additional steps, such as washing after solid/liquid separation, detoxification of

hydrolysates, and development of genetically engineered microbes tolerant to inhibitors, have been widely studied (Jung and Kim 2014).

Even with the awareness of the inhibitors from pretreatment, quantitative information about the degree of inhibition of enzyme and yeast performance remains limited because the toxicity level of each compound is significantly affected by microorganisms and culture conditions, such as media components and pH (Zaldivar and Ingram 1999; Kwon *et al*. 2011; Jönsson *et al*. 2013). In this study, five model inhibitors derived from lignocellulose carbohydrates, such as 2-furaldehyde (furfural), 5-hydroxymethyl-2-furaldehyde (HMF), acetic acid, formic acid, and levulinic acid, were evaluated using cellulase and *Saccharomyces cerevisiae* to simulate simultaneous saccharification and fermentation (SSF) of whole slurry of pretreated lignocellulose. The influence of these compounds on enzymatic hydrolysis yield and yeast fermentability during the SSF process was evaluated under the optimized culture conditions.

EXPERIMENTAL

Materials

For the formulation of SSF media, yeast extract and peptone were purchased from Becton (Dickinson and Company, Franklin Lakes, NJ, USA). Citric acid monohydrate and Avicel® were from Sigma-Aldrich (St. Louis, MO, USA). All the inhibitory chemicals such as furfural, HMF, acetic acid, formic acid, and levulinic acid were also purchased from Sigma-Aldrich. Enzyme (Accellerase® 1000; Genencor, Rochester, NY, USA) and *S. cerevisiae* D_5A (ATCC 200062) were used for ethanol fermentation, respectively.

Methods

SSF

The SSF was performed under optimized conditions determined by response surface methodology (RSM) to evaluate the ethanol production by *S. cerevisiae* (ATCC 200062). After autoclaving the SSF medium (1%, w/v, yeast extract; 2%, w/v, peptone; and 0.05 M citrate buffer) containing each 10 mM inhibitory compound at 121°C for 15 min, Accellerase® 1000 (15 FPU/g of glucan) and 1% (v/v) *S. cerevisiae* D_5A were added. The SSF was conducted microaerobically in a 250-mL flask at 180 rpm with a needle-pierced silicone stopper to release CO_2 produced during fermentation. The ethanol yield was expressed as a percentage of the theoretical maximum yield of glucose (0.51g of ethanol/g of glucose). All of the experiments were performed in triplicate.

RSM

The RSM was performed to determine the optimal conditions for SSF in the presence of various types of inhibitory compounds, namely, furfural, HMF, acetic acid, formic acid, and levulinic acid. Using a Box-Behnken design, three independent variables at three different levels, such as pH (4, 5, and 6), temperature (30 °C, 35 °C, and 40 °C), and Avicel® concentration (2%, w/v; 6%; and 10%) were investigated (Table 1). The optimal culture conditions for achieving the maximum ethanol yield after 24 h of fermentation were determined by using the ridge analysis because they were at a saddle point. A statistical program, SAS software (Version 9.3, SAS Institute, Cary, NC, USA), was used to analyze the data using the response surface regression procedure.

Table 1. Box-Behnken Design for *S. cerevisiae* Ethanol Yield for 24 h of SSF

Run	pH	Temperature (°C)	Avicel Concentration (%, w/v)	Ethanol Yield (%, w/v)
1	6	40	6	22.1 ± 0.2
2	4	40	6	13.8 ± 0.2
3	6	30	6	16.4 ± 0.3
4	4	30	6	19.3 ± 0.1
5	5	40	10	14.1 ± 0.7
6	5	30	10	20.1 ± 0.2
7	5	40	2	28.0 ± 0.3
8	5	30	2	25.1 ± 2.2
9	6	35	10	18.1 ± 0.4
10	6	35	2	17.5 ± 0.4
11	4	35	10	11.6 ± 0.3
12	4	35	2	30.3 ± 1.6
13	5	35	6	23.1 ± 0.6
14	5	35	6	21.7 ± 0.1
15	5	35	6	22.9 ± 0.1

Effect of inhibitory compounds on enzymatic hydrolysis and ethanol fermentation

To investigate the effect of each inhibitory compound on the performance of enzyme and yeast, enzymatic hydrolysis and SSF were performed in the SSF medium containing different concentrations (0 mM to 90 mM) of each inhibitory compound for 50 h. An Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was used for high-performance liquid chromatography (HPLC, Agilent 1260, Agilent Technologies, Waldbronn, Germany) with a refractive index detector (RID, G1362A, Agilent Technologies, Waldbronn, Germany). The HPLC was conducted using 0.01 N H_2SO_4 as a mobile phase (0.5 mL/min flow rate) with the column oven temperature at 65 °C and the RID temperature at 55 °C to measure the concentrations of glucose, ethanol, furfural, HMF, acetic acid, formic acid, levulinic acid, and glycerol. All of the analyses were performed in duplicate.

RESULTS AND DISCUSSION

The inhibitory effects of well-known byproducts from lignocellulose pretreatment, such as furfural, HMF, acetic acid, formic acid, and levulinic acid, on ethanol production and on enzymatic hydrolysis were investigated to possibly cope with the toxicity of those inhibitors under SSF processes utilizing whole slurry of pretreated lignocellulose containing inhibitors.

Optimization of SSF Conditions by RSM

General conditions of temperature (38 °C) and pH (4.8) were originally established for SSF using raw biomass or washed and pretreated biomass (Dowe and McMillan 2008). Thus, in this study, simulated whole slurry SSF that contained five different pretreatment byproducts, such as furfural, HMF, acetic acid, formic acid, and levulinic acid, and culture variables, such as pH, temperature, and substrate concentrations, were optimized (Fig. 1), following the ridge analysis of the Box-Behnken design (Table 1). According to the

second-order polynomial predictive equation, which had a high regression coefficient (R^2 = 0.95), the ethanol yield was calculated as follows,

$$Y = 0.965 + 8.634X_1 + 0.901X_2 + 3.605X_3 + 3.545X_1^2 + 0.556X_1X_2 + 0.044X_2^2 + 1.210X_1X_3 + 0.111X_2X_3 + 0.025X_3^2 \quad (1)$$

where X_1, X_2, and X_3 represent pH, temperature, and cellulose concentration, respectively, and Y represents ethanol yield after 24 h of SSF. As a result, the optimum pH, temperature, and Avicel concentration obtained were 4.7, 35.5 °C, and 2.2% (w/v), respectively, and the predicted theoretical maximum ethanol yield was 28.5%.

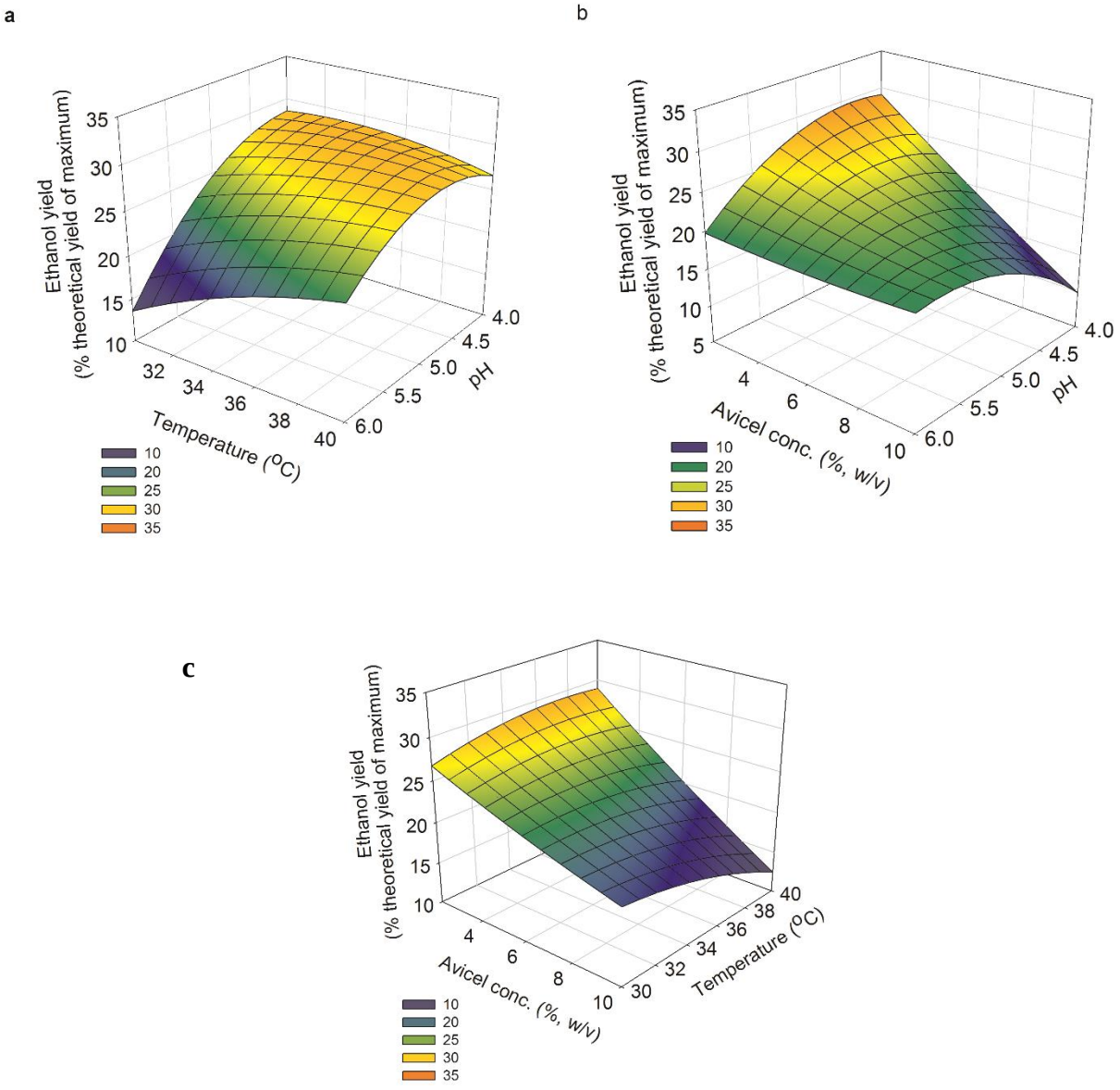

Fig. 1. Response surface plots showing the effect of (a) temperature and pH at a fixed Avicel® concentration, (b) pH and Avicel® concentration at a fixed temperature, and (c) Avicel® concentration and temperature at a fixed pH, on ethanol fermentation yield based on the theoretical maximum glucan yield; SSF was conducted using *S. cerevisiae* D_5A in medium containing 10 mM inhibitory compounds and 15 FPU of Accellerase® 1000/g glucan for 24 h

To determine the validity of the model optimized by RSM, the statistical significance of the model was evaluated by the analysis of variance of the model (Table 2). In addition, in the experimental validation using the inhibitor-containing media under the optimized conditions, an ethanol yield of 26.6% was obtained after 24 h (Fig. 2). This value was in good agreement with the theoretical value of 28.5%. The final ethanol yield after 60 h of SSF was 36.2% due to the significant inhibitory effects of the added compounds on either enzyme or microbial activities.

Table 2. Analysis of Variance for the Response Surface Model

Factor	DF[a]	SS[b]	MS[c]	F value	Prob > F
pH	4	513.3	128.3	76.0	<0.0001
Temperature	4	168.8	42.2	25.0	<0.0001
Avicel® conc.	4	858.2	214.5	127.0	<0.0001

[a] DF: degree of freedom
[b] SS: sum of squares
[c] MS: mean square

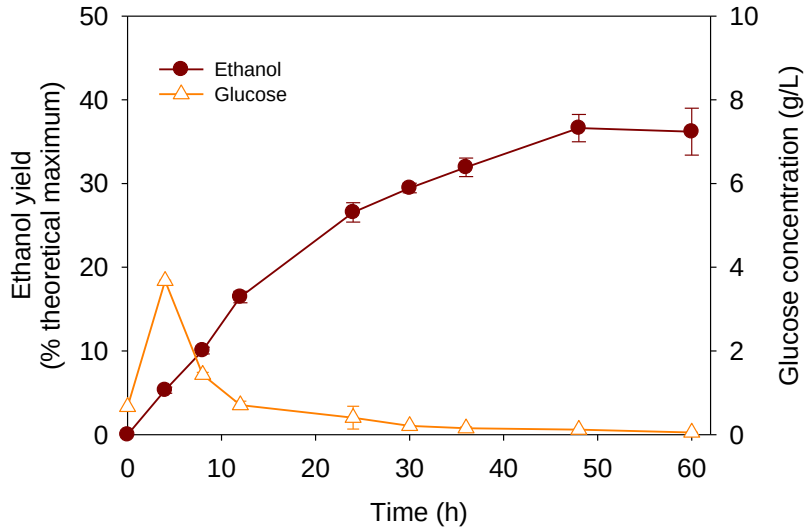

Fig. 2. Time course of ethanol fermentation by *S. cerevisiae* under optimized conditions determined by RSM analysis; SSF was conducted in the medium containing 2.2% Avicel®, 0.05 M citrate buffer (pH 4.7), and 10 mM inhibitory compounds at 35.5 °C for 60 h

Effect of Inhibitory Compounds on Cellulose Hydrolysis

Although inhibitors from pretreatment are well known to affect fermentative microorganisms, quantitative information on the degree of inhibition of both enzyme and yeast performance are limited. Thus, this study was performed using sugar-derived inhibitory compounds in the range between 0 mM to 90 mM, which can simulate the inhibitors in real pretreatment hydrolysates.

The negative effects caused by individual compounds during cellulose hydrolysis in the SSF medium without yeast were investigated (Fig. 3). The largest reduction in hydrolysis yield was observed when HMF was added to the SSF medium. After the addition of 10 mM HMF, a 15.8% decrease in the hydrolysis yield was noted, and when the HMF concentration was increased to 90 mM, an approximate 28% decrease was noted.

This was probably because HMF is derived from glucose, a monomeric sugar of cellulose, which resulted in strong inhibition of cellulase activity. Additionally, furfural and formic acid at 70 mM concentration showed more than a 10% reduction in glucan conversion yield. However, neither acetic acid nor levulinic acid showed any decrease in hydrolysis yield. This was likely because of the stronger polar groups in the formic acid structure and smaller molecular weight of formic acid in comparison with those of other weak acids (Klinke *et al*. 2004; Feng *et al*. 2012). Overall, the toxicity of sugar-derived inhibitory compounds was observed in the following order: HMF > formic acid > furfural > acetic acid ≈ levulinic acid. Thus, from an enzymatic perspective, the generation of HMF should be primarily managed during the pretreatment step.

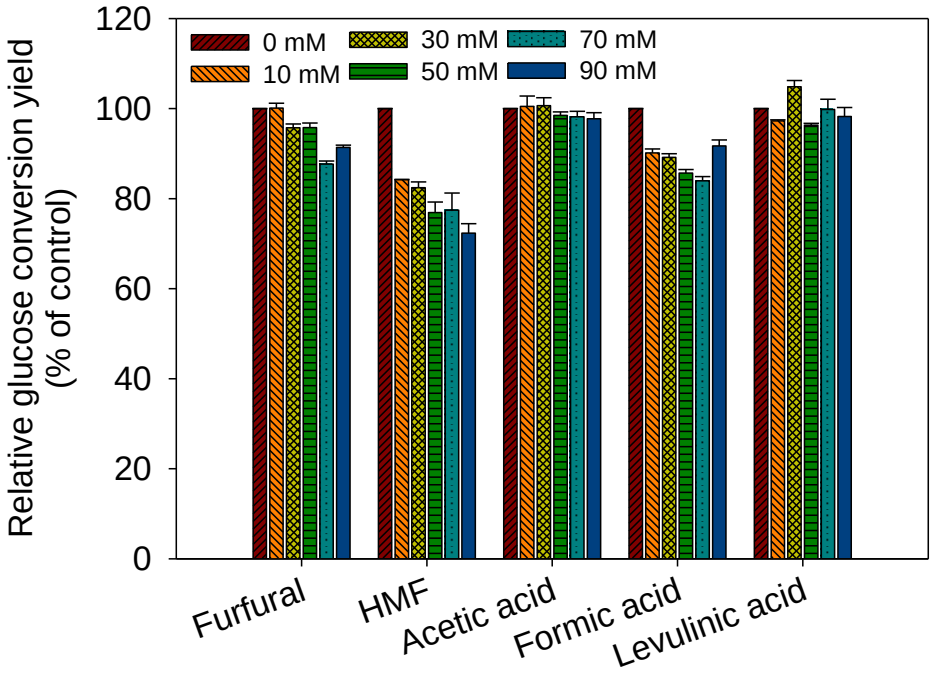

Fig. 3. The effect of different concentrations of inhibitory compounds on enzymatic hydrolysis by cellulase; the SSF medium containing 2.2% Avicel® and various concentrations of each inhibitory compound, including furfural, HMF, acetic acid, formic acid, and levulinic acid, was incubated with 15 FPU Accellerase® 1000/g glucan without *S. cerevisiae* D_5A at pH 4.7 and 35.5 °C for 50 h

Effect of Inhibitory Compounds on Ethanol Fermentation

The negative effects caused by individual inhibitory compounds on ethanol fermentation during SSF were also investigated (Fig. 4). The most rapid reduction in yeast fermentation ability was observed with the addition of furfural. After the addition of 30 mM furfural, an approximate 83% decrease in ethanol yield was noted. Additionally, 30 mM HMF, 50 mM formic acid, and 70 mM acetic acid all substantially reduced the ethanol producing ability (over 50%). Meanwhile, although levulinic acid did not show a noticeable inhibitory effect until at a concentration of 70 mM, it showed approximately a 26% reduction in ethanol yield when 20 mM levulinic acid was added to the media. Thus, it should be considered in the process operation. Interestingly, the addition of the minimum amount of the inhibitory compounds (*i.e.*, 10 mM) was noted to stimulate ethanol production, which is likely related to the improvement of cell growth by organic acids or the enhancement of cofactor balancing by furan compounds (Palmqvist *et al*. 1999; Huang *et al*. 2011; Feng *et al*. 2012).

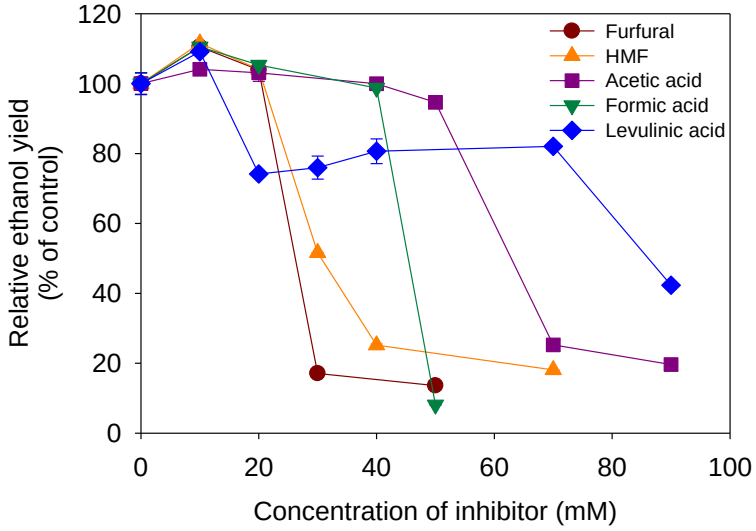

Fig. 4. The effect of different concentrations of inhibitory compounds on ethanol production by yeast; the SSF medium containing 2.2% Avicel® and various concentrations of each inhibitory compound, including furfural, HMF, acetic acid, formic acid, and levulinic acid, was incubated with 15 FPU Accellerase® 1000/g glucan and *S. cerevisiae* D_5A at pH 4.7 and 35.5 °C for 50 h

Overall, in terms of both enzymatic hydrolysis and microbial fermentation in SSF, furan aldehydes showed the greater toxicity than organic acids at the same concentration (Table 2). This is likely because furan compounds can directly affect cell metabolism, while the toxicity of organic acids is mostly correlated with the pH of the fermentation broth (Warnecke and Gill 2005; Almeida *et al*. 2009). Furthermore, the actual inhibition of ethanol production by pretreatment inhibitors was greater than that of sugar degradation products by the commercialized enzyme in this study. Also, considering that the actual amounts of each compound generally produced after pretreatment are less than 40 mM furan and 100 mM organic acid (Klinke *et al*. 2004; Huang *et al*. 2011; Jung *et al*. 2013), a considerable reduction in enzyme and yeast performance is possible under the realistic pretreatment conditions. Thus, the generation of inhibitors during the pretreatment process should be regulated at an appropriate level to suit the final purpose of the subsequent steps.

Table 2. Critical Concentration of Each Inhibitor in the Case of Enzyme and Yeast

Inhibitors	Critical Concentration (mM)	
	Yeast[a]	Enzyme[b]
Furfural	30	70
HMF	30	10
Acetic acid	70	ND[c]
Formic acid	50	30
Levulinic acid	90	ND[c]

[a] More than 50% inhibition
[b] More than 10% inhibition
[c] Not detected in the tested range

CONCLUSIONS

1. The inhibitory effects of the main pretreatment inhibitors, namely furfural, 5-hydroxymethylfurfural (HMF), acetic acid (AA), formic acid (FA), and levulinic acid (LA), on enzymatic hydrolysis and ethanol production were determined.
2. The toxicity of the inhibitors was in the following orders: HMF > FA > furfural > AA ≈ LA for enzymatic hydrolysis, and furfural > HMF > FA > AA > LA for the ethanol production by yeast.
3. The most potent inhibitors of enzymatic hydrolysis were HMF and formic acid, and those of yeast ethanol production were furfural and HMF at optimized SSF conditions.

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REFERENCES CITED

Almeida, J. R. M., Bertilsson, M., Gorwa-Grauslund, M. F., Gorsich, S., and Lidén, G. (2009). "Metabolic effects of furaldehydes and impacts on biotechnological processes," *Appl. Microbiol. Biot.* 82(4), 625-638. DOI: 10.1007/s00253-009-1875-1

Dowe, N., and McMillan, J. (2008). *SSF Experimental Protocols -Lignocellulosic Biomass Hydrolysis and Fermentation* (NREL/TP-510-42630), National Renewable Energy Laboratory, Golden, CO.

Feng, Y., Qi, X., Jian, H. L., Sun, R. C., and Jiang, J. X. (2012). "Effect of inhibitors on enzymatic hydrolysis and simultaneous saccharification fermentation for lactic acid production from steam explosion pretreated *Lespedeza* stalks," *BioResources* 7(3), 3755-3766. DOI: 10.15376/biores.7.3.3755-3766

Huang, H., Guo, X., Li, D., Liu, M., Wu, J., and Ren, H. (2011). "Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids," *Bioresource Technol.* 102(16), 7486-7493. DOI: 10.1016/j.biortech.2011.05.008

Jönsson, L. J., Alriksson, B., and Nilvebrant, N. O. (2013). "Bioconversion of lignocellulose: Inhibitors and detoxification," *Biotechnol. Biofuel.* 6(1), 16. DOI: 10.1186/1754-6834-6-16

Jönsson, L. J., and Martín C. (2016). "Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects," *Bioresource Technol.* 199, 103-112. DOI: 10.1016/j.biortech.2015.10.009

Jung, Y. H., Kim, I. J., Kim, H. K., and Kim, K. H. (2013). "Dilute acid pretreatment of lignocellulose for whole slurry ethanol fermentation," *Bioresource Technol.* 132, 109-114. DOI: 10.1016/j.biortech.2012.12.151

Jung, Y. H., and Kim, K. H. (2014). "Acidic pretreatment," in: *Pretreatment of Biomass: Processes and Technologies*, A. Pandey, S. Negi, P. Binod, C. Larroche (eds.), Academic Press, MA, USA, 27-50. DOI: 10.1016/B978-0-12-800080-9.00003-7

Jung, Y. H., Park, H. M., Kim, I. J., Park, Y. C., Seo, J. H., and Kim, K. H. (2014). "One-pot pretreatment, saccharification and ethanol fermentation of lignocellulose based on acid-base mixture pretreatment," *RSC Adv.* 4(98), 55318-55327. DOI: 10.1039/C4RA10092A

Klinke, H. B., Thomsen, A. B., and Ahring, B. K. (2004). "Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass," *Appl. Microbiol. Biot.* 66(1), 10-26. DOI: 10.1007/s00253-004-1642-2

Kwon, Y. J., Ma, A. Z., Li, Q., Wang, F., Zhuang, G. Q., and Liu, C. Z. (2011). "Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*," *Bioresource Technol.* 102(17), 8099-8104. DOI: 10.1016/j.biortech.2011.06.035

Palmqvist, E., Grage, H., Meinander, N. Q., and Hahn-Hägerdal, B. (1999). "Main and interaction effects of acetic acid, furfural, and *p*-hydroxybenzoic acid on growth and ethanol productivity of yeasts," *Biotechnol. Bioeng.* 63(1), 46-55. DOI: 10.1002/(SICI)1097-0290(19990405)63:1<46::AID-BIT5>3.0.CO;2-J

Warnecke, T., and Gill, R. T. (2005). "Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications," *Microb. Cell Fact.* 4(1), 25. DOI: 10.1186/1475-2859-4-25

Yang, B., and Wyman, C. E. (2008). "Pretreatment: The key to unlocking low-cost cellulosic ethanol," *Biofuel. Bioprod. Bior.* 2(1), 26-40. DOI: 10.1002/bbb.49

Zaldivar, J., and Ingram, L. O. (1999). "Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01," *Biotechnol. Bioeng.* 66(4), 203-210. DOI: 10.1002/(SICI)1097-0290(1999)66:4<203::AID-BIT1>3.0.CO;2-#

Zhu, J. Y., and Pan, X. J. (2010). "Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation," *Bioresource Technol.* 101(13), 4992-5002. DOI: 10.1016/j.biortech.2009.11.007

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