EFFECT OF INHIBITORS ON ENZYMATIC HYDROLYSIS AND SIMULTANEOUS SACCHARIFICATION FERMENTATION FOR LACTIC ACID PRODUCTION FROM STEAM EXPLOSION PRETREATED *LESPEDEZA* STALKS

Yue Feng, Xiang Qi, Hong-lei Jian, Run-cang Sun, and Jian-xin Jiang *

The effects on both cellulose conversion rate and lactic acid yield were studied by adding inhibitors, including formic acid, acetic acid, furfural, and vanillin into the hydrolysate of steam-pretreated *Lespedeza* stalks. The results suggest that formic acid has a significant influence on the enzyme activity and poisoned bacterial cells, resulting in the reduction of cellulose conversion rate and lactic acid yield by 21% and 16.4%, respectively. Acetic acid showed a strong inhibition on simultaneous saccharification fermentation (SSF) process, but little effect on enzymatic hydrolysis. Hydrolysis and SSF were less affected by furfural and vanillin compared with weak acids. The lactic acid yield of *Lespedeza* stalks rinsed with water increased from 64.0% to 89.4%, and the time to reach the maximum concentration was shortened from 96 hours to 48 hours when compared with the unwashed materials.

Keywords: Lactic acid; Inhibitors; Lespedeza stalk; Enzymatic hydrolysis; Steam explosion pretreatment; Simultaneous saccharification and fermentation

*Contact information: Department of Chemistry and Chemical Engineering, Beijing Forestry University, Beijing 100083, China; * Corresponding author: jiangjx2004@hotmail.com*

INTRODUCTION

Lactic acid is commonly recognized as one of the most versatile organic acids, with a long history of usage for the preservation of foodstuffs. It has a broad range of applications in the textile, pharmaceutical, cosmetic, and chemical industries (Davison *et al.* 1995), especially as a feedstock monomer in the polymer industry for the manufacture of poly-(lactic acid) (PLA). The level of lactic acid production was estimated at around 68 million kg per year, and worldwide growth is believed to be 12 to 15% per year (Wassewar 2005). Current research and developments are being directed at the substitution of higher-cost sugar and starch by low-cost lignocellulosic biomass as a way of increasing the production of energy. Examples of lignocellulosic biomass include woody materials, sugar cane bagasse, corn stover, and purpose-grown energy crops.

The efficient bioconversion of lignocellulosic materials to lactic acid calls for some form of pretreatment, but this step itself can introduce two major problems for the subsequent conversion of lignocellulosic hydrolysate to lactic acid. The first is that the hydrolysate contains a broad range of compounds that have inhibitory effects on fermentation strains (Oliva *et al.* 2006). The composition of these compounds depends on the type of lignocellulosic material, as well as the chemistry and the nature of the pretreatment process (Cantarella *et al.* 2004). The second problem concerns the fact that

hemicellulose hydrolysates contain not only hexose, but also pentose sugars. Hexose sugars are readily fermentable using established procedures and industrial strains; however, the pentose sugars are more difficult to ferment and the processes are not economical (Boussaid *et al.* 2001). Steam explosion pretreatment is one of the most attractive pretreatment processes owing to its low chemical usage and energy consumption (Heitz *et al.* 1991). It disrupts the lignin barrier and makes enzyme contact with cellulose more available by removing hemicellulose. In spite of these advantages, there are also some limitations; steam explosion pretreatment, at least partially, degrades hemicellulose-derived sugars and transforms lignin compounds into chemicals that can inhibit downstream processes (Li *et al.* 2009).

The properties and concentrations of the final inhibitors are influenced by the pretreatment conditions (such as chemicals, temperature, and residence time) and the variety of raw materials (hardwood, softwood, herbaceous plants). These inhibitors can be classified into weak acids, furan derivatives, and phenolic compounds according to their chemical structure (Wahlbom and Hahn-Hägerdal 2002; Martin *et al.* 2007). The weak acids commonly include acetic acid, formic acid, and levulinic acid. Furan derivatives mainly contain furfural and 5-hydroxymethylfurfural (5-HMF), which are products of pentose and hexose degradation, respectively. Phenolic compounds are generated from partial breakdown of lignin and are mostly referred to as vanillic acid and vanillin (Cantarella *et al.* 2004). The type of phenolic compounds strongly depends on raw material. The main degradation pathways are schematically presented in Fig. 1 (Palmqvist and Hahn-Hägerdal 2000). The furan derivatives and phenolic compounds will react further to form some polymeric materials (Tengborg *et al.* 2001).

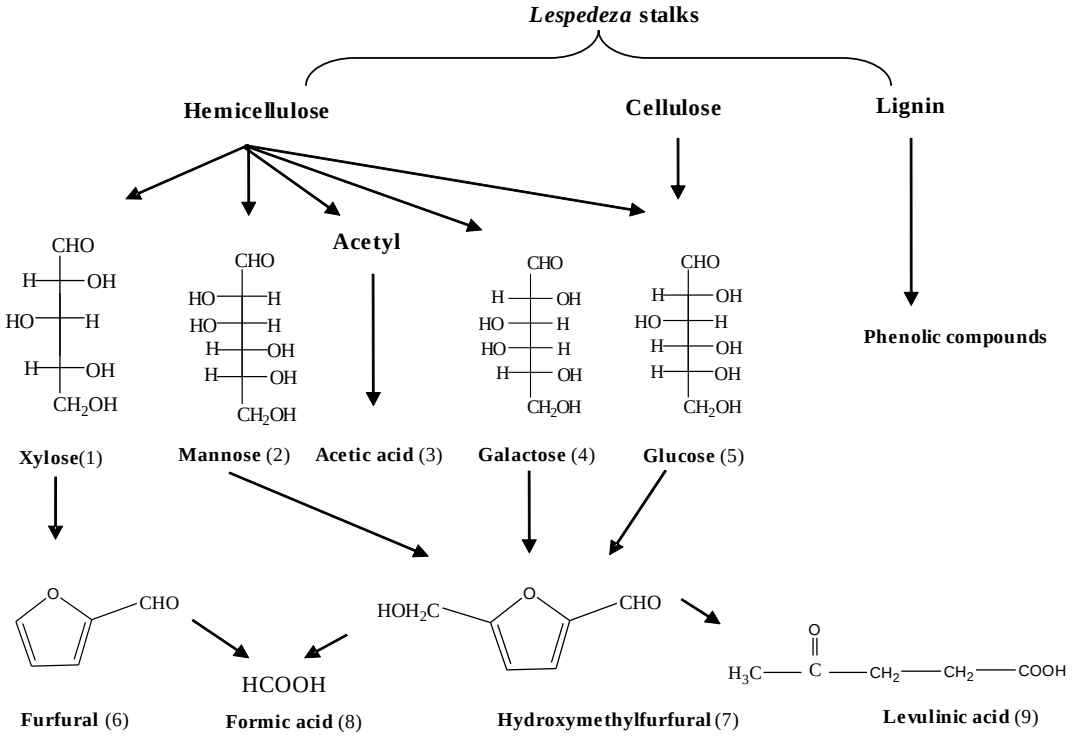

Fig. 1. Reactions occurring during hydrolysis of lignocellulosic materials (Palmqvist and Hahn-Hägerdal 2000)

Lespedeza crytobotrya, a perennial shrub species of the leguminous genus *Lespedeza*, has the properties of substantial biomass, anti-sterility, drought-resistance, and frost-hardness with a well-developed root system (Feng *et al.* 2011). Its branches and leaves could be used as a fertilizer, and its stalks could potentially be an appropriate substrate for bioconversion (Jiang *et al.* 2006; Wang *et al.* 2009). Hence, *Lespedeza* stalks are a potential alternative source of biomass. The objective of this study was to examine the generation of inhibitors degraded from pretreated stalks, and the effects of these inhibitors both on cellulose conversion rate and lactic acid yield. Weak acids (formic and acetic acid), products of xylose degradation (furfural), and lignin degradation products (vanillin) were selected to investigate their effects on hydrolysis and SSF. If the inhibitors are identified and the mechanisms of inhibition elucidated, fermentation can be improved by developing specific detoxification methods, choosing an adapted microorganism, or optimizing the fermentation strategy.

EXPERIMENTAL

Steam Explosion Pretreatment

Lespedeza stalks employed in this work were obtained from an experimental farm of Beijing Forest University. The stalks were air-dried and cut into an average size of 50 mm × 30 mm × 5 mm. They were then pretreated in a flash hydrolysis laboratory pilot unit (7.5 L reactor) at a temperature of 210 ºC with a residence time of 4 min. Using only water as a catalyst has been classified as an autohydrolysis process due to the hydrolytic acid liberated from acetyl xylan. The pretreated materials were stored at 4 ºC in a sealed plastic bag for further analysis. The cellulose was determined by the nitric acid-ethanol method, lignin by 72% H_2SO_4 method, and hemi-cellulose by the double-bromination method (Liu 2004).

The *Lespedeza* stalks with steam explosion pretreatment were washed as follows: two samples of 50 g each were washed with 600 mL of distilled water and stirred at 200 rpm for 3 h at room temperature. The suspensions were filtered under vacuum through filter paper to remove the liquid produced. One solid sample was recovered and labeled as WR1, and the other one was rinsed thoroughly with 3.5 L of distilled water to further remove inhibitors (WR2). Washed samples were stored at 4 ºC.

Enzymes and Microorganisms

A commercial cellulase was used in the experiments as the sole enzymatic complex; this was kindly supplied by Sunson Corporation (Ningxia, China). The cellulase activity was measured and expressed as filter paper units FPU/g. The filter paper activity, endoglucanase activity and β-glucosidase activity of the commercial enzyme were 100 FPU/g, 87.7 U/g, and 24.7 U/g, respectively. Freeze-dried lactobacilli used in this study mainly consist of *Lactobacillus thermophilus* and *Lactobacillus bulgaria*, which were purchased from Meihua Company (Haerbin, China). The number of living cells at packing was above 2.0×10^{10}/g, as defined by the manufacturer.

Enzymatic Hydrolysis

Enzymatic hydrolysis experiments were performed in 100 mL Erlenmeyer flasks containing 60 mL deionized water (pH 5.5) at a substrate loading of 6% DM (w/v) and using an enzyme loading of 15 FPU (g substrate)$^{-1}$. The flasks were placed on an air-bath shaker at 43 ºC and 100 rpm for 96 h. Samples were withdrawn periodically and centrifuged at 2500 g for 10 min. The cellulose conversion rate was calculated according to the following expression,

$$\text{Conversion rate (\%)} = \frac{C \times V}{M \times G_{\text{n}} \times 180/162} \times 100 \quad (1)$$

where the Conversion rate is cellulose conversion rate, C is the glucose concentration expressed as g glucose /L, V is the volume of medium (in all cases this value is 0.06 L), G_{n} is the glucan content of the substrate expressed as g glucan/g substrate, and M is the substrate loading expressed as g.

Inhibitions study of enzymatic hydrolysis by selected compounds, formic acid (0.1 to 0.5 g/L), acetic acid (0.5 to 2.0 g/L), furfural (0.5 to 2.0 g/L), and vanillin (0.1 to 0.5 g/L) were carried out in substrate of WR2.

Simultaneous Saccharification and Fermentation (SSF)

All fermentation experiments with 6 % DM (w/v) steam pretreated solids were conducted in 100 mL flasks containing 60 mL of medium in a rotary shaker. The composition of the SSF medium was 5 g/L yeast extract, 0.5 g/L $MgSO_4$, 0.1 g/L NaCl, and 0.5 g/L KH_2PO_4. The pH was approximately controlled at 4.8 with $CaCO_3$. The SSF experiments were conducted by adding 0.5 % (w/v) dry lactobacilli cells of the total liquid volume and 15 FPU cellulase per gram of substrate, and then incubated in a shaker at 43 ºC and 100 rpm for 96 h. Samples were withdrawn periodically, and lactic acid was analyzed by high-performance liquid chromatography (HPLC). All experiments were performed in duplicates, and the average deviation was less than 5.0 %. The lactic acid yield was calculated according to the following expression,

$$\text{Yield (\%)} = \frac{L \times V}{M \times \text{G}_{\text{n}} \times 180/162} \times 100 \quad (2)$$

where the *Yield* is lactic acid yield, L is the lactic acid concentration expressed as g lactic acid per L, V is 0.06 L, G_{n} is the glucan content of the substrate expressed as g glucan/g substrate, and M is the substrate loading expressed as g.

Inhibition studies of SSF for lactic acid by selected compounds, formic acid (0.1 to 0.5 g/L), acetic acid (0.5 to 2.0 g/L), furfural (0.5 to 2.0 g/L), and vanillin (0.1 to 0.5 g/L) were carried out with the substrate of WR1.

Analyses

The filter paper activity, endoglucanase activity, and β-glucosidase activity were evaluated following the standard method of IUPAC (Ghose 1986). Weak acids (lactic, formic, and acetic acid), furfural, and vanillin were analyzed with an HPLC system

(Gilson Unipoint, France) equipped with 7725i tunable absorbance detector set to 210 nm. A C_{18} YMC-Pack ODS-A ion-exclusion column (250×4.6 mm, A-303) was used with 0.01 mol/L H_3PO_4-NaH_2PO_4 buffer (pH 2.5) as mobile phase at a flow rate of 1 mL/min, while the column temperature was maintained at 35 °C. Glucose in the fermentation broth was determined by HPAEC system (Dionex ICS3000, USA) with pulsed amperometric detector and an ion exchange Carbopac PA-1 column (250×4 mm). The neutral sugars were separated in 18 mM NaOH (carbonate free and purged with nitrogen) with post-column addition of 0.3 M NaOH at a rate of 0.5 mL/min. Run time was 45 min, followed by a 10 min elution with 0.2 M NaOH to wash the column and then a 15 min elution with 18 mM NaOH to re-equilibrate the column.

All experiments were performed in duplicate under the same conditions and average values were reported. The standard deviations were less than 2.8%.

RESULTS AND DISCUSSION

Lespedeza Stalks with Steam Explosion Pretreatment and Water Rinsing

The composition of native and steam explosion pretreated *Lespedeza* stalks was determined based on the wet matter content. Results are reported in Table 1. Comparison of the stalks composition before and after pretreatment indicated that the hemi-cellulose component was mainly degraded during steam explosion pretreatment.

Table 1. Chemical Composition of Native and Steam Explosion Pretreated *Lespedeza* Stalks (dry matter content)

Composition	*Lespedeza* Stalks	
	Native	Steam Pretreated
Lignin (%)	17.0	22.2
Cellulose (%)	37.6	44.0
Hemicellulose (%)	29.3	4.7

Table 2. Composition of Inhibitors from Steam Explosion Pretreated *Lespedeza* Stalks

Inhibitors	Concentration (mg/L)		
	Un-washed Biomass[a]	WR1[b]	WR2[c]
Formic acid	130	11	0.10
Acetic acid	590	52	0.64
Furfural	400	34	0.23
Vanillin	110	10	0.087

[a]Un-washed biomass contained 31.05 % of total solids
[b] WR1: water-rinsed material with 10 fold water
[c] WR2: water-rinsed material with 1000 fold water

The *Lespedeza* sample was washed with a different volume of distilled water after steam explosion. WR1 and WR2 possessed moisture contents of 62.0% and 57.0%, respectively; the cellulose content was 62.2% and 63.1%, respectively. The inhibitor concentrations in flasks are shown in Table 2. One can observe that the reductions in inhibitor concentrations for WR1 and WR2 were approximately 10- and 1000-fold when compared with un-washed biomass. Thus, WR2 could be regarded as steam explosion pretreated sample with little inhibitors, which was the model substrate in this research.

Effect of Water Rinsing on SSF for Lactic Acid

Figure 2 shows the results of the SSF experiments with unwashed material, WR1, and WR2, as substrates. There were two stages observed in the SSF process. With the initial stage lasting about 12 h (except unwashed biomass), fermentation was the limiting step of the overall kinetics, since the rate of enzymatic hydrolysis was comparatively faster, resulting in the accumulation of glucose. In the second period, the enzymatic hydrolysis became the limiting step. Glucose was gradually depleted, while lactic acid concentration was sharply increased. For unwashed biomass, WR1, and WR2, the maximum lactic acid concentration of 18.6, 24.4, and 25.0 g/L were achieved at 96, 48, and 48 h, respectively. It could be found that the lactic acid yield could be promoted from 64.0 to 89.4% of theoretical by water-rinsed pretreatment.

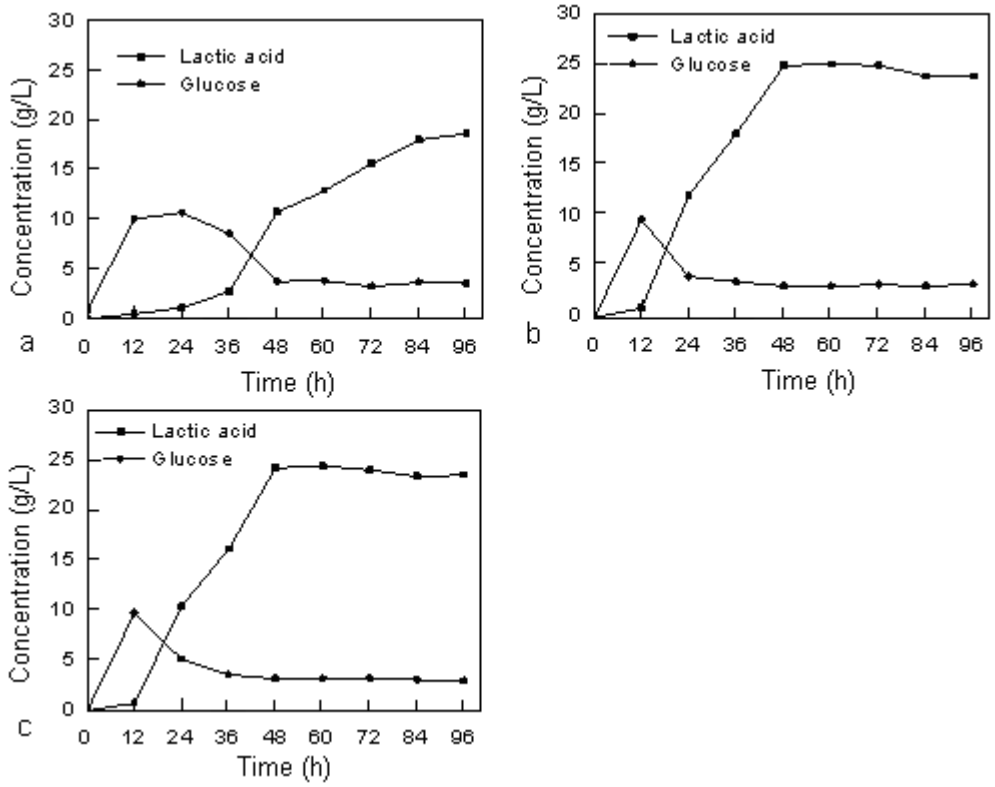

Fig. 2. Comparison of SSF experiments carried out with different conditions pretreated biomass as substrate. (a) unwashed material, (b) WR1 (water-rinsed material with 10 fold water), and (c) WR2 (water-rinsed material with 1000 fold water)

Meanwhile, the time of reaching maximum concentration was greatly decreased from 96 to 48 h, which suggests that water processing is a suitable method to remove inhibitors to improve lactic acid yield. WR1 and WR2 gave similar maximum lactic acid concentration and fermentation time, which indicated that effects of inhibitors would be eliminated practically after 10-fold water rinsing. However, there are some disadvantages for washing procedure such as producing large volumes of process water, removing the soluble sugars, and causing high costs of detoxification (Von Sivers *et al.* 1994).

Effect of Toxic Compounds on Enzymatic Hydrolysis

The following cases were designed in order to assess the inhibiting effects of toxic compounds at different concentrations of hydrolysis steps: (i) using WR2 as a model substrate due to its negligible toxicity, (ii) using the model substrate and adding one type of inhibitor with the same concentration as original slurry (unwashed biomass in flask), (iii) using the model substrate and adding one type of inhibitor with 4-fold higher concentration than the original. The production of sugar was monitored, and cellulose conversion rate was simultaneously determined based on the amount of cellulose supplied to enzymatic hydrolysis step.

Acetic acid (Fig. 1, no. 3) was derived from hemicellulose degradation, and formic acid (Fig. 1, no. 8) was formed when furfural and HMF were broken down. The effect of various concentrations of formic acid (Fig. 3a) and acetic acid (Fig. 3b) on cellulose conversion rate in the hydrolysis step was investigated. The highest cellulose conversion rate (87.9 % at 96 h) was obtained from model substrate (as control). The cellulose conversion rate decreased with increasing formic acid concentration and declined to 68.9 % at a formic acid concentration of 0.5 g/L. This indicated that formic acid strongly restrained the activity of cellulase complex. However, acetic acid did not exert any effect on enzyme hydrolysis, and the cellulose conversion variation trend was similar to the control at the experimental concentrations. This was in accordance with the findings of Cantarella *et al.* (2004) that acetic acid (2.0 g/L) did not significantly affect the enzyme activity from steam pretreated poplar wood. A significant difference was observed in cellulose conversion rate between formic acid and acetic acid at the same concentration. This may be due to the differences of their molecular structures. There was a stronger polar group in formic acid compared with acetic acid, which could exert distinct inhibitory effect on enzymatic hydrolysis.

Xylose was liberated into the liquid during hemicellulose degradation. Xylose was further degraded to furfural (Fig. 1, no. 6) under high temperatures. Figure 3c shows the effect of furfural on enzyme hydrolysis at the concentration of 0.5 g/L and 2.0 g/L. The highest concentration of furfural was determined to be 2.0 g/L based on the literature (Rudolf *et al.* 2005) and its concentration in the original slurry. One can observe that there was a small reduction of approximately 7.5% when the furfural was added. However, cellulose conversion rate exhibited little decrease along with the increment of furfural concentration, which suggested that furfural slightly depressed enzyme activity.

Phenolic compounds are generated from partial breakdown of lignin and carbohydrate degradation. Vanillin is one important kind of lignin degradation byproduct formed by the degradation of the guaiacylpropane units of lignin; this has been detected in hydrolysate from willow, spruce, poplar, red oak, and pine (Nilvebrant *et al.* 1997;

Jonsson *et al.* 1998). Therefore, the influence of vanillin on enzyme hydrolysis was investigated here. As shown in Fig. 3d, the cellulose conversion was the same as for the control. The behavior of the enzyme in the presence of vanillin was similar to that with acetic acid, with no apparent effects on the glucose yield. In fact, in the whole range of concentration explored, vanillin and acetic acid did not affect enzymatic hydrolysis, even at five times the initial concentration.

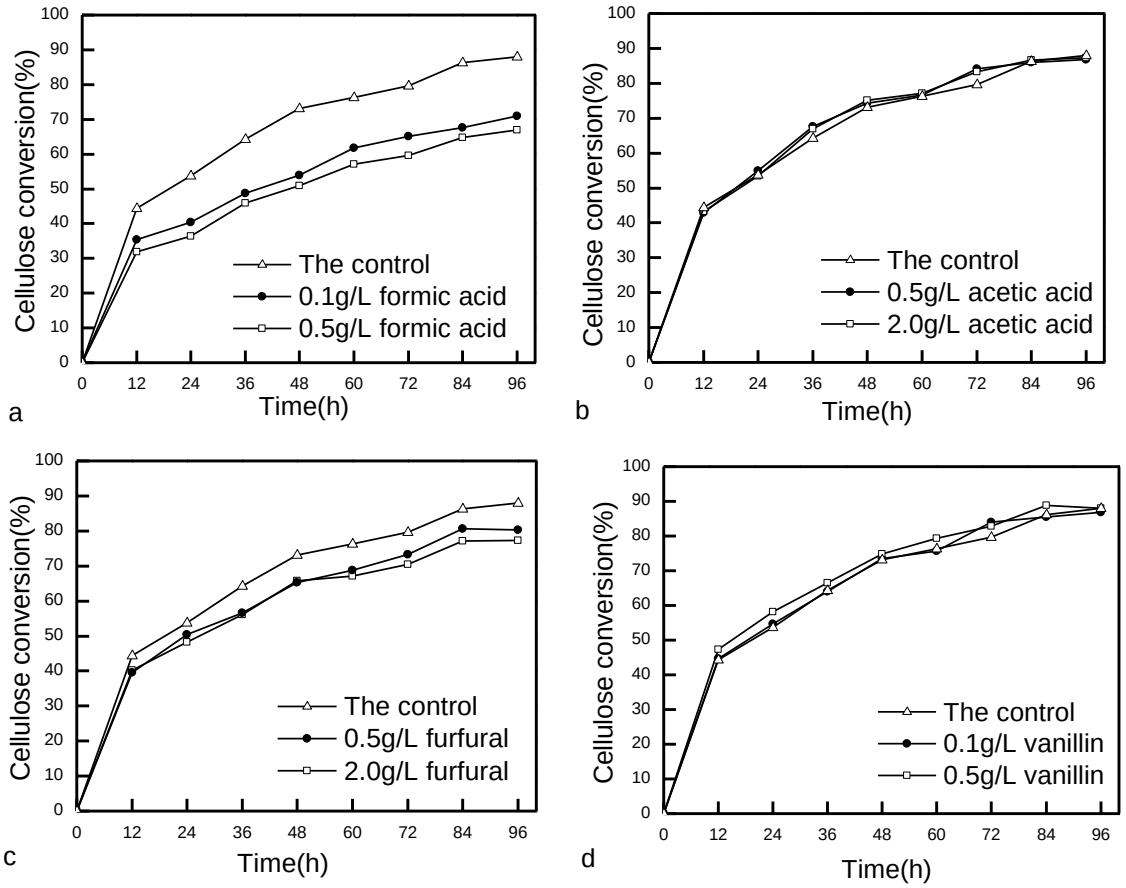

Fig. 3. Effect of inhibitors on cellulose conversion in enzymatic hydrolysis. (a) formic acid, (b) acetic acid, (c) furfural, (d) vanillin

Effect of Toxic Compounds on SSF

The lactic acid yield variation with time in the presence of formic acid (Fig. 4a) and acetic acid (Fig. 4b) is shown. The maximum yield of lactic acid (87.9 % at 48 h) was observed in the model substrate (as control). An addition of 0.1g/L or 0.5 g/L formic acid led to a 16.4% reduction of lactic acid yield, while a reduction of 21% for cellulose conversion rate was found in previous hydrolysis. Such results may be because high concentrations of sugars derived from hydrolysis step had a great inhibition on the enzyme activity, preventing some cellulose from being liberated.

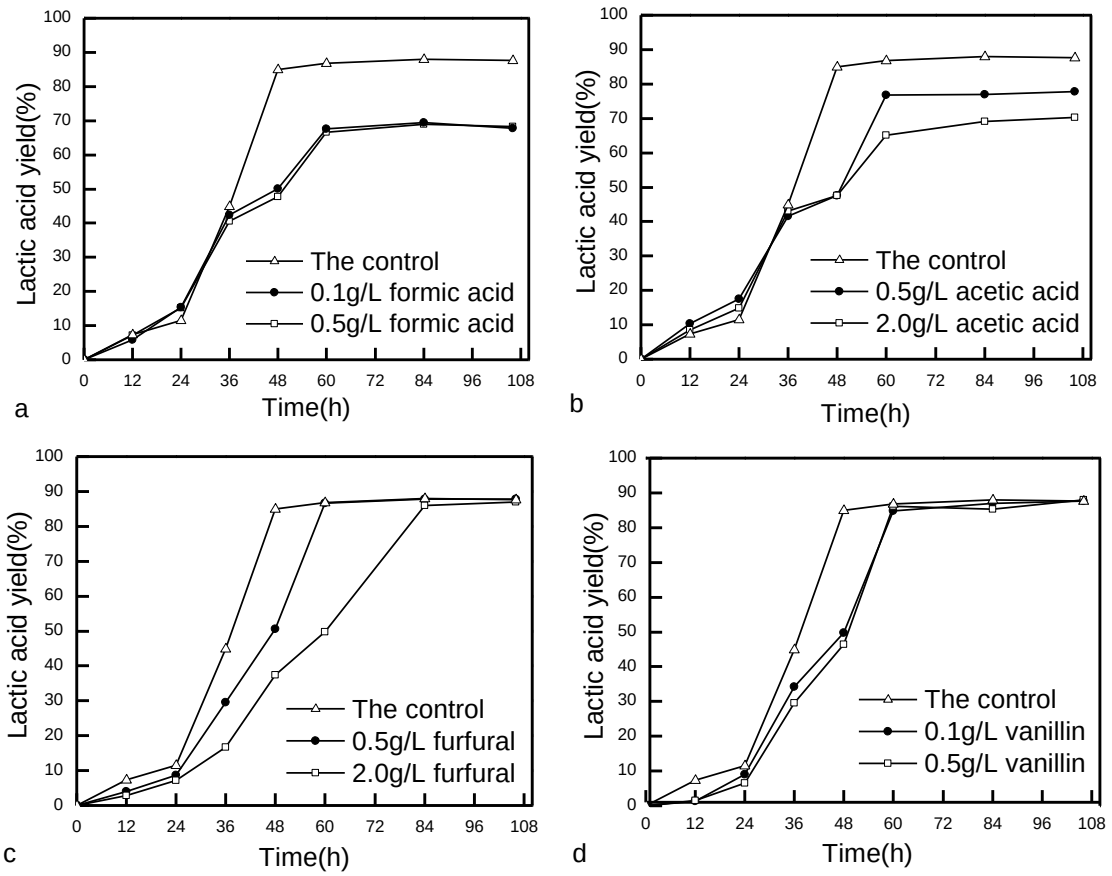

Fig. 4. Effect of inhibitors on lactic acid yield in SSF. (a) formic acid, (b) acetic acid, (c) furfural, (d) vanillin

A similar phenomenon of lactic acid yield reduction was observed in the presence of acetic acid with the concentration of 2.0 g/L. The maximum yield of lactic acid was 77.7%, and the yield was reduced with increasing concentration of acetic acid, resulting in a minimal lactic acid yield of 70.3% at the acetic acid concentration of 2.0 g/L. As can been seen in Fig. 4, a noticeable difference in toxicity between formic acid and acetic acid was observed at the same concentration (0.5 g/L). Such results may have arisen from the differences in membrane permeability or in toxicity of the anionic form of the acids once they have entered the cell. This effect could be explained by the uncoupling theory, which was proposed by Russell (1992). The drop in intracellular pH resulting from inflow of weak acids is neutralized by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. At high acid concentration, the proton pumping capacity of the cell reaches a maximum, resulting in the depletion of ATP. Therefore, less ATP is available for biomass production. The results demonstrated that the reduction of lactic acid yield was attributable to the toxicity of formic acid and acetic acid to bacterial cells.

The effect of furfural with different concentrations on lactic acid yield is shown in Fig. 4c. The most pronounced effect of furfural was a prolonged lag phase. The 12 h and 36 h were lagged in the presence of furfural at the concentration of 0.5 g/L and 2.0 g/L, respectively. Growth was more sensitive to furfural than lactic acid yield (Tu *et al.* 2009).

It was reported that furfural prevented the formation of glycerol, which was necessary to regenerate the excess NADH during fermentation and to maintain the intracellular redox balance. Furfural inhibition of glycolytic enzymes *in vitro* has also been reported, and direct inhibition of ADH might have contributed to acetaldehyde excretion. These findings were in accordance with those of Oliva *et al.* (2006), who pointed out that the presence of furfural and HMF at the experimental concentrations did not affect the final ethanol concentration, although affected the respiration and oxidative phosphorylation and slow down the electron transport system. As a result, ATP production in the cells decreased and cell growth ceased, but it had no influence on the final product yield (the same phenomenon of our research in Fig. 4c). Similarly, furfural has been suggested to be more sensitive to cell growth than ethanol production (Palmqvist *et al.* 1999a).

Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysate, with low molecular weight phenolic compounds being most toxic. However, the mechanism of the inhibiting effect has not been elucidated, largely due to a lack of accurate qualitative and quantitative analyses. The influence of vanillin with different concentrations on SSF was studied, and the results are shown in Fig. 4d. There were no obvious differences in the maximum yield of lactic acid compared to the control, and the time of reaching the maximum yield was delayed 12 h. This finding suggests that vanillin prolonged the lag phase. The research of Palmqvist *et al.* (1999b) showed that no significant effects on either growth or volumetric ethanol productivity were detectable during fermentation with 2 g/L vanillin. Vanillin has been found to be less toxic than 4-hydroxybenzoic acid (1g/L caused a decrease of 25 % in the ethanol yield). Considering the effects on hydrolysis and fermentation, vanillin appeared to be the most nontoxic to lactic acid fermentation compared to the other inhibitors.

CONCLUSIONS

1. The results of hydrolysis experiments in the presence of inhibiting compounds indicated that formic acid strongly depressed the glucose yield. On the contrary, acetic acid did not affect enzymatic hydrolysis within the whole range of investigated concentrations. Furfural slightly reduced the glucose yield. A reduction of 7.5 % for cellulose conversion rate was observed in the hydrolysis step. Vanillin displayed little inhibitory effect on hydrolysis.
2. The data of SSF experiments suggested Formic acid and acetic acid exerted a strong inhibiting action on lactic acid yield. Furfural and vanillin appeared to have no effect on the final lactic acid yield. However, it could significantly extend the lag phase of lactic acid production. It follows that all the inhibitors generated in the course of processing should be removed or diluted prior to use.

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