Detoxification of Steam-Exploded Corn Stover Prehydrolyzate with Organobentonite Enhances Ethanol Fermentation by *Pichia stipitis*

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The inhibitors derived from degradation of lignocellulose have adverse impacts on fermentation, which is considered to be a fundamental problem in bioethanol production. Fermentation of steam-exploded corn stover prehydrolyzate by *Pichia stipitis* showed that phenolic compounds had much higher inhibitory effects than weak acids and furan at high fermentation pH. Two types of organobentonite (cetyltrimethylammonium (CTMA)- and benzyltrimethylammonium (BTMA)-modified bentonite) were used to remove phenolic compounds in prehydrolyzate. The effectiveness of organobentonite treatment was evaluated by ethanol fermentation, which indicated that the organobentonite treatment improved the fermentability substantially, even though a noticeable difference was found in the phenol removal by the two organobentonites. Without organobentonite treatment, the sugar utilization ratio was only 68.1%, and the produced ethanol was 15.36 g/L. After CTMA- and BTMA-bentonite treatment, the sugar utilization ratios were beyond 95%; meanwhile, the ethanol production increased by 45.5% and 42.8%, respectively. This indicated that organobentonite treatment was a potential detoxification method.

Keywords: Detoxification; Steam-exploded prehydrolyzate; Organobentonite; Adsorption; Fermentation

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INTRODUCTION

Growing concerns about the depletion of fossil resources and carbon emission issues have led to the investigation of lignocellulose-derived bioethanol, which is considered to be a good alternative to gasoline (Galbe and Zacchi 2002; Limayem and Ricke 2012; Mood *et al.* 2013). Lignocellulosic materials are the most abundant resources of carbohydrates, typically containing 25% to 55% cellulose and 20% to 35% hemicellulose (Sun and Cheng 2002). The fermentable sugars can be liberated during enzymatic hydrolysis of lignocellulose and then converted to ethanol by microorganisms. Normally, to reduce the recalcitrance of lignocellulose, pretreatment is conducted prior to enzymatic hydrolysis (Chang and Holtzapple 2000; DeMartini *et al.* 2013). After pretreatment, the lignocellulosic materials are typically fractionated into a cellulose-rich solid portion (pretreated substrate) and a hemicellulose-rich liquid portion (prehydrolyzate). However, the pretreatment simultaneously generates a wide range of inhibitory compounds from the degradation of carbohydrates, lignin, and extractives (Palmqvist and Hahn-Hägerdal 2000a). It has been reported that these degradation compounds inhibit enzyme catalysis reactions as well as microbial fermentation (Palmqvist and Hahn-Hägerdal 2000b; Michelin *et al.* 2015).

The inhibitors are usually divided into three major groups based on their origin: weak acids, furan compounds, and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000a). The inhibitory mechanisms of these three groups of inhibitors have been studied extensively, but findings are still far from comprehensive, especially concerning the phenolic compounds (Palmqvist *et al.* 1999; Palmqvist and Hahn-Hägerdal 2000b; Mussatto and Roberto 2004).

As for weak acids, the inhibitory activity is pH-dependent (Pampulha and Loureiro-Dias 1989); uncoupling and intracellular anion accumulation have been proposed as two potential inhibition mechanisms. Furan compounds have been found to be consumed by yeasts and converted into the corresponding alcohols, most likely by reduced nicotinamide adenine dinucleotid (NADH)-dependent enzymes. Phenolic compounds, mostly from lignin degradation, are considered to be the most toxic inhibitors, especially phenolic aldehydes, because they have the potential ability to destroy the integrity of biological membranes (Palmqvist and Hahn-Hägerdal 2000b). To identify the most toxic inhibitors, the inhibition of these degraded compounds has been evaluated for ethanol fermentation by adding model compounds to pure sugar fermentation (Larsson *et al.* 2000; Klinke *et al.* 2004; Cao *et al.* 2014). However, the complex composition of inhibitors and their low concentrations make the identification process more difficult.

Significant efforts have been made for the development of detoxification methods to improve the fermentability of lignocellulosic hydrolyzates. The current reported detoxification methods are classified into physical, chemical, and biological approaches. Evaporation or steam stripping can be used to remove volatile inhibitors, but the non-volatile inhibitors remain untouched (Larsson *et al.* 1999b). Wood charcoals and resin adsorption can be used to remove the phenolic compounds (Miyafuji *et al.* 2003; Schwartz and Lawoko 2010), but the loss of sugar might occur. Various chemical treatments, such as lime, ammonium hydroxide, and sulfite treatments, can be applied to improve the fermentability of hydrolyzates (Larsson *et al.* 1999b; Martinez *et al.* 2001), although lime treatment is reported to cause over 10% sugar loss from softwood dilute acid hydrolyzates (Hodge *et al.* 2009). Laccase, peroxidase, or specific fungi could oxidize the phenolic compounds, which reduces their inhibitory effects but requires a long treatment time (Jönsson *et al.* 1998). In addition, enzymatic treatment would generate by-products that, in some cases, are more toxic to microorganisms (Wagner and Nicell 2002).

A preferred detoxification method would remove the inhibition of lignocellulose degradation products, without sugar loss and long treatment time. Compared to sugars, phenolic compounds possess relatively high hydrophobicity, which suggests that adsorbents with high selectivity for phenol adsorption could be designed. Bentonite is one kind of cheap natural adsorbents. The modified bentonite, organobentonite, has been applied in wastewater treatment for the phenols removal (Nayak and Singh 2007; Lin and Juang 2009). Moreover, the spent organobentonite could be regenerated, which makes the organobentonite treatment a commercially feasible process (Zhu *et al.* 2009). In this study, two organobentonites were introduced to remove the phenolic compounds from steam-exploded corn stover prehydrolyzate. The adsorption conditions were investigated based on the phenol removal. The effects of organobentonite adsorption on the fermentability of prehydrolyzate by *Pichia stipitis* were then evaluated.

EXPERIMENTAL

Preparation of Steam-Exploded Corn Stover Prehydrolyzate

Corn stover was obtained from Huhehaote, Inner Mongolia Autonomous Region, China, and was cut into lengths of 3 cm before pretreatment. The corn stover was immersed in 1.33% (w/w) sulfuric acid solution at 50 °C for 2 h and then pretreated by steam explosion at 170 °C for 5.5 min. The pretreated slurry was washed with distilled water in a liquid to solid ratio of 7.5:1 (v/w). The pH of the mixture was adjusted to 5 with aqueous ammonia. The prehydrolyzate was collected by filtration and thereafter concentrated 2-fold at 70 °C and 160 mbar with a rotary evaporator (BÜCHI R-200, Switzerland) to increase the sugar concentration. The obtained prehydrolyzate was stored at 4 °C and is referred to as steam-exploded corn stover prehydrolyzate in this work. The contents of sugars and inhibitors were determined by HPLC, as shown in Table 1.

Preparation of Organobentonite

To prepare two types of organobentonite, natural Na^+-bentonite was modified with two cationic surfactants (cetyltrimethylammonium (CTMA) bromide and benzyltrimethylammonium (BTMA) bromide) by cationic exchange. Na^+-bentonite with a cation exchange capacity (CEC) of 84 meq/100 g was obtained from Tangshan, Jiangsu, China. First, 20 g of Na^+-bentonite was dispersed in 200 mL of distilled water with surfactant, and the mixture was stirred continuously at 70 °C for 2 h. Various loadings of surfactant, from 0.168 to 1.344 mmol/g bentonite, were added to reach 20% to 160% CEC of the Na^+-bentonite. After modification, the bentonite was collected by centrifugation and washed with distilled water at least five times to remove the loosely attached CTMA bromide or BTMA bromide. The final modified bentonite was dried at 80 °C, ground to pass a 100-mesh sieve, and designated as CTMA- or BTMA-bentonite.

Organobentonite Adsorption Experiments

To test the phenol adsorption capacity of the organobentonite, 10 mL of corn stover prehydrolyzate and a set amount of organobentonite were mixed at room temperature and shaken at 150 rpm. Effects of surfactant packing density (20% to 160% CEC) in organobentonite, pH (2 to 12), adsorption time (1 to 120 min), and organobentonite loading (2% to 14% (w/v)) on phenol removal were investigated. After each adsorption experiment, a sample was taken to analyze the concentration of phenols in the suspension using the Folin-Ciocalteu method (Singleton *et al.* 1999). The adsorbed phenol concentration was calculated as the difference between the phenol concentration after adsorption and the initial phenol concentration. The phenol removal is presented as the ratio of adsorbed phenol concentration to the initial phenol concentration in prehydrolyzate.

Detoxification of the Prehydrolyzate with Organobentonite

The prehydrolyzate was detoxified by treatment with organobentonites before the yeast inoculation. In detail, 8% (w/v) organobentonite was mixed with the prehydrolyzate at room temperature and the mixture was shaken at 150 rpm for 60 min. After that, the organobentonite was separated by centrifugation at 8000 rpm for 10 min. The obtained supernatant was inoculated with *Pichia stipitis* to evaluate the effectiveness of detoxification in the following fermentation experiment.

Fermentation

Inoculum culture

The yeast *Pichia stipitis* CBS 5776 was grown in a medium containing 15 g/L glucose, 30 g/L xylose, 5 g/L peptone, and 3 g/L yeast extract. The culture was shaken at 170 rpm and 30 °C in three batches (24 h each) to prepare the inoculum. The yeast cells were harvested by centrifugation and used as the inoculum in the following fermentation experiments.

Fermentation of synthesized media

To evaluate the effects of weak acids and 5-hydroxymethylfurfural (HMF) on ethanol fermentation, three types of synthesized media (M1, M2, and M3) were fermented by *P. stipitis*, respectively. The three synthesized media contained 10 g/L glucose and 50 g/L xylose each and were supplemented with or without weak acids and HMF. The concentrations of inhibitors were set based on those found in prehydrolyzate. M1 was the pure sugar fermentation medium without inhibitors, used as a control. M2 contained 0.83 g/L formic acid, 3.79 g/L acetic acid, and 0.37 g/L levulinic acid, while M3 contained these weak acids along with 0.42 g/L HMF. These three synthesized media were supplied with KH_2PO_4, 3 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; EDTA, 30 mg/L; $ZnSO_4 \cdot 7H_2O$, 9 mg/L; $MnCl_2 \cdot 2H_2O$, 2 mg/L; $CoCl_2 \cdot 6H_2O$, 0.6 mg/L; $CuSO_4 \cdot 5H_2O$, 0.6 mg/L; $Na_2MoO_4 \cdot H_2O$, 0.8 mg/L; $CaCl_2 \cdot 2H_2O$, 9 mg/L; $FeSO_4 \cdot 7H_2O$, 6 mg/L; H_3BO_3, 2 mg/L; and KI, 0.2 mg/L, with pH controlled at 6.0 by adding a buffer. Fermentation was started with 10 g/L (cell dry weight) of initial inoculum and carried out in a 250-mL shaking flask with a 50 mL working volume, at 30 °C and 150 rpm for 30 h. Samples were withdrawn at the end of the fermentation to analyze the concentrations of residual sugar and produced ethanol.

Fermentation of non-detoxified and detoxified prehydrolyzate

Similarly, fermentation was carried out with 50 mL of non-detoxified or detoxified prehydrolyzate. The initial pH of prehydrolyzate was adjusted to 6.0 by adding dilute NaOH and H_2SO_4. Because of the addition of aqueous ammonia in prehydrolyzate preparation, the fermentation did not require an extra nitrogen source. The fermentation started with 10 g/L of initial inoculation, incubated at 30 °C and 150 rpm for 30 h. Samples were taken from the fermentation medium at 0, 6, 12, 18, 24, and 30 h to monitor the fermentation process.

Analysis

Sugars (glucose and xylose), ethanol, weak acids, and furan compounds (formic acid, acetic acid, levulinic acid, furfural, and HMF) were determined by high performance liquid chromatography (HPLC) (Agilent Technologies Inc., USA) with a refractive index detector and a Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Laboratories Inc., USA) at 55 °C. The lignin degradation products were analyzed using the Agilent HPLC with a variable wavelength UV detector and a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm, Agilent Technologies Inc., USA) at 30 °C (Jiang *et al.* 2011). The structures of natural bentonite and organobentonite were characterized using Fourier transform infrared spectrum (FTIR) spectroscopy. The IR spectra of the natural bentonite and organobentonite were obtained to determine the surface functional groups using a FTIR spectrophotometer (PerkinElmer 1600, USA) in the range of 4000 to 600 cm^{-1}.

RESULTS AND DISCUSSION

Composition of Steam-Exploded Corn Stover Prehydrolyzate

The components of steam-exploded corn stover prehydrolyzate were analyzed, including mono-sugars and inhibitors (Table 1). Glucose (11.02 g/L) and xylose (49.49 g/L) were the main mono-saccharides in the prehydrolyzate that could be utilized by *P. stipitis*. In addition to the fermentable sugars, toxic compounds were formed during the pretreatment process. Weak acids were the most abundent inhibitors in prehydrolyzate. Acetic acid (3.79 g/L) was derived from the deacetylation of hemicellulose; formic acid (0.83 g/L) and levulinic acid (0.37 g/L) were possibly derived from the degradation of furan compounds (Larsson *et al.* 1999a). Furural was nearly removed during the concentration process in this study, leaving only 0.42 g/L HMF in the steam-exploded prehydrolyzate.

Another group of inhibitors were phenolic compounds. Because of the complexity of phenolic compounds, two methods were applied to quantitate the phenolic compounds in this study. Folin-Ciocalteu reagent was used to detect the total amount of phenolic compounds (3.77 g/L). HPLC analysis was applied to determine the specific six mono-phenolic compounds in this study. A substantial difference was found between the results from these two methods. This might be because the phenols in the prehydrolyzate are such a diverse group, having many individual compounds within each subgroup (Singleton *et al.* 1999). The phenolic compounds identified by HPLC were mono-phenols, so the determined amount was much lower than that determined by the Folin-Ciocalteu method, which included the quantity of both mono-phenols and poly-phenols. Furthermore, there were other reducing compounds existing in prehydrolyzate, which may react with Folin-Ciocalteu reagent.

Table 1. Concentrations of Monosaccharides and Inhibitors in Steam-Exploded Corn Stover Prehydrolyzate with or without Organobentonite Treatment

Components (g/L)	Untreated	Treated with CTMA-bentonite*	Treated with BTMA-bentonite*
Glucose	11.02	11.09	11.11
Xylose	49.49	49.72	49.64
Formic acid	0.83	0.78	0.82
Acetic acid	3.79	3.60	3.75
Levulinic acid	0.37	0.25	0.31
5-Hydroxymethylfurfural	0.42	0.27	0.34
Phenolic compounds	3.77	1.63	3.42
p-Hydroxybenzoic acid	0.0077	0.0037	0.0078
Vanillic acid	0.0158	0.0081	0.0158
Syringic acid	0.0169	0.0114	0.0169
p-Hydroxybenzaldehyde	0.0153	0.0015	0.0019
Vanillin	0.1530	0.0512	0.1529
Syringaldehyde	0.0211	0.0020	0.0184
* Organobentonite treatment conditions: 8% organobentonite with 120% CEC of surfactant packing density, pH 5, 60 min, room temperature, shaking at 150 rpm			

Inhibitory Effects of Weak Acids and Furan Compounds on the Fermentation of Steam-Exploded Corn Stover Prehydrolyzate

To evaluate the inhibitory effects of weak acids, furan compounds, and phenolic compounds on yeast fermentation, three types of synthesized media (M1, M2, and M3, respectively) and the prehydrolyzate were fermented by *P. stipitis* for 30 h. The synthesized media (M2 and M3) was supplemented with weak acids and/or furan compounds, to distinguish the inhibition of weak acids and furan compounds. The toxicity of phenolic compounds was indirectly estimated by comparing the fermentability of synthesized media M3 and prehydrolyzate. The complexity of phenolic compounds composition makes it impossible to simulate the inhibition of phenolic compounds in prehydrolyzate by adding model compounds. The concentrations of residual xylose and produced ethanol after 30 h of fermentation are shown in Fig. 1. The results suggested that the addition of weak acids and furan compounds had limited inhibitory effects on fermentation under pH 6. The lowest sugar utilization was observed in prehydrolyzate fermentation, which could be due to the presence of phenolic compounds. Compared with the fermentation of pure sugar (M1), the addition of weak acids in the synthesized media (M2) reduced the produced ethanol concentration from 23.07 g/L (M1) to 21.79 g/L (M2). As HMF was added along with the weak acids in the synthesized media (M3), the produced ethanol concentration was reduced further to 21.33 g/L (M3). An obvious decrease in xylose utilization and ethanol production was found in the fermentation of steam-exploded prehydrolyzate, in which there were weak acids, furan compounds, and also phenolic compounds. The produced ethanol concentration dropped from 23.07 g/L (M1) to 14.92 g/L (prehydrolyzate), with 18.57 g/L xylose remaining untouched. Therefore, it was reasonable to deduce that under this fermentation pH, the presence of weak acids and HMF did not exhibit obvious inhibitory effects on fermentation with *Pichia stipitis*; the presence of complex phenolic compounds might predominantly affect the fermentability of the prehydrolyzate. The result could be different with disparate strains because microorganisms differ in their tolerance to inhibitors. The study should be done with other industrial strains in the future, such as *Saccharomyces cerevisiae.*

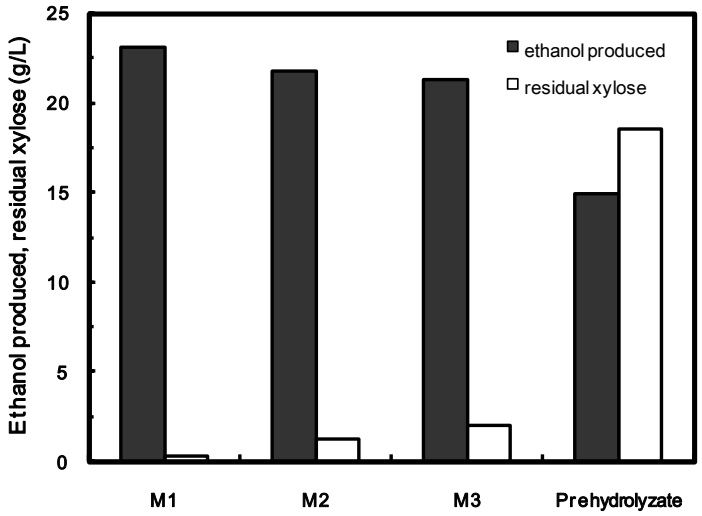

Fig. 1. Comparison of fermentation performance in three synthesized media (M1, M2, M3) and steam-exploded corn stover prehydrolyzate

Previous work indicated that the inhibition of weak acids was strong when the pH was below 6 (data not shown). Because the fermentation pH was beyond the pKa of weak acid compounds, the weak acids appeared in the dissociated form and could not diffuse across the plasma membrane (Zaldivar and Ingram 1999). Therefore, the weak acids inhibited the fermentation slightly under the relatively high fermentation pH in this study (pH 6). A similar result has been reported wherein the inhibitory effect of weak acids could be greatly overcome by increasing the fermentation pH (Huang *et al.* 2011).

The inhibition of phenolic compounds has also attracted attention. Phenolic compounds have been considered as the most inhibitory compounds among the inhibitors, and their inhibition extent depend on their chemical structure (Xie *et al.* 2012; Cao *et al.* 2014). Effective methods should be applied to remove the phenolic compounds to improve the fermentability of the steam-exploded prehydrolyzate.

Adsorption of Phenolic Compounds Present in Steam-Exploded Corn Stover Prehydrolyzate by Organobentonite

To remove the hydrophobic phenolic compounds from the prehydrolyzate, the cation surfactant-modified organobentonite was applied to adsorb the phenols. Natural Na^+-bentonite was modified with two surfactants, cetyltrimethyl-ammonium (CTMA) bromide and benzyltrimethylammonium (BTMA) bromide, to produce two kinds of organobentonite (CTMA-bentonite and BTMA-bentonite) by exchanging Na^+ on the bentonite surface with a surfactant. FTIR analysis results confirmed the modification of bentonite (data not shown). As the bentonite was progressively intercalated by the surfactant (Rawajfih and Nsour 2006), the surface of the bentonite changed to hydrophobic, at which point the organobentonite could adsorb the phenols by hydrophobic interaction. The effects of several factors on phenolic compound adsorption by CTMA-bentonite were investigated (Fig. 2).

Surfactant packing density is the surfactant saturate ratio of the bentonite matrix's cation exchange capacity (CEC) in the process of organobentonite preparation. The results indicated that increasing surfactant packing density of CTMA-bentonite enhanced the phenol removal ratio (Fig. 2a). As the surfactant packing density increased from 20% CEC to 120% CEC, the phenol removal ratio increased from 18.2% to 54.8%. The adsorption of hydrophobic organic compounds on surfactant-modified bentonite was predominantly controlled by the partition of hydrophobic compounds to the adsorbed surfactant phase.

The adsorption capacity has been suggested to be proportional to the amount of the adsorbed surfactant (Zhu *et al.* 2007). However, as the surfactant packing density increased further to 160% CEC, the phenols removal did not increase obviously. As the surfactant packing density increased to a high level, most of the interlayer spaces of the bentonite were filled with the adsorbed surfactant; thus, the phenol adsorption did not increase (Senturk *et al.* 2009). Therefore, 120% CEC was chosen as the appropriate surfactant packing density of CTMA-bentonite used in the following adsorption experiments.

To study the effect of pH on adsorption of phenolic compounds in the prehydrolyzate, 6% CTMA-bentonite with 120% CEC was suspended with prehydrolyzate and shaken for 2 h at different pH values. In the pH range of 1 to 8, the adsorption of phenols was almost constant (55%) (Fig. 2b). However as the pH value exceeded 10, the adsorption of phenols decreased sharply. As the pH of the reaction mixture exceeded 9.8 (the pKa of phenols), the ionization degree of phenol increased,

which might contribute to the increasing electrostatic repulsion between the negatively charged surface sites of the adsorbent and phenol ions (Senturk *et al.* 2009). Therefore, the prehydrolyzate without adjusted pH (pH 5) was used in the following adsorption experiments.

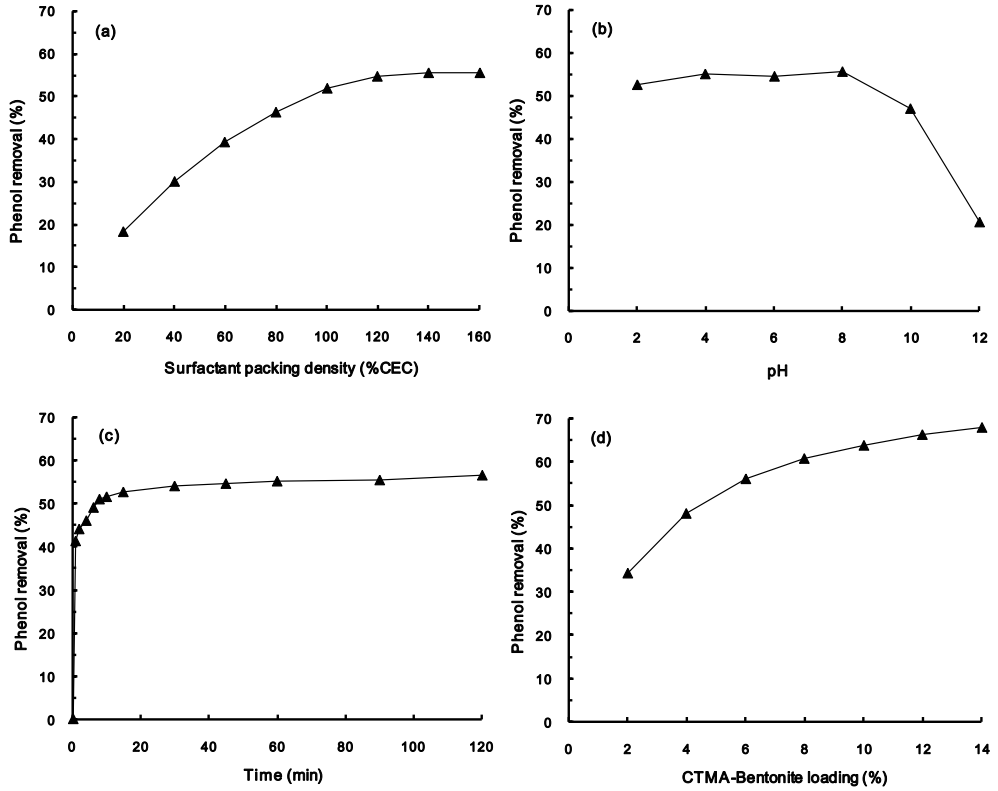

Fig. 2. Effects of (a) surfactant packing density, (b) adsorption pH, (c) adsorption time, and (d) CTMA-bentonite loading on the adsorption of phenolic compounds in the steam-exploded corn stover prehydrolyzate. Adsorption conditions: (a) CTMA-bentonite loading 6%; pH 5.0; reaction time 120 min; (b) surfactant packing density 120% CEC; CTMA-bentonite loading 6%; reaction time 120 min; (c) surfactant packing density 120% CEC; CTMA-bentonite loading 6%; pH 5.0; (d) surfactant packing density 120% CEC; pH 5.0; reaction time 60 min

Figure 2c shows the relationship between the adsorption time and phenol removal. In the first few minutes, the adsorption ratio increased dramatically because of the abundant available adsorption sites. After that, the adsorption reached an equilibrium within 60 min or less. Therefore, the following adsorption experiments were carried out for 60 min.

The effect of CTMA-bentonite loading on the removal of phenols was evaluated with dosages of CTMA-bentonite in the range of 2% to 14% (Fig. 2d). The removal of phenolic compounds increased with increasing dosage of CTMA-bentonite; this could be attributed to more adsorption sites being supplied by the increasing CTMA-bentonite loading. Based on the results, 8% CTMA-bentonite was chosen as a reasonable CTMA-bentonite loading for the following detoxification experiment because the removal ratio of phenolic compounds had reached a considerably high level.

The BTMA-bentonite performed similarly in the adsorption of phenolic compounds, even though it adsorbed markedly less phenolic compounds than CTMA-bentonite did (data not shown). To sum up, the optimal conditions of adsorption

experiments were chosen as follows: the corn stover prehydrolyzate was mixed with 8% organobentonite with 120% CEC surfactant packing density, and the mixture was kept on a rotary shaker at 150 rpm and pH 5.0 for 60 min.

Fermentation of Steam-Exploded Corn Stover Prehydrolyzate with or without Organobentonite Detoxification

To enhance the fermentability of steam-exploded corn stover prehydrolyzate, the prehydrolyzate was detoxified with CTMA- or BTMA-bentonite. After organobentonite treatment, changes in the concentrations of sugars and inhibitors were determined (Table 1). The results indicate that the concentrations of sugars and weak acids did not change obviously, while the HMF and the hydrophobic phenols were removed by CTMA- and BTMA-bentonite to different extents. The concentration of HMF decreased from the original 0.42 g/L to 0.27 g/L and 0.34 g/L using CTMA- and BTMA-bentonite, respectively. The total amount of phenolic compounds in the prehydrolyzate decreased from 3.77 g/L to 1.63 g/L after treatment with CTMA-bentonite, whereas a slight decrease was observed in phenolic compounds, from 3.77 g/L to 3.42 g/L, after treatment with BTMA-bentonite. This might be due to the different chemical structures of CTMA bromide and BTMA bromide, which resulted in the different adsorption mechanisms and compounds adsorbed. CTMA bromide has long alkyl chains, which forms a hydrophobic environment on the surface of bentonite and shows high affinity to hydrophobic organic compounds (Zhu *et al.* 2007). BTMA bromide has a small molecular weight with a benzene ring, which plays an important role in adsorption, probably through π-π type interactions with phenolic compounds (Shen 2002).

Different adsorption results for the six HPLC-detected lignin degradation products on CTMA- and BTMA-bentonite were observed (Table 1). After treatment with CTMA-bentonite, 51.9% p-hydroxybenzoic acid, 48.7% vanillic acid, 32.5% syringic acid, 90.2% p-hydroxybenzaldehyde, 66.5% vanillin, and 90.5% syringaldehyde were removed from the prehydrolyzate. However, BTMA-bentonite treatment removed only 87.6% p-hydroxybenzaldehyde and 12.8% syringaldehyde, while the other four lignin degradation products remained untouched.

The fermentability of steam-exploded corn stover prehydrolyzate with and without organobentonite detoxification was compared (Fig. 3). The results indicated that both CTMA- and BTMA-bentonite treatments improved the ethanol production remarkably. In the fermentation of untreated prehydrolyzate, glucose was consumed completely in 12 h, whereas xylose was utilized slowly. After 30 h of fermentation, 18.01 g/L of xylose was left; the sugar utilization ratio was only 68.1% (Fig. 3a). The concentration of ethanol was 15.36 g/L with an ethanol yield of 82.7%. Extending the fermentation time did not obviously improve the sugar utilization or ethanol production (data not shown).

The fermentability of the prehydrolyzate treated with the two organobentonites was improved substantially (Fig. 3b and 3c), even though a distinct difference existed in the removal of phenolic compounds. The rates of sugar utilization and ethanol production were greatly increased, which indicated that organobentonite treatment could relieve the inhibitory effects on yeast.

After treatment with CTMA-bentonite, glucose and xylose in the prehydrolyzate were consumed completely in 6 h and 30 h, respectively; the sugar utilization ratio reached 97.1%. The concentration of ethanol reached 22.35 g/L and the ethanol yield was 85.9%, much higher than the untreated one. The sugar utilization ratio and ethanol

production were improved by 42.6% and 45.5%, respectively. The fermentation of prehydrolyzate treated with BTMA-bentonite showed similar results. After 30 h of culture, the sugar utilization ratio reached 95.8%, with an ethanol concentration of 21.94 g/L.

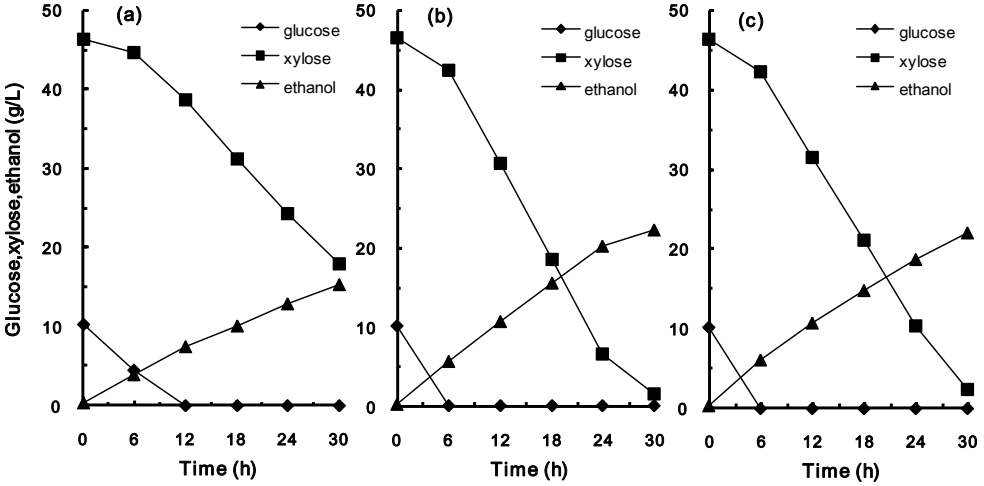

Fig. 3. Fermentation of steam-exploded corn stover prehydrolyzate by *Pichia stipitis* CBS 5776: (a) untreated prehydrolyzate, (b) prehydrolyzate treated with CTMA-bentonite, and (c) prehydrolyzate treated with BTMA-bentonite.

This result showed that ethanol production from detoxified prehydrolyzates (22.35 and 21.94 g/L) was fairly close to that of pure sugar fermentation (23.07 g/L), which suggests that organobentonite treatment is an efficient detoxification method for steam-exploded corn stover prehydrolyzate. Although BTMA-bentonite adsorbed less of phenolic compounds than CTMA-bentonite did, the fermentability of the prehydrolyzate treated with the two organobentonites was similar. These results may indicate that the most toxic inhibitory compounds were a small group of phenolic compounds. p-Hydroxybenzoic acid, vanillic acid, syringic acid, and vanillin did not inhibit the fermentation noticeably because good fermentability was obtained with BTMA-bentonite-treated prehydrolyzate, even with the fairly low removal of these four mono-phenols. A similar result has been reported that the addition of these six phenolic compounds did not show the strong inhibition on ethanol fermentation, except when the addition amount was sufficient; the inhibitions might be caused by other phenolic compounds, or the synergism of various types of phenols (Zhu *et al.* 2014).

Usually, the inhibitory effects of phenolic aldehyde are higher than that of phenolic acid. Ortho-phthalaldehyde, vanillin, syringaldehyde, *etc*., have been selected as model phenolic aldehydes to investigate the inhibitory effects on ethanol fermentation (Nishikawa *et al.* 1988; Xie *et al.* 2012; Cao *et al.* 2014). It has been shown that the inhibitory activities of phenolic aldehydes are related to their lowest unoccupied molecular orbital (E_{LUMO}), which suggests a possible electrophilic reaction between phenolic aldehydes and biological macromolecules, such as the proteins in membranes (Cao *et al.* 2014). More studies are needed to identify and qualify the most toxic phenolic compounds in lignocellulosic prehydrolyzates, which will provide insight into the inhibition mechanism and the development of effective detoxification methods.

CONCLUSIONS

1. Organobentonite treatment was found to be an effective detoxification method for prehydrolyzate medium. After CTMA-bentonite treatment, the sugar utilization ratio and ethanol production of corn stover steam-exploded prehydrolyzate using *P. stipitis* were improved by 42.6% and 45.5%, respectively.
2. An obvious difference in phenol removal was observed with CTMA-bentonite and BTMA-bentonite. However, the two treatments led to similar prehydrolyzate fermentability.
3. Weak acids and furan compounds (0.83 g/L formic acid, 3.79 g/L acetic acid, 0.37 g/L levulinic acid, and 0.42 g/L HMF) exerted a slight inhibition on ethanol fermentation at pH 6. Phenolic compounds could be the most toxic inhibitors in prehydrolyzate.

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