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Cause analysis of the effects of acid-catalyzed steam-exploded corn stover prehydrolyzate on ethanol fermentation by *Pichia stipitis* CBS 5776

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**Abstract** The prehydrolyzate obtained from acid-catalyzed steam-exploded corn stover (ASC) mainly contains xylose and a number of inhibitory compounds that inhibit ethanol fermentation by *Pichia stipitis*. In this study, the effects of the ASC prehydrolyzate, specifically those of the carbohydrate-degradation products, lignin-degradation products (which were extracted from ASC prehydrolyzate using ethyl acetate), and six major phenolic compounds (added to pure-sugar media individually or in combination), on ethanol fermentation were investigated. Results indicate that the effects of the carbohydrate-degradation products were negligible (10 h delayed) compared with those of pure-sugar fermentation, whereas the effects of the lignin-degradation products were significant (52 h delayed). Meanwhile, the inhibitory effects of the major phenolic compounds were not caused by certain types of inhibitors, but were due to the synergistic effects of various inhibitors.

**Keywords** Phenolic compounds · Xylose · Ethanol · Inhibitor · *Pichia stipitis*

**Introduction**

To produce a large amount of fermentable sugars, bioethanol production from lignocellulosic biomass requires pretreatment to disrupt the three-dimensional structure and promote enzymatic hydrolysis [1–3]. Steam explosion with or without acid catalysis is considered as one of the most promising pretreatment methods [4]. However, during the pretreatment, a range of degradation products (also called inhibitors) are inevitably produced and consequently inhibit the subsequent enzymatic hydrolysis and ethanol fermentation [5–8]. These inhibitors are generally classified into three major groups: weak acids (formic acid, acetic acid, and levulinic acid), furaldehydes (5-hydroxymethylfurfural and furfural), and phenolic compounds [9, 10]. Specifically, acetic acid is formed by hydrolysis of acetyl groups of hemicellulose. Furaldehydes include furfural and 5-hydroxymethylfurfural, which result from pentose and hexose dehydration, respectively. Subsequent degradation of furfural and 5-hydroxymethylfurfural generates formic acid and levulinic acid, respectively. Various phenolic compounds are formed from lignin breakdown [8, 11]. The inhibition effects of these inhibitors on ethanol fermentation have been extensively investigated [12–15]. However, different microorganisms exhibit varying resistances to these inhibitors [12]. Thus, identifying the most effective inhibitor has been difficult.

To develop a cost-effective process for lignocellulosic ethanol production, the xylose from hemicellulose hydrolysis must be fermented to produce ethanol. In addition, glucose fermentation technology in industry and laboratory is highly mature, which utilizes high glucose concentrations (above 200 g/L), has a short fermentation time (within 24 h), and high ethanol yield (above 90 %) [16]. However, xylose fermentation in a mixture of glucose and
xylose remains a challenge. Therefore, the separate fermentation of glucose and xylose can be more practical than cofermenting glucose and xylose. The inhibitors in xylose solutions increase the difficulty of fermenting xylose to ethanol. Only a small number of microbes can ferment xylose to ethanol. Among these microorganisms, *Pichia stipitis* is one of the most promising species for industrial application because of its ability to ferment xylose to ethanol efficiently and with low yield of the xylitol by-product [17].

In this study, corn stover was pretreated with acid-catalyzed steam explosion. After washing and filtration, the obtained liquid fraction, which was called prehydrolyzate and mainly contained xylose, a small amount of glucose, and different types of inhibitors, was used for the subsequent experiments. The effects of carbohydrate-degradation products (formic acid, acetic acid, levulinic acid, furfural, and 5-hydroxymethylfurfural), lignin-degradation products extracted from acid-catalyzed steam-exploded corn stover (ASC) prehydrolyzate with ethyl acetate, and six major phenolic compounds (vanillin, syringaldehyde, 4-hydroxymethylbenzaldehyde, vanillic acid, syringic acid, and 4-hydroxybenzoic acid; added to pure-sugar media individually or in combination) on ethanol fermentation by *Pichia stipitis* CBS 5776 were investigated to determine the reason for the low ethanol fermentability of prehydrolyzate. A pure-sugar medium was also fermented, and the results were used as reference.

**Materials and methods**

**Chemicals**

Formic acid and acetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Levulinic acid, furfural, 5-hydroxymethylfurfural, vanillin, syringaldehyde, 4-hydroxymethylbenzaldehyde, vanillic acid, syringic acid, and 4-hydroxybenzoic acid were all purchased from Sigma Co. (Shanghai, China). All of the reagents were analytical or HPLC grade.

**Preparation of acid-catalyzed steam-exploded corn stover prehydrolyzate**

ASC was obtained from Jiangsu Kangwei Biologic Co., Ltd. (Jiangsu Province, China). Steam explosion was performed by preimmersing the corn stover in 1.29 % (w/v) sulfuric acid at 0.8 MPa (gauge pressure) and at 175 °C for 5 min in a 3-L reactor. Subsequently, the ASC prehydrolyzate was collected by filtration. The solid residue was washed three times at a solid-to-liquid ratio of 1:7.5, and the filtrates were added to the ASC prehydrolyzate. The ASC prehydrolyzate was then concentrated in a BÜCHI rotary evaporator R-200 from BÜCHI Shanghai Trading LLC (Shanghai, China) at 70 °C and 160 mbar until the xylose concentration reached approximately 45 g/L, as required in the subsequent study. The condensed ASC prehydrolyzate was called CASC prehydrolyzate.

**Extraction of lignin-degradation products from the CASC prehydrolyzate using ethyl acetate**

Lignin-degradation products were obtained by extracting 100 mL of the CASC prehydrolyzate three times with ethyl acetate (1:1, v/v) [18]. The extracts were then concentrated in a BÜCHI rotary evaporator R-200 at 70 °C and 160 mbar to remove residual solvent. The residue was then dissolved in 5 mL of distilled water for the subsequent experiment.

**Yeast strain and media**

The yeast *Pichia stipitis* CBS 5776 was maintained at 4 °C in a medium containing the following (in g/L): xylose, 20; yeast extract, 5; peptone, 3; and agar, 20.

The inoculation medium (at natural pH) contained the following (in g/L): xylose, 30; peptone, 5; and yeast extract, 3. The xylose or pure-sugar (glucose and xylose) fermentation medium was supplied with (per liter) 45 g of xylose or pure sugars (with concentrations the same as that of the CASC prehydrolyzate), 5 g of (NH₄)_2SO₄, 3 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, and 10 mL of trace elements; the pH was adjusted to 6.0 [19].

For inoculum preparation, the shaking flask was incubated on a rotary shaker at 170 rpm and 30 °C for several batches (24 h per batch). When the optical density (OD) of yeast reached 10, the cells were harvested by centrifugation, and the pellet was inoculated into the fermentation media. Fermentation was performed in a 250 mL shaking flask containing 100 mL of the medium. Incubation was performed on a rotary shaker at 150 rpm and 30 °C.

**Fermentation experiments of different samples**

A control experiment was performed by fermenting 45 g/L of a xylose medium, pure-sugar (glucose and xylose) medium, or CASC prehydrolyzate with *Pichia stipitis* CBS 5776 to produce ethanol.

To investigate the effects of lignin-degradation products on the ethanol fermentation, the ethyl acetate extract of the CASC prehydrolyzate was back-added to 45 g/L of the xylose fermentation medium (with the total volume kept at 100 mL) for ethanol fermentation.

To investigate the effects of carbohydrate-degradation products on the ethanol fermentation, a mixture of pure
components of carbohydrate-degradation compounds, namely, formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural, and furfural, were back-added (at concentrations the same as that of the CASC prehydrolyzate) to pure-sugar fermentation media (with the total volume kept at 100 mL) for ethanol fermentation.

To determine the individual and synergistic effects of six types of phenolic compounds (vanillin, syringaldehyde, 4-hydroxybenzaldehyde, vanillic acid, syringic acid, and 4-hydroxybenzoic acid) on ethanol fermentation, different concentrations of individual phenolic compounds and mixtures of the six phenolic compounds (with proportions the same as that of the CASC prehydrolyzate) were separately back-added to 45 g/L of the xylose fermentation medium (with the total volume kept at 100 mL) for ethanol fermentation.

Approximately 1.0 mL of samples of the aforementioned fermentation experiments were withdrawn at different periods. The OD and the sugar, inhibitor, ethanol, and xylitol concentrations were then determined.

Analysis

The concentrations of sugars (cellubiose, glucose, xylose, and arabinose), fermentation product (ethanol), fermentation by-product (xylitol), and inhibitors (formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural, and furfural) were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 system equipped with a refractive index detector [19]. Separations were performed on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm i.d.) at 55 °C using 0.005 mol/L sulfuric acid as the mobile phase (0.6 mL/min). At least three parallel samples were used in all analyses. Data are presented as the mean of the replicates. Relative errors were determined to be within 5 % for the sugar, ethanol, and xylitol analyses and 10 % for the inhibitor assay.

The phenolic compounds (vanillin, syringaldehyde, 4-hydroxybenzaldehyde, vanillic acid, syringic acid, and 4-hydroxybenzoic acid) were analyzed by reversed-phase HPLC (RP-HPLC) using an Agilent 1100 system and a UV detector [20]. Separations were performed on a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm i.d.) at 30 °C using gradient elution with acetonitrile–water (containing 1.5 % acetic acid) as the mobile phase. The flow rate was 0.8 mL/min, and the detection wavelengths were 254 and 280 nm. Relative errors were determined to be within 10 % for the phenolic compound assay.

The absorbance of the prehydrolyzate at 280 nm was determined using an Amersham Biosciences UV/visible-light (UV/Vis) spectrophotometer (Amersham Biosciences). The sample was placed in quartz cuvets with a path length of 10 mm. The prehydrolyzate was diluted 100 times with double-deionized water. Water was used as the blank control for the measurements [8].

The OD at 600 nm was determined using an Amersham Biosciences UV/Vis spectrophotometer in glass cuvets with a path length of 5 mm. The samples were diluted in a linear range using double-deionized water when necessary. Water was also used as the blank control for the measurements [19].

Calculations

The ethanol yield was calculated as the percentage of determined ethanol to the theoretically calculated ethanol produced from the consumed glucose (0.51 g ethanol/g glucose) and/or xylose (0.46 g ethanol/g xylose) [8].

The sugar utilization ratio of ethanol fermentation was used to indicate the percentage of consumed glucose and/or xylose to the total glucose and/or xylose.

Results and discussion

Compositional analysis of the ASC and CASC prehydrolyzates

The ASC prehydrolyzate was concentrated to increase the sugar concentration for ethanol fermentation. The chemical components of the ASC prehydrolyzate before and after vacuum evaporation are shown in Table 1. When the ASC prehydrolyzate was condensed to half of its original volume, the fermentable sugar, glucose, and xylose levels all showed nearly onefold of their original concentrations increases, and the xylose concentration increased from 24.80 to 49.50 g/L. However, the non-volatile inhibitors, namely, levulinic acid and 5-hydroxymethylfurfural, from carbohydrate degradation were not removed during the concentration process. Meanwhile, the levels of volatile inhibitors, namely, formic acid, acetic acid, and furfural, from the carbohydrate degradation decreased by 38.89, 28.57, and 100.00 %, respectively, compared with the unconcentrated ASC prehydrolyzate. The contents of the six major lignin-degradation products, namely, 4-hydroxybenzaldehyde, vanillin, syringaldehyde, 4-hydroxybenzoic acid, vanillic acid, and syringic acid, were extremely low; the highest concentration was that of vanillin (357.25 mg/L) in the CASC prehydrolyzate. These compounds all inhibit ethanol fermentation [10, 11, 13].

Fermentabilities of the CASC prehydrolyzate

and of the pure sugars by P. stipitis

To determine the fermentability of the CASC prehydrolyzate, pure-sugar fermentation with P. stipitis CBS 5776
was also performed. Figures 1 and 2 show the fermentation data, including the concentration profiles of glucose, xylose, and the produced ethanol, as well as the sugar utilization ratio and ethanol yield.

During the fermentation of the pure-sugar medium, glucose was exhausted within 4 h (Fig. 1). Xylose was also rapidly consumed, and only 0.60 g/L of xylose remained after 20 h. Ethanol reached its highest concentration of 23.13 g/L at 24 h and achieved a sugar utilization ratio of 99.84 % and an ethanol yield of 91.58 %. These results indicate that \( P. stipitis \) CBS 5776 is highly effective in fermenting the pure-sugar media.

The fermentation of the CASC prehydrolyzate was more difficult than those of the pure sugars (Fig. 2). Glucose utilization was complete within 12 h, whereas that of xylose was highly difficult and slow. Xylose was nearly completely utilized within 96 h (95.91 %) and achieved an ethanol yield of 65.17 %. Ethanol reached its highest concentration of 16.74 g/L at 60 h, with a sugar utilization ratio of 87.96 % and an ethanol yield of 74.63 %. Compared with the fermentation of pure sugars (Fig. 1), the fermentation time for the CASC prehydrolyzate was prolonged. Moreover, the sugar utilization ratio and ethanol yield significantly decreased. The concentration of the fermentation by-product, xylitol, reached 2.61 g/L. This result indicates that some inhibitors in the CASC prehydrolyzate can inhibit the ethanol fermentation of \( P. stipitis \) CBS 5776 and disrupt the activity of certain key enzymes.

### Table 1 Composition of the ASC prehydrolyzate before and after concentration by rotary evaporation

<table>
<thead>
<tr>
<th>Item</th>
<th>Component</th>
<th>Prehydrolyzate before concentration</th>
<th>Concentrated prehydrolyzate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentable sugar (g/L)</td>
<td>Glucose</td>
<td>4.93 ± 0.19</td>
<td>9.80 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>24.80 ± 0.68</td>
<td>49.50 ± 0.72</td>
</tr>
<tr>
<td>Carbohydrate-degradation products (g/L)</td>
<td>Formic acid</td>
<td>0.09 ± 0.00</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>0.63 ± 0.04</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Levulinic acid</td>
<td>0.06 ± 0.00</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxymethylfurfural</td>
<td>0.19 ± 0.01</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Furfural</td>
<td>0.03 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Major lignin-degradation products (mg/L)</td>
<td>4-Hydroxybenzaldehyde</td>
<td>17.50 ± 0.78</td>
<td>32.59 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>Vanillin</td>
<td>182.85 ± 3.39</td>
<td>357.25 ± 4.48</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>17.09 ± 0.77</td>
<td>36.07 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxybenzoic acid</td>
<td>5.14 ± 0.31</td>
<td>10.31 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Vanillic acid</td>
<td>10.05 ± 0.15</td>
<td>23.54 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Syringic acid</td>
<td>6.71 ± 0.16</td>
<td>9.91 ± 0.28</td>
</tr>
</tbody>
</table>

Fig. 1 Changes in the parameters during the fermentation of pure-sugar medium by \( P. stipitis \) CBS 5776

Fig. 2 Changes in the parameters during the fermentation of the CASC prehydrolyzate by \( P. stipitis \) CBS 5776
or the balance of some coenzymes in the xylose metabolic pathway to produce xylitol [21].

Effects of the addition of carbohydrate-degradation products to pure-sugar media on ethanol fermentation

Based on the components of the CASC prehydrolyzate, five carbohydrate-degradation products (formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural, and furfural) were artificially added to the pure-sugar media at concentrations similar to that of the CASC prehydrolyzate. The fermentation data, including the concentration profiles of glucose, xylose, and the produced ethanol as well as the sugar utilization ratio and ethanol yield, are shown in Fig. 3.

Glucose utilization was nearly complete within 4 h, whereas that of xylose was be delayed by the consumption of glucose. Xylose was exhausted within 30 h. The ethanol concentration reached its peak of 21.05 g/L at 30 h and achieved a sugar utilization ratio of 100.00 % and an ethanol yield of 86.95 %. The ethanol yield slightly decreased, whereas the sugar utilization ratio remained the same, compared with those during fermentation of the pure sugars (Fig. 1). Meanwhile, the ethanol yield and sugar utilization ratio significantly improved compared with those during the fermentation of the CASC prehydrolyzate (Fig. 2). These results indicate that the carbohydrate-degradation products had negligible effects on ethanol fermentation. Therefore, other causes that may have led to the low fermentability of the CASC prehydrolyzate must be determined.

Effects of the addition of lignin-degradation products (ethyl acetate extracts from the CASC prehydrolyzate) to pure-sugar media on ethanol fermentation

The inhibitors generated from biomass pretreatments were classified into two main groups, namely, carbohydrate-degradation products and lignin-degradation products, based on origin. The carbohydrate-degradation products showed negligible effects on ethanol fermentation. Therefore, the effects of the lignin-degradation products on ethanol fermentation were investigated. Lignin-degradation products can be extracted from the CASC prehydrolyzate by three ethyl acetate extractions (1:1), followed by vacuum evaporation to remove the residual solvent [18]. The extracts were then dissolved in water and back-added to pure-sugar media to determine their effects on ethanol fermentation. The fermentation data, including the concentration profiles of glucose, xylose, and the produced ethanol as well as the sugar utilization ratio and the ethanol yield are shown in Fig. 4. Glucose was nearly completely consumed within 8 h. Xylose was gradually utilized after glucose and was exhausted within 72 h. Meanwhile, the ethanol concentration reached its peak of 18.97 g/L at 72 h and achieved a sugar utilization ratio of 100.00 % and an ethanol yield of 77.24 %. The ethanol yield significantly decreased, whereas the sugar utilization ratio remained constant, compared with those in the fermentation of pure sugars (Fig. 1). Moreover, the inhibitory effects of the lignin-degradation products were stronger than those of the carbohydrate-degradation products (Fig. 1). Therefore, the effects of the lignin-degradation products on ethanol fermentation were further investigated.
Effects of individual additions of six major lignin-degradation products to a xylose medium on ethanol fermentation

Various phenolic compounds are formed by lignin degradation during the acid-catalyzed, steam-explosion pretreatment of corn stover. Six major lignin-degradation products, namely, vanillin, syringaldehyde, 4-hydroxymethylbenzaldehyde, vanillic acid, syringic acid, and 4-hydroxybenzoic acid, were used as the model compounds. Various concentrations of these six lignin-degradation products were individually added to a xylose medium. The effects of these additions on ethanol fermentation were then investigated. The sugar utilization ratios, ethanol yields, and by-product xylitol concentrations obtained from the fermentation of 45 g/L of xylose by \textit{P. stipitis} CBS 5776 in the presence of three phenolic aldehydes and three phenolic acids are listed in Tables 2 and 3, respectively.

Table 2 shows that the addition of the three phenolic aldehydes to a 45 g/L xylose medium reduced the sugar utilization ratio and ethanol yield after 24 h. The inhibitory effects of vanillin and 4-hydroxybenzaldehyde on sugar utilization and ethanol production were stronger than those of syringaldehyde. These results indicate that less heavily ring-substituted phenolics have stronger inhibitory effects than phenolics with higher ring substitutions [22]. During prolonged fermentation (36 h), most of the cultures exhibited at least a partial recovery of the sugar utilization ratio while showing a reduced ethanol yield. Meanwhile, the main by-product, xylitol, was generated during the fermentation. We determined that when used as the substrate for the ethanol fermentation of \textit{P. stipitis} CBS5776, the CASC prehydrolyzate contained vanillin, syringaldehyde, and 4-hydroxybenzaldehyde at 357.25, 36.07, and 32.59 mg/L concentrations, respectively (Table 1). The relatively high concentrations of individual phenolic aldehydes were required for inhibiting ethanol fermentation. This result indicates that these components are expected to accumulate, and that no single compound is solely responsible for the inhibition.

The data in Table 3 show that the phenolic acids exerted similar inhibitory effects as the phenolic aldehydes. The order of inhibitory strength was as follows: vanillic acid > 4-hydroxybenzoic acid > syringic acid. Overall, the aldehydes (vanillin, 4-hydroxybenzaldehyde) showed weaker inhibitory effects than the corresponding acids (vanillic acid, 4-hydroxybenzoic acid), except for syringaldehyde and syringic acid.
Effects of the addition of a combination of six major lignin-degradation products to a pure-sugar medium on ethanol fermentation

According to “Effects of individual additions of six major lignin-degradation products to a xylose medium on ethanol fermentation”, the individual additions of six major lignin-degradation products to pure-sugar media should reach significantly high addition concentrations that can inhibit ethanol fermentation. In the current section, combinations of six major lignin-degradation products were added to pure-sugar media at concentrations that were 1, 10, 25, and 50 times higher than those in the CASC prehydrolyzate. The effects of these additions on ethanol fermentation were then investigated. The sugar utilization ratios, ethanol yields, and xylitol concentrations obtained from the fermentation of pure-sugar media by *P. stipitis* CBS 5776 in the presence of different concentrations of the six phenolic compounds are listed in Table 4.

The inhibitory effects (sugar utilization ratio, ethanol yield) were enhanced with increasing inhibitor concentrations. When the inhibitor concentrations were increased 25-fold, the sugar utilization ratio decreased to 44.16 %, and the ethanol yield was 80.43 %. During prolonged fermentation (36 h), the sugar utilization ratio significantly increased to 68.57 %, whereas the ethanol yield slightly decreased.

Moreover, ethanol fermentation was completely inhibited when the inhibitor concentrations were increased 50-fold. These results indicate that the six major phenolic compounds have certain inhibitory effects on ethanol fermentation. However, the other phenolic inhibitors in the prehydrolyzate also showed certain degrees of inhibitory effects. Therefore, the inhibitory effect was not caused by certain types of inhibitors, but is due to the synergistic effects of various inhibitors.
Conclusions

Acid-catalyzed steam explosion is one of the most promising pretreatment methods. After pretreatment, the obtained prehydrolyzate mainly contained xylose as well as a wide range of inhibitors that hindered ethanol fermentation by *P. stipitis*. Analyses showed that the carbohydrate-degradation products had negligible effects, whereas the lignin-degradation products significantly inhibited the ethanol fermentation. The six major phenolic compounds also affected the fermentation to certain degrees. These results indicate that the inhibition is the synergistic effect of a combination of different inhibitors. The components of the ethyl acetate extracts must be further analyzed to identify other potential inhibitors of ethanol fermentation.

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