Studies on Different Detoxification Methods for the Acid Hydrolysate of Lignocellulosic Substrate Saccharum spontaneum

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ABSTRACT

Pretreatment is an important step in the conversion of lignocellulosic substrates to ethanol. Acid hydrolysis of lignocellulosic biomass results in the generation of fermentable sugars and also compounds that are inhibitory to the fermenting organism. In the present study, the acid hydrolysate of the lignocellulosic substrate (Saccharum spontaneum) was subjected to different detoxification methods. Treatment with alkali (sodium hydroxide and calcium hydroxide), treatment with reducing agent i.e. sodium sulfite, the use of ammonium hydroxide for simultaneous detoxification and addition of nutrients was experimented. Analysis of reducing sugars, furans and total phenolic compounds was performed before and after different treatments. Treatment with calcium hydroxide followed by active charcoal was the most efficient detoxification method, which resulted in 80% reduction in total phenolics and 90% reduction in furans with 10% sugar loss. Treatment with sodium hydroxide and ammonia resulted in substantial decrease in the concentrations of inhibitors but the sugar loss was more when compared to calcium hydroxide treatment, treatment with reducing agent has not given considerable results.

Keywords: acid hydrolysis, cellulose, hemicellulose, inhibitors, pretreatment
Abbreviations: NH4OH, ammonium hydroxide; Ca(OH)2, calcium hydroxide; NaOH, sodium hydroxide; Na2SO3, sodium sulphite; H2SO4, sulphuric acid

INTRODUCTION

It is evident that lignocellulosic hydrolysates contain a variety of inhibitory products (furans, furfural and weak acids) along with sugars. Adaptation of fermenting biocatalyst to these hydrolysates prior to using them in fermentation of lignocellulosic hydrolysates will yield much improved ethanol productivities (Parawira and Tekere 2010). Lignocelluloses are the most abundant organic mass in the biosphere, which accounts for approximately 50% of the biomass. In nature, the annual production of biomass is estimated to 10 to 50 x 109 tons (Chandel et al. 2010a). Hemicelluloses constitute an important fraction of lignocelluloses and the conversion of hemicelluloses into fuel ethanol with utmost yields is the deciding factor for the overall economization of the process (Chandel et al. 2010b). Unlike cellulose, hemicelluloses (also a polysaccharide) consist of shorter chains 500-3,000 sugar units as opposed to 7,000-15,000 glucose molecules per cellulose polymer (Saha 2003; Chandel et al. 2010a). To make the fermentation process more economic, it is necessary to either remove these fermentation inhibitors by less expensive methods or use less severe conditions for hemicellulose breakdown such as auto hydrolysis coupled with enzymatic degradation (Chandel et al. 2010b).

Lignocellulosic materials are the largest source of hexose and pentose sugars with potential use for the production of ethanol, chemicals and protein for food and feed purpose (Kuhad and Singh 1993; Kuhad et al. 1997; Herrera 2004). Thermoochemical pretreatment disrupts the lignocellulosic substrates and partially solubilized polysaccharides to release the fermentable sugars (Gray et al. 2006). Acid hydrolysis is a fast and easy method to perform but it is hampered by non-selectivity and byproduct formation (Gray et al. 2006). During acid hydrolysis of lignocellulosic substrates, in addition to the sugars, aliphatic acids (acetic, formic and levulinic acid), furan derivatives [furfural and 5-hydroxymethylfurfural (HMF)] and phenolic compounds are formed. These compounds affect ethanol fermentation performance (Larsson et al. 1999). Several detoxification methods like neutralization, over liming with calcium hydroxide, activated charcoal, ion exchange resins and enzymatic detoxification using laccase (Jönsson et al. 1998) are known for removing various inhibitory compounds from lignocellulosic hydrolysates. Treatment of the hydrolysate with Ca(OH)2 prior to fermentation is a well established method to improve fermentability. It is one of the most efficient detoxification methods known (Larsson et al. 1999). One drawback with overliming is the formation of a calcium sulfate precipitate (gypsum). Another drawback is that if the treatment is done under too harsh conditions (high pH and temperature), a considerable degradation of fermentable sugars occurs (Martinez et al. 2001; Millati et al. 2002). Chemical analysis of overliming combined with fermentation experiments suggest that it is difficult to find conditions that separate degradation of fermentable sugars from degradation of inhibiting furan aldehydes. A potential approach to overcome the problems associated with overliming is to use another form of alkali. Ammonium does not form poorly soluble salts and previous results suggest that treatment with ammonium hydroxide (NH4OH) compares favourable with overliming (Persson et al. 2002; Alriksson et al. 2005). Sodium hydroxide (NaOH) would be another option, but in comparisons performed under similar conditions, NaOH treatment has so far been less efficient than overliming (Larsson et al. 1999; Alriksson et al. 2005). Treatment with reducing agents, such as sodium sulphite (Na2SO3) has also been reported to be an efficient detoxification method. Some methods using charcoal, lime, ion exchange, and microorganisms were proposed to solve the effect of inhibitory compounds produced during the pretreatment of wood. In the present study four chemicals [calcium hydroxide (Ca(OH)2), NaOH, Na2SO3, ammonium hydroxide (NH4OH)] compares favourable with overliming (Persson et al. 2002; Alriksson et al. 2005). Sodium hydroxide (NaOH) would be another option, but in comparisons performed under similar conditions, NaOH treatment has so far been less efficient than overliming (Larsson et al. 1999; Alriksson et al. 2005). Treatment with reducing agents, such as sodium sulphite (Na2SO3) has also been reported to be an efficient detoxification method. Some methods using charcoal, lime, ion exchange, and microorganisms were proposed to solve the effect of inhibitory compounds produced during the pretreatment of wood. In the present study four chemicals [calcium hydroxide (Ca(OH)2), NaOH, Na2SO3,
and NH₄(OH))] were used to detoxify the acid hydrolysates of a lignocellulosic substrate namely that of Saccharum spontaneum.

**MATERIALS AND METHODS**

**Raw materials**

S. spontaneum was collected from the outskirts of Hyderabad. The species was identified by the authors according to the information provided by Suresh et al. (2010). Dry stem pieces including leaf sheath were processed by using pulverizer to attain a particular size between 4 and 10 mm followed by washing with tap water until the washings were clear and dust free and then gently dried at 50 ± 0.5°C overnight.

**Analysis of chemical composition of S. spontaneum**

The cellulose, lignin and hemi-cellulose fractions of pulverized S. spontaneum were determined according to Technical Association of the Pulp and Paper Institute (TAPPI) test methods (1992).

**Delignification**

100 g of dry S. spontaneum was soaked in 0.2M NaOH (1:10 ratio w/v) and kept at room temperature i.e. 30 ± 2°C for 16 h. The contents were filtered with two layers of muslin cloth and the solid residue was repeatedly washed with water until the pH of the filtrate become neutral. The residue was dried at 50 ± 0.5°C for overnight and subsequently used for acid hydrolysis experiments.

**Acid hydrolysis of delignified substrates**

Acid hydrolysis of delignified S. spontaneum was carried out in two phases. In first phase, fifty grams of delignified substrate was taken in 1L conical flask and 500 ml of 3% H₂SO₄ was added. Hydrolysis was performed at 121°C for 60 min. The hydrolyzed substrate was filtered and again treated with 5% H₂SO₄ at 130°C for 90 min in the second phase and the hydrolysates from two phases were mixed in equal volumes and detoxified.

**Neutralization with 1N NaOH**

The acid hydrolysates were neutralized with 1N NaOH and the samples were estimated for total phenolics and furans in the acid hydrolysates.

**Treatment with alkali**

Treatment with alkali was performed by adding Ca(OH)₂, NH₄OH and NaOH till the pH reached 10.0. Then after 1 h the hydrolysates were filtered. The pH was then readjusted to 5.5 with H₂SO₄ and centrifuged at 10,000 × g for 15 min to remove the precipitates formed during neutralization after which the hydrolysates were filtered again. Then the hydrolysates were further detoxified by treating with activated charcoal with constant stirring at room temperature (30 ± 2°C) for 30 min and the sugar syrup was recovered through filtration and subjected to analysis of sugars, total phenolics and furans.

**Treatment with reducing agent (Na₂SO₃)**

The neutralized hydrolysates were treated with Na₂SO₃ till the pH reached 10. The samples were kept for 1 h and then filtered. The pH of the samples was readjusted to pH 5.5 with H₂SO₄ and then subjected to charcoal treatment.

**Treatment with charcoal**

After treatment with Ca(OH)₂/NaOH/NH₄OH/Na₂SO₃ the hydrolysates were further detoxified by treating with activated charcoal [1.5%, w/v; Gong et al. 1993] with constant stirring at room temperature for 30 min and the sugar syrup was recovered through filtration and subjected to analysis of sugars, total phenolics and furans.

**Analytical methods**

Total reducing sugars present in the acid hydrolysates were estimated by the dinitrosalicylic acid method of Miller (1959). The phenolics in the hydrolysates were estimated by the Tanner and Brunner method (1987). Estimation of total Furans in the hydrolysates was done by UV absorbance method described by Martinez et al. (2000).

**Experimental design**

50 ml of acid hydrolysate was taken separately in four beakers and pH was adjusted to 10 by using detoxifying agents (Ca(OH)₂/NaOH/NH₄OH/Na₂SO₃) and incubated. After 1 h, the hydrolysates were filtered. The pH was then readjusted to 5.5 with H₂SO₄ and centrifuged at 10,000 × g for 15 min to remove the precipitates formed during neutralization after which the hydrolysates were filtered again. Then the hydrolysates were further detoxified by treating with activated charcoal with constant stirring at room temperature (30 ± 2°C) for 30 min and the sugar syrup was recovered through filtration and subjected to analysis of sugars, total phenolics and furans. The experiment was carried out three times. To assess whether there was any significant differences among the mean values before and after treatments, dependent t-tests were performed.

**RESULTS AND DISCUSSION**

S. spontaneum was characterized with regard to its chemical composition. The pulverized material was found to contain 45.10 ± 0.35% cellulose, 22.75 ± 0.28% hemicellulose, 24.38 ± 0.22% Klason lignin and 2.82 ± 0.15% ash. In the present study the acid hydrolysates were treated with NaOH, Ca(OH)₂, NH₄OH and sodium sulphite at room temperature (30 ± 2°C, pH 10.0). Thereafter it was treated with active charcoal (1.5%). Adjustment of pH with Ca (OH)₂ has been reported to increase the detoxification as compared with NaOH (Van Zyl et al. 1988), which is in agreement with our results. However, there was no significant variation in the amounts of measured compounds. Treatment with reducing agents, such as sulfite has also been reported to be an efficient detoxification method, but in our studies treatment with sulphite has not shown any satisfactory results. Fig. 1 shows that 30% of phenolics were decreased and 40% of furans were decreased when the hydrolysate was neutralized with 1N NaOH. Treatment of acid hydrolysate with Ca(OH)₂ followed by charcoal treatment lead to less amount of sugars (10%) when compared to other three treatments i.e., NaOH, ammonia and sodium sulfite loss of sugars were 15, 25 and 35%, respectively (Fig. 2). Optimal over liming

Fig. 1 Concentration of phenolics and furans in hydrolysate before and after neutralization with 1N NaOH. (n=5)
In the present study, treatment of lignocellulose hydrolyzates with Ca(OH)2 and NH4OH in comparison with NaOH was studied. Persson et al. (1999) compared detoxification of hydrolysates of Norway spruce with Ca(OH)2 and NaOH and found better results with Ca(OH)2. These results show that detoxification of lignocellulosic hydrolyzates by alkali treatment (increasing pH to 9–10 with NaOH or Ca(OH)2) followed by pH readjustment to 5.5 with H2SO4 is a well-known method (Larsson et al. 1999). The test yielded positive t-values for both the pairs (Table 1).

Table 1

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<th>inhibitors concentration (mg/ml)</th>
<th>Detoxification with different alkali followed by charcoal treatment</th>
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<tr>
<td>Phenolics before detoxification</td>
<td>Reducing sugars before detoxification</td>
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<td>Phenolics after detoxification with calcium hydroxide followed by charcoal</td>
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<td>Phenolics after detoxification with sodium hydroxide followed by charcoal</td>
<td>Reducing sugars after detoxification with sodium hydroxide followed by charcoal</td>
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<td>Furans before detoxification</td>
<td>Reducing sugars after detoxification with sodium sulphite followed by charcoal</td>
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<td>Furans after detoxification with calcium hydroxide followed by charcoal</td>
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To assess whether there is any significant changes among the mean values of phenolics and furans before and after neutralization, a dependent t-test was performed (Fig. 1). There was a significant difference between the mean values of two pairs i.e., phenolics before and after neutralization and furans before and after neutralization (sig. 0.000, P < 0.05). The test yielded positive t-values for both the pairs.

Statistical evaluation of different detoxification methods using acid hydrolyzate of Saccharum spontaneum

To assess whether there is any significant changes among the mean values of phenolics and furans before and after neutralization, a dependent t-test was performed (Fig. 1). There was a significant difference between the mean values of two pairs i.e., phenolics before and after neutralization and furans before and after neutralization (sig. 0.000, P < 0.05). The test yielded positive t-values for both the pairs (Table 1). Table 2 shows the t-test which was performed to find the significant differences between the mean values of reducing sugar before and after detoxification using different alkali treatments i.e., Ca(OH)2, NaOH, NH3, and Na2SO3 followed by charcoal treatment. The results showed that there is a highly significant difference among all the pair of treatments except the pair 4 i.e., between reducing sugar before detoxification and reducing sugar after detoxification with Na2SO3 and the t values yielded are all positive (P < 0.05). Similarly, study of dependent sample t test resulted in a 51 ± 9% reduction of total furans, a 41 ± 6% reduction in phenolic compounds (Martinez et al. 2001). Chandel et al. (2007) reported that overliming of hydrolysate led to removal of furans by 45.8% and phenolics by 35.87%. Chandel et al. (2011) reported that after overliming of S. spontaneum acidic hydrolysate, there was a significant decrease in furfurals (41.75%) and total phenolics (33.21%). Further, Srilekha et al. (2011) studied that when acid hydrolysate was treated with calcium oxide and activated charcoal it brought about maximum reduction in furans from 0.2 mg/l to 0.025 mg/l (88.4% removal). However, in the present study, 80% of phenolics and 90% furans were removed when hydrolysate was treated with Ca(OH)2 followed by charcoal whereas 65, 55 and 45% of phenolics and 70, 65 and 50% of furans were removed when treated with NaOH, ammonia and sodium sulfite, respectively (Fig. 3). Larsson et al. (1997) compared detoxification of hydrolyzates of spruce with Ca(OH)2 and NaOH and found better results with Ca(OH)2. These results showed that detoxification of lignocellulosic hydrolyzates by alkali treatment (increasing pH to 9–10 with NaOH or Ca(OH)2) followed by pH readjustment to 5.5 with H2SO4 is a well-known method (Larsson et al. 1999). Peron et al. (2002) compared the fermentability of two-step dilute acid hydrolyzate of Norway spruce (Picea abies) after treatment with NaOH, Ca(OH)2 and NH4OH at pH 10.0. The present study agrees with the above two studies.

Fig. 2 Concentration of reducing sugars before and after detoxification with Saccharum spontaneum hydrolysate. (n=3)

Fig. 3 Concentration of phenolics and furans before and after detoxification with Saccharum spontaneum hydrolysates. (n=3)
on furans before and after detoxification and phenolics before and after detoxification followed by different alkali treatments had shown that there was a significant difference among all the pairs of treatments (Table 3).

**CONCLUSIONS**

From the detoxification studies it can be concluded that treatment with Ca(OH)$_2$ for 1 hr followed by active charcoal treatment for 30 min showed a significant decrease in the levels of inhibitors (phenolics 80% and furans 90%) with minimum loss of sugars (10%). However, the choice of detoxification method must be made after considering the composition of hydrolysate and type of raw material. The composition of softwood and hardwood hydrolyzates differs, so an appropriate method must be chosen to remove relevant groups of inhibitors.

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