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.

INTRODUCTION

It is evident that lignocellulosic hydrolysates contain a variety of inhibitory products (furans, furfurals 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 50×10^9 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). Thermochemical 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 (Martínez *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 (NH_4OH) 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 (Na_2SO_3) 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, Na_2SO_3

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and $NH_4(OH)$] 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 $50 \pm 0.5°C$ 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. $30 \pm 2°C$ 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 $50 \pm 0.5°C$ for overnight and subsequently used for acid hydrolysis experiments.

Acid hydrolysis of deliginified substrates

Acid hydrolysis of deliginified *S. spontaneum* was carried out in two phases. In first phase, fifty grams of deliginified substrate was taken in 1L conical flask and 500 ml of 3% H_2SO_4 was added. Hydrolysis was performed at 121°C for 60 min. The hydrolyzed substrate was filtered and again treated with 5% H_2SO_4 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 hydrolyzates were neutralized with 1N NaOH and the samples were estimated for total phenolics and furans in the acid hydrolyzates.

Treatment with alkali

Treatment with alkali was performed by adding $Ca(OH)_2$, NH_4OH 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 H_2SO_4 and centrifuged at $10,000 \times g$ for 15 min to remove the precipitates formed during neutralization after which the hydrolyzates were filtered again and estimated for reducing sugars, phenolics and furans.

Treatment with reducing agent (Na_2SO_3)

The neutralized hydrolysates were treated with Na_2SO_3 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 H_2SO_4 and then subjected to charcoal treatment.

Treatment with charcoal

After treatment with $Ca(OH)_2$/NaOH/NH_4OH/Na_2SO_3 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 hydrolyzates were estimated by the Tanner and Brunner method (1987). Estimation of total Furans in the hydrolyzates was done by UV absorbance method described by Martínez *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/NH_4OH/Na_2SO_3) and incubated. After 1 h, the hydrolyzates were filtered. The pH was then readjusted to 5.5 with H_2SO_4 and centrifuged at $10,000 \times g$ for 15 min to remove the precipitates formed during neutralization after which the hydrolyzates were filtered again. Then the hydrolysates were further detoxified by treating with activated charcoal with constant stirring at room temperature ($30 \pm 2°C$) 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 $45.10 \pm 0.35\%$ cellulose, $22.75 \pm 0.28\%$ hemicellulose, $24.38 \pm 0.22\%$ Klason lignin and $2.82 \pm 0.15\%$ ash. In the present study the acid hydrolyzates were treated with NaOH, $Ca(OH)_2$, NH_4OH and sodium sulphite at room temperature ($30 \pm 2°C$, 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
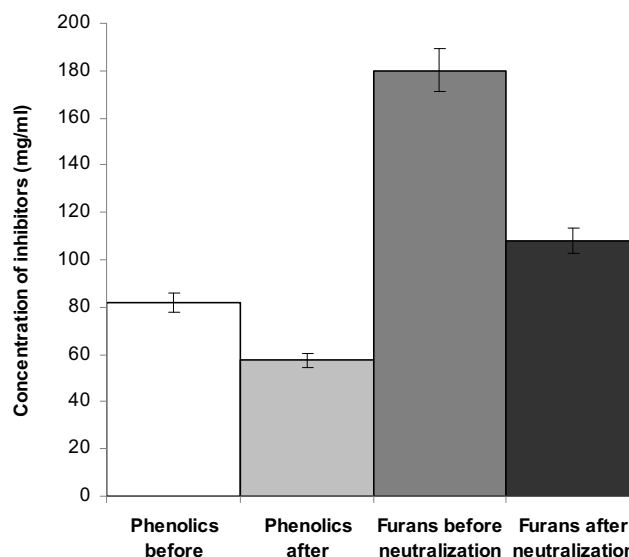

Fig. 1 Concentration of phenolics and furans in hydrolysate before and after neutralization with 1N NaOH. (n=3)
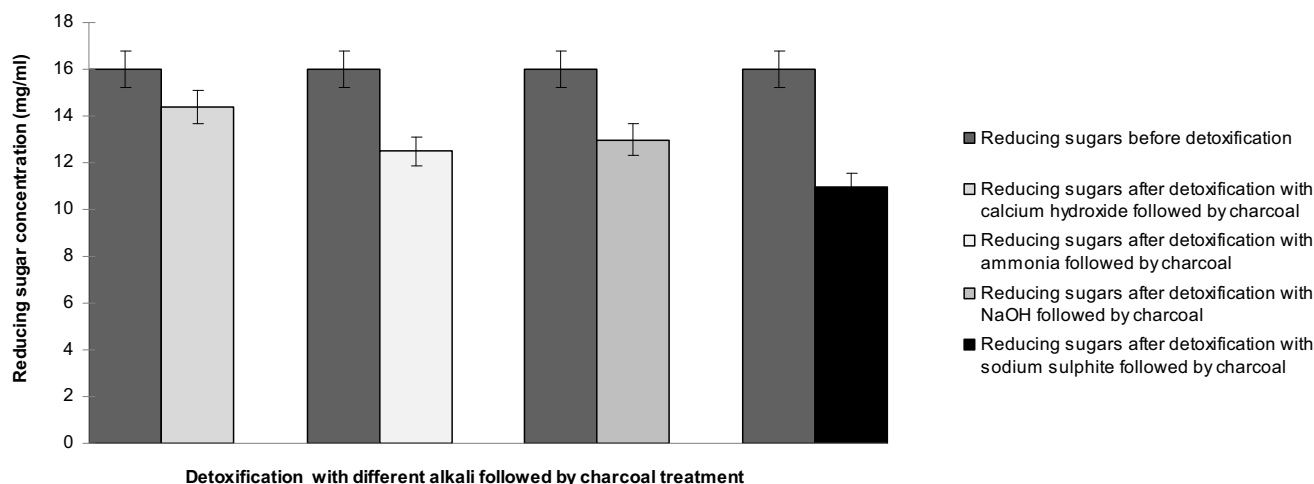
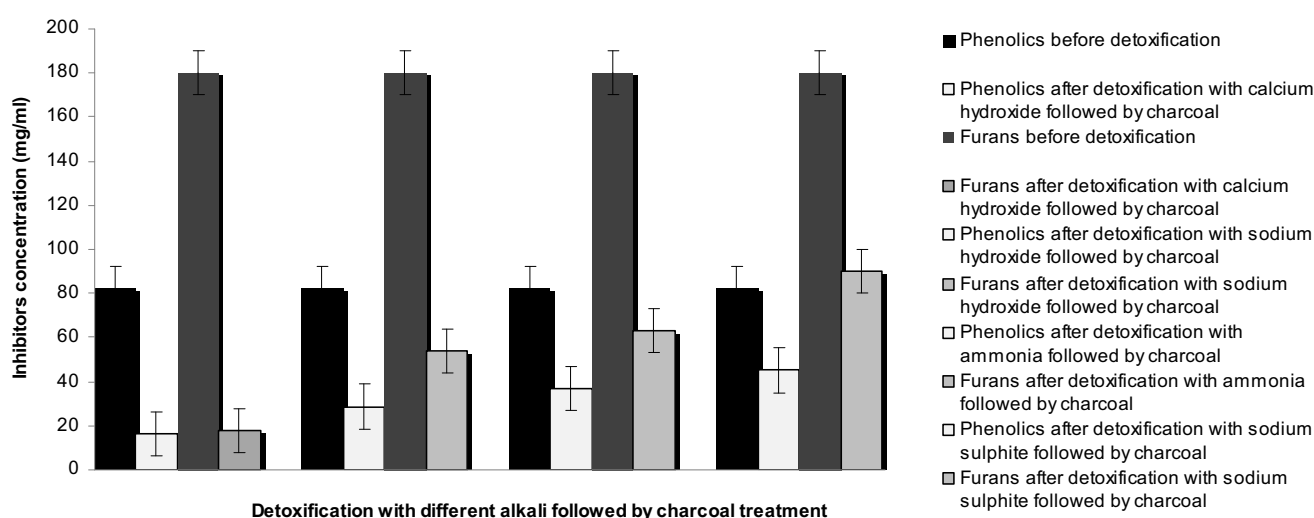**Fig. 2 Concentration of reducing sugars before and after detoxification with *Saccharum spontaneum* hydrolysate.** (n=3)

Legend:
- Reducing sugars before detoxification
- Reducing sugars after detoxification with calcium hydroxide followed by charcoal
- Reducing sugars after detoxification with ammonia followed by charcoal
- Reducing sugars after detoxification with NaOH followed by charcoal
- Reducing sugars after detoxification with sodium sulphite followed by charcoal

Fig. 3 Concentration of phenolics and furans before and after detoxification with *Saccharum spontaneum* hydrolysates. (n=3)

Legend:
- Phenolics before detoxification
- Phenolics after detoxification with calcium hydroxide followed by charcoal
- Furans before detoxification
- Furans after detoxification with calcium hydroxide followed by charcoal
- Phenolics after detoxification with sodium hydroxide followed by charcoal
- Furans after detoxification with sodium hydroxide followed by charcoal
- Phenolics after detoxification with ammonia followed by charcoal
- Furans after detoxification with ammonia followed by charcoal
- Phenolics after detoxification with sodium sulphite followed by charcoal
- Furans after detoxification with sodium sulphite followed by charcoal

resulted in a $51 \pm 9\%$ reduction of total furans, a $41 \pm 6\%$ 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*. (1999) compared detoxification of hydrolyzates of 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 H_2SO_4 is a well-known method (Larsson *et al*. 1999). Persson *et al*. (2002) compared the fermentability of two-step dilute acid hydrolysate of Norway spruce (*Picea abies*) after treatment with NaOH, $Ca(OH)_2$ and NH_4OH at pH 10.0. The present study agrees with the above two studies.

Fermentability of the hydrolyzates was better after treatment with $Ca(OH)_2$ and NH_4OH in comparison with NaOH. In the present study, treatment of lignocellulose hydroly-

sates with $Ca(OH)_2$ and NaOH followed by active charcoal has showed better results than with other agents. For instance, it has been shown that hardwood hydrolyzates contain higher concentrations of acetic acid and furfural than softwood hydrolyzates (Taherzadeh *et al*. 1997). Knowledge of the chemical effects of different detoxification methods may be helpful for rational design of detoxification procedures.

Statistical evaluation of different detoxification methods using acid hydrolysate 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, NH_3 and Na_2SO_3 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 Na_2SO_3 and the *t* values yielded are all positive ($P < 0.05$). Similarly, study of dependent sample *t* test

Table 1 Statistical evaluation of phenolics and furans before and after neutralization.

		Paired differences (dependent sample *t* test)					
				95% confidence interval of the difference			
Sl No.	Mean	Mean differences	Std. Deviation	Lower	Upper	*t*	Sig (2 tailed)
1	82.00	24.60	0.81	22.58	26.61	52.60	0.00**
2	57.40						
3	180.00	72.00	1.96	67.13	76.86	63.62	0.00**
4	108.00						

Table 2 Statistical evaluation of reducing sugar before and after detoxification with different alkali treatments.

		Paired differences (dependent sample *t* test)					
				95% Confidence interval of the difference			
Samples	Mean	Mean differences	Std. Deviation	Lower	Upper	*t*	Sig (2 tailed)
Pair 1	16.00	1.60	0.28	0.90	2.29	9.89	0.010**
	14.40						
Pair 2	16.00	3.00	0.32	2.20	3.79	16.23	0.004**
	13.00						
Pair 3	16.00	3.50	1.32	0.22	6.77	4.59	0.04**
	12.50						
Pair 4	16.00	5.00	1.32	1.72	8.27	6.56	0.22
	11.00						

**p<0.05
1. Phenolics before neutralization
2. Phenolics after neutralization
3. Furans before neutralization
4. Furans after neutralization
**p<0.05
Pair 1: Reducing sugar before detoxification; Reducing sugar after detoxification with $Ca(OH)_2$
Pair 2: Reducing sugar before detoxification; Reducing sugar after detoxification with NaOH
Pair 3: Reducing sugar before detoxification; Reducing sugar after detoxification with NH_3
Pair 4: Reducing sugar before detoxification; Reducing sugar after detoxification with Na_2SO_3

Table 3 Statistical evaluation of phenolics and furans before and after detoxification with different alkali treatments.

		Paired differences (dependent sample *t* test)					
				95% Confidence interval of the difference			
Samples	Mean	Mean differences	Std. Deviation	Lower	Upper	*t*	Sig (2-tailed)
Pair 1	180.0	162.0	0.38	161.05	162.94	738.40	0.00**
	18.00						
Pair 2	180.0	126.0	0.02	125.50	126.49	1091	0.00**
	54.00						
Pair 3	180.0	117.0	0.20	116.95	117.04	1013	0.00**
	63.0						
Pair 4	180.0	90.0	0.03	89.92	90.07	5196	0.00**
	90.0						
Pair 5	82.00	65.60	0.51	64.33	66.86	222.7	0.00**
	16.4						
Pair 6	82.00	53.30	0.19	52.82	53.77	485.88	0.00**
	28.7						
Pair 7	82.00	45.10	0.28	44.40	45.79	278.98	0.00**
	36.9						
Pair 8	82.00	36.90	0.87	34.73	39.06	73.463	0.00**
	45.1						

**p<0.05
Pair 1: Furans before detoxification; Furans after detoxification with $Ca(OH)_2$
Pair 2: Furans before detoxification; Furans after detoxification with NaOH
Pair 3: Furans before detoxification; Furans after detoxification with NH_3
Pair 4: Furans before detoxification; Furans after detoxification with Na_2SO_3
Pair 5: Phenolics before detoxification; Phenolics after detoxification with $Ca(OH)_2$
Pair 6: Phenolics before detoxification; Phenolics after detoxification with NaOH
Pair 7: Phenolics before detoxification; Phenolics after detoxification with NH_3
Pair 8: Phenolics before detoxification; Phenolics after detoxification with Na_2SO_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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