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Potential of Sodium Hydroxide Pre treatment of *Prosopis juliflora* for Fermentable Sugar Production

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ABSTRACT

The present study aimed at investigating the potential of sodium hydroxide (NaOH) as a viable alkaline reagent for lignocellulosic pre treatment considering its various benefits of pre treatment. *Prosopis juliflora* was pre treated with different NaOH concentrations of 1–3 % for varying treatment times of 15–60 min at temperatures of 100, 120 and 140 °C in an autoclave. Maximum lignin reductions at different temperatures were all obtained at the combinations of highest NaOH concentrations and longest treatment times, which indicated a close relationship between pre treatment severity and lignin reduction. The highest carbohydrate retention of 65.09 per cent was observed in the sample pre treated at 120 °C with 2% NaOH and 60 min. Three pre treatment conditions were selected for subsequent enzymatic hydrolysis with Cellic CTec2® enzyme complex for fermentable sugar production along with untreated biomass as control to study the soaking effect. The biomass pretreated with 2.0 % NaOH for 1 h at 120 °C and loaded with 30% enzyme was determined to be the most effective as it resulted in generation of 583.9 mg sugar/g biomass for a corresponding carbohydrate conversion of 90.86 %.

Key words : *Acid insoluble lignin, Enzymatic hydrolysis, Fermentable sugars, NaOH,* Prosopis juliflora

Introduction

Lignocellulose-to-ethanol conversion has been investigated intensively around the world over the last two decades. Lignocellulosic biomass is a complex substrate that typically contains 50–80 % [dry basis (db)] carbohydrates that are polymers of 5C and 6C sugar units (Carlo *et al*., 2005). Lignocellulosic materials have been considered as alternative energy sources because they can capture CO_2 during growth so that their combustion does not generate net CO_2 (Klass, 1998). Cellulose and hemicellulose are polysaccharides that can be used for ethanol production, while lignin is a complex aromatic polymer that stiffens and surrounds the fibres of polysaccharides (Fan *et al*., 1987). The conversion of lignocellulosic biomass to ethanol involves three main steps: pretreatment, the hydrolysis of carbohydrate components present in pretreated biomass to fermentable sugars, and the fermentation of the sugars to ethanol. However, recalcitrant structure of lignocellulosic material necessitates a pretreatment step to break it up, thus making cellulose and hemicellulose more accessible to hydrolytic enzymes for fermentable sugar production (Xu, 2009).

Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/ autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents,

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and organic solvents), biological, electrical, or a combination of these. A number of pretreatments such as concentrated acid hydrolysis (Liao *et al*., 2006), dilute acid hydrolysis (Cara *et al*., 2008), alkali treatment (Carrillo *et al*., 2005), sodium sulphite treatment (Kapoor *et al*., 2008), steam explosion (Ohgren *et al*., 2005), ammonia ber explosion (Teymouri *et al*., 2005), lime treatment (Kim and Holtzapple, 2005), and organic solvent treatment (Xu *et al.,* 2006) have been used frequently to remove lignin and improve the saccharification of the cell wall carbohydrates.

Among all the pretreatment methods, alkaline pretreatment has received greater attention because it is relatively inexpensive and less energy intensive (Chang *et al*., 2001) (Sun and Cheng, 2002). Alkaline solutions lead to a disruption of lignin structure, an increase in internal surface area, and a decrease in cellulose crystallinity (Sun and Cheng, 2002). The major effect of alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, alkali pretreatment removes acetyl and the various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface (Chang and Holtzapple, 2000).

Silverstein *et al.,*(2007) investigated chemical pretreatment of cotton stalks and reported that, among four pretreatment methods (NaOH, H_2SO_4, H_2O_2, and ozone pretreatments), NaOH pretreatment resulted in the highest level of delignification (65.63 % at 2 % NaOH, 90 min, 121 °C) with cellulose conversion of 60.8 %. Sodium hydroxide pretreatment of switchgrass for ethanol production was investigated (Xu *et al*., 2010) and reported that the yield of total reducing sugars was 453.4 mg/g raw biomass, which was 3.78 times that from untreated biomass at the most suited pretreatment condition (50 °C, 12 h, and 1.0 % NaOH). Sodium hydroxide pretreatment of lignocellulosic materials results not only in significant lignin reduction but also excellent retention of the total reducing sugar content per gram of biomass treated. Performer Switchgrass was pretreated at KOH concentrations of 0.5–2 % for varying treatment times of 6–48 h, 6–24 h, and 0.25–1 h at 21, 50, and 121 °C, respectively. The highest percent sugar retention of 99.26 % at 0.5 %, 21 °C, 12 h while delignification up to 55.4 % was observed with 2 % KOH, 121 °C, 1 h (Sharma *et al*., 2013).

After pretreatment, the hydrolysis is to be carried out during which the cellulose is converted into fermentable sugars through catalyst. There are two methods of hydrolysis viz., acid catalyzed and enzymatic catalyzed hydrolysis. Among these, enzymatic method is more effective as it could result into sugar yields closer to 100 % at 50 °C (Wyman, 1994).

Now a day research on non-food crops and cellulosic materials is getting great attention worldwide because they are cheap, easily available, and profitable as compared to food crops and also reduces inflation of the cost of food crops used for bioethanol production. With a huge population to feed and limited land availability, the nation needs to develop bio-ethanol technologies which use the biomass feedstock that does not have food or feed value.

One of the fast growing trees which have the potential to substitute food crops for bioethanol production is *Prosopis juliflora*. It is a tree species native to Northern Mexico and the Southern U.S. that survives droughts and thrives in sunny arid regions. The plant fixes its own nitrogen, requires no seeding, fertilization or irrigation, and grows on dry, nutrient-poor soils. It is a truly promising tree for droughts, because of its multiple and important potential and actual uses, as well as of its remarkable resistance to drought, heat, and poor soils. Most often, the tree is a thorny shrub, but its complex and deep-ranging root system allows it to tap different water tables, both at the surface and deep underground, which makes it a very hardy crop. The roots also act as an energy storage mechanism; because once a tree is cut down, new shoots spring up rapidly from the existing roots (Pasiecznik *et al*., 2001).

Prosopis juliora, a perennial deciduous thorny shrub, the common vegetation of semi-arid region of Indian subcontinent and considered to be as a problematic weed has been suggested recently to use as one of the alternative lignocellulosic biomass materials for long and sustainable production of cellulosic ethanol (Hopkins, 2007). Its very nature to drought tolerance, grazing, could be grown in heavy sandy and saline soils of dryland tracts and not much competence to animal feed demand made it a potential low value substrate for ethanol production.

Hence, keeping the above facts in view, an attempt was made to study the effect of NaOH pretreatment on subsequent hydrolysis of *Prosposi juliflora*. A comparison between pretreatment effectiveness based on delignification and carbohydrate availability in samples treated at high temperatures was made to better understand the mechanism of NaOH in modifying lignocellulose structure. The

effect of NaOH concentration (1–3 %) at various combinations of residence times including 15, 30 and 60 min at temperatures of 100, 120, and 140 °C in an autoclave was investigated. Selected samples with the maximum delignification or maximum carbohydrate availability after pretreatment were hydrolyzed to estimate fermentable sugar yield.

Materials and Methods

Biomass

The *Prosopis juliora* wood available in the University of Agricultural Sciences, Raichur campus, Karnataka, India was used as feedstock. The stems of *Prosopis juliora* were harvested up to 6 in. stubble and were dried in open sun for about a week. The wood were cut into small chips and oven dried at 70 °C in a forced air oven in cloth bags for 72 hours. Then oven dried samples were ground to pass through a 2-mm sieve in a hammer mill (Crompton Greave Ltd., NDA 2 TOP) and stored at room temperature in zip-locked plastic bags at the Department of Farm Machinery and Power Engineeirng, College of Agricultural Engineering, University of Agricultural Sciences, Raichur, Karnataka, India for use in further studies.

Pretreatment

In this experiment, the effect of sodium hydroxide (NaOH) pretreatment of *Prosopis juliflora* woody substrate at different elevated temperatures ranging from 100 to 140 °C with various combinations of residence times and NaOH concentrations was explored. Pretreatment of *Prosopis juliflora* woody substrate samples were performed at 100, 120 and 140 °C in an autoclave at 15 psi, with residence times of 15, 30, and 60 min each. All the temperature–time pretreatment combinations were performed with sodium hydroxide (NaOH) concentrations of 1, 2 and 3% (w/v) in a 3^3 factorial complete randomized block design. The pretreatment conditions selected for the study are summarized in Table 1.

Five grams of *Prosopis juliflora* woody substrate samples was mixed with 50 ml of NaOH solution in 125 ml bottles using glass rods, and the bottles were sealed before pretreatment and kept in an autoclave. The pretreated samples were filtered through preweighed filter paper (*Whatman* filter paper No. 1) in vacuum flask using a vacuum pump. The bottles were rinsed with 50 ml DI water to recover the residual solids. All solids accumulated on the filter papers in the filtration set up were quantified by oven drying and considered in solid recovery calculations. Approximately 5 g of wet biomass was drawn from each pretreated sample and dried at 105 °C in conventional hot air oven for estimation of solid recovery. A similar amount was placed for vacuum drying at 40 °C in vacuum oven to obtain samples for estimation of acid insoluble lignin to study the effect of pretreatment conditions on delignification of *Prosopis juliflora*. Filtrate from the AIL acid hydrolysis was utilized to study the effect of sodium hydroxide (NaOH) pretreatment on reducing sugar content generated in each pretreated sample at various temperature-time combinations (100 – 140 °C and 15 – 60 min) using 1 to 3% NaOH concentrations.

Table 1. Details of conditions selected for pretreatment of *Prosopis juliflora*

Temperature, °C	Concentration, %	Residence time, min
100120140	123	153060

Hydrolysis

Hydrolysis was carried out at 8% solid loading (of total volume 20 ml) to examine the effect of enzyme loading levels (0, 15 and 30%) on the untreated sample and selected pretreated samples for fermentable sugar production with a 3×4 factorial design. The CTec2® Cellulase enzyme complex sponsored by Novozymes, Beijing, China was used for conducting research on hydrolysis of samples for fermentable sugar production. The enzyme complex was reported to have an activity of 108.3–168.8 floating-point unit/ml (Eckard *et al*., 2012 and Kodaganti, 2011) and protein content 117–185.2 mg protein/ml (Eckard *et al*., 2012 and Eylen *et al*., 2011).

To generate enough biomass for hydrolysis at the various conditions, pretreatments were performed in six replicates and two replicates each were combined randomly and mixed thoroughly to generate three larger replicates. This was done to avoid the impact of any scale changes during pretreatment of larger amounts. Untreated samples with equivalent enzyme loading were also hydrolyzed as control. Pretreated and untreated samples with no enzyme were prepared to determine the effect of soaking. Hydrolysis was performed for 72 h at 50 °C in a

shaking water bath (KEMI Make) at 150 rpm. The Laboratory Analytical Procedure (LAP) adopted by National Renewable Energy Laboratory (NREL) for enzymatic saccharification of lignocellulosic biomass (Selig *et al*., 2008) was followed for conducting enzymatic hydrolysis. The samples were withdrawn at regular intervals of 12 hours and centrifuged at 4,000 rpm for 10 min in a high speed refrigerated centrifuge (KEMI Make), and the filtrate was collected for sugar analysis. Upon termination of hydrolysis, the samples tubes were kept in high speed refrigerated centrifuge for centrifugation. The filtrate was collected and stored separately at 4 °C in a freezer for estimation of total sugar generated.

Analytical Methods

The chemical composition of *Prosopis juliflora* woody substrate samples before and after pretreatment was analyzed using Laboratory Analytical Procedures (Sluiter *et al*., 2005a, Sluiter *et al*., 2005b and Sluiter *et al*., 2008) adopted by National Renewable Energy Laboratory (NREL) for the measurement of ash, total solids, acid insoluble lignin (AIL) and acid soluble lignin (ASL). Briefly, AIL was measured by a two-step sulfuric acid hydrolysis, and filtrate from the AIL acid hydrolysis was utilized for the estimation of ASL and total sugars in untreated biomass and solids recovered after pretreatment. ASL was estimated through absorbance measurements at 205 nm in an ultraviolet–visible spectrophotometer (ELICO make). Total reducing sugars in the AIL filtrate and enzyme hydrolysate were estimated by the 3,5-dinitrosalycylic acid (DNS) method (Miller, 1959 and Ghose, 1987) through absorbance measurements at 540 nm. The saccharification rate (mg/g/h) at regular intervals was calculated as the ratio of the sugar yield (mg/g dry biomass) and saccharification time (h) (Gupta *et al*., 2009). Carbohydrate conversion was calculated as the ratio of reducing sugar concentration obtained to potential sugar concentration in the substrate (Sharma *et al*., 2013).

Statistical Analysis

All treatments in this study were conducted in triplicate. Design expert-7 Software was used for data analysis at 99% confidence level. The experimental design was balanced and completely randomized, but with a rather complex factorial structure. There were a total of 28 different experimental conditions. These conditions were comprised of 27 combinations of three factors plus an untreated control.

Results and Discussion

The results pertaining to the composition of biomass, effect of pretreatment, enzymatic hydrolysis of selected samples and fermentation of hydrolyzate are presented and discussed in the following sections.

Composition of biomass

The initial composition of *Prosopis juliflora* used in this experiment is presented in Table 2. The total solids content present in *Prosopis juliflora* woody substrate selected for the study was 98.80 per cent. This in agreement with the moisture content (2.68%) reported by Gupta *et al*. (2009) which represents total solids. The total lignin of *Prosopis juliflora* was observed to be 31.85 per cent which is in close agreement with the lignin present in *Prosopis juliflora* **(**31%) reported by Rajput and Tewari (1986). Similar results (29.10%) were reported by Gupta *et al*. (2009) for *Prosopis juliflora* and 20-32% for dry wood (Alriksson, 2006). The total carbohydrate portion (cellulose and hemicelluloses) represented by total reducing sugar content of *Prosopis juliflora* was reported to be 64.26 per cent. This is in close agreement (66.20%) with the findings of *Prosopis juliflora* wood and also for *P. juliflora* pod flour (69.20%) (Gupta *et al*., 2009 and Choge *et al*., 2007). Ash content present in the *Prosopis juliflora* sample was 2.01 per cent which is fairly close in agreement with the ash content of *Prosopis juliflora* (2.02%) as reported by (Gupta *et al*., 2009). Undefined components are believed to be mainly non-structural compounds including protein, waxes, fats, resins, and chlorophyll (Kuhad and Singh, 1993 and Sluiter *et al*., 2005).

Table 2. Composition of *Prosopis juliflora*

Component	Dry weight (%)
Total solids	98.80 ± 0.71
Acid insoluble lignin	30.18 ± 0.33
Acid soluble lignin	1.67 ± 0.14
Carbohydrates (Total sugars)	64.26 ± 0.53
Ash	2.01 ± 0.23
Others	1.88

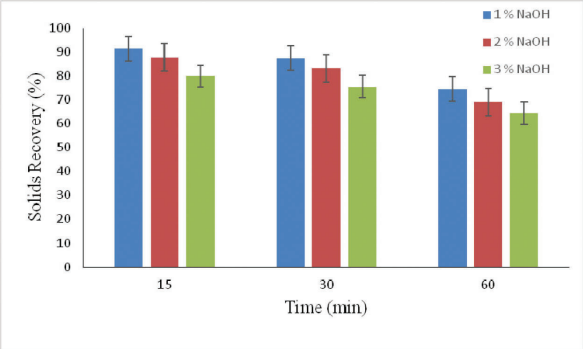
Effect of Pretreatment Conditions

Pretreatment conditions had varying effects on solid recovery, lignin reduction, and sugar availability in the biomass. Intensity of treatment increased with

increasing NaOH concentration and treatment temperature.

Solids Recovery

On average, solid recoveries after pretreatment ranged between 64.31–91.43 %, 62.94–78.50 % and 49.77–74.40 % respectively at 100, 120 and 140 °C (Fig. 1). Almost 51 per cent of solids were dissolved at 140 °C after 60 min pretreatment with 3% NaOH concentration. This is in comparable with that of



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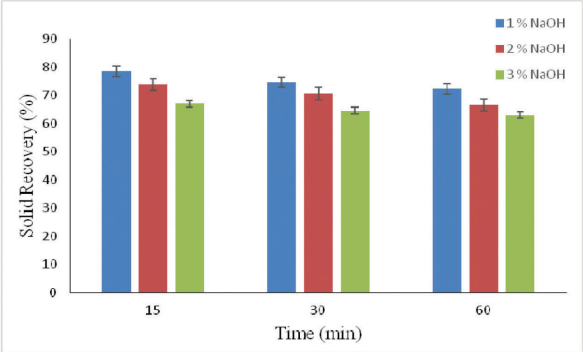

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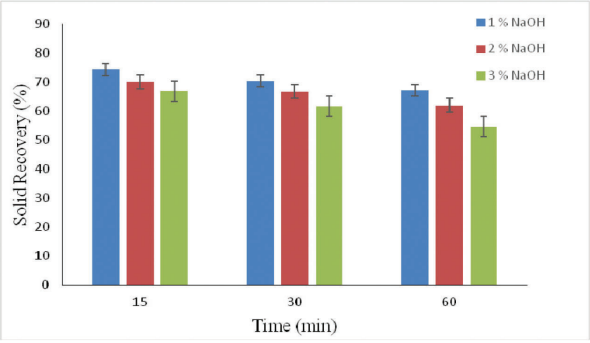


(c)

Fig. 1. Per cent solids recovered in *Prosopis juliflora* pretreated with 1.0–3.0% NaOH at (a) 100 °C, (b) 120 °C and (c) 140 °C

65% of the corn stover dissolved after 1 h in 2% sodium hydroxide at 150 °C (MacDonald *et al*., 1983). It was observed that lesser solids were recovered as intensity of the pretreatment increased. The similar results were reported by (Xu *et al*., 2010 and Sharma *et al*., 2013).

The main effect of both treatment time and NaOH concentration had significant ($p<0.01$) impact on solid recovery. The interaction effect between temperature and concentration also had a significant ($p<0.01$) impact on loss of solids. NaOH and KOH pretreatment of switchgrass followed a similar trend of increased solid loss with increasing intensity treatments in terms of temperature and high concentration (Xu *et al*., 2010 and Sharma *et al*., 2013).

Lignin reduction

Lignin is a three-dimensional complex aromatic that acts as a strong barrier for the release of sugars from lignocellulosic biomass. This makes it imperative to degrade lignin without major disruption of the reducing sugars needed for bioconversion into fuels and chemicals (Fan *et al*., 1987). On an average, AIL ranged between 7.96–28.25% after pretreatment at different temperature-time combinations using various concentrations of NaOH. The AIL was maximum (28.25%) in the sample pretreated at 100 °C, 15 min combination with 1% NaOH, whereas, a minimum of 7.96 per cent was recorded in the sample pretreated with 3% NaOH at 140 °C, 60 min (Table 3). Pretreatment with 2% NaOH concentration for 60 min resulted in 59.72% delignification at 120 °C which is comparable with that of highest level of delignification (65.63% at 2% NaOH, 90 min, 121 °C) by NaOH pretreatment (Silverstein *et al*., 2007). The corresponding maximum lignin reductions of 48.39, 67.01 and 74.79 per cent were obtained at 100, 120 and 140 °C, respectively for 1 h, 3.0% NaOH concentrations. Maximum lignin reductions at different temperatures were all obtained at the combinations of highest NaOH concentrations and longest treatment times, which indicated a close relationship between pretreatment severity and lignin reduction (Xu *et al*., 2010 and Sharma *et al*., 2013).

Statistical analysis indicated that the main effect of both treatment time and NaOH concentration had significant ($p<0.01$) impact on lignin reduction at all the three temperatures. The interaction effect between temperature and concentration also had a significant ($p<0.01$) impact on delignification. However, the combined effect of all the three factors on

acid insoluble lignin was not significant at 1% level of confidence. Further, at 120 and 140 °C, residence time had a significant impact ($p<0.01$) on lignin reduction at all three NaOH concentrations. However, at 100 °C, residence time had significant impact ($p<0.01$) on delignification of sample at higher concentrations only.

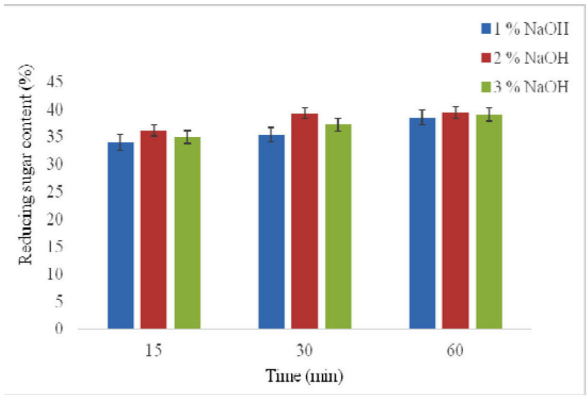
Reducing Sugar Content

Carbohydrate (cellulose and hemicellulose), which is the key component in pretreated biomass for generation of fermentable sugars during hydrolysis, was estimated in this study through total reducing sugar measurement. The carbohydrate availability in pretreated biomass decreased with increase in the intensity of pretreatment conditions (concentration, temperature, and residence times; Fig. 2). The maximum carbohydrate retention of 61.34 and 65.09 % was observed after treatment at 100 and 120 °C respectively at 2 % NaOH, 1 h treatment. The highest carbohydrate availability after treatment at 140 °C was 55.44 %, with 1 % NaOH, 15 min pretreatment. The highest carbohydrate retention of 65.09 per cent observed in the sample pretreated at 120 °C with 2% NaOH and 60 min were more pronounced than the sugars retained (60.6%) in KOH-pretreated samples of switchgrass and (Sharma *et al.*, 2013) and lesser than the sugars retained (74.8%) in NaOH pretreated switchgrass (Xu *et al.*, 2010). The main effect of temperature, time and concentration was significant ($p<0.01$) on reducing sugar released. Also, their interaction effect and combined effect of all the factors were significant ($p<0.01$) on carbohydrate availability. It was observed that 34.90–53.98 % of the original untreated reducing sugar content was lost during various combinations of pretreatments depending on severity.

Selection of optimal pretreatment conditions

The upper and lower limits of all the factors and also, the minimum and maximum values of solids recovered, acid insoluble and soluble lignin and reducing sugars generated were given as input to the statistical software. Selections were based on maximum solids recovery, minimum AIL *i.e.*, maximum delignification and maximum carbohydrate (reducing sugar) retention.

The desirability index of solids recovery, acid insoluble lignin, acid soluble lignin and reducing sugar content retention were found to be 0.9935, 0.7348, 0.4009 and 0.9719, respectively for pretreatment combination of 2.0% NaOH, 60 min at 120 °C which has highest combined desirability of 0.7303 (Fig. 3). Hence, this pretreatment combination of 120 °C, 60 min, 2.0% NaOH was chosen as the optimum for further enzymatic hydrolysis for fermentable sugar production.



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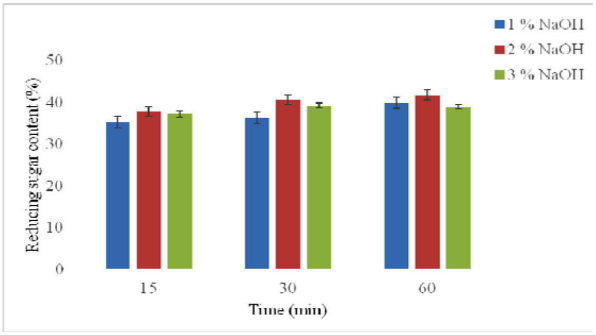

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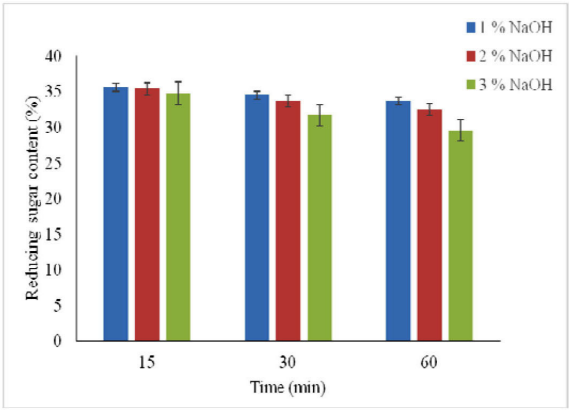


(c)

Fig. 2. Reducing sugars content of *P. juliflora* pretreated with 1.0–3.0% NaOH at (a) 100 °C, (b) 120 °C and (c) 140 °C

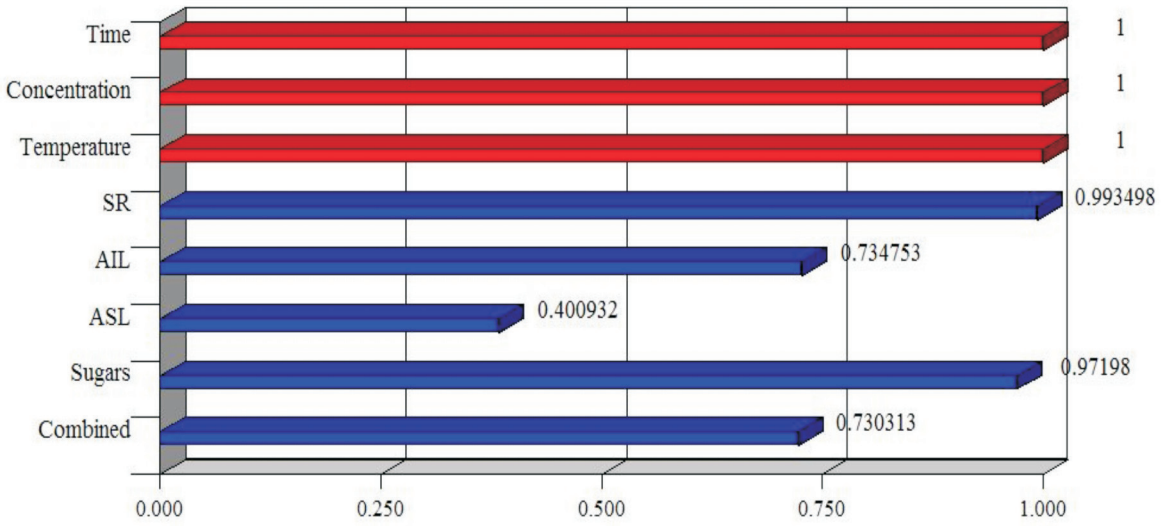


Fig. 3. Desirability index of solids recovery, lignin reduction and reducing sugar retention for optimal pretreatment condition (120 °C, 60 min, 2% NaOH)

However, in order to examine the enzyme loading levels on samples pretreated at 100 and 140 °C for fermentable sugar production, other two pretreatment conditions viz., 100 °C, 60 min, 2.0% NaOH having desirability of 0.712 and 140 °C, 30 min, 1.0% NaOH with 0.614 desirability were also selected for further enzymatic hydrolysis.

Hydrolysis

The enzyme Cellulase *CTec2* (Novozymes, China) was loaded at different levels of 0, 15 and 30% (g enzyme protein/g dry biomass) on the untreated sample and selected pretreated samples to examine its effect for fermentable sugar production.

Saccharification profile

The saccharification profile of the untreated sample without enzyme (0%) and with enzyme (15 and 30%) loading is depicted in Fig. 4. The sugar yield ranged from 12.5 to 373.2 mg/g dry biomass. A maximum sugar yield of 373.2 mg/g biomass was obtained with 30 per cent enzyme loading at the end of 72 h of hydrolysis. While, it was minimum (12.5 mg/g) at 0 per cent i.e., without enzyme loading after 12 h. It was observed that the sugar yield increased as the hydrolysis time prolonged. A maximum saccharification rate of 1.07 mg/g/h was observed for 0% enzyme loading at 36 h, at which the sugar yield was 38.6 mg/g biomass. While with 15% enzyme loading, it was maximum (8.06 mg/g/h) at 12 h of incubation with sugar yield was 96.7 mg/g biomass and a maximum rate of saccharification (15.66 mg/g/h) was attained at 12 h for 30% enzyme loading at which the sugar yield was 187.9 mg/g biomass.

The fermentable sugars generated in the hydrolyzate obtained from the sample pretreated at 120 °C for 60 min with 2% NaOH concentration at 0, 15 and 30 % enzyme loading ranged from 9.3 to 583.9 mg/g dry biomass (Fig. 5). It was observed that a maximum sugar yield of 583.9 mg/g biomass was obtained with 30 per cent enzyme loading at the end of 72 h of hydrolysis, while it was minimum (9.3 mg/g biomass) after 12 h without enzyme loading. At 0% enzyme loading, a maximum saccharification rate of 0.79 mg/g/h was observed at 24 h, at which the sugar yield was 18.9 mg/g biomass. Whereas, for the hydrolyzate loaded with 15% enzyme, the saccharification rate was maximum (14.03 mg/g/h) at 24 h of incubation with sugar yield of 336.8 mg/g biomass. The maximum rate of saccharification (26.07 mg/g/h) was attained at 12 h for 30% enzyme loading, at which the sugar yield of 312.8 mg/g biomass was obtained indicating that the higher dosage of enzyme resulted in faster rate of saccharification.

The saccharification profile of the hydrolyzate obtained from the sample pretreated at 100 °C for 60

min with 2% NaOH concentration at 0, 15 and 30 % enzyme loading is depicted in Fig. 6. A maximum sugar yield of 541.5 mg/g biomass was recorded at the end of hydrolysis with 30% enzyme loading, while it was minimum (7.2 mg/g) for 0% enzyme loading at 12 h of hydrolysis. A maximum saccharification rate of 0.61 mg/g/h was observed at 24 h without enzyme loading, at which the sugar yield was 14.6 mg/g biomass, while at 15% enzyme loading, it was maximum (12.44 mg/g/h) at 24 h of in-

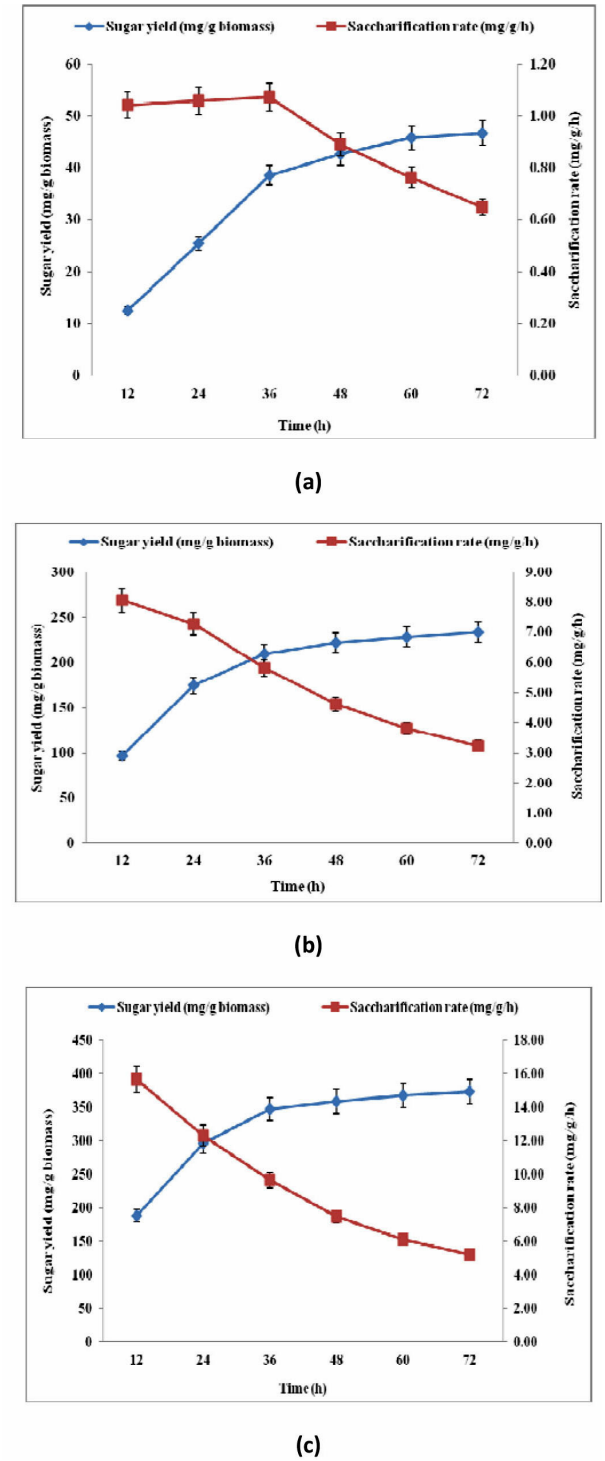


Fig. 4. Saccharification profile of untreated sample hydrolyzed with (a) 0 %, (b) 15 % and (c) 30% enzyme loading

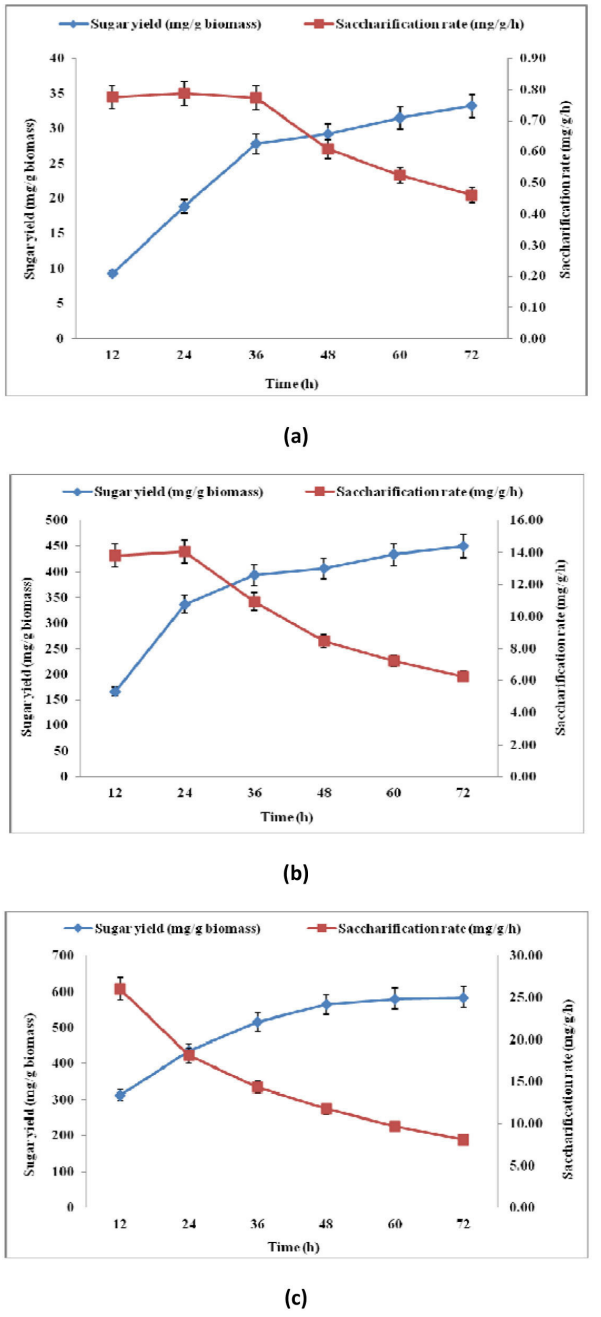
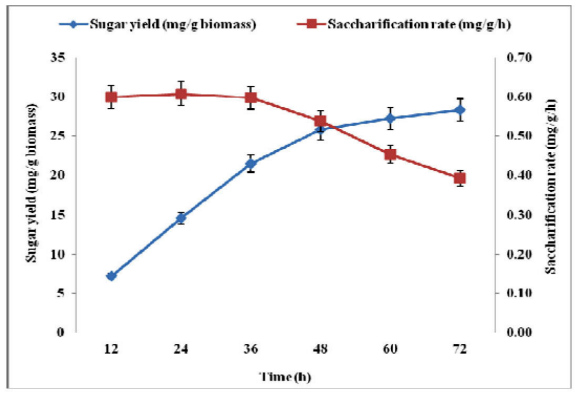


Fig. 5. Saccharification profile of pretreated sample (120 °C, 60 min, 2 % NaOH) hydrolyzed with (a) 0 %, (b) 15 % and (c) 30% enzyme loading

cubation at which the sugar yield was 298.6 mg/g biomass. The maximum rate of saccharification (23.72 mg/g/h) was attained at 12 h for 30% enzyme loading with sugar yield of 284.6 mg/g biomass.



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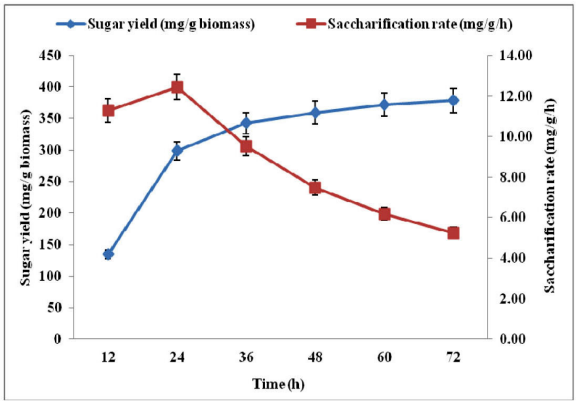

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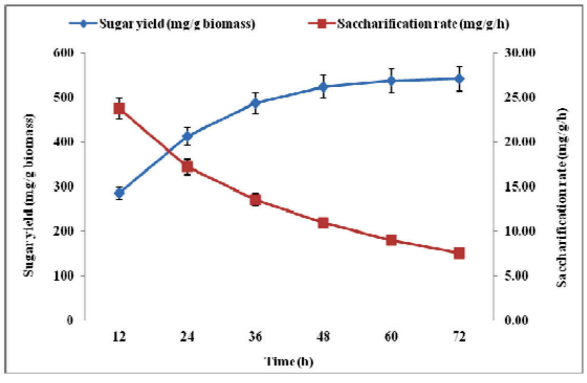
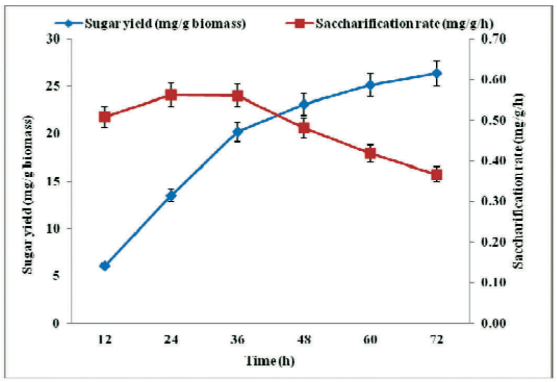


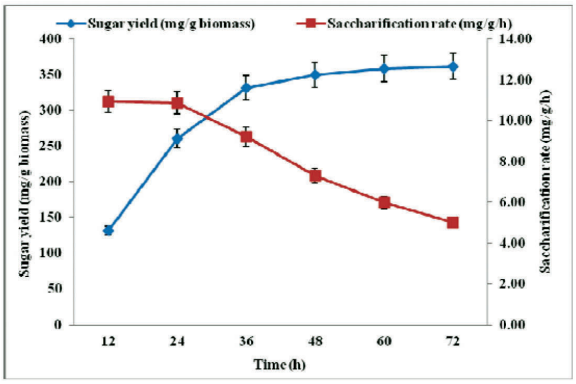
(c)

Fig. 6. Saccharification profile of pretreated sample (100 °C, 60 min, 2 % NaOH) hydrolyzed with (a) 0 %, (b) 15 % and (c) 30% enzyme loading

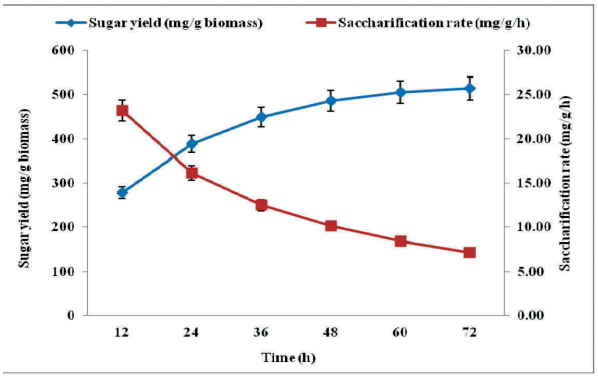
The fermentable sugars generated in the hydrolyzate obtained from the sample pretreated at 140 °C for 30 min with 1% NaOH concentration at 0, 15 and 30 % enzyme loading ranged from 6.1 to 514.4 mg/g dry biomass (Fig. 7). At 0% enzyme loading, a maximum saccharification rate of 0.56 mg/g/h was



(a)



(b)



(c)

Fig. 7. Saccharification profile of pretreated sample (140 °C, 30 min, 1 % NaOH) hydrolyzed with (a) 0 %, (b) 15 % and (c) 30% enzyme loading

observed at 24 h, at which the sugar yield was 13.5 mg/g biomass. While, the saccharification rate was maximum (10.93 mg/g/h) at 24 h of incubation for the hydrolyzate loaded with 15% enzyme with sugar yield of 131.2 mg/g biomass. For 30% enzyme loading, a maximum rate of saccharification of 23.21 mg/g/h was attained at 12 h at which the sugar yield was 278.5 mg/g biomass. The results are in agreement with sugar yields of delignified cellulosic substrate of *Propsopis juliflora* after hydrolysis (Gupta *et al*., 2009).

Total sugar yield

The total sugar yield in the hydrolyzate of untreated and pretreated samples ranged from 26.4 to 583.9 mg/g biomass [Fig. 8]. A maximum total sugar yield of 583.9 mg/g biomass was recorded in the hydrolyzate obtained from the sample pretreated at optimal conditions (120 °C for 60 min with 2% NaOH) when loaded with 30% enzyme. Whereas, it was minimum (26.4 mg/g biomass) in the hydrolyzate of the sample pretreated at 140 °C for 30 min with 1% NaOH without enzyme loading. As the enzyme loading level increased, the per cent total sugar yield also increased for both untreated and pretreated samples (Fig. 5.27). The total sugar yield increased drastically with 15% enzyme loading and thereafter increased gradually with 30% enzyme loading for all the samples. More than 70 per cent of sugars were released with 15% enzyme loading. The effect of enzyme loading was significant ($p<0.01$) on total sugar yield obtained from all the samples hydrolyzed at 1% level of significance.

However, the untreated sample recorded higher total sugar yield than all the pretreated samples at 0 per cent enzyme loading. The results are comparable with the earlier studies, this may be due to the fact that only soaking effect could not recover the sugars without enzyme loading which may be attributed to the loss of sugars incurred during the pretreatment conditions (Sharma *et al*., 2013).

Carbohydrate conversion

The per cent carbohydrate conversion of different samples hydrolyzed with various enzyme loading levels ranged from 4.11 to 90.86 (Table 4). The maximum carbohydrate conversion of 90.86 per cent was recorded for the sample pretreated at optimal conditions (120 °C, 60 min, 2% NaOH) which was loaded with 30% enzyme. The results are comparable with the maximum carbohydrate conversion (91.8%) for KOH pretreated switchgrass at 30% enzyme loading

Table 3. Effect of sodium hydroxide (NaOH) pretreatment on acid insoluble lignin of *Prosopis juliflora*

Temperature (°C)	Time (min)	Acid insoluble lignin (%) NaOH Concentration (%) 1	2	3
100	15	28.25 ± 0.38	25.10 ± 0.61	23.20 ± 0.47
	30	25.51 ± 0.23	23.27 ± 0.80	21.98 ± 0.81
	60	22.64 ± 0.47	18.83 ± 0.58	16.30 ± 0.66
120	15	22.10 ± 0.39	18.87 ± 0.75	15.49 ± 0.72
	30	20.34 ± 0.64	16.74 ± 0.59	14.66 ± 0.56
	60	18.36 ± 0.28	12.72 ± 0.37	10.42 ± 0.90
140	15	18.78 ± 0.96	15.94 ± 0.42	13.79 ± 0.64
	30	15.03 ± 1.04	11.94 ± 0.31	9.98 ± 0.99
	60	12.87 ± 0.29	9.32 ± 0.29	7.96 ± 0.10

Table 4. Carbohydrate conversions of untreated and pretreated samples hydrolyzed with different enzyme loadings

Pretreatment	Carbohydrate conversion (%) Enzyme loading, % (g enzyme protein/g biomass) 0	15	30
Untreated	7.28±0.68	36.34±0.89	58.08±0.94
120°C, 60 min, 2 % NaOH	5.17±0.45	69.97±1.05	90.86±1.02
100°C, 60 min, 2% NaOH	4.41±0.26	58.87±0.93	84.27±0.73
140°C, 30 min, 1% NaOH	4.11±0.67	56.27±0.75	80.05±1.28

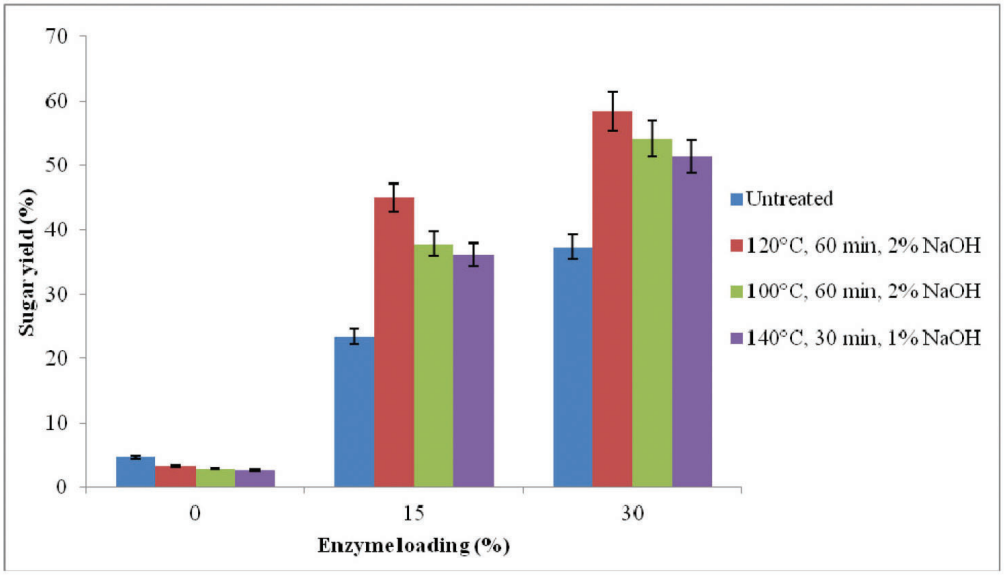


Fig. 8. Total sugar yields of untreated and pretreated samples hydrolyzed with 0, 15 and 30% enzyme loadings

with Cellic(R) CTec2 cellulase enzyme (Novozymes, North America, Franklinton) (Sharma *et al*., 2013). As the enzyme loading level increased, the per cent carbohydrate conversion also increased for both untreated and pretreated samples. More than 70 per cent of carbohydrates were converted with 15% enzyme loading.

The statistical analysis of per cent carbohydrate conversion achieved from hydrolysis of selected samples with different enzyme loadings indicated that there was a significant difference between the per cent carbohydrate conversion obtained from the samples hydrolyzed. The effect of enzyme loading was significant ($p<0.01$) on per cent carbohydrate conversion obtained from all the samples hydrolyzed at 1% level of significance. It was noted that the high enzyme loading (30 % g enzyme protein/g biomass) generated a high amount of sugars from the untreated samples compared to previous studies [17, 39], which have utilized lesser loadings. This seems to suggest higher efficacy of the enzyme and its ability to generate considerable sugars from the untreated biomass. However, this aspect needs further exploration.

Conclusion

Pretreatment of ground *Prosopis juliflora* with NaOH at higher temperatures resulted in promising delignification ranging from 40-60% and reducing sugar conversions of over 85 % after hydrolysis. The maximum rate of saccharification (26.07 mg/g/h) was attained at 12 h for sample pretreated at 120 °C, 60 min, 2% NaOH loaded with 30% enzyme. A total maximum sugar yield of 583.9 mg/g was achieved after 72 h of incubation, with a saccharification rate of 8.11 mg/g/h. The maximum carbohydrate conversion of 90.86% was recorded for the sample pretreated at optimal conditions (120 °C, 60 min, 2% NaOH) loaded with 30% enzyme. Overall, the generation of high fermentable sugars from NaOH pretreated samples of *Prosopis juliflora* at higher temperatures suggests that this alkaline pretreatment reagent has considerable potential but needs to be extensively investigated for comprehensive cost analysis.

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References

Alriksson, B. 2006, Ethanol from lignocellulose: Alkali

detoxification of dilute-acid spruce hydrolysates. Licentiate thesis, Karlstad University Studies, p. 30.

Cara, C., Ruiz, E., Oliva, J. M., Felicia, S. and Castro, E. 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. *Bioresource Technol.* 99 : 1869–1876.

Carlo, N. H., Geertje, V. H. and Andre, P. C. 2005. Ethanol from lignocellulosic biomass: Technoeconomic performance in short-middle and long term. *Biomass and Bioenergy.* 28 : 384–410.

Carrillo, F., Lis, M. J., Colom, X., Valldeperas, M. and Valldeperas, J. 2005. Effect of alkali pretreatment on cellulose hydrolysis of wheat straw: Kinetic study. *Proc. Biochem*. 40 : 3360– 3364.

Chang, V. and Holtzapple, M. T. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Appl. Biochem. Biotechnol.* 4 (6) : 5–37.

Chang, V. S., Nagwani, M., Kim, C. H. and Holtzapple, M. T. 2001. Oxidative lime pretreatment of high-lignin biomass: poplar wood and newspaper. *Appl. Biochem. Biotechnol.* 94 : 1-28.

Choge, S. K., Pasiecznik, N. M., Harvey, M., Wright, J., Awan, S. Z. and Harris, P. J. 2007. *Prosopis pods* as human food, with special reference to Kenya. Nairobi, *Kenya Forest Research Institute* 33 (3): 419-424. (http://www.wrc.org.za).

Eckard, A. D., Muthukumarappan, K. and Gibbons, W. 2012. Pretreatment of extruded corn stover with polyethylene glycol to enhance enzymatic hydrolysis: optimization, kinetics, and mechanism of action. *Bioenergy Research*. 5 : 424–438.

Eylen, D. V., Femke, V. D., Kabel, M. and Bont, J. 2011. Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. *Bioresour. Technol.* 102 : 5995–6004.

Fan, L. T., Gharpuray, M. M. and Lee, Y. H. 1987. Cellulose hydrolysis, in: *Biotechnol. Mono*. Springer, Berlin.

Fan, L. T., Gharpuray, M. M. and Lee, Y. H. 1987. Cellulose hydrolysis. In: S. Aiba, L. T. Fan, A. Fiechter, J. Klein, & K. de Schügerl (Eds.). *Biotechnology Monographs* (p. 8). Berlin: Springer.

Ghose, T. K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*. 59 : 257–268.

Gupta, R., Sharma, K. K. and Kuhad, R. C. 2009. Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis* -NCIM 3498. *Bioresour. Technol.* 100 : 1214–1220.

Hopkins, M. 2007. Cooking up a smoky solution, Nature News, doi:10.1038/ news070813-1, News.

Kapoor, M., Nair, L. M. and Kuhad, R. C. 2008. Cost-effective xylanase production from free and immobilized Bacillus pumilus strain MK001 and its application in saccharification of *Prosopis juliflora*. *Biochem. Eng. J.* 38 : 88–97.

Kim, S. and Holtzapple, M. T. 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. *Bioresour. Technol.* 96 : 1994-2006.

Klass, 1998. Lignocellulosic materials as alternative energy sources. *Encyclopedia of Energy.* 1 : 136-215.

Kodaganti, B. P. 2011. Simultaneous saccharification and fermentation of Arundo donax—Comparison of feeding strategies. www.chemeng.lth.se/E655.pdf. Accessed 24 May 2012.

Kuhad, R. C. and Singh, A. 1993. Lignocellulose biotechnology: Current and future prospectus. *Critical Reviews in Biotechnology*. 13 : 151–172.

Liao, W., Liu, Y., Liu, C., Wen, Z. and Chen, S. 2006. Acid hydrolysis of fibers from dairy manure. *Bioresour. Technol*. 97 : 1687–1695.

MacDonald, D.G., Bakhshi, N.N., Mathews, J.F., Roychowdhury, A. and Baypai, P. 1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. *Biotechnol. Bioeng.* 25 : 2067-2076.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry.* 31 : 426–428.

Ohgren, K., Galbe, M. and Zacchi, G. 2005. Optimization of steam pretreatment of SO2 impregnated corn stover for fuel ethanol production. *Appl. Biochem. Biotechnol*. 124 : 1055– 1067.

Pasiecznik, N. M., Felker, P., Harris, P. J., Harsh, L. W., Cruz, G., Tewari, J. C., Cadoret, K. and Maldonado, L. J. 2001. The *Prosopis juliflora – Prosopis pallida* Complex: A Monograph. HDRA, Coventry, UK. pp 162.

Rajput, S. S. and Tewari, M. C. 1986. *The role of prosopis in wasteland development*. In. Patel, V.J. (ed.), Javrajbhai Agroforestry Center, Surendrabag, Gujarat, India.

Selig, M., Weiss, N. and Ji, Y. 2008. Enzymatic saccharification of lignocellulosic biomass. *Laboratory Analytical Procedure* (LAP). Golden: National Renewable Energy Laboratory.

Sharma, R., Vijaykumar Palled, Ratna R. Sharma-Shivappa and Jason Osborne, 2013. Potential of potassium hydroxide pretreatment of switchgrass for fermentable sugar production. *Appl Biochem Biotechnol*. 169: 761-772.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D. and Osborne, J. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology*. 98 : 3000–3011.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. and Wolfe, J. 2005b. Determination of total solids in biomass and total dissolved solids in liquid process samples. Laboratory Analytical Procedure (LAP). Golden: National Renewable Energy Laboratory.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. and Templeton, D. 2005a. Determination of ash in biomass. Laboratory Analytical Procedure (LAP). Golden: National Renewable Energy Laboratory.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. and Templeton, D. 2008. Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure (LAP). Golden: National Renewable Energy Laboratory.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J. and Templeton, D. 2005. Determination of extractives in biomass. Laboratory Analytical Procedure (LAP). Golden: National Renewable Energy Laboratory.

Sun, Y. and Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* 83 : 1-11.

Teymouri, F., Laureano-Perez, L., Alizadeh, H. and Dale, B. E. 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydroluysis of corn stover. *Bioresour. Technol.* 96: 2014-2018.

Wyman, C. E. 1994. Ethanol from lignocellulosic biomass: Technology, Economics, and Opportunities. *Bioresour. Technol.* 50 : 3-16.

Xu, F., Sun, J. X., Liu, C. F. and Sun, R. C. 2006. Comparative study of alkali and acidic organic solvent-soluble hemicellulosic polysaccharides from sugarcane bagasse. *Carbohyd. Res*. 341 : 253–261.

Xu, J. 2009. *Alkaline Pretreatment of Switchgrass for Ethanol Production.* Unpublshed Ph.D. thesis of Biological and Agricultural Engineering department, NCSU, Raleigh, North Carolina, p22.

Xu, J., Cheng, J. J., Sharma-Shivappa, R. R. and Burns, J. C. 2010. Sodium hydroxide pretreatment of switchgrass for ethanol production. *Energy & Fuels*. 24: 2113–2119.

Xu, J., Cheng, J. J., Sharma-Shivappa, R. R. and Burns, J. C. 2010. Lime pretreatment of switchgrass at mild temperatures for ethanol production. *Bioresource Technology.* 101 : 2900–2903.