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High temperature pretreatment and hydrolysis of cotton stalk for producing sugars for bioethanol production

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ABSTRACT

The aim of this work was to evaluate cotton stalk (waste plant material after harvesting the cotton) as feedstock for bioethanol production. Different pretreatment strategies were tried using sodium hydroxide in a high pressure reactor equipped with a pitch blade turbine stirrer, followed by enzymatic hydrolysis using cellulases; the process optimization was carried out using Taguchi experimental design. Best results were achieved when the pretreatment was carried out at 180 °C for 45 min with mixing of substrate at 100 rpm. The sugar yield was evaluated based on pretreatment severity. The hydrolysis efficiency of pretreated cotton plant waste was very good (96%), showing the excellent efficiency of the method in removing the lignin. The material balance in each stage of the process was estimated and the total process efficiency was found to be 53% based on glucose conversion.

1. Introduction

The commercial production of second generation bioethanol is still in development stage as it requires a more complex know-how than corn-ethanol technology due to the complex structure of the plant cell wall. More over the composition lignocellulosic biomass differs from each other; hence it is very difficult to adopt a common method for all types of biomass.

Fast and efficient technologies are desirable to reduce investment and operating cost, thus making ethanol competitive with gasoline. Largely abundant lignocellulosic materials such as wastes of agricultural, forestry and paper-mill industries or dedicated energy crops (e.g. switch grass) may be a sufficient and cheap feedstock for ethanol production. Based on the structural complexity of lignocellulosic biomass, more severe methods may need to adopt for deriving fermentable sugars. Hence, selection of lignocellulosic biomass is very important in this process as it finally affect the total process economy.

The most important criterion for selecting a suitable biomass for bioethanol production is its availability in a particular locality. The composition of the particular biomass is also very important before it can be considered for biofuel production [1]. A particular biomass containing more than 70% cellulose and hemicelluloses can be considered as a good candidate. The lignin content should be less than 20%.

Agriculture in India has always been the most important economic sector with huge amount of biomass residue generation. Though the generation of biomass residues in the country from agriculture is considerably large, the actual availability of a major share of these residues for bioethanol production is questionable. A significant part of the agro-residues generated is consumed for fodder and other applications resulting in a low amount of surplus material available for fuel production [2]. Thus selection of feedstock for any commercial bioprocess needs a careful evaluation.

India occupies first place among cotton growing countries of the world in respect of area (9.4 million hectares in 2009–2010) which is about 35% of world cotton area [3]. India is the only country to grow all four species of cultivated cotton *Gossypium arboreum* and *herbaceum* (Asian cotton), *Gossypium barbadense* (Egyptian cotton) and *Gossypium hirsutum* (American Upland cotton) [4]. After harvesting the cotton balls, the entire plant, consisting of stalk and leaves, is a residue which remains in the field and the farmers usually destroy it by burning. It is estimated that for every hectare of cotton production, 2 MT of cotton stalks are generated. It has reported that India generated 18.9 MMT of cotton plant waste in 2007–2008 out of which 7.4 MMT residue is used by farmers itself as firewood for household energy needs. Hence there will be a surplus of 11.4 MMT available as feedstock for commercial bio-based processes [5].

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In this scenario, utilization of the cotton plant waste for bioethanol production seems to be very promising. Even though the farmers utilize a small portion of generated residue for house hold fuel needs, there is no other commercial value. This biomass cannot be used a fodder also. All these makes advantages to this plant waste for use as a raw material for bioethanol production. As it is a lignocellulosic material, proper pretreatment for the removal of lignin followed by enzymatic hydrolysis would generate fermentable sugars which can be converted into fuel ethanol. The objective of the present study was to evaluate the best pretreatment condition of cotton stalk for maximum sugar yield. NaOH was used as the pretreatment reagent. The material flow and mass balancing in each process stage has been evaluated.

2. Materials and methods

Cotton stalk (whole plant waste without leaves after harvesting the cotton) was obtained from a field in Andhra Pradesh, India. It was milled in a knife mill to reduce the particle size in the range of 200 μm to 1 cm. The milled material was dried at 40 °C and stored in an air tight container.

2.1. Composition analysis

The composition analysis of cotton plant waste was carried out as per the NREL protocols [6]. Briefly, 0.3 g was dissolved in 10 ml of 64% (v/v) sulfuric acid in a stoppered conical flask and left to hydrolyze at 30 °C in a shaking water bath until complete dissolution of the sample had occurred. Distilled water (90 ml) was added and kept at 121 °C for 1 h in a laboratory autoclave. Small amount of the solution was filtered through 0.2 μ filters for HPLC analysis. A sugar verification standard for glucose, xylose and arabinose were also analyzed by the same procedure to find out the loss in sugar after the treatment. The remaining solution and residue were retained for lignin estimation. Carbohydrate concentrations in the hydrolysates were determined using BioRad Aminex HPX-87P HPLC column using RI detector. This analysis was performed at 85 °C column temperature and 60 °C detector cell temperature with a flow rate of 0.6 ml/min using degassed Milli Q water as mobile phase. Glucose, xylose and arabinose were used as standard for HPLC analysis.

The acid soluble and acid insoluble lignin were determined as per NREL procedure [7,8].

2.2. Pretreatment

Fifteen grams of dried and powdered cotton plant waste was taken for each pretreatment experiment. It was mixed with 285 ml 4% NaOH so as to obtain the solid loading of 5%. Pretreatment experiments were carried out in a high pressure reactor (Amar Equipments Pvt. Ltd., New Delhi, India). The reactor had a maximum working temperature and pressure of 250 °C and 100 bar, respectively. The reactor was equipped with a pitch blade turbine stirrer with 50–1450 rpm. The heating in the instrument was carried out by ceramic heating mantle. After the pretreatment, the samples were withdrawn from the reactor and neutralized to pH 7.0 using IN H_2SO_4. The solid–liquid separation was carried out by filtration and the solid portion was washed with tap water and kept for drying in room temperature (around 30 °C). The pretreated liquor was analyzed for lignin and soluble sugars.

2.3. Taguchi's experimental design

Taguchi statistical design was used to find the best pretreatment condition. This approach has found widespread use in industrial process design, principally in the development trials to generate sufficient process information to establish optimal conditions for a particular process using a minimal number of experiments possible. According to these orthogonal array three variables in 16 experiments was used to evaluate the pretreatment efficiency. Experiments were performed according to an experimental plan given in Table 1A. The pretreatment was carried out in four different temperatures such as 120, 150, 180 and 200 °C with variable residence time and rpm. The statistical software Minitab 15 (Minitab Inc. USA) was used for experimental design and validation.

2.4. Enzymatic hydrolysis

The enzymatic saccharification of pretreated cotton plant waste was carried out using commercial cellulase from Zytex (Zytex India Private Limited, Mumbai, India). The hydrolysis was carried out in a 600 ml reactor with working volume of 300 ml. Twenty-five gram (dry weight) of pretreated residue was taken after moisture correction. The amount of enzyme used was 60 FPU/g (FPU-Filter Paper Unit) of dry pretreated biomass. The samples were incubated at 50 °C for 48 h with 120 rpm. After incubation, the samples were centrifuged to remove the unhydrolyzed residue. The supernatant was estimated for carbohydrates by HPLC.

Table 1A
Taguchi's robust experimental design and corresponding reducing sugar yield.

Run	Factors				Sugar yield (g/g)		
	Temperature	Time	rpm	Ro	Experimental	Predicted	Standard error
1	120	15	50	58.21	0.118	0.020	0.069
2	120	30	100	116.41	0.194	0.033	0.043
3	120	45	150	174.62	0.192	0.165	0.052
4	120	60	200	232.82	0.294	0.181	0.062
5	150	15	50	444.91	0.149	0.255	0.075
6	150	30	100	889.83	0.214	0.267	0.037
7	150	45	150	1334.74	0.338	0.400	0.168
8	150	60	200	1779.65	0.337	0.415	0.055
9	180	15	50	3400.84	0.338	0.377	0.043
10	180	30	100	6801.69	0.63	0.590	0.028
11	180	45	150	10202.53	0.635	0.721	0.061
12	180	60	200	13603.38	0.752	0.738	0.010
13	200	15	50	13196.56	0.53	0.399	0.093
14	200	30	100	26393.11	0.409	0.410	0.001
15	200	45	150	39589.67	0.655	0.543	0.008
16	200	60	200	52786.23	0.515	0.558	0.101

2.5. Estimation of carbohydrates

The sugars present in the hydrolyzed supernatant were estimated by HPLC. Biorad Aminex HPX-87P columns were used for the analysis. The hydrolyzed samples were filtered through 0.2 μ filters and injected to the column. The deionized water was used as the mobile phase with flow rate of 0.6 ml/min. The column temperature was maintained at 85 °C. The compounds were detected using refractive index detector. Glucose, xylose and arabinose (Sigma–Aldrich, India) were used as calibration standards.

2.6. FTIR analysis

Fourier Transform Infrared spectroscopic analysis of untreated and pretreated residue was carried out to detect changes in functional groups. FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu Spectrometer with detector at 4 cm^{-1} resolution and 25 scan per sample. Discs have been prepared by mixing 3 mg of dried sample with 300 mg of KBr (Spectroscopic grade) in an agate mortar. The resulting mixture was successfully pressed at 10 MPa for 3 min.

3. Results and discussion

The chemical composition of cotton plant waste varies depending on the growing location, season, harvesting and processing methods [9]. The cotton plant waste used in the present study mainly consisted of dried stem. The compositional analysis showed that the present variety contains 30% cellulose and 13% hemicelluloses. The lignin content was about 31%. Silverstein et al. [10] also showed a similar result, where the cotton stalk (*G. hirsutum*) collected from United States contains 41.8% holocellulose and 30.1% lignin. The studies conducted by Ververis et al. [11] showed that the cotton (*G. hirsutum*) stalk from Greece contains 40% α-cellulose and 17% lignin. A similar compositional analysis results were presented by Agblevor et al. [9] for cotton gin residue which include immature bolls, cotton seed, hulls, sticks, leaves and dirt.

Based on HPLC carbohydrate analysis, the samples used in the present study showed glucan as the major carbohydrate content (33.3%) followed by Xylan (14.8%). Arabinan and mannan were accounted for only a small portion of the biomass and galactan was not detected. Since the carbohydrate content of the cotton plant waste determined by the HPLC analysis was more likely to be representing the actual sugars available after treatments, subsequent calculations and analysis of data in this study was performed on the basis of HPLC analysis.

The amount of lignin present in the cotton stalk was higher than expected. A similar result was also noted by Silverstein et al. [10]. Literature reports indicate that the lignin content of herbaceous plant materials ranges from 10% to 20%, for hardwoods it ranges from 18% to 25% and for soft wood contains 26% to 34% [10,12]. Silverstein et al. [10] has given an explanation for this increased lignin content in cotton stalk. According to him, the acid insoluble material from woody biomass is generally classified as lignin, however, it would be incorrect to classify all the acid insoluble material from cotton stalks as lignin because other condensable compounds such as proteins condense and become insoluble in concentrated sulfuric acid. An independent nitrogen analysis would be required to determine the acid-insoluble lignin content separate from the condensed protein fraction. Since the major objective of this work is to derive sugars from the cotton plant waste, the accurate lignin estimation is beyond the scope of this study.

3.1. Pretreatment

Alkali pretreatment is regarded as an efficient pretreatment method for removing lignin from lignocellulosic biomass. Lignin due to its ability to absorb enzymes is known to have adverse effects on action of cellulases. The Cellulose Binding Domains (CBD) of cellobiohydrolases have been shown to be the major contributing factor responsible for lignin adsorption, but both the structure and properties of the CBD as well as the catalytic domain are involved in the binding affinity. So it is very necessary for lignin removal before enzymatic hydrolysis of lignocellulosic biomass and alkali is known to be the best chemical for lignin removal. In the study NaOH was used as the chemical reagent for lignin removal. The various concentrations of NaOH, such as 1, 2, 3, 4 and 5% (w/w), was tried for initial pretreatment experiments and it was found that 4% was best for maximum lignin removal as well as sugar yield after enzymatic hydrolysis (data not shown). Further experiments were carried out using this concentration of NaOH by statistical methods.

3.2. Optimization of pretreatment parameters by Taguchi's experimental design

Because the selected Taguchi design [13] is orthogonal, it was possible to resolve the effect of each individual factor on the response and control factor-noise factor interactions, enabling optimization of the process response. Effects of pretreatment temperature, residence time and rpm were studied by Taguchi method employing 16 experimental trials (Table 1A). The minimum and maximum experimental sugar yields were observed to

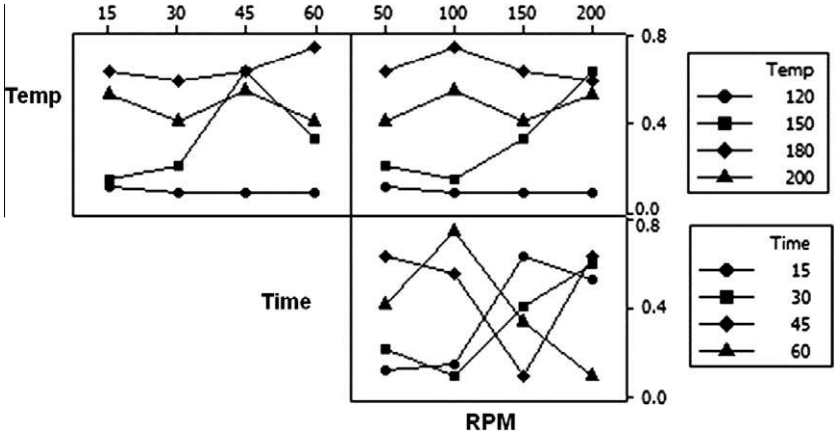

Fig. 1. Interaction plot for time, temperature and rpm.

be 0.092 and 0.752 g/g dry pretreated biomass respectively. The maximum sugar yield, as per the predicted model, showed at pretreatment temperature of 180 °C at 200 rpm for 60 min residence time. As the pretreatment temperature increases, the sugar release decreases. This may be due to the degradation of glucan and xylan present in the biomass at high temperature. The interaction plot for time and temperature (Fig. 1) shows that the sugar yield is having a direct relation with temperature up to 180 °C. At 120 °C, there was very low sugar release (nearly 0.1 g/g dry biomass). At 150 °C there was very low yield at lower residence time and when the residence time increases, the sugar yield also increases. At 150 °C for 45 min residence time the sugar yield reaches up to 0.68 g/g, and further increase in time reduced the sugar yield. The present data suggested a significant improvement in reducing sugar yield after enzymatic hydrolysis from pretreated cotton plant waste.

Understanding of the impact of each individual factor is the key for a successful process development. ANOVA result shows that all the factors considered in the experimental design were statistically significant at 89% confidence limit (Table 1B). Analysis of the sugar yield from pretreated cotton plant waste after enzymatic hydrolysis in above experimental design revealed that among all selected factors temperature contributed the maximum impact.

To validate the determined optimized methodology, further pretreatment experiments were conducted using the obtained optimized conditions. The Taguchi design provided the predicted information. Based on this information, optimum sugar yield from NaOH pretreated cotton plant waste after enzymatic hydrolysis could be achieved at 180 °C, 100 rpm and 45 min treatment time. At this condition it was predicted a sugar yield of 0.74 g/g and it could able to achieve 0.75 ± 0.05 g/g sugar.

3.3. Pretreatment severity

In case of hydrothermal processing such as biomass-pretreatment, the severity of the process is often expressed by a severity index [14]. It is an expression that combines the independent variables of temperature and time into a single independent variable. This is a mathematical expression of the observation that reaction rates double for every 10 °C increase in temperature. The severity index is expressed mathematically as

$$\int_0^t \exp((T-100)/14.75)\mathrm{d}t$$

where T is temperature in degrees Celsius, t is time in minutes and 14.75 is the conventional energy of activation assuming the overall reaction is hydrolytic and the overall conversion is first order.

The severity factor R_0 ($R_0 = t * \exp\left[\frac{(T-100)}{14.75}\right]$ combines the experimental effects of temperature and reaction time to enable an easy comparison of results and to facilitate process control [15]. A modified severity parameter was later developed by Chum et al. [16];

$$M_0 = t * C^n * \exp\left(\frac{T_r - T_b}{14.75}\right)$$

where M_0 is the modified severity parameter; t is the residence time (min); C is the chemical concentration (wt.%); T_r is the reaction temperature (°C); T_b is the base temperature (100 °C); n is an arbitrary constant. This equation can be adapted for application to sodium hydroxide pretreatment by replacing the acid concentration with the alkali concentration and calculating a different n-value [17].

Earlier authors tried standard severity factor (combination of temperature and residence time) and the combined severity factor (combination of acid catalyst concentration, temperature, and residence time) to present the pretreatment data more cohesively [15–19]. In the present experiment, the concentration of NaOH was 4% throughout the experiment and the experimental variables were temperature, time and rpm. Hence combined severity was expressed by including the factor for rpm. The following equation was used to calculate the combined severity factor;

$$R_0 = t * f * \exp\left(\frac{T_r - T_b}{14.75}\right)$$

where t is the residence time (min); f is the factor for rpm (m^{-1}); T_r is the reaction temperature (°C); T_b is the base temperature (100 °C).

The relation between sugar yield and standard severity factor was linear. The model equation for determination of sugar yield during NaOH pretreatment was developed by plotting $\log R_0$ vs. sugar yield (g/g) (Fig. 2A) and the model equation for the sugar yield was;

$$\text{Sugar yield } (g/g) = 0.179 * \log R_0 - 0.209$$

A similar plot of modified severity factor with rpm and $\log R_0$ showed a logarithmic relation (Fig. 2B). The R^2 from the plot was 0.83 indicating good predictive ability of the model. This result is consistent with previous work on the applicability of the severity function for the prediction of sugar as well as degradation products [19].

Table 1B
ANOVA for sugar yield.

Factor	DF	Sum of square	F	P	S	R^2
Temperature	3	0.664	15.03	0.003	0.121	89.43%
Time	3	0.051	1.16	0.399		
rpm	3	0.032	0.73	0.569		
Error	6	0.088				
Total	15	0.836				

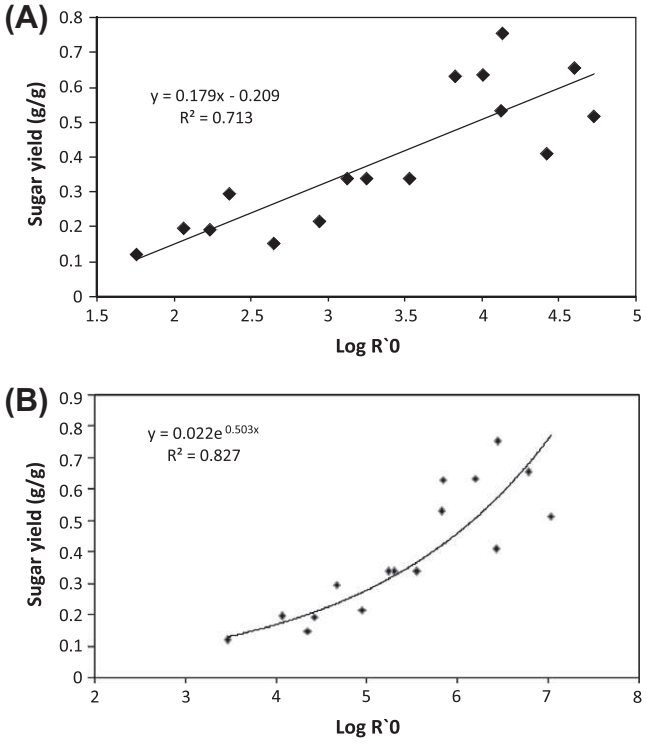

Fig. 2. The sugar yield after pretreatment of cotton plant waste as a function of standard severity (A) and combined severity (B). The lines show only trends.

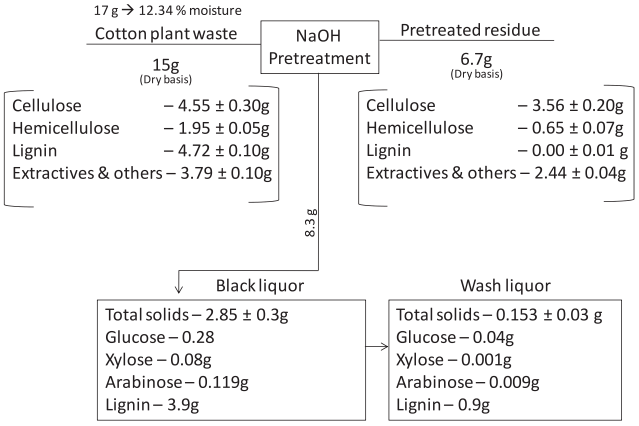

Fig. 3. Material balancing during NaOH pretreatment of cotton plant waste.

3.4. Material flow during pretreatment

During every process stage in biomass to bioethanol technology, there will be loss of materials in each phase. Hence, it is very necessary to estimate the material loss during every stage of operation in order to derive a valid conclusion of total process economy. In the present study, the flow of materials in every unit operation in pretreatment is checked. Initially, 17 g of raw cotton plant waste was taken so as to obtain 15 g dry weight as the milled and stored biomass contained 20% moisture. The biomass loading for pretreatment was adjusted to 5% by adding 285 ml 4% sodium hydroxide. During pretreatment there was a loss of 7 g water as evaporation loss. After pretreatment, 220 ml of 1N H_2SO_4 was added to maintain the pH near neutral. After separation of pretreated biomass from the filtrate (this filtrate is designated as black liquor), one step washing was carried out by adding 150 ml water. After washing 6.7 ± 0.97 g of dry pretreated material was collected by filtration (this filtrate is indicated as wash liquor). Analysis of black liquor revealed 2.40 ± 0.46 g total suspended solids. The black liquor removed some of the sugars from the solid residue. In wash liquor the amount of sugar present were negligible. So, there occurred 55.4% material recovery during the entire pretreatment process.

The sugar analysis of black liquor by HPLC method showed 0.525 ± 0.13 mg/ml glucose, 1.085 ± 0.27 mg/ml xylose and 0.775 ± 0.07 mg/ml arabinose. This indicates that about 1.57 g carbohydrates were removed in the black liquor of which major portion was from hemicellulose. Hemicellulose removal is also evident by residual solid analysis as shown in Fig. 3.

3.5. Evaluation of lignin removal by NaOH pretreatment

The main effect of sodium hydroxide pretreatment on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity of the biomass [17] which helps in increased surface area for enzyme action. Lignin is one of the major inhibitor for enzymatic hydrolysis. It was found that almost 100% lignin removal was observed after sodium hydroxide pretreatment at 180 °C for 45 min. The total carbohydrate content has increased to 60.6% after pretreatment along with 55.4% solid recovery.

The removal of lignin was also evident from Fourier Transform Infrared spectrum of untreated and pretreated cotton plant waste. In the FT-IR spectra the range of 1850–500 cm^{-1} known to encompass lignin related information [20]. Earlier studies on FT-IR spectra of soft wood lignin showed absorption bands at regions 1720, 1670, 1590, 1500, 1463, 1423, 1360, 1330, 1270, 1215, 1140, 1125, 1087, 1031, 970, 855, and 815 cm^{-1} [21]. The FT-IR spectral analysis of untreated cotton plant waste also showed absorption bands in these regions. For NaOH treated residue, the corresponding bands in these regions were absent. This shows significant removal of lignin during pretreatment. The band at 1490 cm^{-1} is assigned to phenolic ring vibration and the bands at 1450, 1210, 1188 cm^{-1} to phenolic hydroxyl groups vibrations. Decreasing aryl-ether bonds can result in dropping the bands at 1140, 1050, and 1030 cm^{-1} [21]. Fig. 4 shows the FTIR spectra of raw as well as pretreated cotton plant waste.

3.6. Enzymatic hydrolysis of pretreated cotton plant waste

The estimation of various carbohydrates generated at different time points during enzymatic hydrolysis of NaOH pretreated cotton waste shows that the hydrolysis rate was maximum during the first 3 h (Fig. 5). During this period 63% glucose yield was recorded. A total of 96% hydrolysis efficiency was found with

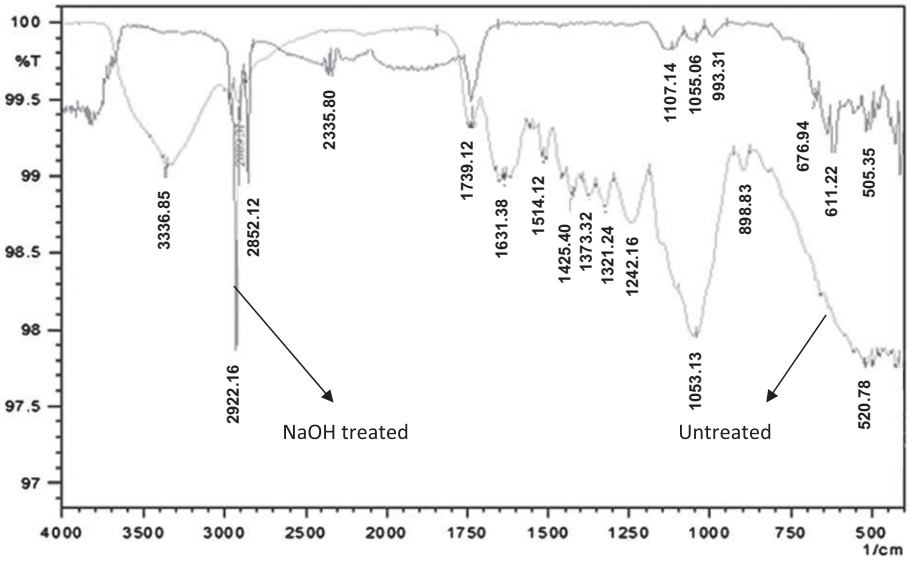

Fig. 4. FT-IR analysis of pretreated and untreated cotton plant waste.

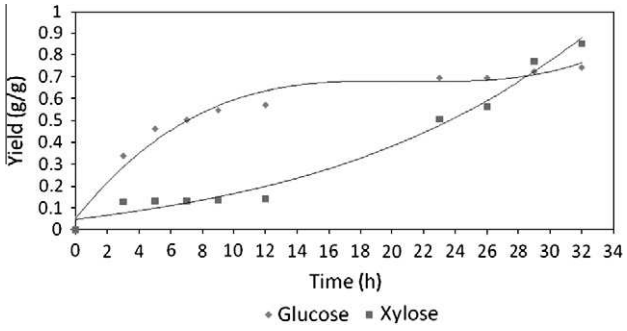

Fig. 5. Glucose and xylose yield during hydrolysis.

pretreated cotton stalk residue. Considering the material loss during pretreatment stage 53% total efficiency can be achieved. All these calculations are only based only on the glucose yield.

3.7. Ethanol production efficiency from cotton plant waste

Based on the pretreatment efficiency in the present study, the ethanol production economy from cotton plant waste has been worked out. From the study it was found 55.4% material recovery during pretreatment process. As mentioned in Section 1, if there is 11.4 MMT cotton plant waste is available in India as surplus, 3.533 billion litres of ethanol can be produced based on the current production process considering 90% fermentation efficiency.

4. Conclusion

Sodium hydroxide pretreatment of cotton stalk (plant waste) was effective for deriving fermentable sugars. The high temperature treatment completely removed lignin which helped to increase the total hydrolysis efficiency up to 96%. The process efficiency, based on glucose recovery was 53% (based on cellulose to glucose conversion), which showed solid losses during the pretreatment stage. About 10% of material was removed as black liquor, a major portion of which include hemicellulosic sugars. Considering the whole carbohydrate present in the cotton plant waste and utilization of xylose and arabinose to any commercial value-added product, the total process economy for the production of ethanol from cotton plant waste can be highly enhanced.

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