Ammonia Pre-Treated Cotton Stalks for Bioethanol Production using *Saccharomyces cerevisiae* CP11

ABSTRACT

Cotton stalks are good raw material for bioethanol production due to its availability throughout the world, abundance, high carbohydrate content and not involved in any food chain. Due to recalcitrant nature of cotton stalks pre-treatment, hydrolysis were not effective. In the present study pre-treatment with ammonia at room temperature for 1 week period and 121^{o}C for 60min were attempted and it is compared with the standard 0.2M NaOH treatment. 1.5% ammonia pre-treatment at room temperature for 1 week found to remove 86% of lignin and subsequently undergone 75.92% acid hydrolysis. The acid hydrolysate obtained consists less lignin and furfurals and fermented to 5.75% ethanol with 91% fermentation efficiency with *saccharomyces cerevisiae.* Hence dilute ammonia pre-treatment for a week was found to be effective for bioethanol production using cotton stalks.

Key words: Ammonia; Pre-Treatment; Cotton Stalk; Bioethanol; Yeast.

1. INTRODUCTION

"Bioethanol is recognized as a clean-burning, non-petroleum liquid fuel. Countries dependence on imported oil, environmental issues, and employment in rural areas has been reasons for the consideration of the replacement of fossil fuels with bioethanol. But only a few countries are successful to use ethanol as fuel, where excessive ethanol is produced from additional or specifically grown raw materials like maize and sugar cane. Biomass, which includes trees, shrubs, yard waste, wood products, grasses and agricultural residue, such as wheat straw, corn stover, rice straw, and cotton stalks, is a renewable resource that stores energy from sunlight" (1). "This can be processed chemically or biologically by breaking chemical bonds and releasing energy in the form of bioethanol. As a substrate, conventional crops such as corn and sugarcane are unable to meet the global demand for bioethanol production due to their primary value of food and feed therefore, lignocellulosic substances such as agricultural wastes have emerged as an attractive feedstock for bioethanol production" (2). "Cotton stalk (CS), remain in the field after harvesting the cotton, as an agricultural residue. It needs to be removed from the field" (3, 4). "It is estimated that for every hectare of cotton production, 2MT of cotton stalks are generated" (5). "A large amount of cotton stalks are used as firewood for household needs or burned on grounds leading to environmental pollution and biomass wastage" (6, 7). "Cotton stalks, which mainly contain lignocellulose, have the potential to serve as a low-cost feedstock to increase the production of fuel ethanol. However, direct saccharification or biotransformation of the cotton stalk is extremely difficult because of the recalcitrant nature of lignocellulosics" (8-

10). "Pretreatment is necessary to change the recalcitrant structure" (11). "Pretreatment is needed to alter the structural and chemical composition of lignocellulosic biomass to facilitate the rapid and efficient hydrolysis of carbohydrates to fermentable sugars" (12). "The pretreatment is perhaps the single most crucial step as it has a large impact on all the other steps in the process, e.g. hydrolysis, fermentation, downstream processing, and wastewater handling. Through many researchers' efforts, current existing pre-treatment techniques of lignocellulosic biomass, including dilute acid, alkali, and biological pre-treatment have been extensively verified in laboratories and under development" (13- 15). "Many pre-treatment strategies focus on lignin removal from biomass to achieve a more efficient substrate hydrolysis process. Alkaline pre-treatment limits the degradation of hemicellulose polymers" (16). "As different lignocellulosic feedstocks have different physicochemical characteristics, suitable pre-treatment techniques based on the raw material must be adopted" (17). "Alkali-based pre-treatment causes the breakdown of ester bonds cross-linking lignin and xylan, removal of lignin, cellulose swelling, and partial decrystallization of cellulose" (18). "Generally, alkaline pre-treatments are more effective on agricultural residues and herbaceous crops than on wood materials" (19). "Alkaline pre-treatment was used on cotton stalks for generating value-added products" (20, 21). In the present study, the cotton stalk was used as feedstock with ammonia pre-treatment at room temperature for bioethanol production.

2. MATERIALS AND METHODS:

2.1.Raw Material and Reagents:

Cotton stalks (CS) of spp. *Gossypium hirsutum* were obtained from the cotton crop field of Mahabubnagar district, Telangana India. Before compositional analysis, the biomass consisted primarily of stalks, which were collected, dried, debarked, and ground to 2mm particle size and stored at room temperature. All chemicals were analytical grade, obtained from MERK.

2.2.Composition analysis:

"The composition of cotton stalks was analyzed for holocellulose, cellulose, pentosens, klason lignin, and ash content. The bark-free cotton stalk was taken and fractioned using a laboratory knife mill to attain a particular size (4–10 mm). The obtained wood dust was passed through by 40 mesh and retained on 60 mesh and was used for proximate chemical analysis and further chemical hydrolysis experiments. The chemical analyses were performed by following the TAPPI test methods" (22).

2.3.Pre-treatment of Cotton Stalks:

Aqueous solutions of NH_3 at concentrations 0, 0.5, 1, and 1.5% (w/v) were used to pre-treat CS samples at a solid loading of 15% (w/v). Treatments were performed in duplicates for three times in an autoclave at 121°C with 15 psi (103.4 kPa) pressure for 60min holding time and at room temperature for a week.

The pre-treated solids were filtered, washed thoroughly with deionized water, dried in an air-circulated oven for 16 h at 85°C, and used for the subsequent hydrolysis and fermentation experiments.

2.4.Acid hydrolysis and detoxification of cotton stalk:

The pre-treated Cotton stalk was subjected to sulphuric acid hydrolysis. In 0.5N sulphuric acid solution, pre-treated biomass (20%w/v) was treated with steam under pressure at 121^{o}C in an autoclave for 30 minutes and four-hour heat treatment at 90^{o}C in the water bath (23). The obtained acid hydrolysate was detoxified by the addition of dried lime up to pH 10 for an hour and then filtered and pH was readjusted up to 6 with sulphuric acid. This is followed by 2% (w/v) charcoal treatment for half an hour with stirring and then filtering (23). The obtained filtrate solution was used as a sole carbon source for fermentation studies.

2.5.Fermentation:

The yeast *Saccharomyces cerevisiae* CP11 strain isolated and maintained in our laboratory was used in the study. The inoculum was prepared by growing yeast on YPD (Yeast, Peptone and Dextrose) media, for 24h at 30°C. The prepared cultures of *Saccharomyces cerevisiae* CP11 were used as inoculum in fermentation.

To acid hydrolysate (100mL^{-1}), the following were added to make fermentation media: 1.5 g yeast extract, 1 g each of peptone and $(NH_4)_2SO_4$, 0.5 g each of K_2HPO_4, $MgSO_4.H_2O$ and $MnSO_4$ at pH 5.5. The medium was sterilized for 25 min at 110°C. After cooling the media, 3% inoculum was added to the flask containing sterilized media. Fermentation was carried out for 96 hours at 30°C. Initially, shaking of 100rpm was provided for 4 hours followed by static anaerobic conditions for 92 hours. The samples were collected at 24h intervals throughout the fermentation process and analyzed for ethanol content and reducing sugars.

2.6.Analytical methods:

Total Reducing sugars were estimated by DNS method of Miller (24).

Hydroxymethylfurfural was determined based on absorbance in spectrophotometer. An aliquot of $5mL^{-1}$ of hydrosylate dissolved in 25mL of distilled water and added to Carrez I solutions (0.5mL) and Carrez II (0.5mL) the solution was filtered and the first 10mL was discarded. From the filtrate, absorbance at 284 and 336 nm was read with an aliquot of solution filtered with 0.2% sodium bisulfite as blank. The HMF is determined by the equation: HMF/ 100mL of hydrolysate = (Abs284 − Abs336) × 14.97 × 5mL of the sample.

Total content of phenolic compound in hydrolysate was determined by FolinCiocalteus (FC) method (25).

Ethanol estimation:

Ethanol estimation in fermented broth was carried out by gas chromatography. The method uses a SHIMADZU GC 2010 with a flame ionization detector. GC was carried out according to NREL procedure LAP # 011, using ZB-Wax column (30mm × 0.25mm).

2.7.Statistical analysis

Experiments were repeated thrice in duplicates (n = 6) and average values with standard deviation was provided.

3. RESULTS

3.1. Chemical composition of raw cotton stalks:

The results revealed that the cellulose content in the raw (untreated) cotton stalk was 44.8 ± 0.55 % and hemicellulose was 13.25 ± 0.50 %; whereas, the lignin content was found to be 29.6 ± 0.75 %. Cellulose and hemicellulose content in a defined combination makes the holocellulose, which was found to be 58.05 ± 0.25%.

3.2. Pre-treatment of cotton stalks:

The cotton stalks were delignified with different concentrations of NH_3 0, 0.5,1, and 1.5 in an autoclave and at room temperature. The results after pre-treatment showed that the lignin content decreased with the increase in the concentration of NH_3. The cotton stalks soaked at a concentration of 1.5% at room temperature showed the highest delignification rate (86 ± 0.31%) and high cellulose content (72.7 ± 0.54%). This was subjected to further processes.

Table 1: Cotton stalks composition after pre-treatment at room temperature for a week

Concentration of NH_3 (%)	Cellulose (%)	Hemicellulose (%)	Holocellulose (%)	Lignin (%)	Delignification (%)	Furfurals(mg/L)
Control NaOH	54.2 ± 0.58	15.20 ± 0.27	69.40 ± 0.19	21.48 ± 0.68	27.43 ± 0.18	2.1 ± 0.14
0	44.8 ± 0.55	13.25 ± 0.57	58.05 ± 0.28	29.60 ± 0.74	00.00 ± 0.00	2.8 ± 0.54
0.5	52.5 ± 0.45	14.80 ± 0.38	67.30 ± 0.47	15.40 ± 0.88	47.97 ± 0.22	3.4 ± 0.42
1	60.6 ± 0.32	15.80 ± 0.21	76.40 ± 0.32	09.30 ± 0.14	68.58 ± 0.47	4.9 ± 0.27
1.5	72.7 ± 0.25	16.20 ± 0.11	88.90 ± 0.18	04.14 ± 0.47	86.01 ± 0.84	5.3 ± 0.97

Table 2: Cotton stalks composition after pre-treatment in autoclave.

Concentration of NH_3 (%)	Cellulose (%)	Hemicellulose (%)	Holocellulose (%)	Lignin (%)	Delignification (%)	Furfurals(mg/L)
Control NaOH	50.0 ± 0.11	14.9 ± 0.15	64.9 ± 0.94	21.49 ± 0.28	27.39 ± 0.36	40.1 ± 0.56
0	48.4 ± 0.57	14.7 ± 0.35	63.1 ± 0.57	23.50 ± 0.37	20.60 ± 0.57	42.4 ± 0.44
0.5	55.4 ± 0.78	15.2 ± 0.47	70.6 ± 0.45	18.50 ± 0.59	37.50 ± 0.85	44.7 ± 0.57
1	61.8 ± 0.24	12.6 ± 0.57	74.4 ± 0.32	12.60 ± 0.89	57.43 ± 0.32	50.2 ± 0.19
1.5	70.1 ± 0.65	11.9 ± 0.17	82.0 ± 0.74	09.40 ± 0.42	68.24 ± 0.18	55.2 ± 0.11

3.3. Acid hydrolysis and detoxification of cotton stalk:

Cotton stalks after pre-treatment at room temperature for a week:

The reducing sugars were 135.02 ± 0.65 g; total sugars were 154.42 ± 0.51 g in 1 liter acid hydrolysate of 200g substrate. The maximum saccharification efficiency was 75.92 ± 0.02 %. Total phenols were 10.6 ± 0.012 mg in litre acid hydrolysate.

Cotton stalks after pre-treatment in autoclave:

The reducing sugars were 118.22 ± 0.15 g; total sugars were 135 ± 0.44 g in 1 litre acid hydrolysate of 200g substrate. The maximum saccharification efficiency was 72 ± 0.04 %. Total phenols were 55.2 ± 0.011 mg in litre acid hydrolysate.

3.4. Fermentation:

Cotton stalks after pre-treatment at room temperature for a week:

Total reducing sugars in 100 ml fermentation medium was 13.5 ± 0.15 g. Maximum ethanol concentrations and reducing sugar consumption was found at 48 h of fermentation. The leftover sugar was 1.26 ± 0.015 g and the consumed sugar was 12.24 ± 0.025 g/100ml fermentation medium. The maximum ethanol concentration produced was 5.75 ± 0.015 % with an ethanol yield of 0.469 ± 0.012 g/g. A fermentation efficiency of 91.78 ± 0.01% was achieved.

Cotton stalks after pre-treatment in autoclave:

Total reducing sugars in 100 ml fermentation medium was 11.82 ± 0.24 g. Maximum ethanol concentrations and reducing sugar consumption rate was found at 48 h of fermentation. The leftover sugar was 3.02 ± 0.012 g and the consumed sugar was 8.8 ± 0.017 g/100ml fermentation medium. The maximum ethanol concentration produced was 4 ± 0.022 % with an ethanol yield of 0.454 ± 0.054 g/g. A fermentation efficiency of 88.84 ± 0.04 % was achieved.

4. DISCUSSION

In the present study, the chemical composition of the cotton stalk was cellulose (44.8%), hemicellulose (13.25%), and lignin (29.6%). The almost similar composition was found in earlier studies from Greece (26), Pakistan (27), and India (28). The hardwoods had similar lignin content (18%-30%) as cotton stalks, whereas the herbaceous plants had lower lignin content (10%-20%) than cotton stalks. The cotton stalks were delignified with different concentrations of NH_3 0, 0.5,1, and 1.5 in an autoclave and at room temperature in our study. Cotton stalks were pre-treated with different concentrations of NaOH ranging from 0 to 10% (w/w, g of NaOH/100 g CS) at 15% (w/v) substrate concentration in autoclave at 121°C /15 psi for 60 min (10). Cotton stalks were soaked for 1 hour in 1L (2%) NaOH solution in 3 flasks and autoclaved at the residence times of 30, 60, and 90 min at constant temperature of 121°C with 15 psi pressure (27). The cotton stalk was subjected to dual-stage sulfuric acid treatment (23). Ammonia pre-treatment at room temperature was found effective in delignification. In this study, the content of reducing sugars in acid hydrolysate was 135.02 ± 0.65 g/L of 200g substrate with the maximum saccharification efficiency of 75.92 ± 0.02 %, the total sugars content was 154.42 ± 0.51 g/L of 200g substrate and phenol content was 10.6 ± 0.012 mg substrate. Maximum values of glucose obtained at enzymatic hydrolysis at different enzyme loads were 9.89g/L to 68.19g/L (29). The highest reducing sugar values were 67.25 ± 1.62 g/L obtained after 72 h of hydrolysis with a saccharification efficiency of 77.39 % (24). The detoxified hydrolysate formed, contained a sugar concentration of 11 g/L, and corresponds to a yield of 0.396 g/g of biomass (23). "The maximum ethanol concentration of 5.75% with the ethanol yield of 0.469 g/g after 48 h of incubation at 30°C with pH 5.5 was achieved. Maximum values of ethanol and ethanol yield according to prehydrolysis time and substrate's concentration ranging 15.95-34.8 and 10.63-17.4" (26). "The ethanol concentration and yield increased at first 48 h and started to decrease after 48 h. The highest ethanol concentration was 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield at 62.2 % reducing sugars consumption rate" (27). "A Peak ethanol concentration of 3.94 g/L (corresponds to a yield of 0.355 g/g of available sugar) was achieved after 36 h of fermentation" as reported by Wendhausen et al. (30). Fermentation efficiency of cotton stalks pre-treated at room temperature and in the autoclave was reported as 91% and 88% respectively. The fermentation efficiency was 55.4% in (NSSF) Non-isothermal Simultaneous Saccharification and Fermentation followed after 14h pre-hydrolysis (26). A fermentation efficiency of 69.53% was reported by Mirza Zaheer Baig and Smita M. Dharmadhikari (23). 78.06% of Saccharification efficiency was reported by K Shahzad et al. (27). Cotton stalks pre-treated at room temperature (5.75%) produced more ethanol than cotton stalks pre-treated in an autoclave (4%) because there is a formation of furfurals at larger amounts in the case of autoclave pre-treated cotton stalks. Traditional methods of pre-treatment at higher temperatures are releasing more furfurals. These furfurals are known to inhibit or reduce fermentation efficiency. So in this connection, it is aimed to perform pre-treatment at ambient room temperature with ammonia. This has yielded high efficiency of delignification, acid hydrolysis, and high ethanol fermentation efficiency.

5. CONCLUSION

Ammonia pre-treatment at room temperature for extended time can a suitable pre-treatment method for lignocellulosic materials in general and cotton stalks in particular for bioethanol production. Ammonia pre-treatment was found to be an effective delignifying agent and ammonia delignified cotton stalk was hydrolysed without much fermentation inhibitors to give more ethanol yield.

COMPETING INTERESTS

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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