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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(10): 1977-1981

www.thepharmajournal.com
Received: 17-08-2023
Accepted: 20-09-2023

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Fermentable sugar production from enzymatic saccharification of alkali pretreated cotton stalks

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Abstract
Some fossil fuels could be replaced by the bioconversion of lignocellulosic biomass into second generation ethanol. From a variety of lignocellulosic biomass, ethanol can be created. An illustration of a lignocellulosic agricultural waste is cotton stem. Ethanol can be produced from a variety of lignocellulosic biomass. Cotton stalk is an example of a lignocellulosic agricultural waste. The enzymatic saccharification of alkali pretreated cotton stalks at varying pretreatment conditions *viz*., temperature, time and alkalinity concentration was investigated. The results indicated that both solids and lignin content were found to be inversely proportional to the severity of the pretreatment conditions. Total sugar released after pretreatment ranged between 268.01-419.51 mg/g with a maximum total sugar content release of 419.51 mg/g at 120 °C with 2% KOH concentration for 1 h, whereas, a minimum of 268.01 mg/g was observed with 1% KOH at 50 °C, 6 h combination. The enzymatic saccharification of untreated and selected pretreated samples was carried out using CTec2® Cellulase enzyme at 8% solid loading rate with 0 to 30% enzyme loading level. Total sugar release peaked when cotton stalks were pretreated at 120 °C with 2% KOH for 1 h and loaded with enzyme loading rate of 30%. The maximum yield of saccharification (518.9 mg/g biomass) was achieved after 72 h incubation, with a saccharification rate of 7.2 mg/g/h. As the enzyme loading level increased, the sugar yield also increased for all the hydrolyzed samples. As the enzyme loading level increased, the carbohydrate conversion percentage also increased for both untreated and pretreated samples with a maximum conversion efficiency of 88.45%.

Keywords: Alkaline pretreatment, cotton stalks, enzymatic saccharification, lignocellulose, potassium hydroxide

1. Introduction
It has been critical to look for alternative energy sources that can take the place of traditional fossil fuels in recent years due to the increased global demand for energy, supply unpredictability, and ever-rising fossil fuel prices that are contributing to greater environmental degradation. Due to the rising need for energy, researchers are looking for affordable, environmentally acceptable, renewable alternative energy sources that can displace fossil fuels. One strategy in this direction is the conversion of plant residues into biofuels, which involves first breaking down and hydrolyzing lignocellulose, the structural framework of plants made up of cellulose, hemicellulose, and lignin, into simple fermentable sugars that, upon fermentation, form biofuels like ethanol. Plant biomass that is made up of cellulose, hemicellulose, and lignin is known as lignocellulosic biomass. The cellulose and hemicellulose carbohydrate polymers are closely linked to the lignin.
The pretreatment procedure aims to lower cellulose's crystallinity, break down lignin and hemicellulose, and increase the porosity of lignocellulosic materials. Following pre-treatment, the cellulose must undergo hydrolysis, which uses a catalyst to transform it into fermentable sugars. Enzymatic catalyzed hydrolysis and acid catalyzed hydrolysis are the two types of hydrolysis. The enzymatic process is the most efficient of these since, at 50 °C, sugar yields could approach 100%. The second phase in the conversion of lignocellulose to ethanol, during which fermentable sugars are created, is the hydrolysis of carbohydrates. Enzymatic hydrolysis is more thoroughly researched than chemical hydrolysis since it is significantly less expensive.

2. Materials and Methods
Pretreatment of cotton stalk samples were performed at 50, 70 with residence times of 6, 12 and 24 h and 120 °C with residence times of 0.25, 0.5 and 1 h each. All the temperature-time pretreatment combinations were performed with potassium hydroxide (KOH) concentrations of 1, 2 and 3 percent (w/v). The process flow chart of pretreatment performed presented in Fig. 1.

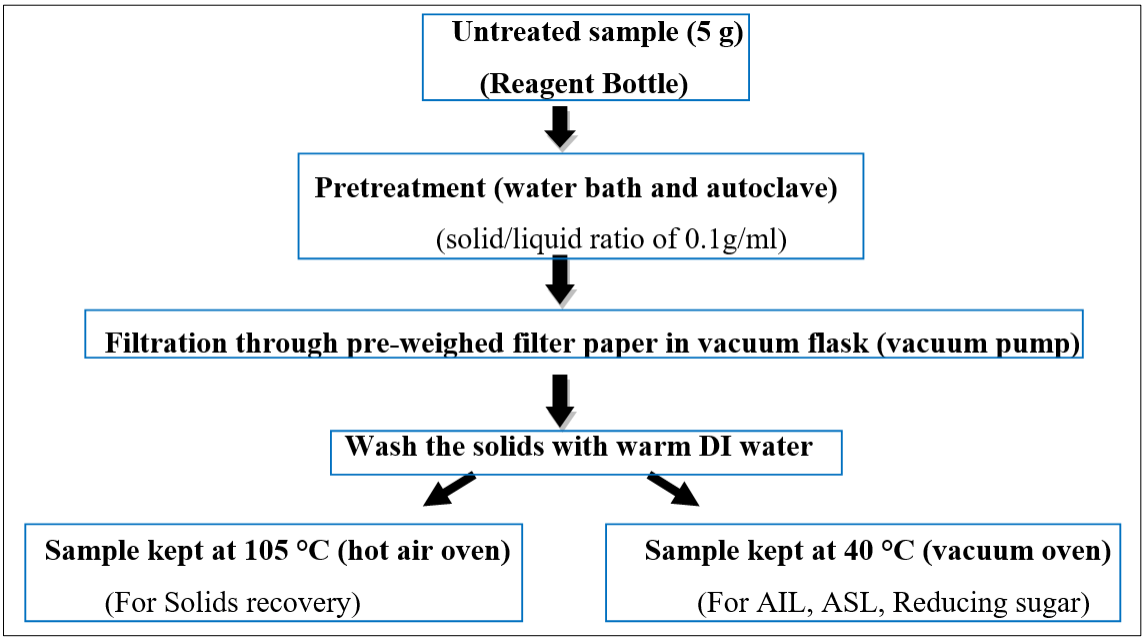

Fig 1: Process flow chart of pretreatment of biomass

Enzymatic hydrolysis of pretreated samples

After pretreatment, 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.

Loading of enzyme for saccharification

The untreated and pretreated samples selected and levels of enzyme loaded for each sample during hydrolysis are given in Table 1. 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 150 rpm (Plate 1).

The Laboratory Analytical Procedure (LAP) adopted by National Renewable Energy Laboratory (NREL) for enzymatic saccharification of lignocellulosic biomass (Selig *et al*., 2008) [17] was followed for conducting enzymatic hydrolysis.

Table 1: Details of samples selected and levels of enzyme loaded during hydrolysis

Sl. No.	Samples selected	Enzyme loading levels (%) (g enzyme protein/g dry biomass)
1	Untreated	0, 15 and 30
2	Pretreated at 50 °C, 24 h, 2% KOH	0, 15 and 30
3	Pretreated at 70 °C, 24 h, 2% KOH	0, 15 and 30
4	Pretreated at 120 °C, 1 h, 2% KOH	0, 15 and 30

The samples were withdrawn at regular intervals of 12 h and centrifuged at 4,000 rpm for 10 min in a high speed refrigerated centrifuge (Plate 2), and the filtrate was collected for sugar analysis.

The fermentable sugars generated during the hydrolysis were estimated by 3, 5-dinitrosalycylic (DNS) acid method. The saccharification rate at regular intervals was calculated using the formula as given below;

$$\text{Saccharification rate (mg/g/h)} = \frac{\text{Sugar yield (mg/g dry biomass)}}{\text{Saccharification time (h)}} \quad \ldots (1)$$

Further, carbohydrate conversion was calculated using the following formula (Gupta *et al*., 2009) [18];

$$\text{Carbohydrate conversion (\%)} = \frac{\text{Reducing sugar concentration obtained}}{\text{Potential sugar concentration in the substrate}} \times 100 \quad \ldots (2)$$

Plate 1: Enzymatic hydrolysis of samples loaded with CTec2 Cellulase enzyme in water bath incubator shaker

Plate 2: Enzymatic hydrolyzed samples kept for centrifugation in high speed refrigerated centrifuge

3. Results and Discussion

The pretreatment at 120 °C for 1 h with 2% KOH concentration was chosen as the optimum as it had highest desirability of 64.9%. However, in order to examine the enzyme loading levels on samples pretreated 50 and 70 °C for fermentable sugar production, other two pretreatment conditions *viz.*, 2% KOH, 24 h at 70 °C having desirability of 63.0% and 2% KOH, 24 h at 50 °C with 58.8% desirability were also selected for further enzymatic hydrolysis.

Effect of enzyme loading levels on saccharification of cotton stalk

Hydrolysis was carried out to examine the effect of enzyme loading levels (0, 15 and 30% g enzyme protein/g dry biomass) on the untreated sample and selected pretreated samples for fermentable sugar production. The enzyme Cellulase *CTec2* sponsored by Novozymes, China was used for the hydrolysis.

The enzymes were loaded and hydrolysis was carried out in a shaking water bath at

50 °C at 150 rpm for 72 h. The samples were drawn at regular intervals (12 h) and analyzed for the fermentable sugars produced. The results of saccharification of cotton stalk**,** total sugar yields and carbohydrate conversion are presented below.

Saccharification profile during hydrolysis

Table 2 displays the results of sugar yields of untreated sample hydrolyzed with 0, 15, and 30% enzyme loadings spaced periodically. The untreated sample had a sugar yield that ranged from 8.1 to 337.29 mg/g dry biomass. At the conclusion of 72 hours of hydrolysis, a maximum sugar yield of 337.29 mg/g of biomass was obtained with 30% enzyme loading. After 12 hours, it was at its lowest (8.1 mg/g) at 0%, or without enzyme loading.

From 7.6 to 498.5 mg/g dry biomass of fermentable sugars were produced during the hydrolysis of the sample after pretreatment with 2% KOH at 50 °C for 24 hours. At the end of 72 hours of hydrolysis, it was discovered that the sugar yield was at its highest level (498.5 mg/g biomass) with 30% enzyme loading. In contrast, after 12 hours without enzyme loading, a minimum sugar yield of 7.6 mg/g of biomass was obtained (Table 3). The sugar yield increased as the hydrolysis time lengthened, and this increase in sugar yield was observed as the level of enzyme loading increased.

The sugar yield of the sample ranged from 8 to 510.4 mg/g dry biomass with different enzyme loadings. A maximum sugar yield of 510.4 mg/g biomass was recorded for 30% enzyme loading at the end of hydrolysis, while it was minimum (8 mg/g) at 12 h of hydrolysis without enzyme loading (Table 4).

The sugar yield in the hydrolyzate of the sample pretreated at 120 °C for 1 h, 2% KOH varied from 10.3 to 518.9 mg/g dry biomass. A maximum sugar yield of 518.9 mg/g biomass was obtained with 30% enzyme loading at the end of 72 h of hydrolysis. While, it was minimum (10.3 mg/g biomass) at 0% *i.e.*, without enzyme loading after 12 h (Table 5). As the enzyme loading level increased, the sugar yield also increased at different intervals. The increased sugar yield was observed with the prolonged hydrolysis time for all the enzyme loading levels.

Total sugar yield

After untreated and pretreated samples were hydrolyzed, the total sugar yield ranged from 29.3 to 518.9 mg/g biomass. The hydrolyzate obtained from the sample pretreated at optimal conditions (120 °C for 1 h with 2% KOH and loaded with 30% enzyme) showed the highest total sugar yield of 518.9 g/g biomass. The hydrolyzate of the sample that had been pretreated at 50 °C for 24 hours with 2% KOH without enzyme loading, however, had the lowest concentration (29.3 g/g biomass) (Table 6). The total sugar yield increased for each sample as the level of enzyme loading increased. This pattern was seen across all hydrolyzed sample sets.

Table 2: Sugar yields of untreated sample hydrolyzed with different enzyme loading levels

Time, h	Sugar yield (mg/g biomass)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
12	8.1	91.3	176.92
24	14.3	149.2	269.91
36	29.7	179.3	316.11
48	37.6	202.6	328.63
60	41.2	216.2	332.37
72	45.7`	221.5	337.29

Table 3: Sugar yields of pretreated sample (50 °C, 24 h, 2% KOH) hydrolyzed with different enzyme loading levels

Time, h	Sugar yield (mg/g biomass)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
12	7.6	126.9	248.3
24	12.8	248.7	338.2
36	19.7	299.6	389.8
48	22.4	329.8	429.6
60	26.1	348.4	466.4
72	29.3	356.6	498.5

Table 4: Sugar yields of pretreated sample (70 °C, 24 h, 2% KOH) hydrolyzed with different enzyme loading levels

Time, h	Sugar yield (mg/g biomass)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
12	8.00	168.5	266.8
24	14.7	271.6	373.6
36	24.5	335.5	402.4
48	29.8	359.3	458.8
60	31.2	369.4	491.3
72	32.9	378.2	510.4

Table 5: Sugar yields of pretreated sample (120 °C, 1 h, 2% KOH) hydrolyzed with different enzyme loading levels

Time, h	Sugar yield (mg/g biomass)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
12	10.3	185.6	318.9
24	19. 8	296.6	389.6
36	26.1	357.7	434.7
48	31.5	401.3	499.0
60	36.2	413.2	503.4
72	40.1	429.7	518.9

In Table 7, the total sugar yield from hydrolysis is shown after statistical analysis. The sugar yields from the hydrolyzed samples were found to differ significantly from one another. All of the samples hydrolyzed at 1% level produced significant sugar yield changes as a result of enzyme loading. Significant was also the result of the factors' combined effect.

Table 6: Total sugar yields of untreated and pretreated samples hydrolyzed with different enzyme loadings for 72 h

Pretreatment	Total sugar yield (mg/g biomass)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
Untreated	45.7	221.5	337.29
50 °C, 24 h, 2% KOH	29.3	356.6	498.5
70 °C, 24 h, 2% KOH	32.9	378.2	510.4
120 °C, 1 h, 2% KOH	40.1	429.7	518.9

Table 7: Analysis of variance for total sugar yield

Source	Sum of squares	df	Mean square	F value	p-value
Model	1305321	11	118665.5	2787.2	< 0.0001
Enzyme loading (A)	1167851	2	583925.5	13715.3	< 0.0001
Samples (B)	85095.2	3	28365	666.2	< 0.0001
AB	52374.8	6	8729.1	205	< 0.0001
Pure error	1021.8	24	42.6	-	-
Cor total	1306342.9	35	-	-	-

SD =0.98, Mean = 304.85, CV = 1.09%, R^2 = 0.99

Carbohydrate conversion

The range of enzyme loading levels used to hydrolyze different samples with varying percentages of carbohydrate conversion was 4.99 to 88.45. The sample pretreated under ideal circumstances (120 °C, 1 hour, 2% KOH), which was loaded with 30% enzyme, produced the highest amount of carbohydrate conversion (88.45%). The sample that was pretreated with 2% KOH at 50 °C for 24 hours saw the least amount of carbohydrate conversion (4.9%) even without adding any enzymes.

The percentage of carbohydrate conversion increased for both untreated and pretreated samples as the enzyme loading level increased. However, compared to all the pretreated samples with 0% enzyme loading, the untreated sample without enzyme loading recorded a higher carbohydrate conversion.

It was discovered that the percent carbohydrate conversions obtained from the hydrolyzed samples varied significantly. At the 1% level of significance, the enzyme loading had a significant impact on the percentage of carbohydrates converted from all the hydrolyzed samples. Table 8 shows that the combined effect of the factors was also significant.

Table 8: Carbohydrate conversions of untreated and pretreated samples hydrolyzed with different enzyme loadings for 72 h

Pretreatment	Carbohydrate conversion (%)		
	Enzyme loading, % (g enzyme protein/g biomass)		
	0	15	30
Untreated	7.79	37.76	57.49
50 °C, 24 h, 2% KOH	4.99	60.79	84.98
70 °C, 24 h, 2% KOH	5.61	64.47	87.01
120 °C, 1 h, 2% KOH	6.83	73.25	88.45

Table 9: Analysis of variance for carbohydrate conversion

Source	Sum of squares	df	Mean square	F value	p-value
Model	38149.51	11	3468.14	351.58	< 0.0001
Enzyme loading (A)	34144.93	2	17072.46	1730.69	< 0.0001
Samples (B)	2477.90	3	825.96	83.73	< 0.0001
AB	1526.68	6	254.45	25.79	< 0.0001
Pure error	236.75	24	9.86	-	-
Cor total	38386.25	35	-	-	-

SD = 0.67 Mean = 48.29 CV = 1.41% R^2 = 0.99

Conclusions

Following the harvest of cotton blows, cotton stalk is a byproduct of the cotton crop. Bioethanol could be produced from cotton stalks. The percentage of carbohydrates converted increased for both untreated and pretreated samples as the enzyme loading level increased. Separate hydrolysis and fermentation processes may be more effective in enhancing the production of ethanol from cellulosic hydrolyzates.

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