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Optimisation of Pre-treatment and Enzymatic Hydrolysis of Cotton Stalk

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Cotton stalk is good source of lignocellulosic biomass which can be utilized for production of alternative energy source like bioethanol. The first step in conversion of lignocellulosic biomass to ethanol is pre-treatment, which exposes more amount of cellulose by removal of lignin covering. The aim behind this research work is to evaluate the cotton stalk as feed stock for bioethanol production. The work is focus on two step process including optimisation of NaOH concentration and time period during pre-treatment for maximum lignin removal and optimisation of concentration of enzyme during enzyme hydrolysis. In first stage optimisation of pre-treatment has been carried out by different concentration of NaOH (0.5%, 1%, 1.5%, 2%) for different time period (30, 60 and90 min) at constant temperature of 121°C and enzyme hydrolysis was carried out by using 40 CMCase (carboxymethyl cellulases) unit of enzyme per gram of substrate.

In the second stage optimisation of enzyme concentration has been studied, by ranged it from 20 CMCase unit of enzyme per gram of substrate to 140 CMCase unit of enzyme per gram of substrate with fixed solid liquid ration of 1:20 (cotton stalk: acetate buffer) at 50°C for 72 hours. The result of this study showed that 2% NaOH treatment, for 60 min at 121°C was found to be optimum procedure for pre-treatment and 100 CMCase unit of enzyme was optimum concentration for maximum sugar yield (.0.49 gram per gram of cotton stalk used) and lignin reduction (0.201g/g of biomass).

Key words: Cotton stalk, Bioethanol, Lignocellulosic material, Pre-treatment, Enzymatic hydrolysis.

Increasing demand and depletion in reservoirs of fossil fuel divert the concentration of research towards alternative energy sources which must be renewable and environmental friendly. Biomass which includes animal and human waste, yard waste, wood product, grasses and agricultural residues such as wheat straw, corn stover, rice straw and cotton stalk etc. are renewable resources that store energy from sunlight in its chemical bond[1]. These agricultural lignocellulosic wastes can be used for the production of bioethanol.

The first step in bioconversion of lignocellulosic biomass to bioethanol is size reduction and pre-treatment[2]. Physical pre-treatment will increase the accessible surface area and size of pores, and decrease the crystallinity and degrees of polymerization of cellulose[3].

Alkaline pre-treatment refers to the application of alkaline solution to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of enzyme

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to the hemicellulose and cellulose[4]. Generally, alkaline pre-treatment is more effective on agricultural residues and herbaceous crop than on wood materials. During alkaline pre-treatment the first reaction taking place are salvation and saphonication. This causes a swollen state of the biomass and makes it more accessible for enzymes and bacteria[5].

Indian economy is agricultural based economy. Cotton is one of the most abundant crops in India with annual residues generation is 18.9 million metric tons[6]. Decomposition of cotton stalk left after harvesting is big problem and other than as substrate of paper industries, use of this potential lignocellulosic material has not takes place properly, mostly these cotton stalks are burned on the field soon after harvesting. Conversion of these feed stocks in to bioethanol can provide an environmental friendly disposal method and achievement of one alternative energy source.

Technologies for biomass to ethanol conversion are also under various stages of development including pre-treatment, hydrolysis and fermentation. The objective of this research work is optimisation of pre-treatment with different concentration of NaOH solution with different time periods followed by enzyme concentration during enzyme hydrolysis.

MATERIALS AND METHODS

Physical Pre-treatment

The cotton stalks were collected from nearby field at Government Institute of Science in Marathwada region. The stalk were shredded and bailed in the field soon after the cotton was picked. These stalks were chopped, over dried and ground to pass 1 mm sieve in laboratory mill. Dried sample were stored in sealed plastic bags at room temp until use.

Compositional analysis of cotton stalk

The composition of cotton stalk like cellulose, hemicellulose and lignin were analysed by using standard NREL (National Renewable Energy Laboratory) protocol.

Chemical pre-treatment

Chemical pre-treatment comprises mainly on delignification process which have been carried out by using different concentration of NaOH solution at different temperature.

Different concentration of NaOH (0.5%, 1%, 1.5%, 2 %) and steam treatment for different time (30, 60 and 90 min) were carried out at 121°C with fixed solid: liquid ratio of 1:10(cotton stalk: NaOH solution) for optimisation purpose. After pre-treatment, the pre-treated biomass has been separated from lignified liquor by centrifugation and liquor was collected for detection of lignin removal.

Enzymatic hydrolysis

Enzymatic hydrolysis of biomass was carried out by using commercial cellulases purchased from Sisco Research Laboratories Pvt. Ltd. Mumbai, India). Initial optimisation of pre treatment was carried out by least amount of enzyme i.e. 40 CMCase (carboxymethyl cellulases) [7] unit of enzyme per gram and finally enzyme concentration was also optimised by taking different concentrations of enzymes per gram of biomass.

Pre treated cotton stalk were incubated with 5% solid loading in 50mM acetate buffer (pH 4.8) with respective concentration of enzymes and was incubated at 50°C and 150 rpm for 72hours.

After incubation time the sample was centrifuged in chilled condition at 5000 rpm for 10 min[5]. Supernatant was collected for detection of fermentable sugar.

Analytical methods

Total Fermentable sugars

After appropriate dilutions the solubilisation of fermentable sugar were determine by DNS (3, 5-dinitrosalicylic acid) method of Miller[8].

Phenolic compounds (Lignin)

The phenolic estimation was carried out by folin ciocalteus methods of Singleton and Rossi[9].

Statistical analysis

An analysis of variance (ANOVA) of fermentable sugar yield was performed for each individual experiments mention above using the statistical analysis system.

RESULTS AND DISCUSSION

Cellulose, hemicellulose and lignin are the main components of plant cell wall. The function of lignin is to cross linking with cellulose and hemicellulose fibres. Reducing the lignin content of biomass helps to expose cellulose and facilitates substrate access by hydrolytic enzymes[10] but the

concentration of this phenolic polymer (lignin) varied from plant to plant. Cotton stalk (*Gossypium hirsutum*) is also good source of lignocellulose biomass. Silverstein (2007) reported that lignin content of cotton stalk is higher (30%) than other agricultural feed stock such as corn stover, wheat straw, switch grass and even softwood.

Compositional analysis of cotton stalk

The major chemical composition of cotton stalk is cellulose, hemicellulose and lignin but their concentration varied, depending on growing locations, seasons, harvesting methods as well as analysis procedure[11]. The stalk used in this study was debarked for the purpose to increase the concentration of cellulose and minimizing the concentration of lignin reported that 40% to 55% lignin is present in bark of soft wood[12]. In the present study compositional analysis shows that debarked cotton stalk contain 57% holocellulose, in which 40.60%was alpha cellulose and 17.24% was hemi cellulose. The lignin content was 24.18%.

Optimisation of pre-treatment

Pre-treatment was carried out by taking various concentration of sodium hydroxide such as 0.5%, 1%, 1.5% and 2.0%. Along with different concentration of NaOH, time period of steam explosion has also been optimised by varying from 30 min to 90 min as shown in table 1.

The main effect of sodium hydroxide pre-treatment on lignocellulosic biomass is delignification by breaking the ester bonds, cross linking lignin and xylan, thus increasing the porosity of the biomass[5] which helps in increasing the surface area for enzyme action[13]. Starting from 0.5% NaOH concentration and steam explosion for 30, 60 and 90min the solubilisation of fermentable sugars ranged from 0.12g to 0.19g per gram of biomass. Similarly the concentration of sugars was continuously increased with increased in concentration of alkali treatment and finally the maximum solubilisation of sugar (i.e. 0.37gram per gram of cotton stalk) was obtained by using 2% NaOH treatment concentration. Yield of sugar was not considerably increased by using more than2% NaOH treatment (data not shown) . For optimisation of pre-treatment, constant amount of enzyme has-been exposed i.e. 40 CMC units per gram of biomass in solid liquid ratio of 1:20(cotton stalk: acetate buffer),it was found that 2% NaOH treatment with 60 min steam explosion at 121°C was optimum procedure for maximum sugar yield.

Table 1. Effect of NaOH concentration and time period on sugar yield (g/g of biomass)

NaOH	Time of steam treatment at 121°C		
concentration	30 min	60 min	90 min
0.5%	0.12	0.15	0.19
1.0%	0.18	0.19	0.28
1.5%	0.27	0.29	0.32
2.0%	0.31	0.37	0.37

Table 2. Effect of NaOH concentration and time period on lignin reduction (g/g of biomass)

NaOH	Time of steam treatment at 121°C		
concentration	30 min	60 min	90 min
0.5%	0.133	0.184	0.1189
1.0%	0.155	0.188	0.192
1.5%	0.178	0.192	0.199
2.0%	0.189	0.201	0.204

Table 3. Optimisation of Enzyme concentration

Concentration of Enzyme in CMCase unit	sugar/g of cotton stalk
20 units	0.31
40 units	0.37
60 units	0.39
80 units	0.42
100 units	0.49
120 units	0.48
140 units	0.49

Table 4. Analysis of variance (ANOVA) of sugar yield

Source Term	NaOH conc.	Time of pre-treatment	Interactions
SEm±	0.013	0.011	0.023
C.D. at 5%	0.039	0.023	0.068(NS)

NS-non significant

The percentage of lignin solubilisation in alkali solution after pre-treatment was presented in Table 2.

The data showed that lignin reduction was varied from 0.133g/g of biomass to 0.189/g of biomass by treating it with 0.5% NaOH solution from 30 min. to 90 min. and these reduction was continuously increased up to 2% NaOH treatment, which ranged from 0.189g/g (after 30 min), 0.201g/g of biomass (after 60 min) and 0.204g/g of biomass (after 90 min) at 121°C. The described data was also supported by Silverstein *et al.,* (2007).

Optimisation of enzyme concentration

After pre-treatment optimisation the enzyme concentration was carried out by using different concentration of enzymes ranged from 20 CMCase units per gram to 140 CMCase units of enzyme per gram of biomass as shown in Table 3. From the data it was shown that, by increasing the concentration of enzyme in hydrolyzates, sugar yield was increase up to 100 CMCase unit (0.49gram per gram of biomass) there after it comes in stationary phase and likely to became as it is and from the data it was found that 100 CMCase unit of enzyme per gram of biomass was optimum concentration for maximum sugar yield.

Statistical analysis

Effect of NaOH concentration and time

Results presented in table 1 elaborate the effect of pre treatment by different concentration of NaOH and time required for pre-treatment at 121°C. Significant increased in the yield of fermentable sugar was recorded with each unit increased in NaOH concentration from 0.5% to 2 % at 30, 60 and 90 min., the highest value of fermentable sugar (0.37g/g of biomass) was noted with 2 per cent NaOH at 60 min. as well as 90 min. and these results were statistically at par with each other. The time require for pre-treatment has also showed its impact on sugar released pattern but 60 min time was found statistically optimum for pre-treatment at 121°C. The interactions between NaOH concentrations and time of pre-treatment was found statistically non significant as shown in Table 4. From the data it can be inferred that 2 % NaOH concentration and 60 min time for pre-treatment might be optimum parameters for better sugar yield.

Effect of enzyme concentration

Effect of enzyme concentration on optimized parameter of pre-treatment (NaOH concentration and time) on released of fermentable sugar from cotton stalk narrated in Table 2.

Scrutiny of the data indicates linear increased in sugar yield with enzyme concentration from 20 CMCase units to 100 CMCase units and 100 CMCase unit enzyme concentrations yielded significantly more sugar (0.49 gram per gram of biomass) over other enzyme concentrations tried. However 120 and 140 CMCase units of enzymes were found statistically at par with 100CMCase unit enzyme concentration.

From the results immerged out 100 CMCase units enzyme concentration can be considered as optimum concentration for maximum sugar yield from cotton stalk.

CONCLUSION

The optimisation of pre-treatment and concentration of enzyme during enzyme hydrolysis has been investigated in two stages. In first stage, the effectiveness of different concentration of NaOH (0.5%, 1%, 1.5%, and 2 %) for different time period (30, 60 and 90 min) at constant temperature of 121°C has been carried out and different concentration of enzyme has optimised in second stage. The result of this study showed that 2% NaOH treatment, for 60 min at 121°C was found to be optimum pre-treatment procedure for maximum delignification and 100 CMCase unit of enzyme (per gram of biomass) during enzyme hydrolysis was optimum concentration for maximum sugar yield.

This study can serve as one step towards successful hydrolysis of cotton stalk for ethanol production. In addition more research efforts are require to optimising the procedure (including optimisation of temperature during pre-treatment and enzyme hydrolysis) for increasing the yield of fermentable sugar and decreasing concentration of by products to make the process more effective for bioethanol production.

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