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Bioethanol Production from Enzymatically Hydrolysed Cotton Stalk: One Approach Towards Sustainable Energy Development

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ABSTRACT:

The lignocellulosic nature of cotton stalk favours to use as renewable material for variety of commercial applications. Present study was evaluating the potential of cotton stalk for bioethanol production. In this regards cotton stalk were subjected to series of treatment including pretreatment, hydrolysis and fermentation. The resultant data shows that physically pre-treated cotton stalk when subjected to 2% alkaline solution at 121°C for 60 minute followed by enzyme hydrolysis with 100 CMC units of enzyme releases sugar of 0.49 g/g and 24.5 g/L of biomass.Furthermore when it goes to fermentation using co culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*,givesan ethanol concentration of 9.56 g/L which corresponds to a yield of 0.191 g/g of biomass, 0.298 g/g of holocelluloses and 0.392 g/g of fermentable sugars while fermentation and sugar consumption efficiencies were recorded as 76.85% and 97.81% respectively.

Key words: Cotton stalk, Bioethanol, Pretreatment, Hydrolysis, Fermentation.

INTRODUCTION

Worldwide increasing energy demand and decreasing fossil reservoir led to the resurgence in development of alternative fuel, which must be renewable and environmental friendly. Unlike fossil fuel, ethanol is renewable energy source produced through fermentation of sugar. Ethanol can be produced from variety of biomass and among various biomasses; lignocellulosic biomass source is plentiful and economical resource that can serve as source of ethanol production at large scale[1]. Lignocellulosic biomass sources include: agricultural wastes, industrial wastes, forestry wastes and municipal solid wastes, etc[2]. Cotton stalk which are left behind after the cotton harvest, is one of the example of a lignocellulosic agricultural waste. There are about 32 million hectares of cotton cultivable area across the world and about 10 million hectares in the country[3]. Since cotton stalk is a by-product of cotton crop; India has an abundance of this lignocellulosic biomass source. The objective of present study is to evaluate the potential of ethanol production from alkali pre-treated and enzymatically hydrolysed cotton stalk by suing co culture of *Saccharomy cescerevisiae* and *Pachysolen tannophilus*.

MATERIALS AND METHODS

Collection of raw material

The cotton (*Gossypiumhir sutum* NHH44) stalkused in this research work was harvested material from the farmer's field of Marathwada region.

Physical pretreatment

The cotton stalk which consist of different unwanted residues were removed mechanically by shredding followed by sundried, debarked, bailed and ground to 1mm particle size with laboratory blender and stored in tightly sealed plastic bags.

Compositional analysis

A major portion of biomass feedstock is made up of carbohydrates, which are polysaccharide in nature. These carbohydrate sub units were quantified by HPLC (Zodiac. Ltd) using laboratory analytical proceure-002(LAP-002), standard protocol of NREL (National Renewable Energy Laboratory)[4]. The lignin was also determined as per NREL procedure.

Alkaline pretreatment and enzyme hydrolysis

Alkaline pretreatment and enzyme hydrolysis was carried out by following the guideline from previous research studies[5].

Alkaline pretreatment

Alkaline pretreatment was performed by treating 2% NaOH at substrate loading of 10% (w/v) and the flask were sterilized for 60 minutes at 121°C. After pretreatment the biomass has been separated from lignified liquor by centrifugation at 10000 rpm for 10 minutes and supernatant (black liquor) was separately collected from each sample for quantitative detection of lignin. The delignified biomass was repeatedly washed with distilled water till to become neutral pH and dried in oven at 60°C and was stored for further studies[6].

Enzyme hydrolysis

Enzymatic hydrolysis of pre-treated biomass was carried out using commercial cellulases purchased from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. Pre-treated cotton stalk was incubated with 5% solid loading in 50mM acetate buffer (pH 4.8) with 100 CMC (Caroxy methyl cellulose) unit of enzyme and was incubated at 50°C with 150 rpm for 72 hours. After incubation, the sample was centrifuged in chilled condition at 5000 rpm for 10 minutes and supernatant was collected as fermentation sugar.

Fermentation of enzyme hydrolysate of cotton stalk

Fermentation of detoxified hydrolyzate of cotton stalk was carried out by using *Saccharomyces cerevisiae* MTCC 36 and *Pachysolen tannophilus* MTCC 1077 purchased from Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Lyophilized culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* were activated separately on yeast and malt extract (YM) medium. The medium was prepared by adding 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose in distilled water. pH of the medium was adjusted to 6.5[7]. The yeast cells were allowed to grow aerobically at 30°C on rotary shaker incubator with 120 rpm for 48 hours or till the culture partially covered the bottom of flask. Completely activated yeast cells were actively transferred to YM agar plates and allowed to grow at 30°C for 48 hours and purity was checked microscopically from isolated colonies.

Cell mass for inoculum development

Biomass require for batch fermentation was obtained by growth of yeast cell on YM medium in Erlenmeyer flask and was sterilized at 110°C for 40 minutes. The flasks were cooled and cells from slants were aseptically transferred into the flask and were allowed to grow aerobically on rotary shaker incubator with 120 rpm at 30°C for 48 hours. After incubation, completely activated yeast cells were harvested by centrifugation at 4000 rpm at 4°C for 10 minutes and repeatedly washed with distilled water and used as cell mass for inoculum development.

Inoculum preparation

Inoculum was prepared in detoxified hydrolyzate solution of cotton stalk supplemented with 0.5% yeast extract, 1% peptone and pH was 5.5%. The yeast cells which were harvested by centrifugation were added in inoculum and incubated on rotary shaker incubator with 150 rpm at 30°C for 24 hours[8] and grown aerobically to promote healthy growth of yeast cells in hydrolyzate and used as inoculum for fermentation. The volume of inoculum again set to 10% to the total volume used for fermentation.

For quantifying the cell mass, One millilitre aliquot from each suspension was taken to performed serial dilution up to 10^5 and 100 µL of diluted culture was spread-plated on to YM agar plates by adding 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2.5% agar and were incubated at 30°C for 48 hours and yeast colonies were counted to ensure that each time the inoculation stayed at

approximately 6.0×10^7cfu/mL corresponding to 10g dry weight/Litre.

Ethanol productions from enzymatic hydrolysate of cotton stalk

The filtrated product obtained from enzymatic hydrolysis of cotton stalk was used as sole carbon source for fermentation and was supplemented with 0.1% (w/v) yeast extract, peptone, NH_4Cl, KH_2PO_4 and 0.05% (w/v) of $MgSO_4.7H_2O$, $MnSO_4$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$ and $ZnSO_4$ in 250 mL Erlenmeyer flasks. Fermentations were performed in semi aerobic mode (250 mL Erlenmeyer flask containing 150 mL of fermentation medium) having pH 5.5 and sterilized at 110°C for 20 minutes[9]. The flasks were inoculated with 10% co-culture of *Saccharomyces cerevisiae* and *Pachysolentan nophilus* at concentration of 6% and 4% respectively. The flasks were sealed with aluminium foil and incubated on rotary shaker with 120 rpm for first 24 hours and then kept in static mode at 30°C for 96 hours. Sample was removed from each flask at one time at the interval of 12 hours and analysed for ethanol, residual sugar and cell biomass concentration.

Determination of ethanol, residual sugars and cell growth

Sample obtained during fermentation was transferred to pre weighted centrifuged tube and were centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and analysed for concentration of ethanol and residual sugars in broth while pellet was repeatedly washed with distilled water and dry in hot air oven at 60°C till constant weight[10].

Analytical method

Analytical tools and methods applied to conduct the study are as follows.

Total reducing sugar

After appropriate dilution the solubilisation of fermentable sugars were determined by DNS (3, 5-dinitrosalicyclic acid) method of Miller[11].

Ethanol estimation by Gas Chromatography

After each experiment, part of supernatant was filtered by 0.22 µm cellulose acetate filter and analyzed by Gas Chromatography (Shimadzu Japan). All analysis was carried out according to NREL (National Renewable Energy Laboratory) procedure LAP # 011 using ZB-Wax column (30mm × 0.25mm) with Flame Ionization Detector[12].

Statistical analysis

Statistical analysis were carried out in factorial completely randomized design (CRD) by software MAUSTAT developed by department of statistics ofVasantraoNaikMarathwada Agriculture University, Parbhani, Maharashtra, India.

Table 1: Ethanol production from enzymatically hydrolysed cotton stalk by using co-culture of *Saccharomyces cerevisiae*and *Pachysolentannophilus*

Time period (hr)	Ethanol conc. (g/L)	Ethanol yield (g/g)			Fermentation efficiency (%)	Sugar consumed (%)	Cell mass conc (g/L)
		Biomass	Holocellulose	Fermentable sugar			
06	00	000	000	000	00.00	18.56	02.42
12	0.86	0.017	0.027	0.035	06.91	41.29	05.38
24	2.61	0.052	0.081	0.107	20.98	78.72	11.79
36	4.29	0.086	0.134	0.176	34.49	97.29	12.14
48	9.56	0.191	0.298	0.392	76.85	97.81	12.20
60	9.54	0.190	0.296	0.391	76.69	97.88	12.24
72	9.28	0.186	0.290	0.380	74.60	97.92	12.25
84	9.07	0.181	0.283	0.371	72.92	98.06	12.29
96	9.04	0.180	0.281	0.370	72.67	98.11	12.31
SEm+	0.654	0.013	0.020	0.024	04.77	0.067	0.759
CDat 5%	1.941	0.039	0.060	0.072	14.28	1.978	2.252

RESULTS AND DISCUSSIONS

Compositional analysis of cotton stalk

The major chemical composition of cotton stalk is cellulose, hemicellulose and lignin but their concentration varied depending on growing location, harvesting methods as well as analysis procedure[13].

Cotton stalk (*Gossypium hirsutum*) used in this study was composed of 42% glycan and 22% xylan while other ingredient of hemicellulose was in very small proportion. The lignin content was 24.18%. Our results are harmony with the results reported earlier[6, 14, 15].

Alkaline pretreatment and enzyme hydrolysis

Alkaline pretreatment significantly delignified cotton stalk and increases the sugar concentration in residual pre-treated biomass. Cellulases can provide huge benefits inutilization of biomass in long term because of the possible high glucose yields and opportunity to apply the modern tools of biotechnology to reduce cost[16]. The result reported that when cotton stalk powder at substrate loading of 10% (w/v) was subjected to 2% NaOH at 121°C steam explosion in steam sterilizer for 60 minutes was significantly removed lignin of 0.201 g/g of biomass and when this delignified biomass was subjected to enzyme hydrolysis by incubating with 5% solid loading in 50mM acetate buffer (pH 4.8) and exposing 100 CMC (Carboxy Methyl Cellulose) unit of enzyme concentration(per gram of biomass) at 50°C with 150 rpm for 72 hours, yielded 0.49 gram of fermentable sugar per gram of biomass corresponds to the concentration of 24.5 g/L [5]. Finally the obtained enzyme hydrolyzate of cotton stalk was used as sole carbon source for ethanol production.

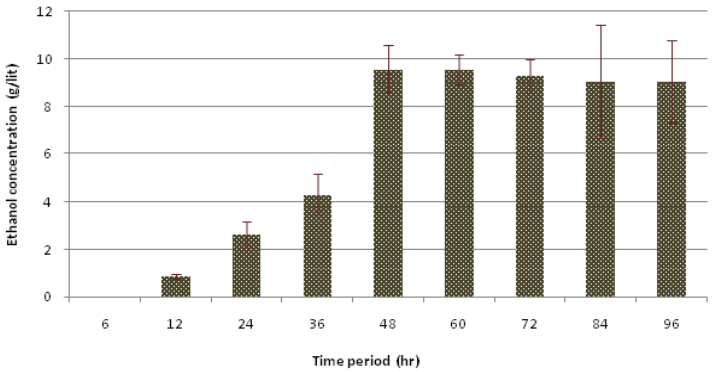

Fig.1: Effect of time on ethanol fermentation from enzymatically hydrolysed cotton stalk by co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

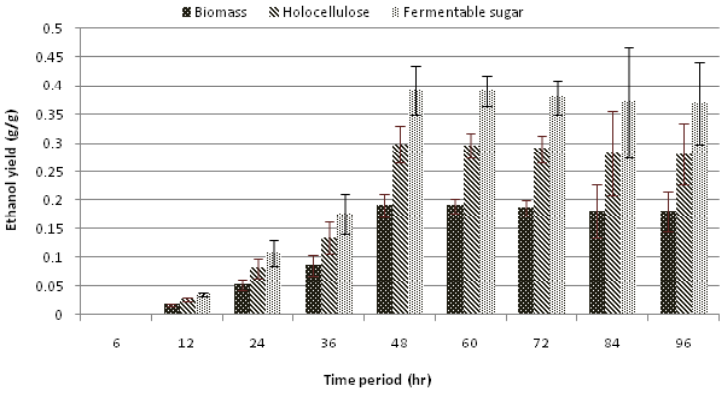

Fig.2: Effect of time on ethanol yield during ethanol fermentation from enzymatically hydrolysed cotton stalk by co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

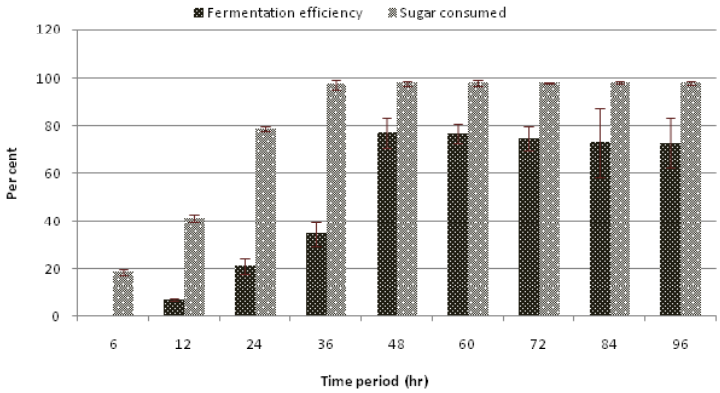

Fig.3: Effect of time on fermentation and sugar consumption efficiencies during ethanolfermentation from enzymatically hydrolysed cotton stalk by co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

Fermentation of enzyme hydrolysate of cotton stalk by co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

The sugar solution obtained from optimized alkaline pre-treated and enzymatically hydrolysed cotton stalk were fermented for analysing the potential of bioethanol production through fixed parameters and outcomes of all experimental setup were evaluated by using three analytical parameters simultaneously including ethanol concentration in the fermentation broth, substrate utilization and growth of cell mass. The reliability of results was checked statistically by passing through ANOVA (analysis of variance). The setup was conducted at 30°C, using 250 mL Erlenmeyer flasks containing 150 mL of fermentation broth (semi aerobic nature) loaded with cotton stalk hydrolysate as sole carbon source having sugar concentration of 24.5 g/L. The fermentation was started with addition of 10% inoculum (6% *Saccharomyces cerevisiae* and 4% *Pachysolentan nophilus*) and were agitated for first 24 hours and then kept in static mode up to 96 hours. Samples were withdrawn from 06 hours onwards followed by at every 12 hours interval of time from separate flask and were analysed.

Resultant data obtained after statistical analysis is presented in Table 1. indicates that, ethanol was not detected in first 6 hours of incubation while sugar consumption and cell mass concentrationgot started at the rate of 18.56% and 2.42 g/L respectively. As for as ethanol production is concern, it commence from 12 hours onwards which gives 0.86 g/L as shown in Fig 1. which corresponds to an yield of 0.017 g/g of native cotton stalk, 0.27 g/g of holocelluloses and 0.35 g/g of fermentable sugar and continuously increases up to 48 hours of incubation and finally maximum ethanol production was recorded at 48 hours which produces 9.56 g/L corresponds to yield 0.191 g/g of biomass, 0.298 g/g of holocelluloses and 0.392 g/g of fermentable sugar, beyond which the ethanol concentration remained constant and show slight fall mainly due to feedback inhibition or catabolic repression. The fermentation efficiency at 48 hours of incubation was recorded as 76.85% while more than 97% sugars of hydrolysate were effectively consumed by yeast cultures as shown in Fig 3. Simultaneously cell mass concentration was also increased up to 36 hours of incubation (12.14 g/L) and after that no significant change was observed. Moreover no ethanol was detected in hydrolysate of pre-treated samples generated in absence of enzyme (control) as no fermentable sugars were available. Interestingly, it was observed that as co-culture was used for fermentation, but no diauxy growth pattern was observed during growth and production. These finding were harmony with results reported earlierduring simultaneous saccharification and fermentation of the alkali-treated cotton stalks resulted in ethanol concentration and ethanol yield was 19.48 g/L and 0.21 g/g of biomass respectively, by using thermo tolerant *Pichia kudriavzevii* HOP-1[17].

CONCLUSION

Conclusively, the maximum ethanol production from enzyme hydrolysate of cotton stalk was recorded at 48 hours of incubation which gives an ethanol concentration of 9.56 g/L with a yield of 0.191 g/g of biomass, 0.298 g/g of holocelluloses and 0.392 g/g of fermentable sugar. The fermentation and sugar consumption efficiencies were recorded as 76.85% and 97.81% respectively. Ethanol production is affected by variety of factors, including concentration of substrate, cellular activity in co culture environment and reaction conditions such as pH, temperature, time etc. and in this regards this study can serve as a one step towards sustainable energy development and more efforts were needed in terms of process optimization to make the process more feasible.

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