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National Academy of Agricultural Science (NAAS) Rating : 3.03

Alkaline Pretreatment an Effective Approach for Saccharifaication of Sugarcane Bagasse

Monika Barkodia*, Sneh Goyal* and Leela Wati*

ABSTRACT: *The aim of this study was to cosign an effective pretreatment approach for production of bioethanol from lignocellulosic materials like sugarcane bagasse. For this sugarcane bagasse (milled and non milled) was treated with different concentration of alkali to make bagasse a suitable candidate for saccharification and libration of fermentable sugar from the bagasse. For the pretreatment of sugarcane bagasse, bagasse was ground to fine particle size of 4mm. Finely ground bagasse (milled bagasse) and as such bagasse was treated with 1-5% sodium hydroxide. Taking into consideration the reaction time and alkali concentration, 5% NaOH pretreated milled bagasse was found to be best, there was 75.9% decrease in lignin content and 28.0% increase in cellulose content was observed. 5% alkali pretreated milled bagasse used as a carbon source for the growth of Trichoderma ressei different cultures. Enzyme activities of different cultures Trichoderma ressei checked on cellulose powder as well as on bag culture MTCC4876 showed maximum FPase (0.18 IU/ml), CMCase (0.40 IU/ml), Xylanase (3.56 IU/ml). asse. On the 5th day of incubation on rotary shaker at 150 rpm,*

Keywords: *Alkali pretreatment, Bioethanol Sugarcane bagasse, Saccharification,*

INTRODUCTION

In the last few decades, demands for alternative fossil fuel increasing continousely because of shortage of fossil fuels and global climate changes. India has 0.5% of the oil and gas resources of the world but 16% of the world's population with the result that the country depends heavily on oil imports to meet the domestic demand. More than 70% of the needs of the country are met from imports of crude oil and natural gas. India is one among the few nations having a separate ministry for renewable energy which address the development of biofuels along with other renewable energy sources. The blending targets for ethanol and biodiesel in gasoline and petroleum diesel, respectively were proposed as 10% and 20% by 2011–2012 and a 5% ethanol blend in gasoline was made mandatory in 11 states and three union territories of the nation. Blending has created an increased demand for fuel grade ethanol (Sukumaran and Pandey, 2009).

For large scale production of fuel ethanol it is desirable to use cheaper and easily available substrates. Producing ethanol from maize or sugarcane, the raw material constitutes about 40-70% of the production cost. Therefore, for large scale production of ethanol suitable substrate with availability and lower cost is required. Lignocelluloses are most abundant biomass with a worldwide annual production of $1x10^7$ MT. It is widely considered as an important source for production of sugar that can be fermented into ethanol. Among lignocelluloses, sugarcane bagasse has immense potential as a renewable substrate. In India total sugarcane production is about 67 million tones which yield about 40 million tones of bagasse. Each tone of raw cane is associated with generation of 130 kg of dry weight of sugarcane bagasse. In India most of the bagasse is burnt by the farmers that causes global warming. Use of bagasse in bioethanol production provides an alternative opportunity for more sustainable development of renewable resources. Each tone of raw cane is associated with generation of 130 kg of dry weight of sugarcane bagasse. In India most of the bagasse is burnt by the farmers that causes global warming. Use of bagasse in bioethanol production provides an alternative opportunity for more sustainable development of renewable resources (Zhao *et al.*, 2011). Lignin is major barrier in conversion of biomass into ethanol. This limitation

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includes close association between lignin and plant cell wall polysaccharides. In general, pretreatment presents the most practical and economic challenges in the attempt to commercialize cellulosic bioethanol since it may affect upstream as well as downstream processes by determining fermentation toxicity, enzymatic hydrolysis rates, enzyme loadings, product concentrations and purification, waste treatment demands, and power generation (Sarkar *et al.* 2012). However, no perfect pretreatment method has been discovered since there are variations in terms of suitability of one method for various materials. Physical and physiochemical methods are not so much efficient. Biological methods are much expensive than other methods. Among chemical methods acid or alkali pretreatments are mostly used for removal of lignin from lignocellulosic materials. Test under acidic conditions are more common but alkaline pretreatment followed by cellulosic pretreatment found to give high sugar yield (Mosier *et al.* 2005). Alkali pretreatments were found to be more effective on agricultural residues. It also increases cellulose digestibility and they are more effective for lignin solublization, exhibiting minor cellulose and hemicelluloses solublization than acid or hydrothermal processes. Enzymatic hydrolysis of cellulose of biomass is considered as the most efficient and least polluting method for generating glucose from lignocellulosics, but the production economics of bioethanol is largely dependent on economical use of all sugars or major sugars. Currently, the mainstream process of bioethanol production makes use of *Saccharomyces cerevisiae* (Sangkhark, 2011). The research investigation was carried out to evaluate saccharification efficiency of different strain of *Trichoderma ressei* on lignocellulosic material like sugarcane bagasse for bioethanol production.

MATERIALS AND METHOD

Materials Preparation and alkaline Treatment

Sugarcane bagasse was procured from sugar mill, Maham (Rohtak). It was dried at 50ÚC, and ground to the size of about 400 microns.

Estimation of cell wall constituents

Cellulose, Hemicellulose and Lignin were estimated by determining acid detergent fibre (ADF) and neural detergent fibre (NDF) in the sample (AOAC, 1970).

Pretreatment of sugarcane bagasse

Four types of pretreatments were done:

T1- Bagasse as it comes from factory was treated with 5% NaOH.

T2- Finely milled bagasse was treated with 5% NaOH.

For 5% NaOH pretreatment, non milled/milled sugarcane bagasse was immersed in 5% NaOH (1:10 solid-liquid ratio) and heated at 60°C. After cooling it was washed with tap water to get alkali free bagasse. Washing was done till pH reached up to 6. It was dried at 80°C in an oven for further use.

Microorganisms

Different cultures of *Trichoderma ressei* were obtained from different sources. *Trichoderma ressei* NCIM 1186 from Pune, *Trichoderma ressei* ITCC 4026 from IARI Delhi, *Trichoderma ressei* MTCC 4876 and MTCC 164 were obtained from IMTECH (Chandigarh) and maintained on malt extract and potato dextrose agar (PDA) slants.

Composition of malt extract agar medium (g/l)

Malt extract	20.0 g
Agar-Agar	20.0 g
pH	6.5 g

Composition of potato dextrose agar medium (g/l)

Potatoes	250.0 g
Dextrose	20.0 g
Agar–agar	20.0 g

Extract from 250 g potatoes was prepared by boiling potatoes in water. After cooling volume was made one litre and then 20 g dextrose and 20 g agar agar-agar was added.

Preparation of crude culture filtrate

For the preparation of crude culture filtrate, different culture of T. ressei were grown on Mandel's and Sternberg (1976) media containing 1% cellulose and pretreated substrate (5% alkali pretreated milled bagasse) for 5 days at 30°C on rotary shaker at 150 rpm.

Composition of Mandel's and Sternberg medium (g/l)

Component	*Quantity (g/l)*
Cellulose	10.0
Potassium dihydrogen orthphosphate	2.0
Ammonium sulphate	1.4
Urea	0.3
Magnesium Sulphate	0.3
Calcium chloride	0.3
Peptone	1.0
Tween 80	0.05%
Trace element solution	1.0ml
pH	6.0

Composition of trace element solution (g/l)

Component	*Quantity (g/l)*
$MnSO_4.H_2O$	1.56
$FeSO_4.7H_2O$	5.00
$ZnCl_2$	1.67
$CoCl_2$	2.00

Culture filtrate was obtained by filtering the contents through G-1 sintered glass Gooch crucible. This clear filtrate was characterized for Filter paper, CMCase and Xylanase activities measured by method of Mandels (1969). Reducing sugars were determined by DNS method (Miller, 1959).

Determination of Filter paper, Carboxymethylcellulase (CMCase) and Xylanase activities

For determining these activities, 100ml of Mandel's and Sternberg medium was taken in 250ml conical flask and inoculated with about 10^7spores/ml from seven days old slant of fungus. The flasks were then incubated at 28±2°C on rotary shaker (150 rpm). The clear culture filtrate obtained by filtration was used for determining these activities (Mandels, 1969).

Estimation of total reducing sugar

Total reducing sugars in the media were determined by the dinitrosalicylic acid (DNS) method as described by Miller (1959).

$$Reducing\,sugars = \frac{Concentration\,of\,standard\,solution}{O.D.\,of\,standard\,solution\,used} \times O.D.\,of\,sample \times Dilution\,Factor$$

RESULTS AND DISCUSSION

Pretreatment

The lignocellulosic complex is made up of a matrix of cellulose, lignin and hemicelluloses. Lignin hinders in microbial attack, so pretreatment is required to make surface area accessible for microbial utilization (Saha *et al.*, 2011). In present study, alkali pretreatment with mechanical treatment (grinding) is employed. The main objective behind mechanical pretreatment is reduction in particle size and crystallinity of lignocelluloses in order to increase the specific surface and reduce the degree of polymerization. This can be produced by a combination of chipping, grinding or milling depending on the final particle size of the material. Sugarcane bagasse is very bulky material and it is necessary to ground the bagasse to appropriate size so that alkali solution effectively alters its chemical composition (Fig.1). Sugarcane bagasse was dried at 50ÚC, and ground to the size of about 400 microns (Fig. 2).

Sugarcane bagasse without pretreatment cooking or untreated bagasse contained 31.7% of cellulose, 22.8% of lignin and 23.7% of hemicellulose (Table 1).Bagasse after grinding contains cellulose 32.5 % lignin 22.0% and hamicellulose 21.7% (Table 2). Initial analysis of non milled and milled sugarcane bagasse did not show much difference in composition. Nearly similar results 23.6% lignin and 22.5% hemicelluose were observed by Ferriera-Leitao *et al* (2010) in sugarcane bagasse.

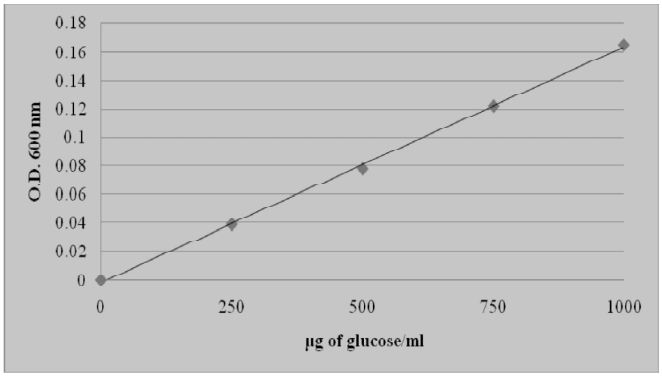


Figure 1: Standard curve of glucose for measurement of filter paper and CMCase activities

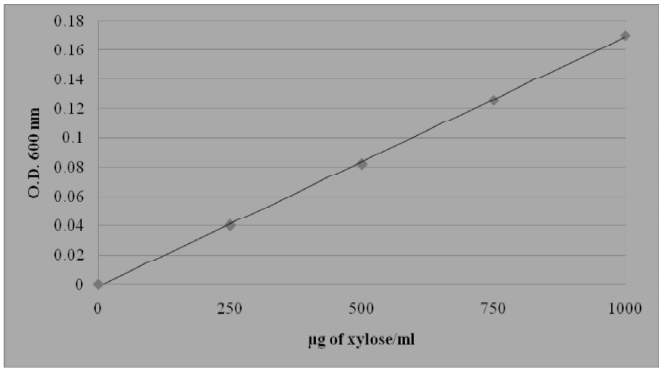


Figure 2: Standard curve of xylose for measurement of xylanase activity. Reducing sugars were determined by using the formula

Figure 1: Non milled bagasse

Figure 2: Milled bagasse

Table 1
Chemical composition of sugarcane bagasse

Chemical composition	*Percentage (%)*
Lignin	22.8
Cellulose	31.7
Hemicellulose	23.7

From initial analysis of composition of sugarcane bagasse it was observed that there is slightly increase in concentration of cellulose and decrease in concentration of lignin and hemicelluloses after grinding. But this grinding (mechanical pretreatment) is not sufficient to remove lignin from bagasse. For maximum liginin removal and makes cellulose accessible, a further treatment of bagasse is become necessary. So, alkali pretreatment is carried out using different concentration of alkali.

Table 2
Changes in chemical composition of sugarcane bagasse after grinding

Chemical composition	*Percentage (%)*
Lignin	22.0
Cellulose	32.5
Hemicellulose	21.7

Alkali pretreatment refers to the application of alkaline solutions to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of enzyme to the hemicellulose and cellulose. The use of an alkali causes the degradation of ester and glycosidic side chains which results into delignification, cellulose swelling, partial decrystallization of cellulose and partial solvation of hemicelluloses. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processess. Sugarcane bagasse was pretreated with 5% NaOH at different time intervals (40, 60 and 80 min) in order to make cellulose and hemicellulose free from lignin. With increase in time duration for pretreatment of both milled and non milled bagasse, lignin content decreased and cellulose concentration increased in each treatment. But the decrease in % lignin content and increase in cellulose concentration after each alkali treatment were in different proportions. With the difference in time interval there is different % of liginin removal i.e. 37.7%, 69.2 and 75.0%. Increase in cellulose content also varies with autocalving time.

Table 3
Composition of sugarcane bagasse after alkali pretreatment at different time intervals

Treatment	*Time(min)*	*Lignin (%)*	*Cellulose(%)*	*Hemicellulose*
5% alkali	**40**	14.2	35.0	20.2
pretreated non	**60**	7.0	39.9	18.8
milled bagasse				
(T1)	**80**	5.7	40.4	14.2
5% alkali pretreated	**40**	13.0	36.9	17.4
milled bagasse	**60**	5.3	41.6	14.0
(T3)	**80**	3.7	42.0	13.5

Lignin removal in non milled sugarcane bagasse was less as compared to lignin removal in milled sugarcane bagasse. It shows that mechanical treatment combined with mechanical treatment is more effective towards lignin removal. With increase in time duration for pretreatment of both milled and non milled bagasse, lignin content decreased. (Table 2). Comparison of the % of lignin, hemicellulose and cellulose content after delignification by 5% NaOH is presented in Figure 3. It was observed that there is significant decrease in lignin and hemicelluose concentration while the cellulose concentration increased

Based on autoclaving time 40, 60, 80 min in 5% alkali pretreated milled bagasse, there is decrease in lignin content 31.0 %, 75.9% and 83.1% respectively and increase in cellulose content 13.5%, 28.0% and 29.2%. Taking into consideration the reaction time 5% NaOH pretreated milled bagasse was used for further studies. Approximately similar results were reported by Irfan *et al.*, 2011 in sugarcane bagasse by using 2% alkali pretreatment. This research further supported by studies of various workers. Sun and coworkers (2002) found 60% removal of lignin and 80% dissolution of hemicellulose in 1.5% alkali pretreated wheat straw. Similarly Silverstein *et al.,* 2007 achieved 65% delignification and 60.8% cellulose conversion using sodium hydroxide pretreatment in cotton stalk. Similarly Silverstein *et al.,* 2007 achieved 65% delignification and 60.8% cellulose conversion using sodium hydroxide pretreatment in cotton stalk.

Activities of *Trichoderma reesei* were assayed in submerged conditions upto 5 days on two insoluble substrates i.e. 1% cellulose powder and 1% bagasse.

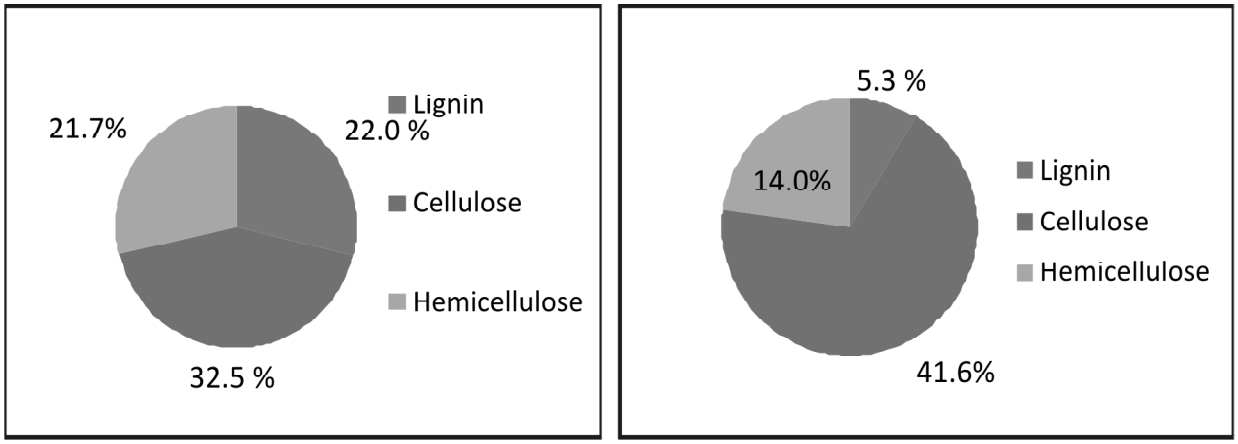
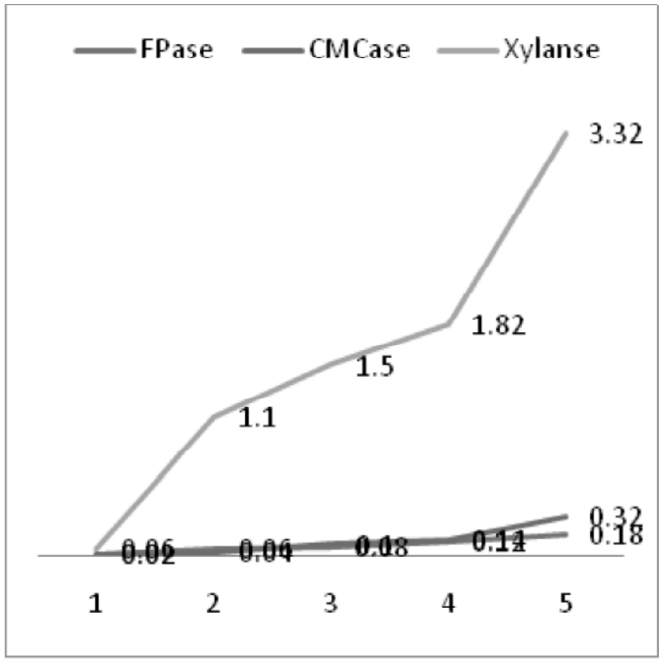


Figure 3: Comparison of chemical composition of milled and 5% alkali pretreated milled bagasse (60 min.)

On 5th day of growth, maximum activities were observed in both cellulose powder as well as 1% bagasse (Fig-4, Fig-5)

Enzyme activities of different cultures were also tested upto 5 days in liquid Mandels and Sternberg mediam containing 1% cellulose powder and bagasse as a carbon source. Between the two substrates, higher enzyme activities were observed on 1% bagasse (Table 4). It was observed that when cellulose powder was replaced by 1% alkali pretreated milled bagasse as a carbon source, CMCase and xylanase activity increased.

This suggested that pretreated bagasse stimulated the appropriate inducers of cellulase production and provided an effective source of metabolic substrates for cellulase as well as xylanase. Similarly, Malik *et al.* (2010) also reported high enzymatic activites in sugarcane bagasse by using bagasse as substrate for cellulase production and they obtained CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min. It may be inferred from the results that *Trichoderma reesei* MTCC 4876 showed good performance for enzyme production. Therefore, Mandels and Sternberg media supplemented with%



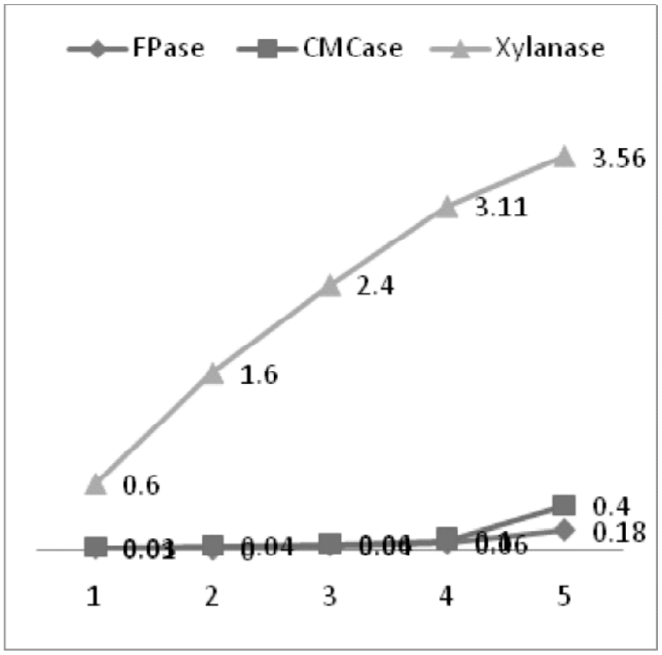


Figure 4: Comparison of enzyme acivites of *Trichoderma ressi* 4876 upto 5 days using cellulose powder as a carbon source,
Figure 5: Comparison of enzyme acivites of *Trichoderma ressi* 4876 upto 5 days using 1% alkali pretreated sugarcane bagasse as a carbon source

Table 4
Enzyme activities of different cultures of *T.reesei* grown on cellulose powder for 5 days

Cultures	*Enzyme activities (IU/ml)*					
	Cellulose powder			*Sugarcane bagasse*		
	FPase	*CMCase*	*Xylanase*	*FPase*	*CMCase*	*Xylanase*
NCIL 1186	0.04	0.06	0.18	0.18	0.26	3.20
MTCC 4876	0.18	0.32	3.22	0.18	0.40	3.56
MTCC 164	0.04	0.26	0.68	0.04	0.38	3.31
ITCC 4026	0.18	0.08	0.26	0.10	0.30	3.40

alkali pretreated milled bagasse as a carbon source was used for further experiments.

CONCLUSION

Non milled sugarcane bagasse procured from sugar mill, Meham (Rohtak) contained 31.7% cellulose, 23.7% hemicellulose and 22.8% lignin. After grinding there was negligible change in composition. Milled and non milled sugarcane bagasse were treated with the 5% NaOH treatment for 1 hour was found to be the best in terms of change in composition of substrate and time of pretreatment. There was 75.9% decrease in lignin content whereas cellulose content was increased to 28.0% in 5% NaOH of milled bagasse treatment for 1 hour and this pretreated bagasse was further used to determine amount of reducing after different time interval. Mandels and Sternberg media supplemented with 1% bagasse as carbon source was for cellulase production by *T.ressei* because more enzyme activites were observed as compared to media containing cellulose powder as a carbon source. After 5 days of incubation at 30^0C under shaking conditions, culture MTCC4876 showed maximum FPase (0.18 IU/ml), CMCase (0.40 IU/ml), Xylanase (3.56 IU/ml). These finding suggest that sugarcane bagasse treated with 5% alkali shows significant lignin removal and also increase the accessible surface area for the activity of cellulolytic enzyme for the conversion of bagasse into ethanol. Further, it was also found that sugarcane bagasse can use as carbon source for the growth of cellulolytic fungus *Trichoderma ressei* and it was a better substrate as compared to cellulose powder for the growth of fungus *Trichoderma ressei.*

ACKNOWLEDGEMENTS

Thanks to Department of Microbiology, CCSHAU, Hisar for providing necessary facilities during research work.

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