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EFFECTS OF PHYSICAL PRETREATMENT (CRUSHING AND BALL MILLING) ON SUGARCANE BAGASSE FOR BIOETHANOL PRODUCTION

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Abstract

In order to obtain ethanol, the C_4 plant stem (Sugarcane bagasse) was physically pretreated by crushing (with sieving) and ball milling prior to the enzymatic hydrolysis by *Trichoderma viride* and fermentation by *Saccharomyces cerevisiae*. Experiments on the comparison of crushed bagasse samples of different mesh size (20-40, 40-60, 60-80, above 80 mesh) with ball milled samples (5, 10, 15, 20 min) of 20-40 mesh, experiments revealed that ball milled for 20 min had shown better result than crushed sample (above 80 mesh) due to more surface area and greater accessibility to hydrolytic enzymes. It was found that at 48 hrs theoretical yield of glucan and xylan for crush (above 80 mesh size) bagasse sample were 9.23, 40.89% and for 20 min ball milled bagasse sample were 68.17 and 54.19%, respectively. Optimum condition for ethanol fermentation was identified by using hydrolysate of 10% substrate (above 80 mesh size sugarcane bagasse) and 8.85% ethanol was obtained after 24 hrs.

Introduction

for the purpose of green energy utilization, Greenhouse Gases (GHG's) emission reduction, lessen global warming and to achieve sustainable development goal (SDG) of Bangladesh, scientists are considering several renewable energy sources such as wind, sun, hydro, geothermal, lignocellulosic biomass etc. Among these sources, lignocellulosic biomass especially agricultural residues have been paid much attention by the researchers due to inexpensive, renewable and abundant source for the production of second generation biofuel production (Long *et al.* 2011). Second generation biofuels are made from non-food crops or agricultural wastes especially lignocellulosic biomass and they do not compete with the use of raw materials as food like first-generation biofuels. An agricultural country like Bangladesh, C_4 plants are receiving a renewed interest due to their high growth rate and better reduction of carbon footprint compared to an equivalent area of woody plants (Mathiyazhakan *et al.* 2013). Sugarcane (*Saccharum officinarum*) is one of the main feed stocks for bioethanol production which is in C_4 grasses. In modern bio-refinery concept industry will need a continuous and reliable supply of lignocellulosic biomass that can be produced at a low cost and with minimal use of water, fertilizer and arable land (Caitlin *et al.* 2011). In Bangladesh sugarcane is cultivated and produced annually about 0.38 million acres of land and 5.5 million metric tons, respectively. (http://en.banglapedia.org/ index.php?title= sugarcane, date: 07/05/2017).

The most important processing challenge in the production of biofuel is pretreatment of the biomass. Lignocellulosic biomass is a complex matrix of cellulose and lignin bound by hemicellulose chains. The pretreatment is done to break the matrix in order to reduce the degree of

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crystallinity of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Nibedita *et al.* 2012). Crushing and Ball milling pretreatment of SB (sugarcane baggase) plays an imperative role to improve the substrates for enzyme production as well as for enzymatic saccharification (Firoz *et al.* 2012).

An attempt has been made to investigate the effects of the proposed pretreatment methods to enhance the enzymatic digestibility of SB and to clarify the optimum condition for sugar yield and finally checking bioethanol production.

Materials and Methods

Sugarcane bagasse (SB) was collected from street sugarcane juice sellers (New market and Science Laboratory area). Five kg of crushed SB were collected and washed 3 times with hot tap water and pressed to remove the residual free sugars. The bagasse was then dried in a drying oven at 65^0C for 16 hrs. The dried bagasse was cut into small pieces and successively crushed using a locally made crusher and fractionated into four levels (A1 = 20-40 mesh, A2 = 40-60 mesh, A3 = 60-80 mesh and A4 = above 80 mesh) by using sieving machine (Retsch, D-42759, HAAN, Germany). Ball milling was carried out using the High Performance Planetary Ball Mill (Pulverisette 6, Germany). In these experiments 10 balls of 10 mm diameter and at about 400 rpm were used for grinding. Bagasse of 20-40 mesh size was then grinded successively for 5, 10, 15 and 20 min which was designated as B1, B2, B3 and B4, respectively and stored in air tight container at room temperature before use.

The moisture content (T 412 om-11), ash content (T 211 om-02), α-cellulose (T 203 cm-99), pentosan (T 223 cm-01), klason lignin (T211 om-83), acid soluble lignin (T UM 250) and extractive content of SB were determined on dry basis by technical association of the pulp and paper industry (TAPPI) method.

Raw SB contained 9.44 % moisture, 1.75% ash and 1.79% extractive. It also contained 34.66% α-cellulose, 22.43% pentosan and 19.57% klason lignin in which 1.75% acid soluble lignin was detected on a dry basis. The composition of SB used in this work was determined by acid hydrolysis and found 45.35% glucan and 30.64 % xylan.

A suspension containing spores (10^6 - 10^7) of *Trichoderma viride* per ml (as determined by Neubauer counting chamber) was prepared by scraping conidiospores from agar slants into sterile saline water. According to Copa-Patino *et al*. (1993) basal medium was prepared which contained (per liter) 0.6 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.74 g $CaCl_2.2H_2O$, 0.4 g K_2HPO_4, 2.32 g $NH_4H_2PO_4$ and 1.0 g yeast extract, and 7.0 ml of trace salts solution (per 100 ml, 500 mg $FeSO_4.7H_2O$, 200 mg $CoCl.7H_2O$, 140 mg $ZnSO_4.7H_2O$, and 160 mg $MnSO_4.H_2O$) and sugarcane bagasse 2.0% (w/v) as a carbon source. The growth medium was sterilized at 121°C for 15 min, then inoculated with 5 ml spore suspension (10^6 - 10^7 spores/ml) and incubated at 30°C on an orbital shaking incubator at 200 rpm for 3 days. After cultivation, the culture filtrate was centrifuged at 10,000 rpm for 15 min. Enzyme activity of clear supernatant (EC 3.2.1.4) was determined as filter paper activity (FPase) according to the method described by Mahmud and Gomes (2012).

Saccharification experiment was done in 100 ml Erlenmeyer flasks with 200 mg (2% dry wt.) substrate and 10 ml enzyme solution in citrate buffer (0.05 M, pH 5.0) at 30°C for 24, 48, 72 and 96 hrs. Hydrolysates were transferred in screw-capped tubes, heated in a boiling water bath for 15 min and centrifuged to remove solid particles. The supernatant was used for analysis of released sugars.

During fermentation 100 ml media together with 0.5 g commercial yeast (10^6 - 10^7 spores/g) were added in a 250 ml Erlenmeyer flask which was placed in a shaking incubator for 48 hrs. Next

10 ml of this medium was added to the autoclaved samples in a laminar air flow chamber. The flasks were properly covered with aluminum foils and then placed in the incubator at 30°C for 48 hrs.

Sugars and ethanol concentration in liquid fraction of hydrolysis and fermentation were determined by HPLC using Hyper Rez XP carbohydrate H^+ 8 µm column (100 × 7.7 mm) equipped with a refractive index detector. The mobile phase was degassed by deionized water with a flow rate of 0.7 ml/min. The column temperature was 70°C. Sugars concentration in hydrolysis liquid fraction can be determined by comparison its peak area detected by HPLC with peak area of 1% standard sugar consisting of two sugars, namely glucose and xylose.

Yields of all sugars are mentioned by comparing the acid hydrolysis which is termed as theoretical yield of sugar (Jamal *et al.* 2011).

$$\text{Theoretical yield} = \frac{\text{Experimental yield}}{\text{Total yield (Yield of acid hydrolysis)}} \times 100$$

Particle size was determined by Malvarn Zetasizer (Model Nano-ZS) through dynamic light scattering (DLS) technique.

Scanning electron microscopy (ZEISS EVO 18 SEM) was used to observe modifications on bagasse fibers. SB samples were adhered to carbon tape and observed in the SEM through the use of an acceleration voltage of 2.0 KV.

Fourier transformed infrared (FTIR) spectrometer (Perkin Elmer, Model Frontier) operated by Perkin Elmer spectrum software version 10.4.4 and detector MIR TGS was used to obtain FTIR spectra of samples. FTIR spectra were collected in frequency 4000-650/cm by co-adding 32 scans and at resolution of 4/cm.

The crystalline structure of the samples was analyzed by X-ray diffraction (XRD) by means of a diffractometer GBC XRD and filtered copper Kα radiation (λ = 0.1542 nm) by a monochromator at 35.50 KV voltage and 28 mA current, with a speed of about 2 degrees per minute and scanning in the range of 10 - 80ºC. The crystallinity index (CI) was obtained from the ratio between the intensity of the 002 peak (I_{002}, 2θ = 22.5) and the minimum dip (I_{am}, 2θ = 18.5) according to the equation i.e. % CI = $[(I_{002} - I_{am})/I_{002}] \times 100$ (Roberta *et al.* 2012) where I_{002} is the intensity of plane 002 and I_{am} is related to the amorphous structure.

Results and Discussion

Saccharification reactions were carried out at 30°C for 24, 48, 72 and 96 hrs. All saccharification reactions were performed by using 10% substrate and 5 ml enzyme solution in citrate buffer (pH 5) (Jamal *et al.* 2011). The effect of different reaction times on hydrolysis of A1- A4 and B1-B4 samples is presented in Table 1. It was observed that the yield of sugars through saccharification increased from 24 to 48 hrs and then gradually decreased up to 96 hrs. It is assumed that after 48 hrs almost all accessible cellulose was converted into sugars and after this period *Trichoderma viride* consumed some sugars by itself. Among the different mesh size samples, B4 A4 gave the highest theoretical yield of sugar i.e. 9.23% glucan and 40.89% xylan at 48 hrs. In all cases of physical treatment the highest theoretical sugar yield was obtained at 48 hrs.

Ball milling pretreatment was used to reduce the particle size and increase the surface area which improves saccharification and fermentation processes (Hyeon *et al.* 2013). Saccharification reaction was carried out of ball milled samples at the same condition of different mesh size samples. It was observed that glucan and xylan production was increased with the increase of ball

milling time from 5 to 20 min. B4 sample was the most effective on enzymatic hydrolysis than others and glucan and xylan were obtained 68.17 and 54.19%, respectively at 48 hrs.

The results showed that the theoretical yield of sugars (glucan and xylan) for B4 sample was more than the A4 sample. For ball milled sample greater accessibility of hydrolytic enzymes was observed due to small particle size and fiber structure was more loosely organized (Zengxiang *et al.* 2010).

Table 1. Theoretical sugar yield with time of different pretreatment samples.

Sample name	Theoretical sugar yield (%), 24 hrs		Theoretical sugar yield (%), 48 hrs		Theoretical sugar yield (%), 72 hrs		Theoretical sugar yield (%), 96 hrs	
	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan
A1	0.75	1.2	3.28	3.72	0.96	1.81	0.64	1.15
A2	1.44	1.85	4.18	4.31	1.13	1.99	0.66	1.75
A3	2.01	2.31	8.95	38.84	4.71	21.52	1.07	1.12
A4	**2.51**	**3.04**	**9.23**	**40.89**	**5.34**	**30.68**	**1.17**	**1.14**
B1	4.35	3.78	9.05	8.76	6.88	4.95	5.4	3.66
B2	14.54	13.9	23.05	22.9	8.54	7.49	5.86	4.36
B3	19.61	17.2	41.47	41.03	25.97	22.12	18.26	13.48
B4	**26.54**	**22.07**	**68.17**	**54.19**	**37.2**	**30.3**	**23.78**	**19.58**

During fermentation A4 sample was used to check ethanol production. Yeast *Saccharomyces cerevisiae* showed good performance to convert C_6 sugar into ethanol when it was incubated at 30°C for 24 hrs and 8.85% ethanol yield was observed.

Ethanol yield was obtained 6.75, 6.46 and 5.15% after 48, 72 and 96 hrs, respectively (Fig. 1). Particle size analysis showed B4 sample was finer and uniform i.e. mono dispersed (Fig. 2a) and A4 sample was poly dispersed (Fig. 2b). The average particle size (in diameter) of B4 and A4 samples was 0.80 and 1.71 μm, respectively. Probably this factor influenced the fermentation process.

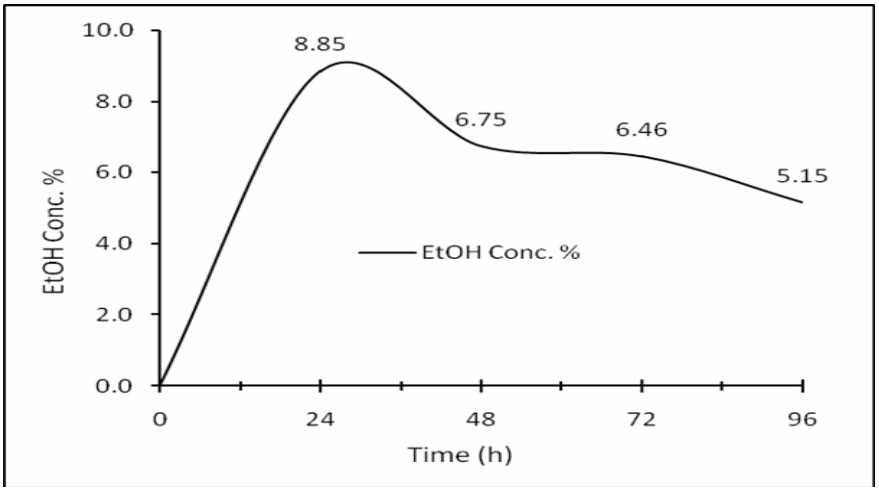

Fig. 1. Fermentation of A4 sample.

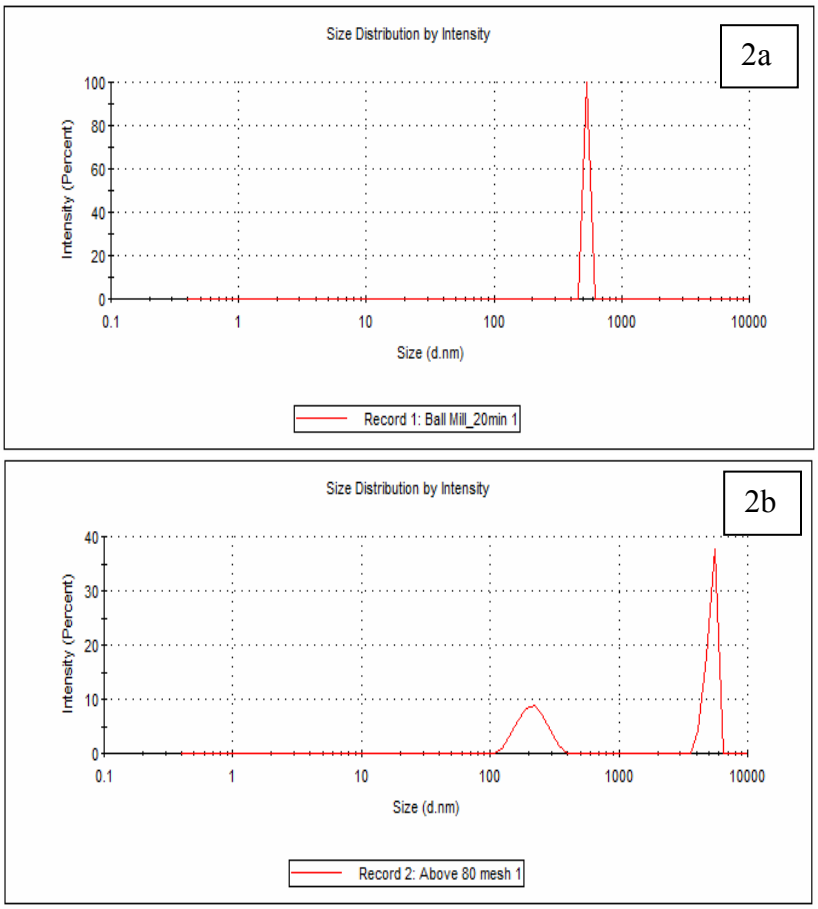

Fig. 2. Particle size: (2a) B4 sample and (2b) A4 sample.

The use of SEM as an analytical technique has significance and versatility for studying the biomass structure (Roberta *et al.* 2012). Figs 3a,b,c (WD 10 mm and magnitude 500×) show the morphological characteristics of sample A1, A4 and B4, respectively. A1 and A4 samples (Fig. 3a, b) present a rigid and compact morphology compared to ball milled sample B4. Increasing ball milling time exhibited a more disorganized morphology with greater exposure of the fibers which allowed for a greater accessibility to hydrolytic enzyme and facilitated the hydrolysis of lignocellulosic biomass.

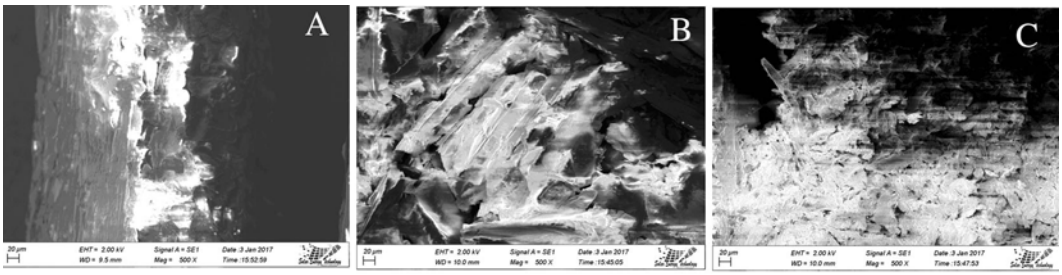

Fig. 3. SEM image: (3a) A1 sample, (3b) A4 sample and (3c) B4 sample.

The main features of FTIR spectra are attributed to the presence of lignin, hemicelluloses and cellulose; the natural components of lignocelluloses fibers. Infrared spectra (Fig. 4) of different samples (A1, A4 and B4) gave very similar absorption spectra which referred that due to physical pretreatment no structural change took place in components of sugarcane bagasse. Slight difference was observed due to concentration of the sample to be analyzed.

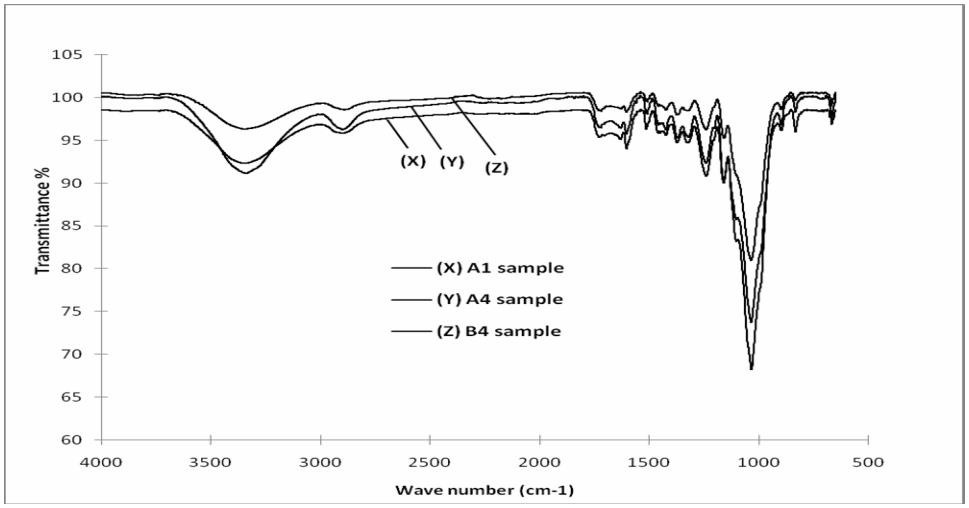

Fig. 4. FTIR spectra: (X) A1 sample, (Y) A4 sample and (Z) B4 sample.

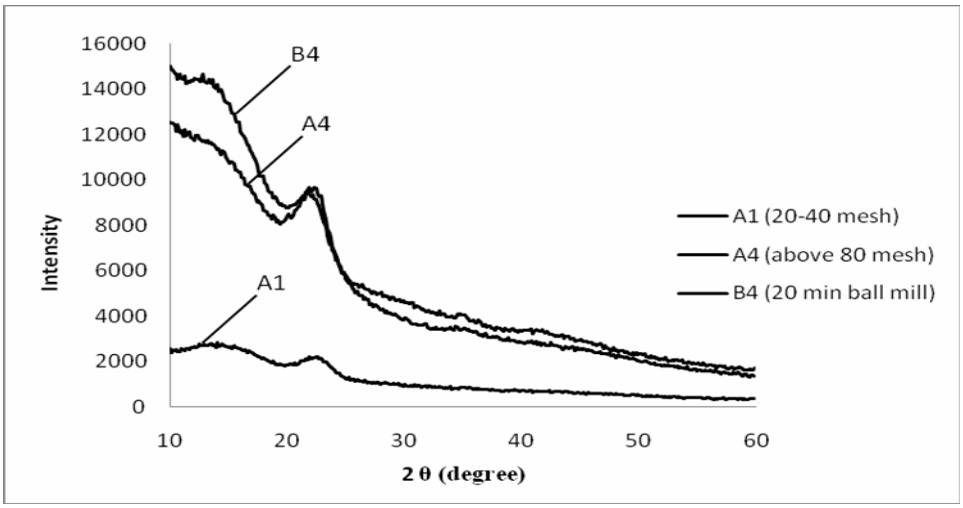

Fig. 5. XRD spectra: (A1) 20-40 mesh, (A4) above 80 mesh and (B4) 20 min ball mill.

Fig. 5 shows diffractograms of A1, A4 and B4 samples. It was observed that all samples exhibited typical cellulose diffraction peaks, where the highest peak corresponded to the 002 crystallographic planes at an angle 22.5^{o}. The crystallinity index was calculated according to equation mention in the methods section. The A1 sample showed a lower crystallinity index

(CI = 6.44%) and physically pretreated samples A4 and B4 showed crystallinity index 8.38 and 9.90%, respectively. Crystallinity index of all samples were close to each other, the fact is no chemical composition change had taken place due to physical pretreatment of sugarcane bagasse. Enhancement of crystalline phase was observed with finer and uniform particle size.

It is assumed that physical pretreatment showed only the deformation of physical structure of lignocellulosic biomass. Thus, to observe the possible change of chemical structure of biomass, FTIR and XRD analyses were done.

Bioethanol production from C_4 plant stem (Sugarcane bagasse) can play a vital role. Pretreatment was the key element which influenced the sugar yield as well as the ethanol production. Experiments and analysis revealed that B4 sample gave better result over A4 due to uniform particle size, more surface area and greater accessibility to hydrolytic enzymes. For saccharification of SB, at 10% substrate concentration and 48 hrs was identified as optimum condition for theoretical yield of sugars in both pretreatments i.e. by using *Trichoderma viridae.* Theoretical yield of glucan and xylan for A4 sample was obtained 9.23, 40.89 and for B4 sample was 68.17, 54.19%, respectively. At fermentation step *Saccharomyces cerevisiae* was used to convert hydrolysate of A4 sample and ethanol yield was obtained 8.85% at 24 hrs. As theoretical sugar yield of glucan and xylan for B4 sample were higher so it produced more ethanol in compared to A4 sample.

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