PRETREATMENT AND ENZYMATIC HYDROLYSIS OF PEARL MILLET STOVER BY MULTI-ENZYMES FROM *ASPERGILLUS NIDULANS* AKB-25

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The pretreatment of pearl millet stover at 3% alkali dose was found to yield 65.85% cellulose, 19.35% hemicelluloses and 9.78% lignin with substantial reduction in extractives (92.5%) and proteins (84.1%). Alkali treatment enhanced the Brunauer-Emmett-Teller (BET) surface area and water retention value (WRV) by 322.92 and 78.66%, respectively, compared to the untreated sample.The crystallinity index was increased with increasing alkali doses and reached the maximum, *i.e.*41.78%,at 3% alkali treatment, compared to the untreated pearl millet stover (27.40%). Likewise, conversion of biomass into reducing sugars increased with increasing alkali doses up to 3% and the yield of reducing sugars was 53.13% at a hydrolysis time of 72 h. An enzyme dose of 15 FPU/g dry substrate was found to produce maximum reducing sugars (57.77%) after 72 h of enzymatic hydrolysis. The addition of Tween-20 and Tween-80 (0.15 g/g dry substrate) increased saccharification yield up to 62.14 and 64.77%, respectively, compared to the control (57.64%).

Keywords:pearl millet stover, alkali pretreatment, saccharification, enzymes, *Aspergillus nidulans*, surfactants

INTRODUCTION

The increasing demand of fuel energy for transportation and industrial processes in a sustainable way is a great challenge for society in the 21st century. Energy consumption is increasing globally, while fossil-fuel reserves are depleting continuously.[1]The production of bioethanol from lignocellulosic biomass is one way to reduce both consumption of fossil fuels and environmental pollution.[2] Lignocellulose is a renewable resource produced by photosynthesis; its annual production is estimated as 1×10^{10} MT in the world.[3]As lignocellulosic materials are inexpensive and abundantly available, they are an attractive feedstock for bioethanol production. Lignocellulosic materials are also advantageous because they are not directly related to food.[3,4] The conversion of lignocellulosic biomass into fermentable sugars by cellulases and hemicellulases may be an alternative for conventional sugarcane or starch based bioethanol. Bioethanol production from lignocellulosic biomass is already used in several countries, such as Brazil, USA, Sweden and Taiwan, either in pure form or as a blend with gasoline.[5,6,7]

Lignocellulosic biomass is mainly composed of three natural polymers: cellulose, hemicelluloses and lignin, which are present in tight and compact association in a plant cell wall.[8,9] Lignin resists the degradation and confers hydrolytic stability and structural robustness to plant cell walls. It provides a physical barrier for the action of cellulases and hemicellulases.[10,11] To overcome the recalcitrance of lignocellulosic biomass, a pretreatment is required, which enables an effective release of monomer sugars from lignocellulosic biomass.[1] The enzymatic hydrolysis of lignocellulosic materials is limited by several factors, such as crystallinity of cellulose, degree of polymerization, moisture content, lignin content, and specific surface area.[10,12,13] Researchers conclude that the purpose of the pretreatment process is to breakdown the lignin structure and disrupt the crystalline structure of cellulose to enhance its accessibility.[10,14] Alkaline pretreatment by

chemicals such as NaOH, KOH, $Ca(OH)_2$, hydrazine and anhydrous ammonia causes swelling of biomass, decreases both the cellulose crystallinity and the degree of polymerization.[10,15] It allows the removal of lignin and hemicelluloses, which increases the accessibility of enzymes.[10,13,15] It is most effective for agricultural residues, which have low lignin content, but becomes less effective as the lignin content of the lignocellulosic biomass increases.[10,15]

In the current study, pearl millet has been selected as feedstock for the production of fermentable sugars. It is considered as an ideal feedstock for sustainable bioethanol production, as it achieves high biomass with minimal or no fertilization and irrigation, and can grow under conditions such as drought, low soil fertility and high temperature.[16,17] Pearl millet is grown in tropical and subtropical areas of Africa and on the Indian sub-continent. India, Nigeria, Niger, Mali and Chinaare world-leading pearl millet producers. Pearl millet is grown on 9 million hectares in India and produces 8.3 million tons annually, which generates approximately 14.76 million tons of stover.[18]

The present study aimed at investigating the effect of pretreatment on the surface area, crystallinity, and changes in the chemical and physical structure of pearl millet stover. The effect of different enzyme dosage on the hydrolysis of pearl millet stover with or without presence of surfactants was investigated.

EXPERIMENTAL

Enzyme production

A fungal strain isolated from a soil sample was identified as *Aspergillus nidulans* AKB-25 at the National Fungal Culture Collection of India, Agharkar Research Institute, Pune, with the accession number NFCCI 2977.The pure culture of the fungal strain was maintained by sub-culturing over potato dextrose agar at 30 ºC and preserved over potato dextrose agar slants at 4 ºC. Enzyme production was carried out on black gram residue of particle size ranging from 250 to 1400 µm as substrate by *Aspergillus nidulans* AKB-25 under solid-state fermentation. Five grams of black gram residue was moistened (80.0% initial moisture content) with Mandel Weber medium as gl^{-1}: $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $CaCl_2$ 0.3, $MgSO_4.7H_2O$ 0.3, Tween-80 0.1 and trace elements such as $FeSO_4.7H_2O$ 0.005, $MnSO_4.7H_2O$ 0.0016, $ZnSO_4.7H_2O$ 0.0014 and $CoCl_2.6H_2O$ 0.002. The initial pH of the Mandel Weber medium for enzyme production was adjusted to 8.0 with 1N NaOH.

Enzyme assays

The filter paper activity (FPase) and endoglucanase (CMCase) activities were determined by standard procedures recommended by the Commission on Biotechnology, International Union of Pure and Applied Chemistry (IUPAC).[19] Endoglucanase and FPase activities were determined by using 2% (w/v) carboxymethyl cellulose of medium viscosity (Sigma Chemical Co. St Louis, MO, USA) and Whatman no. 1 filter paper strip of 50 mg (approximately 1×6 cm), respectively. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µmole of glucose from the respective substrate per min per millilitre under assay conditions. β-glucosidase activity was assayed by using 5mM p-nitrophenyl-β-D-glucopyranoside according to Wood and Bhat.[20] One unit of β-glucosidase activity is defined as the amount of enzyme that releases 1 µmole of *p*-nitro phenol per min per millilitre under assay conditions. Xylanase activity was assayed by estimating the reducing sugars released by 1% (w/v) of beech wood xylan (Sigma) at 50 ºC for 15 min.[21]One unit of xylanase activity corresponds to the amount of enzyme that releases 1 µmole of xylose per min per millilitre under reaction conditions. All the enzyme assays were performed using 50mM citrate buffer (pH 5.0) at 50ºC. Reducing sugars were analyzed by the dinitrosalicylic acid (DNS) method.[22]

Pretreatment of pearl millet stover

Pearl millet stover was collected from district Muzaffarnagar in Uttar Pradesh, India, which was chopped into 1-2 cm pieces and dried. Chopped millet stover was milled in a Wiley mill to reduce the size before pretreatment. Particle size ranging from 250 to1400 µm was selected for hydrolysis and pretreated with different doses of NaOH varied from 1 to 4% with an interval of 1%, maintaining a solid to liquid ratio of 1:8. Alkali pretreatment of millet stover was performed at 121 ºC for 20 min in an autoclave. Pretreated pearl millet stover was washed several times with tap water to neutral pH and finally rinsed in distilled water. The treated samples were used immediately for enzymatic hydrolysis without drying.

Analytical methods

For the compositional analysis, pearl millet stover samples were milled and the portion that passed through a sieve of -14 mesh size and was retained on a ±40 size mesh was used for proximate chemical analysis, including extractives (T204 cm 97), ash content (T 211 om-2), and acid insoluble lignin (T222 om-88),as per TAPPI standard methods.[23] Acid insoluble lignin with ash (AIA) was calculated gravimetrically and acid insoluble lignin was measured by subtracting the ash content from AIA.[23] Acid soluble lignin was calculated in hydrolyzed liquor by spectroscopy at 280 nm wavelength.[24] Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were

determined as per Van Soest method.[25] Cellulose and hemicelluloses contents were calculated by subtracting AIA from ADF and ADF from NDF, respectively. The nitrogen content of the samples was estimated by an organic elemental analyzer (Thermo scientific, Flash 2000) and the protein content was calculated as N × 6.25.[26] For BET surface area measurements, untreated and alkali-treated pearl millet stover samples were dried in a vacuum oven at 90±2 °C for 6 h. Dry samples were first de-gassed at 90 ºC for 6 h and isotherms of nitrogen adsorption-desorption were recorded at liquid nitrogen temperature, using AutosorbiQ from Quantachrome Instruments. Water retention value (WRV) was measured by a SIGMA centrifuge, according to TAPPI useful method UM 256. A water saturated pearl millet stover sample with an oven dry weight of1400 g/m^2 was centrifuged at a relative centrifugal force of 900 G for 30 min. G is the relative centrifugal force, which is given in multiples of earth gravity. After centrifugation, the samples were dried at 105±2 ºC for 2 h in a preheated oven.[23]WRV was calculated as follows:

$$WRV\ (\%) = 100 \times (W_w - W_d)/W_d \quad (1)$$

where W_w is the weight of wet samples after centrifugation and W_d is the weight of oven dried samples.

Fourier transform infrared spectroscopy (FTIR) analysis was performed for untreated and alkali-treated pearl millet stover. The FTIR spectra were obtained by the KBr pellet method in a Perkin-Elmer Infrared spectrophotometer. 10 mg of sample was mixed with 200 mg of KBr for the preparation of pellets. Sample spectra were recorded in the wavenumber range of 4000-400 cm^{-1} at 1cm^{-1} resolution, with 16 scans per sample. XRD analysis was carried out to determine the crystallinities of untreated and alkali-treated pearl millet stover by anUltima IV Rigaku X-Ray Diffractometer, using Cu K_α radiation (λ=1.5405 Å) at 40 kV and 40 mA. Samples were scanned at a 2θangle ranging from 5 to 60º with a speed of 2º/min^{-1} and a step size of 0.02º. The crystallinity index was calculated as the ratio between the area of the crystalline contribution and total area by the XRD amorphous subtraction method.[27] Morphological changes in the pearl millet stover samples during the pretreatment were visualized by FE-SEM (Zeiss Ultra Plus). Images were taken at 5.00 kV voltage and 500X to 2000X magnifications. Samples were dried before analysis. Before sample injection into the sample chamber, the samples were gold coated by a standard sputtering technique for 30 s.

Saccharification of pearl millet stover

The hydrolysis experiments were conducted at 2% (w/v) insoluble solid substrate in 50 mL of reaction volume at pH 5.0, using crude enzyme with enzyme loading of 5 to 20 FPU per gram of dry millet stover. The hydrolysis experiments were carried out in an incubator shaker (New Brunswick Scientific, Innova® 43, USA) at 50 ºC and 120 rpm for 96 h. In all experiments, the hydrolysis mixture was supplemented with 0.01% sodium azide to prevent microbial contamination. Hydrolysate samples were taken from the reaction mixture at regular intervals of 12 h and centrifuged at 9000 rpm for 15 min to remove solids. The supernatant was analyzed for reducing sugars.

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars} \times 0.9}{\text{Carbohydrates in substrate}} \times 100 \quad (2)$$

The effect of sodium hydroxide concentration, enzyme dosages, and surfactants was analyzed during pearl millet hydrolysis. Enzyme dosages 5 to 20 FPU/g of dry substrate were tested with the difference of 5 FPU/g of dry substrate. Surfactants Tween-20 and Tween-80 at 0.15 g/g dry substrate were used during saccharification.[2]

Statistical analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of values.

RESULTS AND DISCUSSION

Chemical composition analysis

It was observed that when pearl millet stover, of particle size varying between 250 and1400 µm, was treated with different doses of NaOH (1.0 to 4.0%), the cellulose yield increased up to an alkali dose of 3%; beyond that, the increase in the cellulose yield was not so pronounced. This might be due to compositional changes in hemicelluloses, lignin, protein and extractives, occurring during the treatment. The increase in the cellulose content at 3% alkali dose was due to partial removal of low molecular weight carbohydrates, *i.e.* hemicelluloses, lignin, protein, and extractives,f rom pearl millet stover. Conversely, hemicelluloses, lignin and protein decreased with an increase in the alkali dose up to 3% and thereafter the increase was insignificant (Table1). In lignocellulosic biomass, cellulose and hemicelluloses are protected by the matrix of lignin, which makes it resistant against enzymatic action. Lignin and its phenolic groups may be involved in the inhibition of enzymatic hydrolysis. Lignin is covalently bonded to carbohydrates and hinders enzyme accessibility and hydrolysis. Lignin may also prevent the swelling of cell wall significantly, thus restricting enzyme accessibility.[28,29]

Alkali pretreatment is aimed at removing lignin and hemicelluloses, which facilitates the conversion of cellulose and hemicelluloses into fermentable sugars.[30]Wet pretreated biomass was

used for enzymatic hydrolysis, otherwise, drying the pretreated lignocellulosic biomass may cause an irreversible collapse of the porous structure, which retards the penetration of enzymes into the matrix and adversely affects enzymatic hydrolysis.[2,10]

Substrate accessibility analysis

The enhanced accessibility of enzymes tothe substrate (pearl millet stover) can be measured in terms of the substrate's specific total pore volume or specific surface area (square meter per gram). Substrate accessibility to the enzymes was measured only underwet conditions. WRV was utilized to measure the accessible volume (porosity) or the surface area of the substrate, using water as the probe molecule under wet conditions. WRV is the measurement of the total surface area accessible to water molecules.[12]The water retention value of untreated pearl millet stover was 125.11% and increased to 203.11% (+78.66%) when treated at an alkali dose of 3%. The treatment of pearl millet stover at 4% alkali dose increased the WRV by 86.09%, compared to the control.

A classic BET surface area analyzer was used for measuring the surface area of the substrate under dry conditions. A BET surface area analyzer measures the adsorption of nitrogen molecules by the substrate. The surface area of untreated pearl millet stover was 3.49 m^2/g, and increased by 3.49 m^2/g when it was treated with a 3% alkali dose, compared to the control. Beyond an alkali dose of 3%, the BET surface area of pearl millet stover reduced drastically. Initially, the alkali treatment broke the compact structure of the cell wall and removed lignin and hemicelluloses, resulting in an increase in the accessibility of the substrate by opening the pores, and thus, increasing the surface area.

The surface area of the substrate can be divided into exterior surface area, affected mainly by the substrate length and width, and interior surface (pore surface) area, which is governed by the size of the lumen and the number of substrate pores and cracks.[31]The interior surface area of cellulose is found to be 1-2 orders higher than the exterior surfacearea.[32]The WRV results followed the same path as in the case of the BET surface area, except the results obtained at a 4% alkali dose. When using a 4% alkali dose, the BET surface area decreased, while WRV increased. The reason is that WRV of alkali treated pearl millet stover was measured on never-dried samples, while BET surface area was measured under dry conditions. Moreover, the alkali treatment increases the flexibility of cellulose fibres. Therefore, drying of pretreated lignocellulosic biomass causes the collapse of pores, resulting in the reduction of pore size, and affects enzymatic digestibility because of hornification. This may be the possible reason for the drastic decrease in the BET surface area at a 4% alkali dose.[12,13] Compared to acid treatment, alkaline treatment causes less sugar degradation and residual caustic salts can be recovered and/or regenerated.[33,34]

FTIR analysis

FTIR spectra of untreated and pretreated pearl millet stover are shown in Figure 1. The absorption band at 3440 $cm^{-1}$was attributed to the stretching of the –OH groups present in cellulose, hemicelluloses and lignin.[35] The broadness of the band decreased when pearl millet stover was treated with 2% NaOH, compared to that for the untreated and 1% alkali treated samples. This might have occurred due to breaking of the hydrogen bonds in the lignocellulosic structure. For the 3 and 4% alkali treated samples, peak broadness increased again. Three and four percent alkali doses removed hemicelluloses and lignin to a great extentfrom pearl millet stover and hydrogen bonding increased as cellulosic fibres came closer to each other due to surface tension forces. The absorption band at 1718 $cm^{-1}$was attributed to C=O stretching of unconjugated ketones, which mainly occurred in the side chain of lignin structural units. The disappearance of the band at 1718 cm^{-1} indicated that the side chain of lignin was broken down during sodium hydroxide pretreatment.[1] The band at 1512 $cm^{-1}$was assigned to the vibration of the aromatic ring in lignin.[35] With an increase in the sodium hydroxide dose in the pretreatment, a prominent decrease in the intensity of the band at 1512 cm^{-1} was found.[1] This might be due to the removal of lignin during the pretreatment. The band at 1239 $cm^{-1}$was assigned to syringyl ring breathing and C–O stretching out of lignin and xylan.[36]The intensity of the band at 1239 cm^{-1} became weaker in the pretreated pearl millet samples, which also indicated the removal of xylan and lignin during the pretreatment, as shown in Table 1. The band at 895 $cm^{-1}$was attributed to the β-glycosidic linkage between sugar units in cellulose.[36,37] The band at 895 cm^{-1} became sharper in the alkali pretreated samples, compared to the untreated

samples. This can be explained by the removal of hemicelluloses and lignin from around the cellulose.[37]

Table 1
Compositional analysis of pearl millet stover before and after the pretreatment

Pearl millet stover	Solid yield (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Protein (%)
Untreated	100.00	35.78±1.20	26.75±0.92	20.32±0.72	8.80±0.34	4.23±0.15	4.02±0.15
1% NaOH	66.85±3.25	43.60±1.39	25.12±0.71	18.45±0.70	4.76±0.16	4.11±0.12	3.18±0.11
2% NaOH	50.92±2.14	62.12±1.46	20.54±0.61	11.76±0.41	0.84±0.03	3.94±0.15	0.75±0.04
3% NaOH	46.63±2.22	65.85±1.90	19.35±0.50	9.78±0.32	0.66±0.03	3.18±0.10	0.64±0.02
4% NaOH	42.68±2.17	66.91±1.97	18.40±0.43	9.27±0.29	0.54±0.02	2.21±0.06	0.64±0.04

± refers to standard deviation

Table 2
Effect of pretreatment on pearl millet stover

Pearl millet stover	WRV (%)	BET surface area (m²/g)	Crystallinity index (%)
Untreated	125.11±3.88	3.49±0.19	27.40±1.30
1% NaOH	134.27±3.75	4.05±0.24	35.81±1.63
2% NaOH	185.00±4.33	8.19±0.45	40.42±1.95
3% NaOH	203.77±5.38	14.76±0.80	41.78±1.75
4% NaOH	211.20±6.23	6.59±0.43	41.93±1.38

± refers to standard deviation

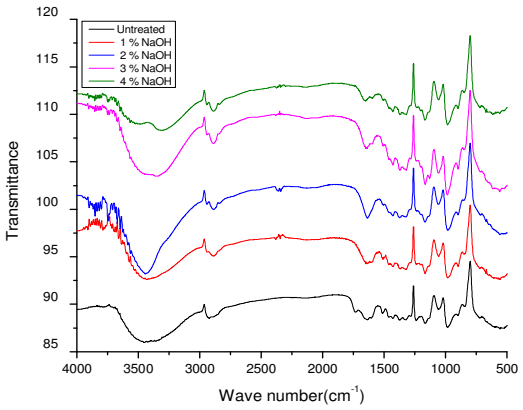

Figure 1: FTIR spectra of untreated and pretreated pearl millet stover

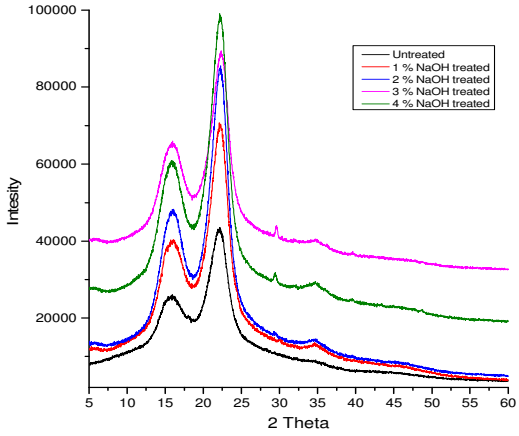

Figure2: XRD spectra of untreated and pretreated pearl millet stover

X-ray diffraction analysis

In lignocellulosic biomass, cellulose consists of crystalline (ordered) and amorphous (less ordered) regions. Cellulose contains a major proportion of crystalline regions and a small proportion of amorphous regions. Amorphous cellulose is more susceptible to cellulase attack.[27,38] XRD spectra of untreated and alkali treated pearl millet stover show the peaks of crystalline cellulose (~16 and 22.5) and the region around 18.5 was due to amorphous cellulose (Fig.2). The crystallinity index of untreated pearl millet stover was of 27.40%, and increased up to 41.78% when pearl millet stover was treated with a 3% alkali dose. Thereafter, the crystallinity index remained almost constant, *i.e.* 41.93% at a 4% alkali dose (Table 2). The solubilization of hemicelluloses and lignin along with less ordered cellulose occurred during alkali pretreatment, which was also observed in the chemical composition analysis of the solid residues (Table1). Due to the removal of these components during the pretreatment, cellulose concentration increased in the pretreated samples.[36,39] The

crystallinity and changes in the crystal structure of cellulose are regarded as the main factors affecting the enzymatic hydrolysis of lignocellulosic biomass. The crystallinity of cellulose results from inter- and intra-chain hydrogen bonds in the cellulosic fibre, which are changed during the pretreatment.[39,40] Several researchers reported an increase in the crystallinity index of solid residue after the pretreatment.[35,39] Maeda *et al.*[41] also reported an increase in the crystallinity index of sugarcane bagasse upon pretreatment with 0.1 to 4% sodium hydroxide.

Morphological studies by FE-SEM

The changes that occurred on the surface of pearl millet stover after the alkali pretreatment were observed by field emission scanning electron microscopy (FE-SEM). In the untreated sample, a complete and compact lignocellulosic structure with smooth surface was clearly visible in Figure 3 (A). In the alkali pretreated pearl millet stover, significant differences were observed in surface morphology (Figure 3 B-E). The lignocellulose structure was broken and the surface structure of the residues became extensively irregular and rough. The removal of hemicelluloses and lignin during the alkali pretreatment indicated the formation of linear cavities on the surface of pearl millet stover. On increasing the alkali dose, these liner cavities became deeper, and because of this cellulose bundles started to separate. Thus, the separation of cellulose bundles increased the specific surface area for enzyme digestibility. The structural modifications that occurred due to the partial removal of hemicelluloses and lignin exposed the cellulosic fibres in the remaining carbohydrate fraction of pearl millet stover, which made it suitable for saccharification. Several similar investigations were reported earlier, where morphological changes occurred during the pretreatment of lignocellulosic biomass, which would be helpful for saccharification.[39,42]

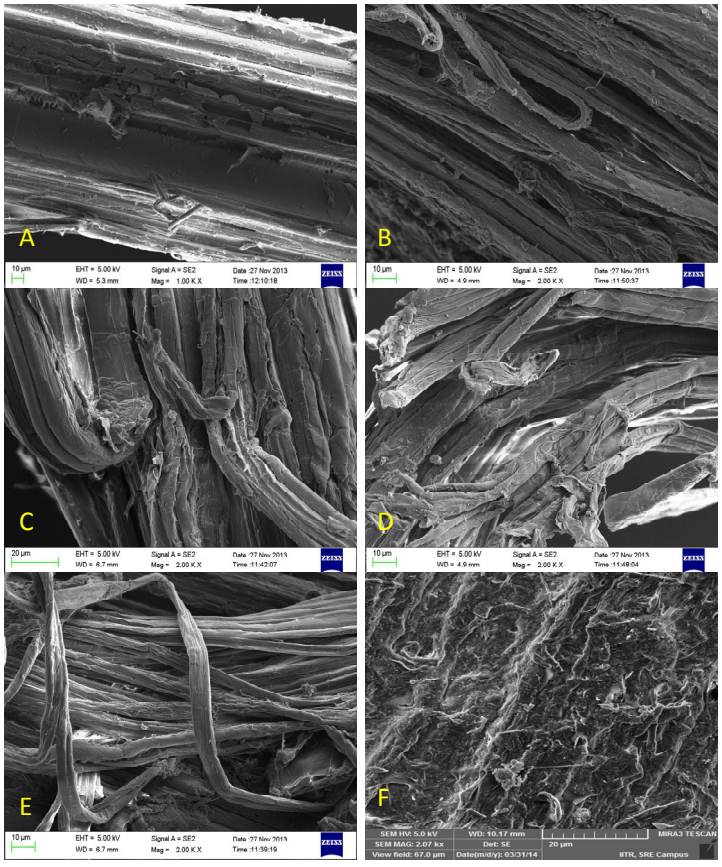

Figure3: FE-SEM images of untreated and pretreated pearl millet stover;
(A) untreated, (B) 1% NaOH treated, (C) 2% NaOH treated, (D) 3% NaOH treated, (E) 4% NaOH treated, (F) after enzymatic hydrolysis

Das *et al.*[1] also observed the formation of cavities on the surface of rice straw due to partial removal of hemicellulose and lignin by the pretreatment with sodium hydroxide.

Effect of pretreatment on enzymatic saccharification of pearl millet stover

The enzymatic saccharification converted the pearl millet stover (without alkali treatment) into reducing sugars by 13.61% after 72 h of hydrolysis (Eq. 2). The lower yield obtained after saccharification might be due to physical barriers for enzyme digestibility, such as compact structure, lower accessibility and lower surface area. The release of reducing sugars after saccharification increased with increasing alkali doses during the pretreatment of pearl millet residue. The curves plotted between reaction time and hydrolysis time at different alkali doses showed that the gap among the curves reduced gradually with increasing alkali doses used for the pretreatment, and the curves for the 3 and 4% alkali pretreatments almost overlapped each other. It indicated that the pretreatment carried out at 3% alkali dose was sufficient for enzyme digestibility to release maximum reducing sugars. These curves also indicated that the release of reducing sugars beyond a reaction time of 72 h was almost constant (Fig. 4). The pearl millet stover produced the maximum total solids yield (+3.95%) at the 3% alkali treatment, compared to the pretreatment carried out with 4% alkali dose (Table 1), but the release of reducing sugars was almost the same in both cases (Table 3). Based on the above-mentioned findings, it can be concluded that a 3% alkali pretreatment and a hydrolysis time of 72 h for saccharification of pearl millet stover may be taken as the optimum. A saccharification yield of 46% was obtained by Chandra *et al*.,[43] who used cellulase from *Tricoderma citrino viride* for the saccharification of marc of *Artemisia annua* under pretreated conditions.

Effect of enzyme loading on saccharification

Crude enzyme produced from *A. nidulans* AKB-25 was evaluated for its ability to hydrolyze pearl millet stover. The curves plotted for reducing sugars released from pearl millet stover (treated with 3% alkali dose) at different hydrolysis time revealed that the gap in the curves became narrower with increasing enzyme doses. The curves plotted for 15 and 20 FPU/g of dry substrate almost overlapped, indicating that the increased dose of enzyme did not increase reducing sugars substantially (Fig. 5). The enzyme loading for saccharification of the substrate depends on the total contents of the carbohydrate present in the substrate.[42] These curves also showed that the hydrolysis of the substrate beyond a hydrolysis time of 72 h became insignificant, because the release of reducing sugars was almost constant.

Table 3
Yields of reducing sugars under different conditions after 72 h of hydrolysis

Particulars			Yield of reducing sugars (%)
Pretreatment	Enzyme dose: 10 FPU/g No surfactant added	Untreated	13.61±0.68
		1% NaOH	40.10±1.96
		2% NaOH	44.44±2.20
		3% NaOH	53.13±2.30
		4% NaOH	53.39±2.13
Enzyme dose	Pretreatment: 3% NaOH No surfactant added	5 FPU/g	44.36±1.76
		10 FPU/g	52.84±2.86
		15 FPU/g	57.77±2.75
		20 FPU/g	58.39±2.48
Surfactants	Pretreatment: 3% NaOH Enzyme dose: 15 FPU/g Surfactant: 0.15% (w/w)	Control	57.64±2.44
		Tween-20	62.14±2.27
		Tween-80	64.77±2.16

± refers to standard deviation

Note:Crude enzyme equivalent of 1 FPU contained 39.39 IU endoglucanase, 11.68 IU β-glucosidase and 642.47 IU xylanase

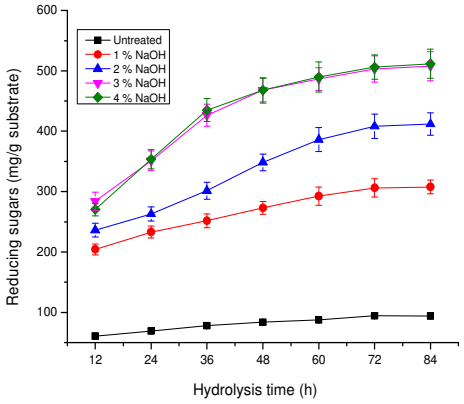

Figure 4: Effect of alkali pretreatment on enzymatic hydrolysis of pearl millet stover

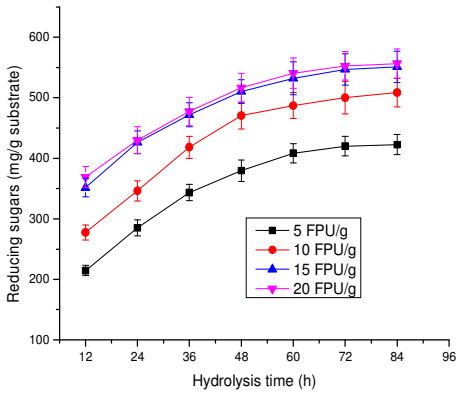

Figure 5: Effect of enzyme loading on enzymatic hydrolysis of pearl millet stover

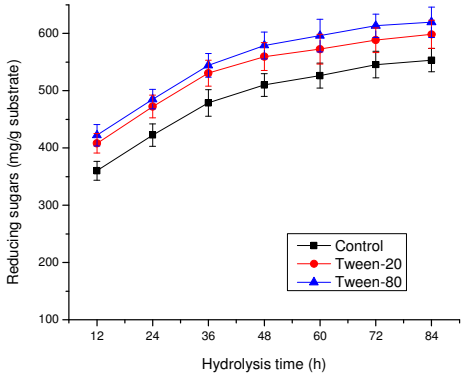

Figure 6: Effect of surfactant on enzymatic hydrolysis of pearl millet stover

Crude enzyme was found effective in the saccharification of pearl millet stover due to a higher amount of β-glucosidase and xylanase, along with cellulase, which plays an important role in the process of saccharification. The accumulation of cellobiose in the reaction mixture inhibits cellulase enzyme during hydrolysis and β-glucosidase reduces this effect of inhibition by converting cellobiose into glucose.[44,45] Moreover, a large amount of β-glucosidase in the hydrolysis system also circumvents the loss due to inhibition and deactivation of β-glucosidase by phenolic compounds produced during the pretreatment.[44,46] A higher level of xylanase activity in the crude enzyme is also helpful in achieving higher conversion of cellulose and hemicellulose. The xylanase attacks xylan in hemicelluloses and converts it into xylose units. The removal of xylan due to enzymatic treatment enlarges pore size.[1,47] Some researchers have observed that xylooligomers inhibit cellulases more strongly in comparison with glucose, cellobiose and xylose.[10,41,47] A higher amount of xylanase in crude enzyme also neutralizes the effect of inhibition by xylooligomers. The hydrolysis of lignocellulosic biomass has been reported in several investigations with commercial and crude enzyme dosages of 5-30 FPU/g of substrate.[2,39,41,48] Chandel *et al.*[42] investigated the hydrolysis of acid pretreated *Saccharum spontaneum* with enzyme from *Aspergillus oryzae* MTCC1846 and achieved a maximum release of sugar (310 mg/g) with 15 FPU/g of the substrate. The efficiency of the enzyme from *A. nidulans* AKB-25 was comparable to commercial enzyme preparations and crude enzyme preparations reported earlier. Harnpicharnchai *et al.*[49] investigated the hydrolysis of rice straw with a combination of β-glucosidase BGL I from *Periconia* sp. and commercial enzyme (Celluclast 1.5L), and found a release of reducing sugars of approximately 132 mg/g of the substrate. Deswal *et al.*[50] also studied the saccharification of wheat straw and obtained a production of reducing sugars of 214.1 mg/g of the substrate, using crude enzyme from *Fomitopsis sp.*RCK2010.

Effect of surfactants on saccharification of pearl millet stover

The effect of non-ionic surfactants, namely Tween-20 and Tween-80, on the release of reducing sugarswas examined at different reaction timesduringthe hydrolysis of alkali pretreated pearl millet stover under optimum conditions. The curves plotted for reducing sugars released as a function of hydrolysis time indicated that the addition of Tween-20 and Tween-80 (0.15 g/g of the dry substrate) increased the saccharification yield from 57.74 to 62.14% and 57.74 to 64.77%, respectively, after 72 h of hydrolysis.Thus, the addition of Tween-80 was more effective than that of Tween-20, and both were more effective than the control (Table 3). The mechanisms involved in the surfactant's action to enhance the release of reducing sugars was based on three independent principles: *i.e.* (i) stabilization of enzymes and prevention of enzyme's denaturation, (ii) modification of surface structure for increasing enzyme accessibility, and (iii) promotion of enzyme-substrate interactions, particularly by preventing non-productive adsorption of enzymes. The hydrophilic portions of the bound surfactant protrude into the aqueous solution and sterically prevent unspecific and non-productive binding of enzymes to lignin, which in turn increases the conversion of holocellulose into reducing sugars.[2,28,51] Kim *et al.*[52] suggested that the adsorption of surfactants at the air-liquid interface prevents enzyme denaturation due to agitation during enzymatic hydrolysis.An enhancement in the hydrolysis of lignocellulosic biomass by the addition of surfactants was reported by several researchers.[48,51,53] Thus, an increase of 7.5% in the hydrolysis of maize straw with the addition of Tween-80 was reported by Chen *et al.*[48] Ferreira *et al.*[2] showed an enhancement in the release of glucose from 4.575 g/L to 7.355 g/L from *Erica* spp. by the addition of 0.15 g/g of the dry substrate of PEG 4000.

Several studies on enzymatic hydrolysis have been reported using different lignocellulosic biomass.[39,43,44,50] To the best of our knowledge, this is the first report on the hydrolysis of pearl millet stover with crude enzyme from *Aspergillus nidulans*.

CONCLUSION

The alkali dose of 3% was found the optimum for the pretreatment of pearl millet stover. Compositional studies and FTIR analysis revealed that lignin and hemicelluloses were removed partially during alkali pretreatment. The removal of lignin and hemicelluloses enhanced the accessibility of pretreated pearl millet stover, which was evidenced by the increased BET surface area and WRV. *Aspergillus nidulans* AKB-25 hydrolyzed the pearl millet stover effectively due to better accessibility and increased surface area obtained after 3% alkali treatment. An enzyme dose of 15 FPU/g of dry substrate was found the optimum for the saccharification of pretreated pearl millet stover. The saccharification was further improved by supplementing surfactants (Tween-80) to the reaction mixture.

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