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Dilute acid pretreatment of Sweet sorghum stalk and its characterization

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Abstract: Sweet sorghum, a promising low- cost lignocellulosic feedstock, is pretreated for the synthesis of microbe utilizable simple monomers that has an economic importance in the secondary biofuels production. The aim of the present study is the pretreatment of sweet sorghum stalk using different dilute acids for effective delignification and hydrolysis, to optimize the simple monomers yield by varying the acid concentrations and further analyze the chemical bonds breakdown in the pretreated stalk through structural and morphological characterization by comparing with untreated sweet sorghum stalk as control. Sweet Sorghum (SS) stalk was pretreated with different acids such as HCl and H_2SO_4 at different concentrations ranging from 0.5% (v/v) to 3% (v/v) to optimize the simple sugars yield. The maximum sugar yield was obtained at 1% (v/v) HCl pretreated SS stalk hydrolysate. Structural and morphological characterizations of the pretreated and untreated SS stalk were carried out using FT-IR and SEM respectively. During FT-IR analysis, the chemical bonds present in the hemicellulose were completely broken down in the pretreated SS stalk which was inferred by comparing with untreated SS stalk. SEM micrographs revealed that the lengthier microfibrils structure of the biomass network was disrupted resulting in the formation of more holes on the annular rings and on the cellulose wall which were the internal structure of the biomass network. This study reveals out the structural composition and monomeric yield during dilute acid pretreatment of SS stalk.
Keywords: Sweet sorghum stalk, pretreatment, dilute acids, FT-IR, SEM.

Introduction

Lignocellulosic biomass such as food processing wastes, municipal solid wastes, wood, paper and pulp industries wastes, agricultural residues are available in large abundance in the world[1]. These lignocellulosic substrates are rich in cellulose, hemicellulose and lignin. Sweet Sorghum (SS) stalk is also one of the prominent lignocellulosic biomass for biofuel production due to its third largest availability among the cereal crops[2,3], high biomass yield and sugar[4]. This crop has wider adaptability due to its faster growth rate, accumulation of more sugars and has the potential for higher biomass yield due to its more photosynthetic efficiency and its stems are usually discarded in the field[5,6]. SS requires less water for its growth and due to this special characteristics, it can be grown even during drought conditions. Compared to sugarcane, sweet sorghum can be harvested within 100 - 120 days whereas the sugarcane can be harvested after a year due to its perennial growth. Average SS production is in the range of 31.25 - 37.5 ton ha^{-1} and the average sugarcane production is 56.25 - 62.50 ton ha^{-1}. SS requires only 4 months to grow in India and it needs 8000 m^3 over two crops of water for its cultivation[7,8,9] whereas sugarcane requires 12-16 months for its growth and need minimum of 3600 m^3 $crop^{-1}$ of water for its

cultivation. Availability of reducing sugars in the SS biomass can prevent the process of crystallization and it has the potent for 90% fermentation efficiency[10].

The main composition of SS stalk are the polymers of carbohydrates especially cellulose, hemicellulose and lignin. Few researchers were focussed on the study of chemical composition, structural and morphological characteristics of the hemicelluloses in SS stalk. Hemicelluloses are heterogenous form of polysaccharides present in the cell wall of the plant which is connected with cellulose through hydrogen bonds. It is linked with lignin by covalent bonding of α- benzyl ether linkages. In Gramineae cell walls, it is connected with hydroxycinnamic acids and acetyl units through ester linkages[11]. These bonds mainly hinder the release of hemicelluloses from the matrix of the cell wall structure. Hence, the cell wall matrix must be disturbed for the effective degradation of hemicelluloses using pretreatment methods and made the substrate, easily utilizable by the microorganisms. In the current scenario, the generation of bioenergy from lignocellulosic substrates can be achieved through different stages such as study of pretreatment, enzymatic hydrolysis of lignocellulosic biomass proceeded by the process of fermentation. The initial step in bioenergy synthesis from the complex substrates is through the gateway of pretreatment strategy that exposes the internal structure of the matrix for further enzymatic attack by the microorganisms. Among the different pretreatment methods, dilute acid pretreatment is much focussed due to its ease availability, capability of hydrolyzing hemicelluloses[12] and simultaneously breaks out the network of lignin polysaccharides to facilitate the conversion of hemicellulose and cellulose[13]. The pretreatment using dilute acid can be helpful in mitigating the structure of lignin, reducing the crystallinity of cellulose which leads to enlargement in its surface area and pore value and thus results in accessing the cellulose for further enzymatic digestion by the microorganisms that synthesizes cellulases[14].

Research on dilute acid pretreatment of different lignocellulosic biomass such as corn stover and grain have been carried out by the researchers. Few literatures have reported on the study of dilute acid pretreatment on SS stalk[15]. Hence, the understanding of dilute acid pretreatment on SS biomass becomes necessary. Most of the literature has mainly focussed on the fermentation inhibitors synthesized during hydrolysis process[15] whereas the monomer yield is an important factor to be considered for effective fermentation process. The components in hydrolysate are diverse due to the lignocellulosic complex substrates. Depending on the severity of pretreatment conditions of different acids, the monomer yield may vary. Hence, optimum concentration of different acids for its hydrolysis process becomes necessary to obtain higher yield of monosaccharides and low byproducts formation. Thus, the hydrolysate could be the rich carbohydrate source utilized for the microbial growth during the course of fermentation in bioethanol and biohydrogen synthesis.

In this study, the total sugars and cellulose content in the SS stalk were estimated. The concentration of different simple sugars present in the hydrolysate after different acids pretreatment at different concentrations was analyzed using High-performance Liquid Chromatography (HPLC). To reveal out the structural and morphological characteristics, the untreated and pretreated SS stalk were characterized using Fourier Transform Infra red Spectroscopy (FT- IR) and Scanning Electron Microscopy (SEM) respectively. This study provides a thorough knowledge on SCB and SS stalk characteristics before and after pretreatment processes.

Experimental

Acid hydrolysis of SS stalk

Three months grown sorghum stalks were harvested and the leaves were stripped off from the stem, chopped into a size of 20 cm and stored at -20 °C for further use. The stalks were coarsely grounded to a size of 1-2 mm using laboratory grinder and the free sugars present in the stalk were extracted using boiled distilled water at 100 °C for one hour. After the extraction process, a solid fraction obtained was air dried in oven at 100 °C. After drying the solid fraction, it was powdered using mixer grinder. The powdered SS stalk was pretreated with its respective acids such as HCl and H_2SO_4 at different concentrations ranging from 0.5 % (v/v) to 3% (v/v) for 60 min at 37 °C. After hydrolysis, the solid residue of the SS stalk was extracted using thin layer cloth[16]. Dried, pretreated SS stalk was used for structural and morphological characterization. The hydrolysate obtained after pretreatment was used for HPLC analysis.

Estimation of total sugars and total cellulose

Total sugars present in the extract of SS stalk before pretreatment were estimated using DNSA method[17]. Total cellulose content of untreated SS stalk were estimated using standard procedure[18].

Analysis of simple sugars in the hydrolysate using HPLC

Reducing sugars present in the hydrolysate of SS stalk was estimated by using HPLC (Shimadzu LC prominence). The composition of different reducing sugars such as glucose, xylose and arabinose present in SS stalk hydrolysate was estimated using HPLC with Zorbox carbohydrate column in the flow rate of 2 mL min^{-1} at 30 ℃ using Shimadzu refractive index detector. HPLC grade acetonitrile and water were used as mobile phase.

Structural characterization using FT-IR

The pretreated SS stalk were subjected to FT-IR analysis to study the structural changes of cellulose, hemicellulose and lignin binding before and after the pretreatment process. 2 mg of the powdered sample was mixed with 200 mg of spectroscopy grade KBr. Spectral measurements were carried out in the wave number range of 400 - 4000 cm^{-1} with a detectable resolution of 4 cm^{-1} and 32 scans per sample.

Morphological characterization using SEM

SEM analysis of the untreated and pretreated lignocellulosic substrates was carried out to predict the microstructure of biomass using JEOL JSM- 6610V SEM from 1.5K to 3K. Sample was mounted on conductive adhesive tape and observed under a voltage range between 15 to 20 kV.

Results and Discussion

Estimation of total sugars and cellulose

Simple sugar extraction from the SS stalk plays an important role in fermentation. The effective and feasible method for sugar extraction in SS stalk is by using distilled water[19]. Thus, SS stalks was finely grounded using distilled water at 100 ℃ and kept undisturbed for one hour to extract the simple sugars present in the stalk. After extraction, the raffinate and filtrate were separated using laboratory filter. Filtrate obtained after filtration was used for total sugars estimation. Total sugars present in the filtrate was 8.180 g L^{-1}. The result obtained from this study was matched with 9.2 g L^{-1} of total sugars in SS stalk[19]. Cellulose present in the SS stalk residue was estimated. 0.41 g g^{-1} of cellulose was present in the SS stalk residue.

Estimation of different sugars in the hydrolysate using HPLC

The concentration of different sugars such as glucose, xylose and arabinose in the pretreated SS stalk were estimated using HPLC. Based on the standard value, the different sugars and its concentrations were analyzed. The different sugar concentrations in the hydrolysate after H_2SO_4 pretreatment were estimated and it is shown in Table 1.

Table 1 Composition of different sugars in SS hydrolysate after different concentrations of H_2SO_4 pretreatment

Different H_2SO_4 concentrations (%) (v/v)	Concentration (g L^{-1})			
	Glucose	Xylose	Arabinose	Total sugars
0.5	10.68	11.25	2.28	24.21
1.0	14.74	15.12	2.96	32.82
1.5	12.85	13.65	2.24	28.74
2.0	9.25	8.45	1.32	19.02
3.0	7.85	5.24	1.91	15.00

*Mean value of the three runs was taken as results. Standard deviation was below 5%

Table 1 shows the composition of SS hemicellulose hydrolysate obtained after 60 minutes of pretreatment at different H_2SO_4 concentrations. The different H_2SO_4 concentrations such as 0.5%, 1%, 1.5%, 2.0% and 3% (v/v) were used to pretreat the SS stalk at 37℃ for 60 min. The hydrolysate obtained after different concentrations of H_2SO_4 pretreatment was underwent to analyze the different sugar concentrations such as glucose, arabinose and xylose and the results are tabulated in Table 1. The main sugars released after pretreatment were glucose and xylose and the least proportion of arabinose was formed during hydrolysis.

Most of the microorganisms can utilize the carbohydrate source in the form of simple hexose sugars. The derivation of glucose can be obtained either due to homopolymers of the cellulosic fraction or due to some of the heteropolymers of the hemicellulosic fraction[20]. Highest glucose concentration of 14.74 g L^{-1} was obtained during the hydrolysis of SS stalk using 1% H_2SO_4 with a retention time of 60 min (Table 1). When the acid concentration increased from 0.5 to 1.0% (v/v), a marked increase in glucose concentration observed from 10.68 g L^{-1} to 14.74 g L^{-1} respectively. Further increase in acid concentrations ranging from 1% to 3% (v/v), the glucose concentration was decreased gradually.

Another major sugar present in SS stalk hydrolysate after hydrolysis is xylose in equivalent to glucose. The highest xylose concentration of 15.12 g L^{-1} was obtained at 1% (v/v) H_2SO_4 pretreatment with a retention time of 60 min. Xylose concentration was little higher than the glucose concentration during 0.5, 1 and 1.5% (v/v) acid pretreatment. Next to glucose and xylose, arabinose is the sugar obtained during hydrolysis. Arabinose is produced either due to hydrolysis of some heteropolymers present in hemicellulosic fractions or due to arabinoxylans. Xylose is the major component present in arabinoxylans rather than arabinose. The highest concentration of 2.96 g L^{-1} of arabinose obtained at 1% (v/v) H_2SO_4 pretreatment. Compared to glucose and xylose, arabinose was in the least proportion and it was in the range of 1.32- 2.96 g L^{-1} (Table 1).

Pretreatment using dilute acid mainly helps in the breakdown of strong chemical bonds in the cellulose resulting in the formation of hydrolyzed products especially simple sugars which can be easily utilized by the microorganism[21]. Higher acid concentration resulted in the conversion of generated simple sugars such as glucose, xylose and arabinose into acetic acid and furfural[21]. Furfural and 5-hydroxymethyl furfural (HMF) are produced under the action of excess H_2SO_4 concentration especially by the degradation of pentose and hexose such as xylose. Furfural and HMF inhibit the growth of the microorganisms by diminishing its biological and enzymatic activities, through hindering the process of transcription and translation by reducing the synthesis of DNA, RNA and proteins[22]. Limited literature reports have been available on the susceptability of furfural and HMF towards microorganism[23]. Increase in H_2SO_4 concentration increases the furfural synthesis[24].

Acetic acid, an inhibitory compound formed due to hydrolysis of acetyl group of the hemicellulosic fraction[21]. Increase in H_2SO_4 concentration increases the formation of acetic acid in the hydrolysate[25]. Acetic acid concentration in the range of 4 - 10 g L^{-1} inhibit the microbial growth by percolating through the cell membrane and reducing its intracellular pH which further disturbs the cell metabolic activities[21,25]. Thus, excess H_2SO_4 concentration must not be preferred for SS stalk pretreatment to reduce the adverse effects of acetic acid and furfural synthesis.

Table 2 Composition of different sugars in SS hydrolysate after different concentrations of HCl pretreatment

Different HCl concentrations (%) (v/v)	Concentration (g L^{-1})			
	Glucose	**Xylose**	**Arabinose**	**Total sugars**
0.5	11.65	12.23	2.96	26.84
1.0	17.74	19.12	4.96	41.82
1.5	13.85	14.65	3.45	31.95
2.0	10.99	11.62	2.32	24.93
3.0	9.85	8.24	1.98	20.07

* Mean value was taken as results. Standard deviation was below 4%.

The composition of different sugars in the SS stalk hydrolysate after different concentrations of HCl pretreatment are tabulated in Table 2. Main simple sugars present in SS stalk hydrolysate after HCl pretreatment were glucose, xylose and arabinose as like the hydrolysate obtained after H_2SO_4 pretreatment. Compared to glucose yield in the SS stalk after H_2SO_4 pretreatment, the highest glucose yield of 17.74 g L^{-1} was obtained at 1% (v/v) HCl pretreatment. Next to glucose, xylose and arabinose are the major sugars present in the SS stalk hydrolysate. Among the different simple sugars obtained after pretreatment, arabinose is in least proportion in the range of 2-4 g L^{-1} as like H_2SO_4 pretreatment.

Compared to the yield of glucose, yield of xylose was higher in all the concentrations studied except at 3% (v/v) HCl pretreatment. Maximum simple sugars yield of 41.82 g L^{-1} was obtained after 1% (v/v) HCl pretreatment. Compared to simple sugars obtained after pretreatment, there was 80% lesser free sugars were present before the pretreatment process. At higher acid concentrations, the presence of least proportion of simple sugars is mainly due to the reduction of simple sugars to further smaller compounds such as acetic acids,

furfurals and HMF. Thus, 1% (v/v) acid concentration has more simple sugar and which was considered as optimum one (Table 2).

Structural characterization using FT-IR analysis

Pretreatment of SS stalk augments the digestibility of the lignocellulosic material and converts the complex structure of cellulose and hemicellulose into simple utilizable substrate for microorganism. Dilute acid pretreatment on lignocellulosic material alters the crystalline nature of the cellulose structure by expanding the surface area of the biomass, allowing the water penetration into the crystalline structure thus improving the solubilization of biomass which results in increasing the production of fermentable sugars by the process of fermentation. After extraction process, pretreatment of biomass was carried out using two different acids such as 1% (v/v) hydrochloric acid (HCl) and 1% (v/v) sulphuric acid. After pretreatment, the biomass was thoroughly washed with distilled water to obstruct the process and to neutralize the pH which in turn used for the structural characterization.

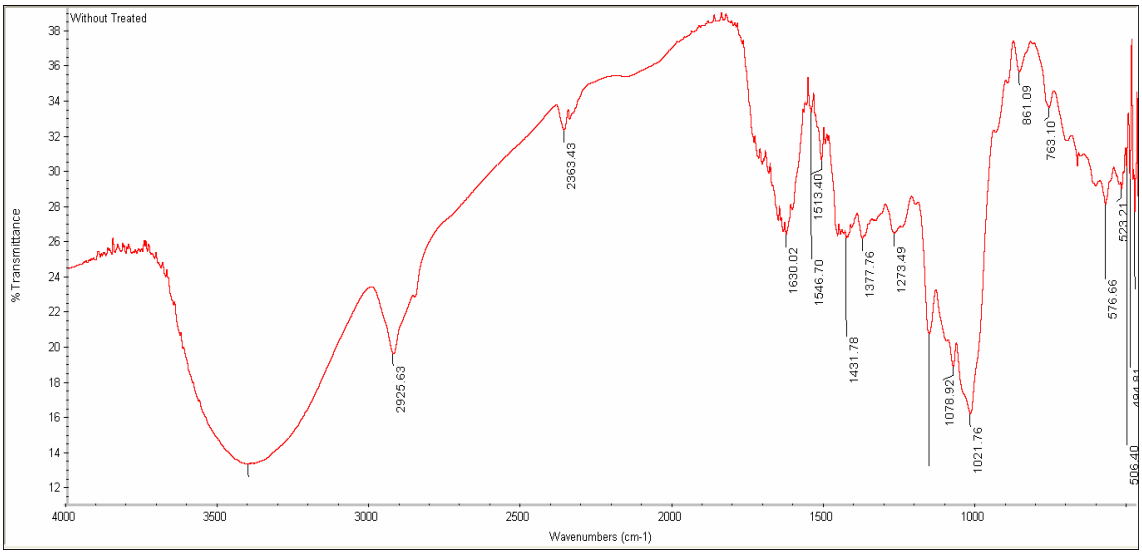
Table 3 Results interpretation of the structural characterization of SS stalk using FT-IR before and after pretreatment

Wavenumbers (cm^{-1})	SS stalk biomass			Assignment	References
	Untreated	Pretreated with 1% HCl	Pretreated with 1% H_2SO_4		
900-895	Y	N	N	Antisymmetric out- of plane ring stretch of amorphous cellulose	[28,29,34,36]
1033-1026	Y	Y	Y	C-H deformation in guiacyl, C-O deformation in primary alcohol	[29,33,34]
1100-1098	Y	Y	Y	Antisymmetric in phase ring stretch of cellulose	[34,36]
1202-1198	Y	N	N	O-H in plane deformation	[34,36]
1239-1232	Y	Y	Y	O-H in plane deformation	[29,34]
1273-1267	Y	Y	Y	C-O of guaiacyl ring and C-O stretching	[29,34]
1337-1329	Y	N	N	O-H in plane deformation, Syringyl ring breathing deformation	[29,33,34]
1423-1417	Y	N	N	C-H deformation (asymmetric) of cellulose, C-H bonds of the methoxyl groups	[33]
1450	Y	N	N	C-H deformation (asymmetric) of cellulose	[33]
1512-1509	Y	N	N	C=C (related to lignin removal) guaiacyl ring of lignin	[29,31-33]
1605-1590	Y	N	N	Aromatic skeletal vibration + C=O stretching (related to lignin removal)	[28,29,33]
1650-1633	Y	Y	Y	Absorbed water in cellulose, C=O with intramolecular hydrogen bond	[27,29,34,36]
1740-1735	Y	N	N	C=O ester; strong carbonyl groups in branched hemicellulose	[27-30]
2905-2850	Y	N	N	C- H stretching (indicates rupture of methyl/ methylene group of cellulose	[29,34]
3400-3200	Y	Y	Y	O-H stretching vibrations of hemicelluloses (indicates break of hydrogen bonds in cellulose)	[29,33,34]

Table 3 interprets the structural characterization of SS stalk before and after pretreatment. To study the conformational and physiochemical properties of carbohydrates, infra red spectroscopy has been widely used. FT- IR spectra show several absorption bands specified to major structural components such as hemicellulose, cellulose and lignin. In the region between 4000- 900 cm^{-1}, band at 3300 cm^{-1} corresponds to O-H group stretching of hemicelluloses due to the breakdown of hydrogen bond in celluloses was appeared in all the spectral results. The band in the range of 2905-2850 cm^{-1} indicated the C- H stretching including the rupture of methyl/ methylene group of cellulose was appeared in the untreated spectral sample (Fig. 1A). The absorption band in the range of 1650-1633 cm^{-1} was appeared in all the untreated and pretreated spectra samples (Fig. 1A-C). This indicated the absorbed water in cellulose and C=O with intramolecular hydrogen bonding. Cellulose have disordered structure in its solid state and thus it was easily hydrated due to its strong affinity towards water by forming intramolecular hydrogen bonding[26].

FT- IR spectra of untreated SS stalk and pretreated SS stalks showed only slight modifications. In all the untreated and treated spectra samples, bending modes of C-H is explicitly visible in the range from 1435-1431cm^{-1}. In comparison with the pretreated samples, there were no peaks observed at 1714, 1567, 1271, 1273, 904 in the untreated spectra sample (Fig. 1A). Stretching of C=O ester strong carbonyl group in hemicelluloses appeared at 1737cm^{-1} was obtained in untreated sample spectrum unlike the pretreated samples[27,28,29,30]. Spectra of untreated and 1% HCl pretreated samples showed similar spectral results. Absence of hemicellulose band after pretreatment indicated that hemicellulose was fully hydrolyzed (Fig. 1A-C).

The two types of rings present in the lignin structure are guaiacyl ring and syringyl ring. Bands appear in the range between 1514-1518 cm^{-1} is responsible for guaiacyl ring and the one which appear at 1435 cm^{-1} indicates the absorption of syringyl ring in lignin structure[29,31,32]. From the spectra results, it was observed that the band responsible for guaiacyl ring structure was appeared in all the samples but band corresponding to syringyl ring structure was not present. Lignin related bands were appeared at 1273, 1518, 1610 and 1715 cm^{-1} in all the samples[28,29,32]. C=C lignin band appeared at 1518 cm^{-1} which was clearly visible even after the pretreatment process. After pretreatment, band at 1715 cm^{-1} corresponding to C=O stretching of the phenyl ester side chains of the lignin structure was clearly seen in 1% H_2SO_4 treated SS stalk. After pretreatment, there were no changes in the lignin structure but the structure of hemicellulose has significant changes which were inferred through this study. Cellulose related bands were appeared at 904, 1381, 1435, 2927 and 3340 cm^{-1} in all the spectra samples[28,29,32,33,34]. Bands at 1633,1159,1112,1516,1158 and 901 cm^{-1} corresponding to O-H bending of adsorbed water, C-O-C stretching in pyranose rings and C=O stretching in aliphatic rings, ring assymmetric valence vibration, stretching of phenyl ring and C-O-C stretching at the β-(1-4)- glycosidic linkages were also appeared in all the spectra samples (Fig. 1A-C).



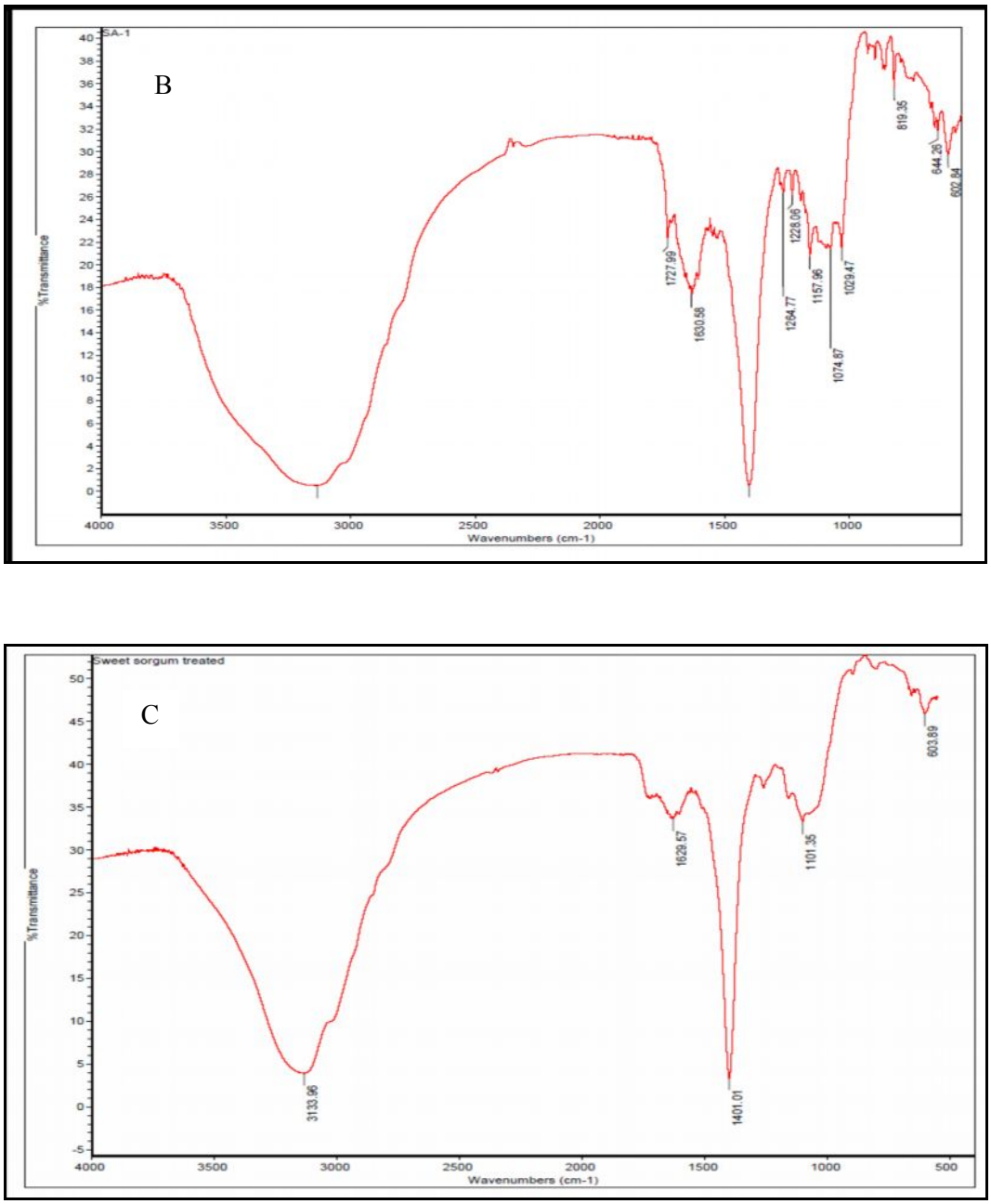


Fig. 1(A-C) FT-IR spectra of SS stalk in the wavenumber region between 4000-900 cm^{-1}. (A) Untreated SS stalk, (B) Pretreated SS stalk with 1% HCl and (C) Pretreated SS stalk with 1% H_2SO_4

Morphological analysis using SEM

Surface layer of the untreated dried powdered SS stalk contains softened surfaces of the waxes, cellulose and hemicelluloses and lignin. The other binding materials on the surface were removed during the grinding process and expose the interior region. Cellulose bundle is made up of various ordered microfibrils which are commonly represented as the backbone of the cell wall. In the untreated dried powdered SS stalk, the individual microfibrils have a diameter of 19 microns (Fig. 2a, 3a). In the untreated sample, the microfibrils length were much longer and in some of the fibers seems endless in its length. The pretreatment of SS stalk by the combination of the alkali and acid chemical pretreatment and extrusion process of thermo- mechanical improved the decrystallation rate and simple sugars yield[35]. The improvement is mainly due to the constant removal of the softened surface of the substrate by applying shear forces during extrusion mechanical process and thereby exposing the interior region of cellulose and hemicelluloses to the process of pretreatment by acid or alkali. Clean and softened surface were observed in SEM images of the acid pretreated SS stalks. The lengthier microfibrils structure of the biomass network was disrupted resulting in the presence of more holes on the annular rings and on the cellulose wall which were the internal structure of the biomass network. Due to this, the lengthier microfibrils before pretreatment were shortened and separated after pretreatment and are clearly observed in Fig. 2b, 2c, 3b and 3c. The results obtained in this study are clearly matched with the SEM

image patterns of corn stover. Thus, the interior surface of the biomass structure has been exposed and the bonds in the hemicellulose were easily disturbed after the pretreatment process.

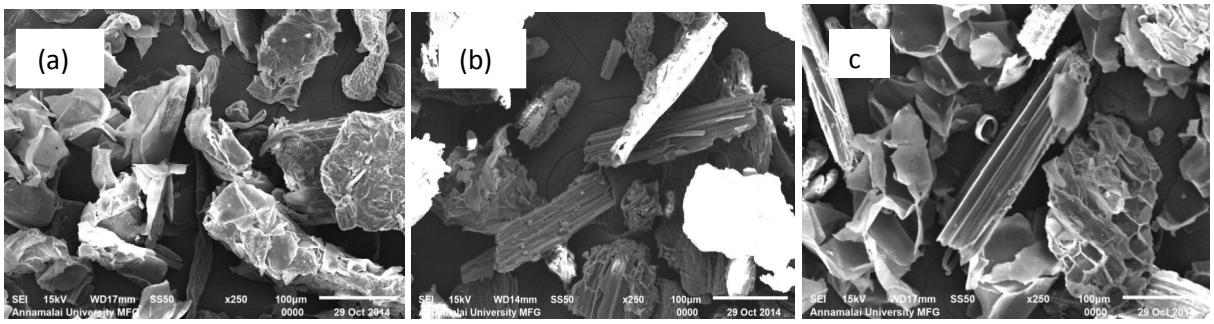


Fig. 2(a-c) SEM images of SS stalk at magnification (× 250) (a) Before pretreatment, (b) After pretreatment with 1% Sulphuric acid and (c) After pretreatment with 1% HCl

Morphological structural difference was explicit during different acid pretreatments. Shortened microfibril structure was clearly visible in SEM images of 1% HCl pretreatment rather than with 1% H_2SO_4 pretreatment (Fig. 2b, 2c, 3b and 3c). This indicates that the bonds in hemicellulose were easily break down during 1% HCl pretreatment (Fig. 2c and 3c).

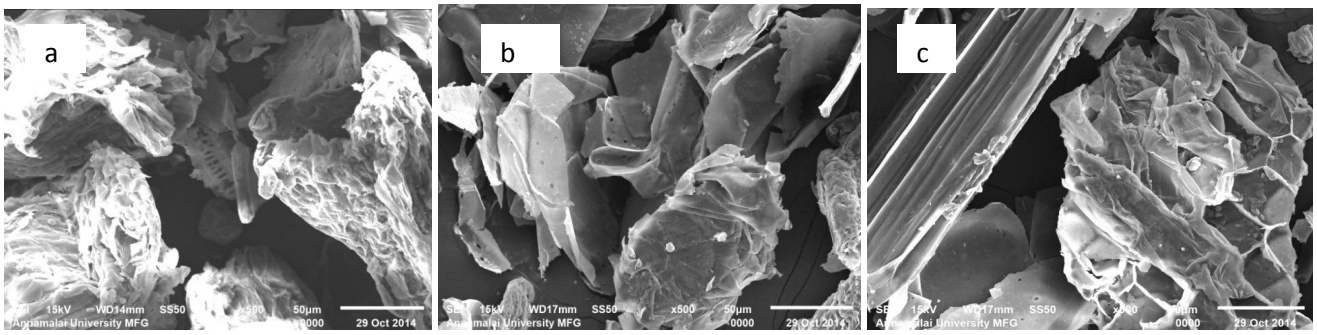


Fig. 3 (a-c) SEM images of SS stalk at magnification (× 500) (a) Before pretreatment, (b) After pretreatment with 1% Sulphuric acid and (c) After pretreatment with 1% HCl

Conclusion

Dilute acid pretreatment of SS stalk was optimized for maximizing the simple monomers yield in the SS stalk hydrolysate. The maximum sugar yield of 41.82 g L^{-1} was obtained at 1% (v/v) HCl pretreated SS stalk hydrolysate. FT- IR analysis showed that the two rings in the lignin structure was not completely cleaved whereas the hemicellulose was completely broken down resulting in the synthesis of free simple sugars. SEM micrographs revealed that the microfibrils become shortened which resulted in exposing the internal structure of the biomass network. This makes the process of an enzymatic treatment by the microorganisms using cellulases in an effective manner. Among the two different acids used to pretreat the SS stalk, HCl showed effective delignification and hydrolysis and obtained the highest monomer yield. All these salient observations revealed that the pretreatment of SS stalk using HCl could be effectively used for the efficient delignification and hydrolysis which finds useful in a broad range of industrial applications.

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