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ORIGINAL RESEARCH

Efficient conversion of cornstalk to bioethanol using dilute H_2SO_4 pretreatment

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Abstract

Corn stalk (CS) is one of the most abundant agricultural residues containing high polysaccharides for low-cost bioethanol production. In this study, dilute acid along with intensified thermal pretreatment of CS and other parameters were optimized for higher yield of bioethanol. CS samples were pretreated using H_2SO_4 concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5% at 100 °C for 1 h reaction time. Optimal conditions of 2% acid-pretreated CS, 5% (w/v) of *Saccharomyces cerevisiae* addition and 48 h fermentation produced highest yield of bioethanol: 32.53 (g/L) which was 1.24-fold increase. Hemicellulose degradation of 75.68% was recorded in the 2% acid-treated substrate. Scanning electron microscope (SEM) images revealed induced porosity and surface area disruption of CS in the treated samples. Crystallinity of the treated samples increased as shown by X-ray diffraction (XRD) analysis. Low concentrated H_2SO_4 coupled with thermal pretreatment could be a viable method of lignocellulosic biomass utilization for efficient bioethanol production.

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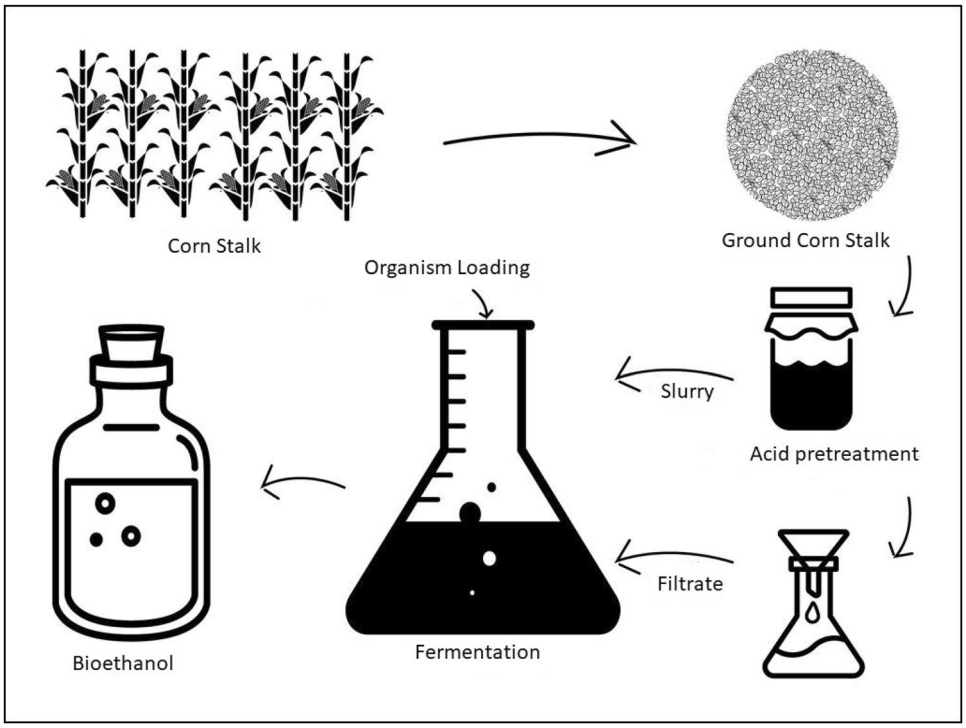
Graphic abstract



Keywords Bioethanol · Acid pretreatment · Biofuel · Corn stalk · Fermentation

Introduction

The industrial development occurring globally has created a surge in the demand for fossil fuels and its derivatives. This demand cuts across lots of sectors such as transportation, manufacturing and urban development, thus making energy demand a pressing issue in the twenty-first century [1]. Fuels from fossil sources are non-renewable energy and a source of environmental pollutants. Consequently, increasing their day by day utilization causes increase in the level of pollution and deteriorates public health. This makes it imperative to find alternative source of energy that is renewable and possess less environmental concerns [2]. Agricultural wastes and biomass have been shown to fit into this scenario and act as a source of renewable feedstock for the production of biofuels [3]. One of the most important biofuels produced from biomass is bioethanol.

Worldwide, bioethanol is a liquid biofuel produced from biological materials and prominently used in the transportation sector [4]. As an alternative fuel, bioethanol could replace the use of petroleum fuel to reduce air pollution and ultimately cut down greenhouse gas emission [5]. Complete combustion property of bioethanol is a significant advantage over fossil fuels. Bioethanol contains 35% oxygen and thereby releases less hydrocarbon, carbon monoxide and particulate matters during combustion. Using 10% ethanol blends with gasoline could reduce greenhouse gas emission by 12–19% compared to conventional fossil fuel [1]. Another advantage of bioethanol as an alternative fuel is its high octane number compared to non-renewable petroleum-based gasoline [6].

Generally, bioethanol produced from non-edible lignocellulosic biomass is referred to as "second-generation" bioethanol. This is of great advantage as it circumvents the stress placed on the food chain by first-generation biofuels that are directly produced from sugar and starchy materials [1]. Among the different lignocellulosic biomass usable for bioethanol production, corn stalk is a valuable agricultural waste annually produced in large amount [7]. The robust cell structure of corn stalks contains cellulose, hemicellulose and lignin which contribute to its recalcitrant nature like other lignocellulosic biomass [8]. This makes pretreatment a prerequisite for the conversion of lignocellulosic materials, as it solubilizes some of the components and releases the necessary chemicals for fermentation and product formation [9].

Numerous studies have utilized an array of pretreatment methods on lignocellulosic materials for bioethanol production in fermentation processes [10–12]. In some of

these studies, the authors go through a pattern of pretreatment with acid/alkali, followed by washing. Although this washing is aimed at removal of inhibitors, it is believed that sugars released during pretreatment are lost during washing [13]. Another disadvantage of this process is the requirement for excessive utilization of water which is not sustainable for large-scale production [13].

The present study was set up to valorize the slurry from acid-pretreated corn stalk for bioethanol production. Various acid pretreatment concentrations and some fermentation conditions were studied to determine the effective conditions for enhanced bioethanol production.

Materials and methods

Microorganism and chemicals

All chemicals used for this study were of analytical grade. Absolute ethanol, hydrochloric acid, sulfuric acid 5-hydroxymethylfurfural (5-HMF) and furfural were purchased from Sigma-Aldrich. Disodium ethylenediaminetetraacetate, sodium borate decahydrate, disodium phosphate anhydrous, sodium dodecyl sulfate, ethylene glycol diethyl ether, potassium di-hydrogen phosphate, ammonium sulfate heptahydrate, magnesium sulfate, anthrone and potassium dichromate were purchased from MerkKGaA (Germany). Commercially procured baker's yeast, *Saccharomyces cerevisiae*, was used as the organism for the fermentation process.

Raw materials collection and sample preparation

CS was collected from an agricultural farm in Dhamrai, near Dhaka, Bangladesh. After sample collection, debris and dust particles were removed by washing with distilled water. The samples were oven dried and crushed with laboratory milling machine to particle size of 2 mm. The materials were stored in air tight containers at room temperature till further use.

Dilute acid (DA) pretreatment of CS

5 g of the solid material for pretreatment was weighed and placed in 250 mL flask. Sulfuric acid pretreatment of CS was carried out at different concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5%. In each case, 120 mL of the diluted acid was added to flasks containing pre-weighed CS. Equal mass of CS was treated with distilled water to serve as control. The 250 mL Erlenmeyer flasks containing the mixture were left standing for 1 h at room temperature. Thereafter, the flasks were heated at 100 °C for 1 h by using a shaker water bath (LABTECH Blue water bath incubator-shaker). The slurry was further incubated at 70 °C for 72 h.

Fermentation and optimization

Fermentation medium containing (g/L) KH_2PO_4—0.75, $(NH4)_2SO_4$—0.15, $MgSO_4$—0.25, yeast extract—9.0 was used as supplement in this study. To determine the effect of acid pretreatment concentration on CS for bioethanol production, the pretreated slurry was supplemented with the media described above. The pH of the aliquot was adjusted to 5 and autoclaved at 121 °C for 15 min. Each fermentation flask was inoculated with 1% (w/v) *Saccharomyces cerevisiae* under aseptic condition and incubated at 30 °C in the dark under static mode. To monitor the effect of filtration of acid-pretreated CS slurry on bioethanol production, slurry of the 2% H_2SO_4-pretreated CS was filtered through a Buchner funnel which was coupled to a vacuum pump. The filtrate was supplemented with the same media described above, autoclaved and inoculated with 1% (w/v) organism. The effect of inoculum size on bioethanol production was carried out by using the media-supplemented-filtrate of 2% pretreated CS. This medium was inoculated with organisms at 1, 2, 3, 4 and 5% w/v. In all cases, pH adjustment, media sterilization and incubation condition followed the same protocol described in the first experiment. All experiments were conducted in triplicate.

Analytical methods

Fermented samples were withdrawn from each flask at regular time interval and centrifuged at 5000 rpm for 5 min. The residual sugar and bioethanol concentration in the supernatant were estimated spectrophotometrically by using a spectrophotometer (Spectroscopy GBC central 2020). Sugar concentration was determined with anthrone reagent (anthrone-sulfuric acid assay, a green color complex), absorbance was read at 600 nm [14]. Acidified potassium dichromate solution (0.1 M $K_2Cr_2O_7$ in 5 M H_2SO_4) was used to determine bioethanol via the oxidation of ethanol to ethanoic acid. The orange color of dichromate turns to green and absorbance was measured at 600 nm [15].

Inhibitors, furfural and 5-HMF, were determined by spectrophotometer GBC central 2020. Furfural and 5-HMF concentrations in the pretreated CS and control samples were determined at 277 and 285 nm, respectively [16].

Chemical composition analysis of CS

Chemical composition (cellulose, hemicellulose and lignin) of CS samples was determined using detergent fiber analysis method. Neutral detergent was added to remove extractives and the remaining residue, neutral detergent fiber (NDF),

was digested with acid detergent reagent (2 M HCl) to quantify hemicellulose. 72% H_2SO_4 was used to digest the acid detergent fiber (ADF) and residual fiber was used to determine the cellulose content. Dried ADF was placed in a muffle furnace at 550 °C for 4 h and the weight loss was used to calculate the percentage of lignin content [17].

Micro image characterization of the surface structure of CS

The morphological alteration of raw (untreated), control (pretreated with water at 100 °C) and acid-treated (2% H_2SO_4, at 100 °C) corn stalk was observed using scanning electron microscopy (SEM model; EVO18, Carl Zeiss, UK). The samples were dried prior to analysis, then fixed into aluminum stubs using carbon tape and coated with a gold layer, with voltage EHT maintained at 5.0 kV and WD at 10.5 mm. All the images were captured within 1.00 kx magnification. X-ray diffraction (XRD) analysis was carried out to determine the crystallinity index of the treated and untreated corn stalk using XRD Instrument, Bruker Germany, Model D8 Advance. All samples analyzed were at 2θ, voltage 40 V and current 40 mA.

Results and discussion

Effect of acid concentration on CS for bioethanol production using *saccharomyces cerevisiae*

The optimization of dilute H_2SO_4 pretreatment concentration for efficient hydrolysis of CS was conducted at varied concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5%. At different acid concentrations, the production of bioethanol sharply increased up to 24 h and reached a maximum level at 48 h. Figure 1 shows that production of bioethanol by *S. cerevisiae* increased with increasing acid concentration from 0.5 to 2%. These pretreatment concentrations led to bioethanol yields of 16.91, 18.72, 20.62 and 21.85 g/L for 0.5, 1.0, 1.5 and 2.0% acid concentration, respectively. Further increase in the acid pretreatment concentration to 2.5% yielded lower concentration of bioethanol (19.77 g/L). The control sample produced bioethanol that was lower than those recorded in all the acid-pretreated CS (Fig. 1). The effects of different acid concentrations on the chemical compositions of the untreated and acid-pretreated corn stalk are shown in Table 1. Increase in the severity of the acid used for pretreatment led to degradation of the hemicellulose and increase in residual lignin and cellulose content.

Dilute sulfuric acid pretreatment can be utilized to solubilize hemicellulose to monomeric sugars and increase the biomass surface area [18]. Cellulose is mainly composed of hexose (glucose) and hemicellulose contains both pentoses

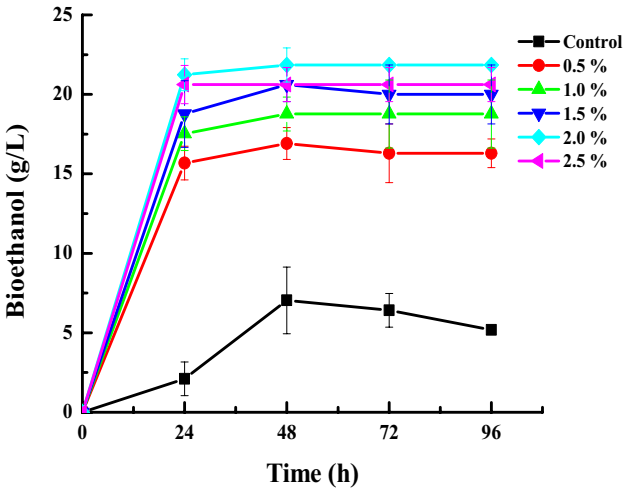


Fig. 1 Effect of acid concentration on bioethanol production using *Saccharomyces cerevisiae*

(xylose and arabinose) and hexoses (mannose, glucose, and galactose) [19]. These sugars can be utilized by the organism for metabolic activities and production of bioethanol during fermentation [20]. In this experiment, the reduction in bioethanol production was observed with increase in the severity of acid treatment; when the acid dose was increased from 2 to 2.5%. Severe acid concentration can easily degrade some of the fermentable sugars into inhibitory compounds, viz., 5-hydroxymethyl furfural (5-HMF) and furfural (Table 1) [21]. These inhibitors could be harmful to the microorganism's metabolic activity. They could retard cell growth and ultimately impede the bioethanol production during the fermentation process [22]. The optimal acid pretreatment concentration (2%) reported in this study could have been due to the organism's tolerance to the level of the inhibitors and the amount of sugars present in the pretreated slurry [23]. The optimal condition for acid pretreatment obtained in this study was similar to the previous report of enhanced bioethanol production from dilute H_2SO_4-pretreated sugarcane bagasse pith [24].

Effect of filtration of acid-pretreated CS slurry on bioethanol production using *Saccharomyces cerevisiae*

The experiment was carried out to determine the effect of using a filtrate of 2% sulfuric acid-treated CS for production of bioethanol. As shown in Fig. 2, the highest bioethanol concentration obtained from the filtrate media was 26.17 g/L at 48 h fermentation time. This represents 19.8% increase when compared to the highest value obtained from the slurry fermentation in the previous experiment under the same conditions. In the control experiment, the highest bioethanol produced from the slurry was 7.04 g/L

Table 1 Compositional analysis and the inhibitors in pretreated samples

	Compositional analysis (%)			Total sugar (g/L)	Inhibitors (mg/L)	
	Hemicellulose	Cellulose	Lignin		5—HMF	Furfural
Untreated	33.55 ± 0.76	38.70 ± 3.44	18.94 ± 2.98	15.16 ± 1.69	0.0611 ± 0.004	0.0508 ± 0.006
Control	29.30 ± 3.01	38.62 ± 1.85	19.74 ± 0.37	18.76 ± 1.25	0.1367 ± 0.021	0.1051 ± 0.078
0.5%	12.72 ± 1.25	39.76 ± 0.76	22.29 ± 1.07	46.98 ± 1.95	0.3094 ± 0.076	0.2157 ± 0.011
1.0%	9.94 ± 0.34	40.18 ± 1.06	25.46 ± 1.73	76.03 ± 4.79	0.3193 ± 0.096	0.2260 ± 0.017
1.5%	9.60 ± 0.79	41.76 ± 0.48	26.03 ± 1.20	78.80 ± 2.77	0.3317 ± 0.087	0.2514 ± 0.009
2.0%	8.16 ± 0.98	42.08 ± 2.41	30.59 ± 1.39	81.35 ± 3.86	0.3571 ± 0.016	0.2769 ± 0.018
2.5%	7.33 ± 0.59	46.50 ± 0.32	32.14 ± 0.75	80.64 ± 1.60	0.4809 ± 0.114	0.3898 ± 0.092

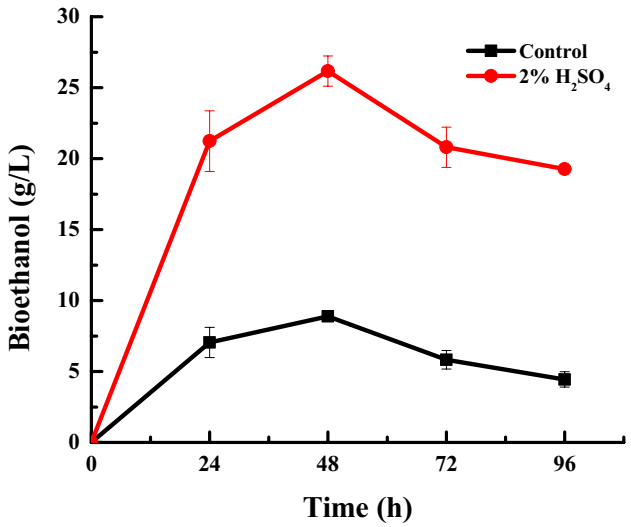


Fig. 2 Bioethanol production with filtrate of 2% acid-pretreated slurry

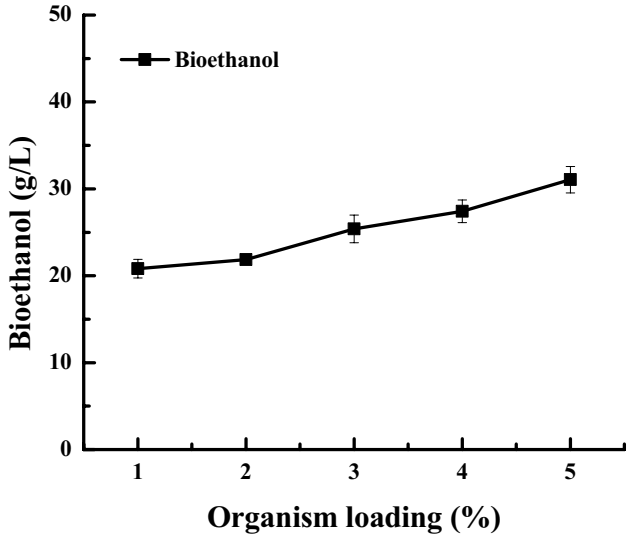


Fig. 3 Effect of inoculum size on the bioethanol yield

(Fig. 1) which increased to 8.88 g/L for filtrate condition (Fig. 2). These results show 26.13% increment in bioethanol production in the filtered control when compared to the control of the unfiltered slurry.

The efficiency of the fermentation process is dependent on a number of factors, of which the nature and composition of fermentation broth is one of them. The rheological characteristic of fermentation broth of residual slurry was primarily controlled by the biomass concentration which controls the magnitude of the internal friction in fermentation media. In flasks containing slurry, the viscosity of the fermentation broth increased due to agglomeration of biomass [25]. The higher viscosity in the slurry fermentation broth makes it non-Newtonian in nature and the negative impact of this is that it prohibited proper mixing, nutrient and mass transfers [26]. Removal of these prohibitions in the filtrated media improved accessibility of the organism to nutrient and enhanced secondary metabolite product formation.

Effect of inoculum size on bioethanol production using *Saccharomyces cerevisiae*

Increase in inoculum size of the organism used for fermentation had direct effect on the bioethanol yield in the fermentation media (Fig. 3). Varying the amount of yeast used for fermentation from 1 to 5% resulted in increased bioethanol concentration from 26.17 to 32.53 g/L. This connotes 1.24-fold increase in bioethanol production. Previous study had shown increase in bioethanol production with increase in the inoculum size of the organism used for fermentation [27].

In this study, the ultimate inoculum size used (5%) gave the highest bioethanol yield. Previous study by Li and coworker showed that *Saccharomyces cerevisiae* gave nearly the same amount of ethanol at 5% compared to 10% inoculum loading when steam-exploded corn stover hydrolysate was utilized for ethanol production [28].

Table 2 shows the comparison of ethanol production by fermentation of different biomasses using different chemical and physical pretreatment methods. For an efficient

Table 2 Bioethanol production from fermentation of lignocellulosic biomass using *S. cerevisiae* (strain) at optimized conditions

Lignocellulosic material	Pretreatment method	*Saccharomyces cerevisiae* strain	Fermentation		Ethanol (g/L)	References
			pH	Temperature (°C)		
Mission grass	NaOH	TISTR 5596	6	30	16	[31]
Rice husk	NaOH	MTCC 174	–	28	14	[32]
Corn stover	Steam explosion	Y5	4.8	35	50	[33]
Coffee pulp	H_2SO_4	Baker yeast	5	30	7.4	[5]
Corn stover	H_2SO_4	DQ1	5	40	48	[34]
Sugarcane leaves	H_2SO_4	TISTR 5596	4.5	30	4.71	[35]
Corn stover	Steam explosion	Y5	–	30	40	[28]
Cassava pulp	H_2SO_4	TISTR 5596	4.5	30	11.90	[36]
Empty palm fruit bunch fibers	NaOH	L2524a	5.2	30	62.5	[37]
Corn stalk	2% H_2SO_4	Baker yeast	5	30	32.53	This study

Table 3 Compositional analysis and bioethanol yield of the dilute acid-pretreated corn stalk under optimal conditions

Hemicellulose		Cellulose		Lignin		Ethanol	
Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Produced (g/L)	Yield (g/g)
33.55 ± 0.76	8.16 ± 0.98	38.70 ± 3.44	42.08 ± 2.41	18.94 ± 2.98	30.59 ± 1.39	32.53 ± 2.18	00.451

bioethanol production, environmentally friendly and less expensive pretreatment method should be considered to break down the chemical components of lignocellulosic materials [29]. The crucial steps (hydrolysis and fermentation) during the bioethanol production process is highly influenced by the use of suitable feedstock along with appropriate pretreatment method [30].

Effect of acid pretreatment on chemical composition of CS used for bioethanol production

The effect of acid pretreatment on the chemical constituents (cellulose, hemicellulose, and lignin) of corn stalk was determined before and after dilute acid pretreatment (Table 3).

Chemical composition analysis of the 2% dilute-acid-pretreated corn stalk compared to the untreated corn stalk shows 75.68% degradation in hemicelluloses content, whereas cellulose and lignin content increased by 8.73 and 61.51%, respectively (Table 3). These results are consistent with the previous report of cellulose and lignin content increase with corresponding hemicellulose removal after dilute sulfuric acid pretreatment of corn stalk and corn cob [7]. Li and co-worker reported increase in lignin and cellulose percentages of acid-pretreated lignocellulosic materials and concluded that the increments in their contents arose as a result of the predominance in the leftover material after degradation [8]. After acid treatment, the recovered fermentable sugars are digested by *Saccharomyces cerevisiae* to produce bioethanol [20].

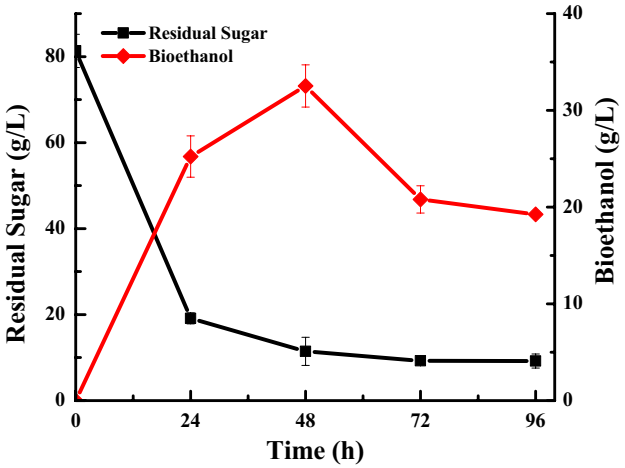


Fig. 4 Sugar consumption and bioethanol production by *Saccharomyces cerevisiae* at optimal fermentation condition

Final optimization condition for the fermentation of 2% acid-pretreated corn stalk filtrate was carried out at 30 °C for 48 h fermentation with 5% (w/v) inoculum. In this study, the initial concentration of total sugar was 81.35 ± 3.86 g/L and this sugar concentration finally reduced to 9.22 ± 1.63 g/L in the fermentation flask after 96 h fermentation period (Fig. 4). These results of bioethanol yield and sugar consumption by *Saccharomyces cerevisiae* are similar to those obtained from the same organism by its immobilization on luffa sponge discs [38]. Generally, organisms tend to utilize

available nutrients in fermentation media to synthesize the growth factors, increase their cell mass and produce secondary metabolites; substantially based on available nutrients [23, 39].

Effect of acid pretreatment on microstructural changes of CS used for bioethanol production using *Saccharomyces cerevisiae*

To compare the morphological changes which occurred in corn stalk as a result of the pretreatment, samples of the untreated and pretreated corn stalk were subjected to SEM analysis. Figure 5a–c shows the surface structure of raw, control (water pretreated, at 100 °C) and acid-treated (2% H_2SO_4, at 100 °C) corn stalk, respectively. Figure 5a shows the SEM image of raw corn stalk with rough, amorphous and compact structure [40]. As shown in Fig. 5b (control) compared with the untreated samples, no significant changes were observed in hot water-treated corn stalks except for the observation of larger surface area which might have occurred due to minimal change that is related to low efficient removal of hemicellulose [41]. A more fractured and less compact surface structure with numerous pores was observed in the acid-pretreated samples (Fig. 5c). Predominantly, dilute sulfuric acid works on plant cells by breaking down its compact structure and causes the biomass to lose the fibrous connection [42]. Figure S1 shows loss of intermolecular bonds in pretreated CS compared to the untreated samples [43, 44]. Dilute acid pretreatment can easily solubilize hemicellulose fraction, develop porosity and increase the surface area of the biomass. Increasing cellulose accessibility is an inevitable factor for efficient conversion of lignocellulosic biomass to fermentable sugars [45].

The degree of crystallinity of the three categories of CS used in this study was determined by X-ray diffraction analysis. XRD analysis showed all samples displayed two distinct peaks: at $2\theta \approx 18°$ and at $2\theta \approx 22°$, which are the respective signatures of the amorphous and crystalline components of the CS samples (Fig. 6). The spectra revealed that the crystallinity of cellulose increased in the 2% acid-pretreated CS when compared to the control. Customarily, lignocellulosic materials are made of both amorphous and crystalline cellulose. Acid pretreatment digested the amorphous cellulose and hemicelluloses, leaving behind the crystalline cellulose portion which explains the increase in the degree of crystallinity. Dilute sulfuric acid pretreatment of lignocellulosic materials can be utilized to solubilize hemicellulose, break the amorphous connection, increase biomass surface area, porosity and crystallinity index of the pretreated material [18].

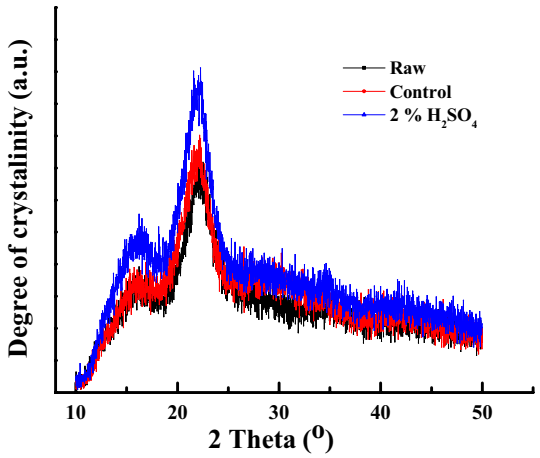


Fig. 6 X-ray diffraction analysis of the untreated corn stalk, control (water-treated corn stalk) and 2% acid-pretreated corn stalk

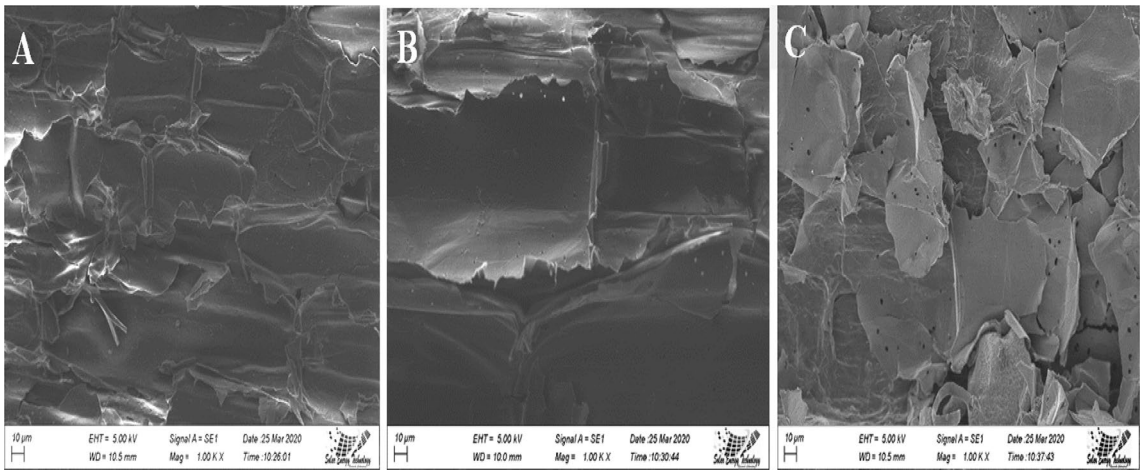


Fig. 5 Scanning electron microscope (SEM) images: **a** untreated corn stalk; **b** control (water-treated corn stalk), **c** 2% acid-pretreated corn stalk

Conclusion

This study investigated the application of dilute sulfuric acid and thermal pretreatment for enhanced bioethanol production from corn stalk. Acid pretreatment of CS with 2% H_2SO_4, followed by fermentation process using *Saccharomyces cerevisiae* yielded 21.85 g/L bioethanol concentration in the fermented slurry. Bioethanol yield was improved by 1.24-fold due to optimization strategies of using the media-supplemented filtrate of acid-treated slurry and 5% inoculum size. Utilization of cheap and abundant lignocellulosic materials for biochemical production is one of the ways of reducing its production cost and encouraging its availability. Low cost production of this biochemical is one of the ways to make it appealing to governments and private investors to embrace bioethanol as a green fuel to avoid the adverse effects of fossil fuels. Developing this process to industrial scale will boost the production and availability of this important biochemical.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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