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Enhancing Enzymatic Hydrolysis and Delignification of Sugarcane Bagasse Using Different Concentrations of Sodium Alkaline Pretreatment

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ABSTRACT

Lignin, being highly resistant, needs to be eliminated in the process of extraction of soluble reducing sugar and bioethanol production from lignocellulosic biomass. In the present work, pretreatment of sugarcane bagasse (SCB) was performed using NaOH of various concentrations (1-5%) to facilitate delignification. The hydrolysis efficiency of pretreated SCB was evaluated at different reaction times by the production of reducing sugar using the Cellic CTec2 enzyme. The maximum cellulose content of 57.6% and lignin removal of 62.04% were observed with 2% sodium hydroxide at 121°C autoclaved for 60 min. The hemicellulose content decreased with increasing NaOH concentration with the maximum decrease of 13.6% from native bagasse having 26.5% xylan content. The microstructure, morphology, and chemical composition of SCB were analyzed using Field Emission Scanning Electron Microscopy (FESEM), Fourier Transform InfraRed (FTIR), and XRD. The hydrolysis with 10 FPU.g^{-1} of enzyme at 48 h of reaction time shows a maximum yield of 12.34 g.L^{-1} corresponding to 55.53 ± 0.45% at 2% NaOH pretreated SCB. This study claims that lignin components exhibited the highest susceptibility to NaOH pretreatment, which directly affects enzymatic hydrolysis.

INTRODUCTION

While first-generation ethanol production from starchy edible grain materials has advanced well, second-generation ethanol production from lignocellulosic biomass is still considering its economic viability. The potential sources of bioethanol production include agricultural and wood processing waste. Lignocellulose feedstock is a complex matrix that is degradation-resistant and provides hydrolytic stabilization and compositional robustness. This is mainly because cellulose, hemicellulose polysaccharides, and lignin are linked together through ester and ether bonds (Sharma et al. 2022, Madadi et al. 2023).

Lignin is present in wood plant tissue as deposited cell walls around cellulose and hemicellulose. It might include 15-20% of the dry matter in the entire feedstock. Lignin is a complex polymer and aromatic matrix containing phenolic compounds along with different types of functional groups like carbonyl, hydroxyl, and methoxyl (Wunna et al. 2017). Lignin's recalcitrant nature became one of the key obstacles to the manufacture of bioethanol economically. It must be removed so that a larger amount of sugar can be extracted from lignocellulosic feedstock, or bioethanol can be produced from them. Sugarcane is the main bioenergy crop with higher production. Brazil ranked first, which has been successfully exploited to make bioethanol. Sugarcane bagasse contains 36-46% of cellulose as the most abundant component, hemicellulose as the second highest with an amount between 15.91-28.7%, lignin composition is between 9.8-26.2%, and a slight amount of pectin (Wunna et al. 2021, Periyasamy et al. 2022).

Several pretreatment strategies can be used depending on the high yield of sugar and fermentable ethanol production through hydrolysis and fermentation procedures. Pretreatment can reduce the level of polymerization and polysaccharide crystallinity, as well as modify the molecular structure of biomass feedstock to increase its accessibility for enzymes. These techniques can be divided into biological, physical, chemical (involving acid and alkaline methods), and physicochemical methods of pretreatment. Compared to different pretreatment techniques, alkali pretreatment is used extensively during biomass pretreatment and is considered efficacious (Kininge & Gogate 2022). It increases the lignin removal percentage and enhances the cellulose yield in pretreated solid residue, which leads to easier saccharification to obtain a greater quantity of

glucose. Alkali pretreatment is preferred over the acidic pretreatment method because it can be performed under milder conditions. This method does not necessitate the need for expensive materials and specific designs to cope with harsh reactions and corrosion conditions. The alkali pretreatment reaction process involves the breakdown of intermolecular ester linkages and lignin, hemicellulose dissolution to break the rigid cell wall structure to facilitate the cellulose hydrolysis by increasing the accessibility for the hydrolytic enzyme (Nasution et al. 2022). Alkali reagents like NaOH, KOH, lime, or NH_4OH are typically used for alkali pretreatments. Sodium hydroxide is thought to be the most efficient chemical due to its greater solubility, lower cost, and more potent alkalinity (Wunna et al. 2021, Wang et al. 2020).

The current study concentrated on the pretreatment of SCB with different concentrations of sodium hydroxide for removing lignin significantly and to improve the amount of glucan in SCB for its application in the hydrolysis of cellulose and hemicellulose to reduce sugar. In this pretreatment experiment, NaOH concentrations were varied at constant temperature (121°C) to evaluate their effect on the lignin removal and hydrolysis yield (%) of SCB. Enzymatic hydrolysis was performed on 1-5% NaOH pretreated SCB using Cellic CTec 2 enzyme, which is a cocktail of cellulase, hemicellulase, and -glucosidase enzymes for the application of degrading cellulose to fermentable sugars. The impact of alkali pretreatment on the modification of lignin monomeric units and its subsequent effects on enzymatic hydrolysis must be thoroughly investigated to maximize the utilization of SCB biomass.

MATERIALS AND METHODS

Sample Collection, Preparation, and Characterization

Sugarcane bagasse (SCB) raw samples were collected from juice vendors in Hisar district, Haryana (India). The substrate was sun-dried and washed thoroughly with running tap water to get rid of debris and then dried in a hot air oven to ensure complete dryness. After this, these 1-2 cm long substrates were then grounded to 0.2 mm size. The sample was then characterized for compositional (Moisture, Cellulose, hemicellulose, and lignin) and proximate properties (ash content, total organic carbon). Organic carbon was estimated by using the dry combustion method (Nelson & Sommers 1983), and ash content was determined using the method given by (Han & Rowell1997). The Goering & Van Soest (1970) approach was used to determine the contents of lignin, cellulose, and hemicellulose by using an acid detergent and neutral detergent solution.

$$\text{Cellulose (\%)} = \frac{\text{Weight of crucible after}\left(\text{reflux}\right) - \left(\text{KMnO}_4\text{treatment}\right)}{\text{Initial sample weight}} \times 100$$

Hemicellulose (%) = NDF (%) – ADF (%)

Lignin (%) = ADF (%) – $KMnO_4$ fiber (%)

[ADF- Acid detergent fiber, NDF- Neutral detergent fiber]

Alkaline Pretreatment of SCB

Alkali pretreatment was performed using NaOH, which was prepared at different concentrations (1-5 wt%). The solution was then added to a substrate at a solid loading of 10% w/v, and the mixture was subjected to pretreatment in an autoclave at 12°C and 15 psi for 60 min. After heating, the sample was allowed to cool by resting it to room temperature and then the pretreated solid residues were separated from liquid hydrolysate by filtration with muslin cloth. The obtained solid residue was then neutralized by washing them thoroughly with distilled H_2O and was dried at 50°C for further analysis of cellulose, hemicellulose, and lignin components, as mentioned earlier. All experimental runs were performed in triplicates.

Enzymatic Saccharification of Pretreated SCB

Saccharification of alkali pretreated SCB (1-5% NaOH) was carried out in a 250 mL Erlenmeyer flask by adding 1g of substrate to 50 mL of citrate buffer having pH 5.0 enclosed with rubber septum and an aluminum cap and placed in a shaker incubator set at 100 rpm, 50°C for a different time interval (12-48 h) (Nosratpour et al. 2018). Then, a 10 $FPU.g^{-1}$ dose of Cellic CTec2 (procured from Sigma Aldrich, USA) was added to a flask having enzyme activity of 127 $FPU.mL^{-1}$ as determined by the Ghose (1987) method. Additionally, sodium azide had been added at a dosage of 0.005% to prevent any growth of microbes during hydrolysis. Samples of hydrolysate were extracted at various time intervals during the hydrolysis process and subjected to analysis for the quantification of total reducing sugar using the dinitro salicylic acid (DNS) method and analyzed by High-performance liquid chromatography (HPLC) using an Agilent system with a refractive index detector (RID) utilizing NH_2 column with a flow rate of $1mL.min^{-1}$. At a temperature of 50°C (Miller 1959). The percentage of saccharification was determined using the equation proposed by Mandels and Sternberg (1976) as follows:

$$\text{Saccharification}(\%) = \frac{\text{Reducing sugars}\left(\text{g/L}\right) \times 0.9}{\text{Initial concentration (g/L)}} \times 100$$

Sugarcane Bagasse Characterization

Morphological and structural characterization of untreated raw bagasse and pretreated SCB has been examined. Characterization of functional groups and change in biomass vibrational frequency has been predicted by Fourier Transform InfraRed (FTIR) spectrophotometer (Perkin Elmer model) within a wavelength range of 400-4000 cm^{-1} using pre-dried KBr powder. To investigate the alterations in biomass surface following pretreatment, the morphological features of both the untreated SCB and the solid residue obtained after pretreatment were examined. Prior to image analysis, which was performed using a high-resolution field emission scanning electron microscope (FESEM) (7610F Plus/JEOL), dried fine powdered samples of SCB were placed on the aluminum stubs and coated with a layer of gold. This gold coating ensures better conductivity and enhances the image quality obtained during the subsequent image analysis process. The X-ray diffraction analysis was performed on untreated and alkali-treated SCB substrate using a monochromatic CuK□ energy basis X-ray diffractometer. The analysis was conducted in the 2□ range value scanned from 10-50º with a phase of 0.04 and a scanning time of 5 min. The Crystallinity Index (CrI) can be calculated using the following equation:

$$\mathrm{CrI}(\%) = I_{002} - \frac{I_{am}}{I_{002}} \times 100$$

where,

I_{002}, the intensity of the crystalline part of the biomass with a peak around 22° (e.g., cellulose)

I_{am} the intensity of the amorphous part of the biomass.

Statistical Analysis

The given experiments were conducted independently in triplicates, and the findings are expressed as the mean value with its corresponding standard deviation.

RESULTS AND DISCUSSION

The raw SCB used in this study has a composition of 43.02% cellulose, 26.5% hemicellulose, and 16.6% lignin content. In addition to these compositional components, lignocellulosic biomass also contains other components such as ash, organic carbon, moisture, nitrogen, and various trace elements. Table 1 presents the proximate and compositional properties of the raw untreated sugarcane bagasse. It is important to consider the complete composition of lignocellulosic biomass, including these additional components, as they can have implications for the bioconversion processes, such as bioethanol production, and may influence the overall efficiency and yield of the process. In the study mentioned, the composition of the sugarcane bagasse (SCB) used is within the reported ranges for cellulose, hemicellulose, and lignin content in other research studies. The hemicellulose and lignin content of the SCB was reported as 26.5% and 16.6%, respectively, which falls within the reported range of 28-47% and 14-22% mentioned in other studies (Hernandez et al. 2019, Melesse et al. 2022, Alarcón et al. 2022).

Pretreatment Effect on Compositional Properties of SCB

The objective of the pretreatment is to enhance lignin removal and glucan content in the SCB, which is important for bioethanol production. The given text describes the results of the alkali (NaOH) pretreatment of SCB, which was employed for enhancing the hydrolysis of lignocellulosic, which further results in bioethanol production. Generally, pretreatment with NaOH is known to cause hemicellulose solubilization and removal from the biomass. The alkali pretreatment using NaOH induces delignification by extensively breaking the cross-ester bonding between complex lignin and hemicellulose carbohydrates like arabinoxylan (Jung et al. 2020). In this study, different concentrations of NaOH (1-5%) were utilized for pretreatment to analyze their effect on bagasse composition. Enhancing the alkali concentration per gram of biomass or extending the pretreatment duration can lead to the significant cleavage of ester-linking in the biomass. In their study, the researchers noted a substantial increase in the extraction of guaiacyl lignin (G type) through NaOH pretreatment in comparison to syringyl (S) and hydroxy phenyl (H) lignins (Laskar et al. 2013).

As shown in Fig. 1, as a result of alkali pretreatment, the cellulose content of SCB increased while hemicellulose and lignin decreased. The cellulose content showed a maximum increase of 57.6% at 2% NaOH concentration. When NaOH concentration was further increased, the cellulose content decreased, indicating that some of the glucan may have been partially decomposed. The obliteration of hemicellulose and lignin components exposes the cellulose fraction, making it

Table 1: Composition of raw SCB [ADL- Acid detergent lignin, AIA= Acid insoluble ash].

Properties	Components	Sugarcane bagasse
Compositional properties	Moisture [%]	6.4±0.2
	Cellulose (ADF-ADL) [%]	43.02±07
	Hemicellulose (NDF-ADF) [%]	26.5±0.6
	Lignin (ADL-AIA) [%]	16.6±0.9
Proximate properties	Ash [%]	4.27±0.4
	Total Organic Carbon [%]	46.6±1.2

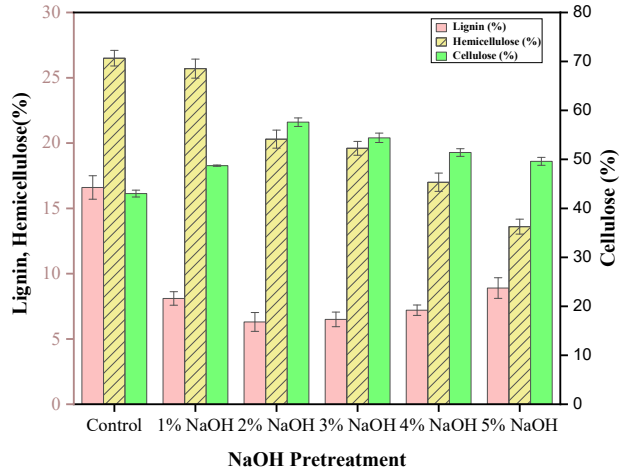

Fig. 1: Variation in composition of raw and NaOH treated (1-5%) sugarcane bagasse.

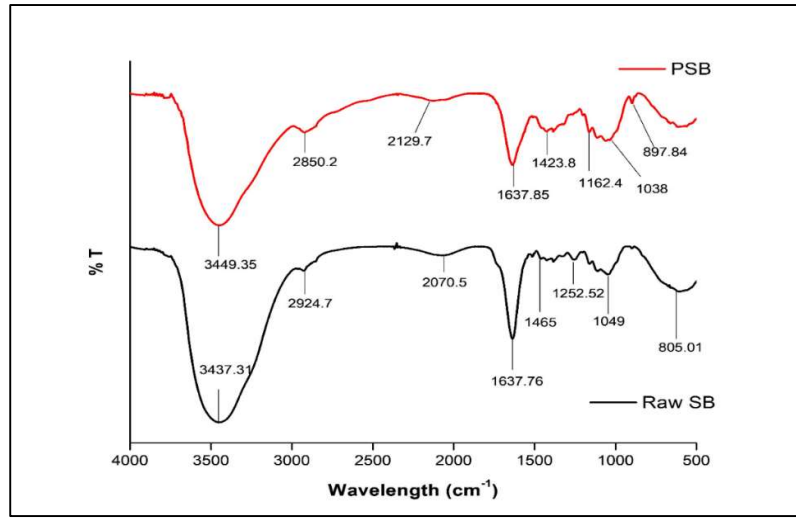

Fig. 2: FTIR spectrum of raw and 2% NaOH pretreated SCB.

more accessible to subsequent enzymatic hydrolysis or other conversion processes.

At lower NaOH concentrations (1-2%), the effect on hemicellulose content may be relatively moderate. At 2% NaOH concentration, the hemicellulose content decreased to 20.3%. These concentrations of NaOH can partially break down hemicellulose, leading to some degree of removal of hemicellulose from the sugarcane bagasse. However, a significant portion of hemicellulose may still remain in the pretreated biomass. As the concentration of NaOH was increased (3-5%), the effect on hemicellulose content became more pronounced. It decreased up to 13.6%. Higher concentrations of NaOH can lead to greater solubilization and removal of hemicellulose from the sugarcane bagasse. The results obtained in this study are consistent with the previous research conducted by Melesse et al. (2022) and Saad et al. (2023), which also displayed a decrease in hemicellulose content with an increased concentration of NaOH.

The lignin content of the SCB was significantly decreased with increased concentration of NaOH. At 2% and 3%, maximum removal of lignin, 62.04% and 60.8% were obtained, respectively. The decrease in lignin content varied from 46-56%, with an increase in NaOH concentration up to 5%. The loss of cellulose was considered while calculating hydrolysis yield efficiency. The present study compositional analysis showed that when NaOH concentrations above 2% were used it did not lead to significant removal of hemicellulose and lignin or enrichment of cellulose in the sample. Excessively high concentrations or prolonged exposure to NaOH can also lead to the degradation of

cellulose and lignin, impacting the overall composition and structural integrity of the biomass (Jin et al. 2020).

Characterization of Raw and Alkali Pretreated SCB

FTIR characterization: The spectra analysis of fresh (untreated) bagasse and bagasse after NaOH pretreatment revealed significant differences, indicating structural changes due to alkaline treatment, as shown in Fig.2. Between 3500 and 600 cm^{-1}, the unaltered fresh SCB and pretreated bagasse spectra showed the most noticeable differences in this range. The raw sugarcane bagasse showed a broad peak at 3400 to 3500 $cm^{-1,}$ which suggests the presence of the functional group -OH. The presence of $-CH_2$ groups was indicated by a stretching vibration of CH bonds at a 2924 cm^{-1} absorbance peak. The absorption at 1049 cm^{-1} represents the C-O-C stretching of the glycoside bond β-(1-4), indicating the presence of cellulose. In the bagasse spectrum, the O-H bending of the adsorbed water produces absorbance maxima at about 1637 $cm^{-1,}$ and similar results were reported by Wang et al. (2020).

The increased peak intensity between 900-1150 cm^{-1} indicates elevated levels of cellulose, suggesting an increase in cellulose content after delignification as presented in Fig. 2b. The absorbances around 1423.8, 1162 cm^{-1} were linked with the typical value of cellulose. The peak observed at 2924 cm^{-1} wavelength was contributed by the $-CH_2$ function group stretching in raw SCB, but after pretreatment of bagasse, the peak absorption shifted to 2850.2 cm^{-1}. After delignification, the NaOH peak at 1252.52 cm^{-1} diminished completely, indicating the effective removal of acetyl groups from hemicellulose. The absorbance at 1038 cm^{-1} contributes to the C-O stretching functional group at C-6, which is attributed to cellulose. The NaOH extracted bagasse spectrum showed an absorption peak at 1162 $cm^{-1,}$ which is related to the asymmetric stretching vibration of cellulose/hemicellulose C-O-C pyranose ring. The absorption frequency signals become very weak after NaOH pretreatment, indicating the breakage of linkages. Similar FTIR spectral results of raw and pretreated SCB were shown in previous studies conducted by Zhu et al. (2016), da Costa et al. (2023), and Saad et al. (2023).

Overall, the spectral analysis provides evidence of structural changes in the bagasse after delignification with NaOH. It suggests a decrease in lignin content, removal of acetyl groups from hemicellulose, and an increase in cellulose content, which are desirable changes for various applications of bagasse biomass.

FESEM morphological analysis: The use of FESEM allowed for the observation of changes in the morphology of biomass throughout the pretreatment process. Alkali-based pretreatments, such as alkaline peroxide or sodium hydroxide, can cause lignin swelling and redistribution, resulting in the exposure of cellulose-rich regions (Thite et al. 2019). The surface of the raw untreated biomass initially appeared to be smooth, compact, rigid, and tightly woven undamaged fibers, as presented in Fig. 3(a,b).

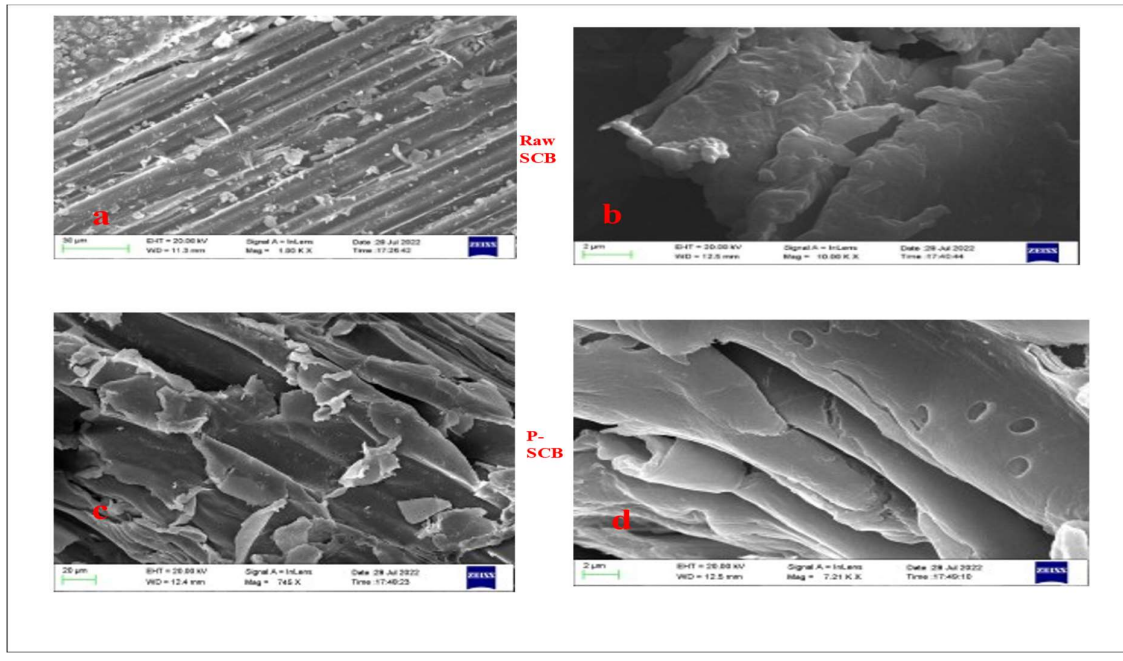

Fig. 3: FESEM images of raw SCB fragments (a,b) and 2% NaOH treated micrograph (c,d).

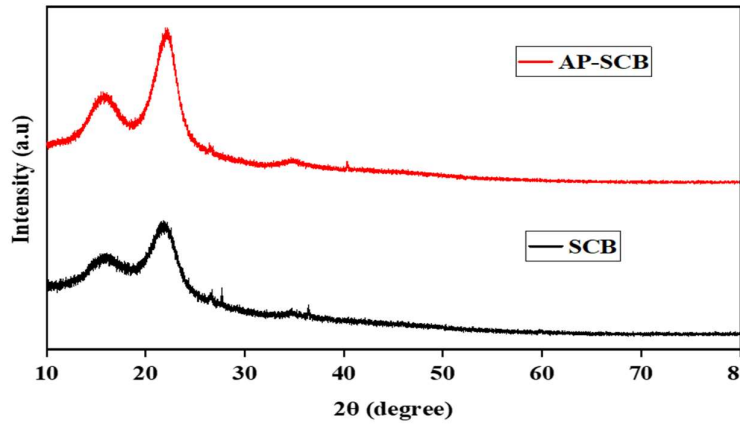

Fig. 4: XRD analysis of raw and alkali-pretreated sugarcane bagasse samples.

However, after alkali pretreatment, the biomass exhibited a disorganized or distorted appearance and also resulted in the formation of pores or holes. Increased porosity and a larger surface area that is accessible to enzymes resulted from the separation and full exposure of the microfibrils inside the biomass. The mild alkaline conditions (>1%) facilitate the breakdown of lignin and detach it from the fibers. As a result, the bundles of bagasse fibers start to dismantle. At higher concentrations of NaOH (2-3%), the bundles of bagasse fibers become more unstructured, resulting in complete detachment, as shown in Fig. 3(c,d). The enhanced porosity and increased enzyme-accessible surface area facilitated the enzymatic hydrolysis of the biomass. As a result of this hydrolysis process, the biomass underwent further degradation. Similar SCB morphological appearances from FESEM analysis have also been reported by Kumari et al. (2015) and Prajapati et al. (2020) after alkali delignification.

X-ray diffraction crystallinity analysis (XRD): The degree of crystallinity in alkali-treated SCB indicates the proportion of amorphous and crystalline cellulose components. The diffraction patterns consistently display peaks at approximately 2θ angles of 16.3 and 22.5° as observed in Fig. 4. These particular peaks correspond to the characteristic (100) and (002) planes of cellulose I. The SCB that was pretreated with sodium hydroxide showed a higher elevated peak compared to the untreated SCB biomass. After undergoing pretreatment, the structure and crystallinity of cellulose in the biomass may change due to the disruption of inter and intra-chain H-bonding of cellulosic fibrils. The cellulose is considered crystalline, while lignin and hemicellulose are amorphous, impacting the crystalline index (CrI) of complex biomass. The Segal method was employed to determine the CrI for the examined SCB samples (Kundu et al. 2023, Sharma et al. 2023). The raw SCB had a CrI value of 38.8%. In contrast, alkaline pretreatment resulted in CrI values of 53.64%. The reported observation underscores that crystallinity plays a pivotal role in enzymatic hydrolysis efficiency. This is attributed to the substantial crystalline structure of cellulose, which renders the biomass less accessible to enzymatic degradation. The pretreatment using dilute sodium hydroxide removed amorphous lignin and hemicellulose components (Alokika & Singh 2020). After pretreatment, the crystallinity index may increase due to the hydrolysis of glycosidic bonds in the exposed cellulose area. These findings align with various studies that have noted an uptick in this index value following biomass pretreatment (da Costa et al. 2023, Kininge & Gogate 2022, Melesse et al. 2022, Sharma et al. 2023).

Enzymatic Hydrolysis of Pretreated SCB

Enzymatic saccharification was conducted on SCB pretreated with different concentrations of sodium hydroxide using the Cellic ctec2 cocktail enzyme. Alkaline pretreatment is attributed to the substantial release of glucose and xylose during enzymatic hydrolysis due to the effective delignification process in biomass, cleaving bonds between complex structures of lignin and carbohydrates (Hans et al. 2021). The glucose yield (%) of delignified SCB at different time intervals is represented in Fig. 4. The hydrolysis of pretreated SCB is influenced by the enzymatic reaction time. The given result showed that with a longer reaction time, there was an increase in the concentration of released soluble reducing sugars. The bagasse that had been processed with 2% NaOH exhibited the highest concentration of glucose, measuring 12.34 ± 0.53 $g.L^{-1}$ at 48 h hydrolysis reaction time. Among the three different enzymatic reaction times applied, hydrolysis yields (%) of the 1% NaOH pretreated samples hydrolyzed at 24 h were found to be the lowest. The hydrolysis yields varied between 14.24 and 55.53% for different concentrations of alkali (NaOH) pretreatment

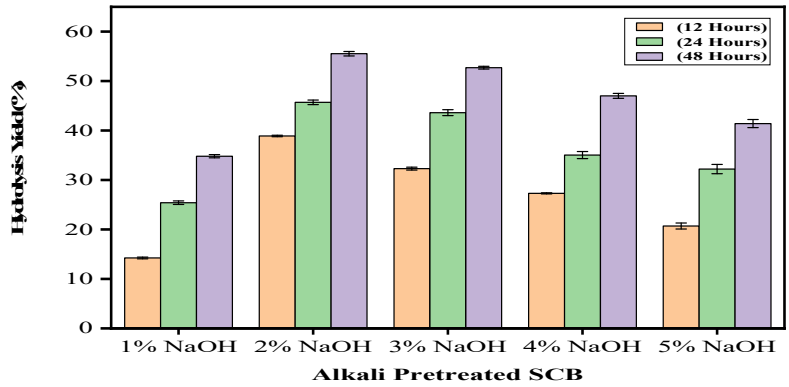

Fig. 5: Hydrolysis yield of pretreated (1-5%) SCB at 12, 24 and 48 hours.

(Fig. 5). Nosratpour et al. (2018) observed that pretreatment of sugarcane with 0.25 M sodium sulfite solution at 180°C provided 12.87g.L^{-1} of glucose when hydrolyzed with 10 FPU.g^{-1} of Cellic CTec2. Throughout the enzymatic hydrolysis process, the saccharified hydrolysates from various pretreated batches exhibited glucose as the primary reducing sugar, with significant amounts of xylose and minor quantities of cellobiose present. Tiwari et al. (2020) observed similar results with 5% and 10% NaOH pretreated sugarcane bagasse when hydrolyzed by Cellulase and xylanase enzymes, which were produced by a bacterial strain of *Pseudomonas sp*. *CVB-10* and *Bacillus paramycoides T4*. The maximum cellulose saccharification was obtained at 30 h of hydrolysis reaction time at a substrate concentration of 2%. The results showed that reducing sugar concentration increased with increasing reaction time. Sakuragi et al. (2018) conducted ammonia pretreatment on hardwood birch, which further resulted in glucose and xylose yields of 53.3% and 64.8%, respectively, after subjecting to 48 h of enzymatic hydrolysis employing a mixture of Cellic CTec2 and Cellic HTec2 enzymes (in a ratio of 1:1) at 4% v/v loading and 1% w/v solid concentrations at 37°C.

CONCLUSION

Among the potential feedstocks for 2nd generation bioethanol production, sugarcane bagasse stands out as a highly promising option. 2% NaOH was found to be an optimum concentration for sugarcane bagasse pretreatment. At this concentration, the maximum amount of delignification and cellulose extraction from biomass occurred. The raw sugarcane bagasse biomass had 16.6% lignin, which was reduced to 6.3%, and cellulose content increased to 57.6% from untreated 43.6%. Hydrolysis of pretreated solid residue was performed by an enzyme cocktail of Cellic CTec2, which is a complex of both cellulase and hemicellulase enzymes. The 12.34 g.L^{-1} of total reducing sugar was produced at 48 h of enzymatic reaction time which showed that saccharification efficiency improved as reaction time was increased from 12 to 48 h. Scanning electron micrograph of 2% NaOH pretreated bagasse showed the increase in porosity and disorderly appearance of biomass fiber fragments compared to the compact structure of untreated biomass. While FTIR results showed enrichment of cellulose content in pretreated SCB. The SCB's crystallinity increased based on the X-ray diffraction graph after treatment. This pretreatment process showed a promising positive effect in reducing biomass recalcitrance and higher glucan content, which can greatly facilitate catalytic hydrolysis and make saccharification easier. This, in turn, enables the production of high sugar concentrations and high ethanol concentrations during the fermentation process.

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