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Impact of pretreatment severity on fungal cellulase production on sugarcane bagasse substrate

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ABSTRACT

On-site production of cellulases using lignocellulosic materials can improve the economic viability of biorefineries. This, however, requires the pretreatment of substrates using thermochemical conditions that can vary in severity. To understand the effect of pretreatment severity on cellulase production by *Aspergillus ustus* S3 on sugarcane bagasse, we applied NaOH pretreatment corresponding to 3 severity factors (SF1.32, SF1.79, and SF3.64) to generate SCB that was used as inducing substrate. The highest cellulase activity (0.681 U/mL) was obtained with the intermediate severity (SF1.79) while significantly lower activities of 0.495 and 0.539 U/mL were recorded with low (SF1.32) and high (SF3.64) severities, respectively. Chemical and structural characterization revealed that low and intermediate severities improved cellulose accessibility and cellulase titres while high severity impaired them, thus limiting substrate suitability for enzyme production. These results show that though high SFs might be desirable in other applications, moderate severities may be more appropriate for cellulase production.

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Cellulase; fungi; lignocellulose; pretreatment severity; sugarcane bagasse; severity factor

1. Introduction

Lignocellulosic biomass has been promoted as a sustainable alternative to fossil sources for the production of chemicals and fuels. This is because of its abundance, availability, and cheap cost. Cellulose and hemicellulose, which are polymeric structures within the lignocellulosic matrix, can be hydrolyzed into simple sugars for downstream fermentation or diversion to other purposes. However, the use of lignocellulosic as raw materials for biofuels and biochemicals is constrained by the high cost of hydrolytic enzymes, mainly cellulases, that are required for the hydrolysis of the complex polysaccharides into utilizable sugars [1]. Hence, one means of achieving the economic viability of biorefineries is to reduce the cost of cellulase [2]. This can be achieved through the on-site production of cellulase using lignocellulosic substrates as inducers or carbon sources in fermentation [3]. Fungi have been shown to produce diverse and efficient cellulases in copious amounts when cultivated on lignocellulosic substrates compared to expensive simple sugars [4,5].

Due to the highly recalcitrant nature of lignocellulose, it needs to undergo pretreatment with various physical, chemical, or biological methods to improve its digestibility and cellulose accessibility for microbial utilization [6]. The severity of this pretreatment can influence the suitability of the substrate for cellulase production. Excessive pretreatment can lead to the degradation of useful nutrients in the substrate and it can also generate chemical inhibitors which can hinder microbial metabolism. Hence, it is important to identify the ideal set of pretreatment conditions (catalyst type and concentration, temperature, residence time, etc.) that are suitable for the substrate and/or organism to be used in fermentation [7]. While the effects of nutrients and growth conditions on fungal growth and cellulase production are well-documented, there is limited understanding regarding the direct influence of substrate structural and chemical properties [3], which are dependent on pretreatment severity, on enzyme production.

Overend et al. [8] derived a simple parameter called the severity factor (Equation (1)), which allowed the comparison of different pretreatment conditions based on reaction temperature and time. Several modifications of this parameter have been developed to accommodate the influence of different catalysts used in the pretreatment [9]. Severity factor has been used widely in studies of biomass pretreatment and enzymatic hydrolysis to understand the effect of relevant factors on product yields and pretreatment efficiency [9–11]. However, with cellulase production, comparisons of the effect of pretreatment severity have not been based on any unified quantitative parameter. This has led to a lack of clarity regarding how pretreatment severity affects cellulase production. While some studies suggested that

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severe pretreatment is detrimental to cellulase production [12,13], others have claimed that it favours high enzyme titres [14,15]. One reason for this disparity is that different types of substrates and pretreatment methods are often compared with each other, thereby introducing several confounding factors from the widely varying substrate characteristics. The use of a single substrate type and the same pretreatment method modified to obtain varied SF values will allow an objective evaluation of the effect of SF on cellulase production.

$$\log(R_0) = \log\left\{t \times \exp\left[\frac{(T - T_{ref})}{14.75}\right]\right\} \quad (1)$$

where log (R_0) is the severity factor, t is the residence time, T is the pretreatment temperature, $T_{ref} = 100°C$, and 14.75 is the activation energy.

Application of favourable pretreatment severities to lignocellulosic substrates used in fungal cellulase production will improve enzyme titres, yields, and productivities due to the generation of substrates with ideal physical and chemical properties that favour growth and metabolism [5]. This can ultimately improve process economics, thereby reducing enzyme production costs. Sugarcane bagasse (SCB) is an abundant type of biomass that is generated from the cultivation and processing of sugarcane. Global production of sugarcane in 2019 was 1.9 billion tonnes, with Brazil, India, Thailand, and China being the highest producers [16]. SCB is rich in cellulose (about 36%) [17] thus, making it a suitable substrate for fungal cellulase production.

In this study, we investigated the effect of pretreatment severity on cellulase production by an efficient fungal strain with sugarcane bagasse as the substrate. Hydrothermal pretreatment and sodium hydroxide (NaOH) pretreatments were selected for the study because of their low cost and mild conditions required. While hydrothermal pretreatment is a chemical-free process that has high hemicellulose recovery, NaOH has high delignification efficiency, especially for substrates like SCB [18,19]. The best among these two options was used to generate SCB of varying SFs, which were then used as carbon sources for cellulase production. This was done to be able to relate the changes in SCB characteristics to enzyme titres. To the best of our knowledge, this is the first report that has utilized SF to study the effect of pretreatment severity on cellulase production.

2. Materials and methods

2.1. Isolation of cellulolytic fungi

Cellulase-producing fungi were isolated from a municipal solid waste dumpsite, a saw dust mill, and a sugarcane plantation in Ilorin, Nigeria, using the spread plate technique. Soil samples were serially diluted and 0.1 mL aliquots of appropriate dilutions were plated on sterile Czapek-sugarcane bagasse (SCB) medium, modified from Lübeck and Lübeck[20], with the following composition (per litre): SCB 2 g, $NaNO_3$ 3 g, KH_2PO_4 1 g, KCl 0.5 g, $MgSO_4{\cdot}H_2O$ 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, agar 15 g, Tween-80 1.5 µL, and 1 mL trace elements solution ($ZnSO_4{\cdot}7H_2O$ 0.01 g/L, $CuSO_4{\cdot}7H_2O$ 0.05 g/L in 100 mL water). Initial medium pH was 6.5. The plates were incubated at 25°C for 5–7 days in the dark. Pure cultures of distinct fungal isolates were subcultured and maintained on potato dextrose agar (PDA) at 4°C until further use. The SCB was washed with tap water to remove debris and dirt and then dried and milled to reduce the particle size.

2.2. Primary screening for cellulase production

Preliminary screening for cellulase production was carried out on carboxymethyl cellulose (CMC) agar medium in which SCB was replaced with CMC in the isolation medium at the same concentration. Spore suspension of each isolate was harvested from a 72-h old culture plate using 10 mL of sterile distilled water. A 0.5 mL aliquot of the suspension containing 8×10^5 spores/mL [21] was inoculated onto the CMC agar and incubated for 96 h. Congo red dye (0.1%) was poured into the plates until the colonies were completely submerged by the dye. After 15 min, the dye was poured off and replaced with 1 M NaCl solution in the same manner and for the same duration. Diameter of the halo zone around each colony, which indicated the zone of CMC hydrolysis, was measured and divided by the original colony diameter to determine the hydrolytic capacity (HC) of each fungal isolate. This parameter was used to select the best three isolates (highest HC) which were then used in the secondary screening.

2.3. Secondary screening for cellulase production

As the plate screening method is limited in terms of accurate prediction of the true cellulolytic capacity of microorganisms, it was used in the primary screening in this study mainly for the purpose of reducing the number of isolates. This enabled the evaluation of fewer number of strains subsequently under submerged fermentation. Hence, the best three isolates from the primary screening were further compared based on total cellulase production (FPase) in the secondary screening using untreated SCB as the sole carbon source. Here, 1 mL of spore suspension of each fungal isolate containing 8×10^5 spores/mL was inoculated into 100 mL of sterile Mandel's medium (pH 6.5), which had the following composition (g/L): untreated SCB 2.0, urea 0.3, KH_2PO_4 2.0, $(NH)_2SO_4$ 1.4, $MgSO_4.7H_20$ 0.3, $CaCl._2H_2O$ 0.3, peptone 1.0, Tween-80 2 mL, $MnSO_4.H_2O$ 0.016, $FeSO_4.6H_2O$ 0.005, $ZnCl_2.2H_2O$ 0.017, and $CoCl_2.6H_2O$ 0.002 [22]. Flasks containing the inoculated medium were incubated at 30°C for 7 days

under an agitation speed of 150 rpm. Culture samples were withdrawn at 24 h intervals and centrifuged at 6000 rpm for 15 min. The supernatants were used as crude samples for enzyme assay.

2.4. Identification of selected fungal isolate

The isolate that produced the highest amount of cellulase in the secondary screening was identified using molecular methods. Genomic DNA of the isolate was extracted using a Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research, USA) following the manufacturer's guidelines. Amplification of the genomic DNA was done by PCR using ITS5F and ITS4R primers. Amplification, PCR product purification, and sequencing were carried out at the Bioscience Center, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, following previously described protocols [23]. The obtained sequence was compared with sequences on the GenBank database using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and identity was confirmed on the basis of highest similarity.

2.5. Selection of pretreatment method for SCB

Hydrothermal and alkali (NaOH) methods were compared for the pretreatment of raw SCB as a substrate for cellulase production by the best isolate from the screenings. A mass of 20 g of raw SCB was added to 200 mL of water (hydrothermal) or 1 M NaOH (alkali) in a 500-mL Erlenmeyer flask to obtain a solid loading of 10%. The flasks were covered with aluminium foil-wrapped cotton plug and were autoclaved at 121°C for 20 min. After cooling, the slurries were filtered, and the residues were washed with distilled water until the pH of the wash liquid turned neutral. The pretreated SCB were then used as a carbon source for cellulase production as done for the secondary screening. The pretreatment method that supported the highest cellulase production was selected for further studies.

2.6. Effect of pretreatment severity on cellulase production

Because NaOH supported higher cellulase production, it was selected as the pretreatment method for the rest of the study. Raw SCB was treated under a combination of different NaOH concentrations, temperatures, and residence times, corresponding to three different severity factors (Table 1). The severity factors (SF) were calculated based on Equation (1). Slurries from the various pretreatment severities were processed, and the solids retained for use as carbon sources in shake flask fermentation. Fifty mL of sterile Mandel's medium in a 250-mL conical flask containing 2 g of pretreated SCB from each SF was inoculated with 2 agar plugs (5-mm diameter each) of 72 h old fungal culture. The flasks were incubated in a shaker incubator (150 rpm) at 30°C for 120 h. Samples were withdrawn at an interval of 24 h and centrifuged at 6000 rpm for 15 min, and the supernatants obtained were used for FPase assay.

Table 1. Pretreatment conditions for different severity factors applied on SCB.

Temperature (°C)	Residence time (min)	NaOH conc (M)	SF
121	5	0.5	1.32
121	15	0.5	1.79
200	5	2	3.64

2.7. Compositional analysis and characterization of pretreated SCB

Structural and chemical changes in the pretreated substrates were studied to relate pretreatment severities to cellulase production. The chemical compositions (i.e. cellulose, hemicellulose, and lignin) of the SCB samples were determined according to the method described by Goering and Van-Soest [24]. The effect of pretreatment severity on the surface and microstructural characteristics of the SCB was analyzed using a JSM 7600F field emission scanning electron microscope (Joel, Japan) while changes in functional groups were analyzed using a Nicolet iS5 FTIR spectrometer (Thermo Scientific, USA) between 4000 and 400 cm^{-1}.

2.8. Enzyme assay

Total cellulase activity (FPase) was determined using the method of Ghose [25]. A strip of filter paper measuring 1 × 6 cm and weighing about 50 mg was placed in a test tube containing 1 mL of 0.05 M citrate buffer (pH 4.8). A volume of 0.5 ml of crude enzyme was added and the tube was incubated for 1 h at 50°C. The reaction was terminated with the addition of 3 mL of dinitrosalicylic (DNS) acid reagent, and liberated sugars were quantified spectrophotometrically at 540 nm by extrapolation from a glucose standard curve. One unit of FPase activity was defined as the amount of enzyme that would release 1 µmol of glucose per mL per min from filter paper under the specified conditions.

3. Results and discussion

3.1. Isolation and screening

To study the effect of pretreatment severity on cellulase production, it is essential to use an efficient cellulase-producing strain that would be able to produce copious amounts of the enzyme of various substrates. In this study, a total of 16 distinct fungal isolates were obtained from various sources (data not shown), and each of them was subjected to primary screening on CMC agar. Isolates with the 3 highest HC values (S6 – 1.52, S4 – 1.54 and S3 – 1.72) were selected for secondary screening in a liquid medium with untreated

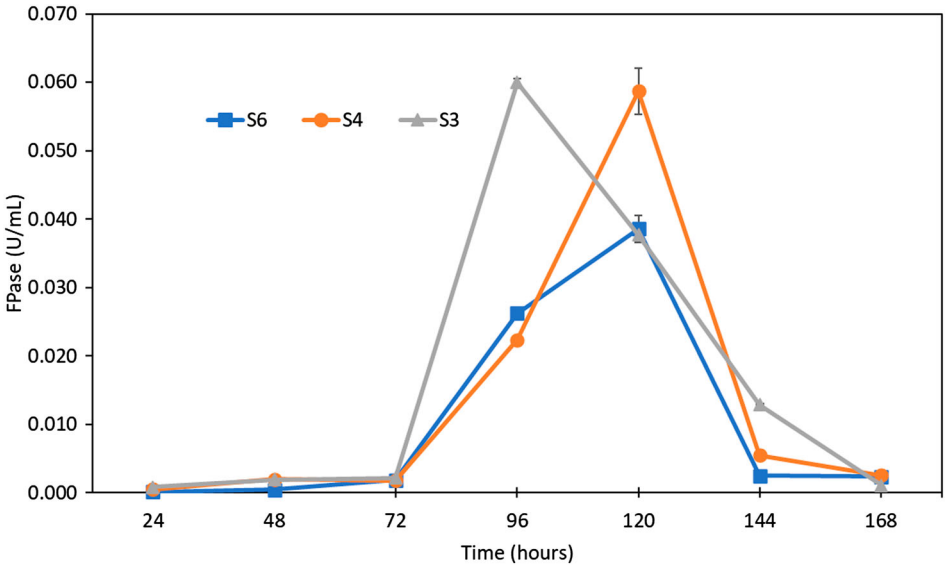


Figure 1. Secondary screening of fungal isolates for cellulase production on untreated SCB. Values represent means of 3 replicates ± standard deviation.

SCB as carbon source. As shown in Figure 1, S3 and S4 both produced significantly higher ($p < 0.05$) amount of FPase than S6, which had maximum enzyme activity of 0.039 U/mL at 120 h. Although S3 and S4 produced similar amount of maximum FPase (0.060 U/mL and 0.059 U/mL respectively), the former was selected for further studies because of its higher productivity. Maximum FPase of S3 was recorded earlier (96 h) than that of S4 (120 h). This isolate was identified as *Aspergillus ustus* using molecular techniques.

Sequential screening has been used by several researchers to select industrial strains for cellulase production [21,26]. It has been applied successfully in this study to select an efficient cellulolytic fungus, *A. ustus*. Strains of this fungus isolated from various environments have demonstrated exceptional cellulolytic ability [27,28].

3.2. Selection of suitable pretreatment method for SCB

Substrates pretreated with alkali and hydrothermal methods were compared for their suitability as carbon sources for cellulase production by S3. Figure 2 presents the results from the comparison. Clearly, NaOH pretreatment supported higher cellulase production than hydrothermal pretreatment. Maximum FPase was 0.075 U/mL at 144 h with NaOH treatment while hydrothermal gave maximum titre of 0.028 U/mL at 128 h. This represents a 2.7-fold increase in cellulase production over that of the hydrothermal treatment. This may be due to the contrasting effects of both treatments on the biomass, which affected its utilization by the fungus. Alkali pretreatment has been shown to remove lignin from sugarcane bagasse with higher efficiency than hydrothermal pretreatment [29]. Higher amount of lignin in pretreated solids may be unfavourable for cellulase production in many microorganisms [30,31]. Oke et al. [12] also reported lower endoglucanase production on hydrothermal pretreated combined substrates compared to the NaOH-treated one. Thus, NaOH was selected as the preferred pretreatment method for the rest of the study.

3.3. Effect of pretreatment severity on cellulase production

The effect of NaOH pretreatment severity on cellulase production by *A. ustus* S3 was investigated by using SCB that was pretreated at different SFs as substrate in the fermentation process (Figure 3). The highest FPase activity (0.681 U/mL) was seen at 72 h with SF1.79, while SF1.32 and SF3.64 had significantly lower ($p < 0.05$) FPase activities of 0.495 and 0.539 U/mL respectively at the same time. There was no significant difference ($p > 0.05$) between FPase activity of SF1.32 and SF3.64. A clear trend from this result is that the ideal SF was between the two extremes of low and high severity i.e. between SF1.32 and SF3.64. This shows that moderate pretreatment severity was the most favourable for cellulase production by *A. ustus* S3.

This observation agrees with some previous studies, which suggested that extremes of pretreatment severity could be detrimental to cellulase biosynthesis in different microorganisms. The study by Oke et al. [12] found that substrates pretreated with harsher treatments had lower enzyme production than those with milder treatments. They attributed this to the degradation of amorphous regions in the substrates because of the severe pretreatments. Similarly, Rodriguez-Zuniga et al. [13] recommended that severe conditions should be avoided for substrates used in cellulase production because such conditions erode the

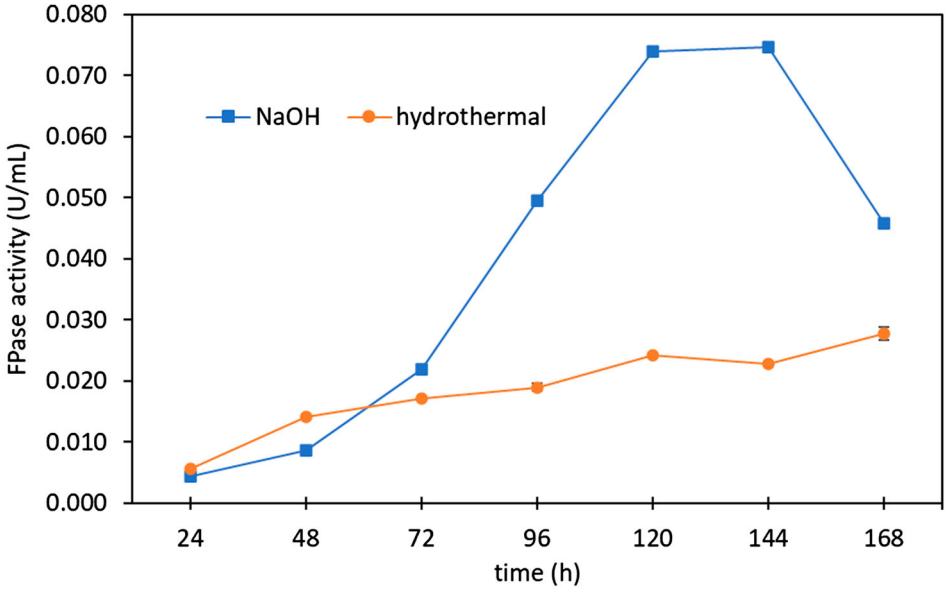


Figure 2. Cellulase production by *Aspergillus ustus* S3 on NaOH- and hydrothermally pretreated SCB. Values represent means of 3 replicates ± standard deviation.

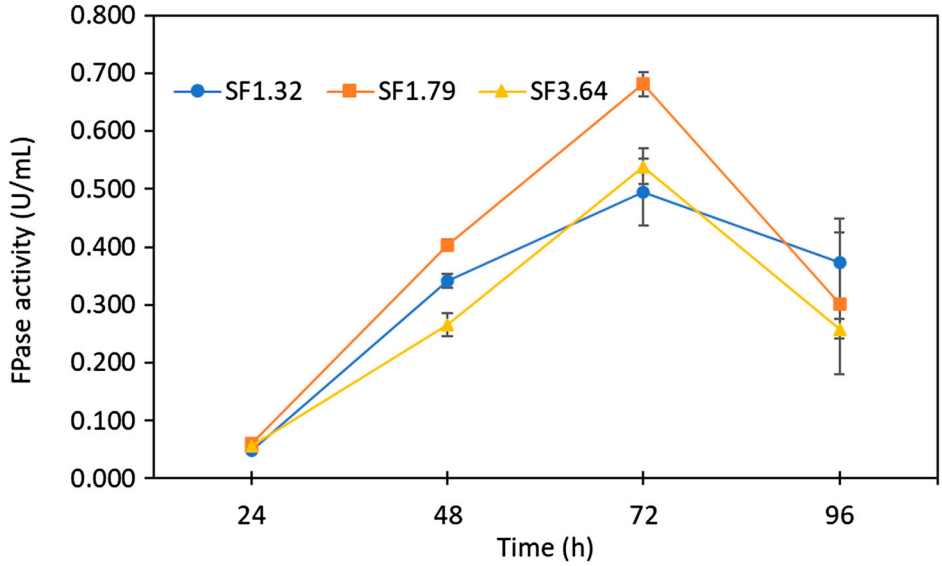


Figure 3. Effect of pretreatment severity on cellulase production by *A. ustus* S3 using NaOH-pretreated SCB as carbon source.

amorphous regions, leading to a substrate with high crystallinity that impedes microbial utilization. In contrast, Sharma et al. [15] reported that severely pretreated wheat straw and cellulose-II produced higher amounts of enzyme. However, they compared these substrates with untreated wheat straw, and not with substrates that had been subjected to same pretreatment method. Although, in agreement with other studies, they found that the increased presence of amorphous regions supported higher enzyme titres. One of the limitations of studies that investigated the effects of pretreatment severity on cellulase production is that most of them compared widely varied substrates and pretreatment methods rather than focusing on a particular substrate or method. In this study, we utilized a standardized parameter, SF, which allowed for a more objective comparison of severity effects on the same substrate. Similar approach has been adopted in studies on lignocellulose pretreatment, saccharification, and bioethanol production [10,32].

3.4. Effect of pretreatment severity on chemical composition and structural characteristics of SCB

3.4.1. Chemical composition

The changes in chemical composition of SCB before and after pretreatment are shown in Table 2. Increased cellulose accessibility was observed across the three SFs used compared to the untreated SCB. Untreated SCB had the lowest cellulose composition of 43.8%, and this increased with increasing severity from SF1.32 (52.1%) to SF1.79 (66.5%). The cellulose composition then decreased to 59.5% in SF3.64. The lignin and hemicellulose contents of the untreated SCB were

Table 2. Chemical composition of untreated SCB and SCB pretreated with different SF.

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated SCB	43.8	31.7	16.0
SF1.32	52.1	27.1	11.5
SF1.79	66.5	10.8	7.23
SF3.64	59.5	5.80	4.31

found to be 16.1% and 31.7%, respectively, which were lower than that of all SFs. Hemicellulose and lignin decreased with increasing pretreatment severity among the treated substrates. The highest cellulase was produced by SF1.79, which had the highest amount of cellulose. Thus, moderate pretreatment severity produced SCB with suitable composition for maximum cellulase production. The changes in the composition of cellulose, hemicellulose, and lignin observed in this study with higher severities are similar to those reported in other studies of alkaline pretreatment of SCB [33, 34].

3.4.2. SEM

It was observed that with increasing severity conditions, the changes that occurred in the SCB became more pronounced as seen in (Figure 4(a-l)). As the pretreatment got more severe, the patches observed on the untreated SCB disappeared, bits of fragments were deposited on the main fibres and the structure became more disrupted. The untreated bagasse (Figure 4(a–c)) can be seen with cellulose fibres that were covered with patches thought to be lignin, and the surface was rough, with the fibres tightly packed together. As the SF increased to SF1.32, the surface of the fibres became smoother (Figure 4(d–f)) than that observed in the untreated SCB. Also, the patches on the surface were observed to have reduced, suggesting lignin removal. For SF1.79, the cellulose fibrils are well exposed with the presence of micro-fibrillated cellulose (Figure 4(g–i)). Droplet-like deposits, which suggest redisposition of lignin, were also observed on the surface of the moderately pretreated SCB (SF1.79). As pretreatment severity increases, droplets of liquefied lignin are forced out of the cell matrix, and then settle on the biomass surface upon cooling [35]. The disruption in the structure of the fibre and a sloughing-off of the top layer, which revealed the internal structures, could be due to the removal of lignin and hemicellulose [36]. This corresponds with the reduction in the percentage lignin and hemicellulose content obtained in the compositional analysis results shown in Table 2. The cellulose fibres were totally disrupted, and pores damaged with SF3.64, indicating a highly severe treatment, which is detrimental to the structural integrity of SCB. There was a total loss of structure, with large openings on the fibres suggesting a loss of part of the cellulose content as indicated in the compositional analysis where the percentage composition of cellulose was lower than was recorded in the milder pretreatments. Similar disruption and loss of cellulose were previously reported by Kazeem et al. [37] with high pressure steam pretreated rice husk at higher pretreatment conditions. Vera et al. [5] showed that altered fibre characteristics of substrate can induce stress and affect fungal micromorphology, thereby impacting enzyme activity and efficiency.

3.4.3. FTIR

FTIR spectroscopy was performed to determine the changes that occurred in the structure of the SCB at different pretreatment severities (Figure 5). The changes observed in the spectra before and after pretreatment indicated that the different severities caused changes in the structural composition of the SCB. The band absorption at 3700-3200 cm^{-1} is assigned to O-H stretching vibration, which is characteristic of the crystalline nature of cellulose [15]. This was found in all samples, but the intensities were higher in the pretreated samples than the untreated SCB, with the intensity of the bands peaking on sample SF1.79. The increase in band intensity of the pretreated biomass is similar to what was reported by Zheng et al. [36] when wheat straw was pretreated with NaOH, H_2SO_4, hot water, and enzyme. This is suggesting that there was an increase in the cellulose content of all the pretreated samples at the different conditions of severity, with SF1.79 having the highest cellulose content. This corresponds with the result obtained from the compositional analysis (Table 2), which showed that this severity factor had the highest amount of cellulose.

The bands around 2800–2950 cm^{-1} are assigned to the aliphatic lignin C–H stretching [38] appears similar for all the samples. The slight prominence of this band in SF3.64 despite its low lignin content may be due to redeposition of lignin due to the high severity. Disappearance of the small shoulder at 1730cm^{-1} representing ketone/aldehyde stretch of hemicellulose [13, 38] in the pretreated samples indicates the removal of hemicellulose in the untreated SCB. The bands around 1630–1640 cm^{-1}, which are assigned to aromatic stretch of lignin [13] was most prominent in the SF1.32 sample, correlating with its high lignin composition relative to the others. The unexpectedly higher peak of SF3.64 sample in relation to SF1.79 may be because of lignin redeposition from the harsh pretreatment. The change in the shape of the spectra around 800–1100 cm^{-1}, which are attributed to the C–O/C–H stretching of cellulose sugars and C–O–C stretching of β-(1,4) glycosidic bond of adjacent sugars [39], may be related to the increased exposure of carbohydrates following the removal of lignin by NaOH. These results indicate that the different pretreatment severities had different effects on the structure of the SCB, with varying impacts on the production of cellulase.

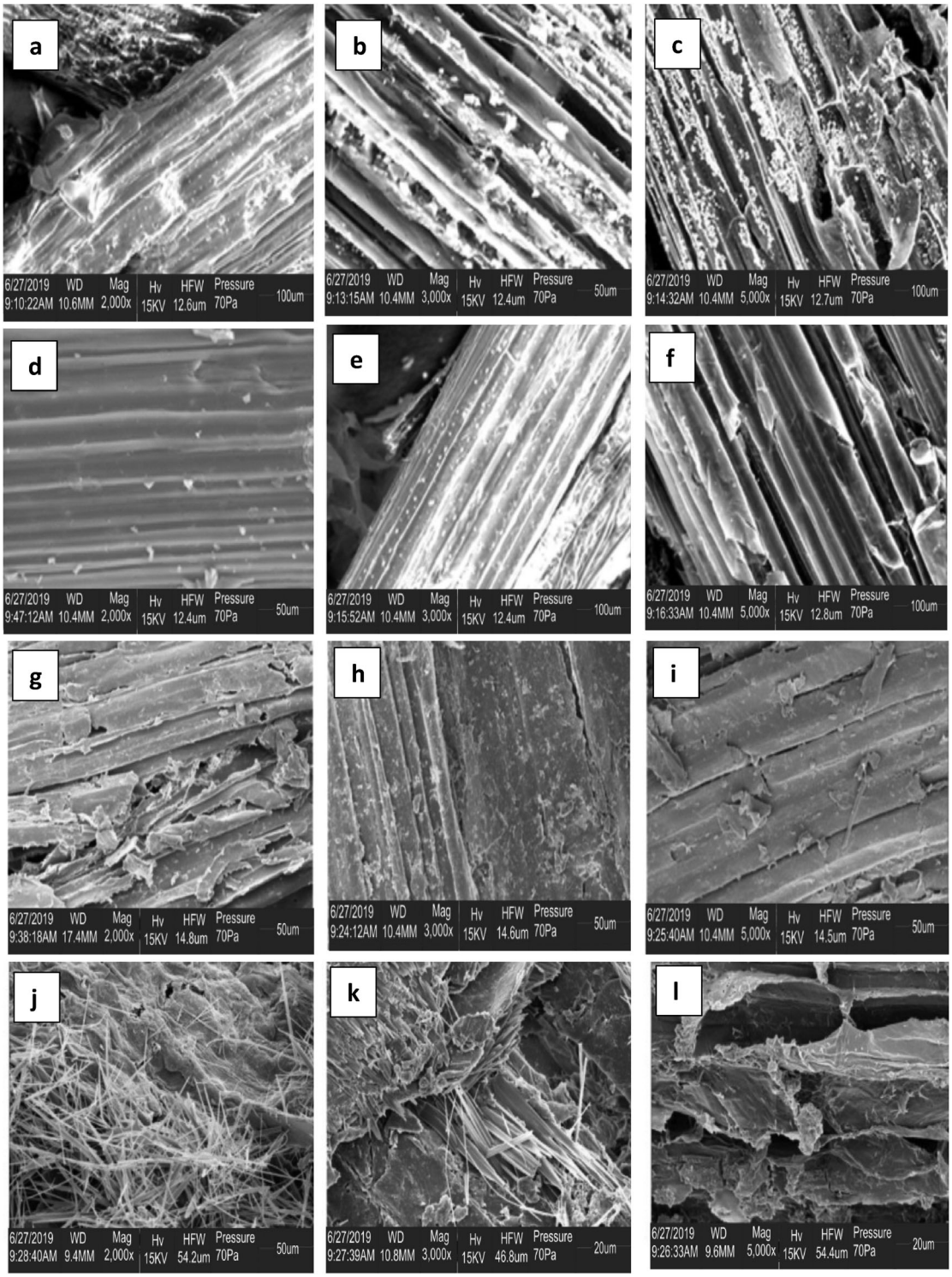


Figure 4. Scanning electron micrographs of SCB at 2000X, 3000X and 5000X magnification. (a–c) untreated, (d–f) SF1.32, (g–i) SF1.79, (j–l) SF3.64.

4. Conclusion

This study sought to determine the effect of pretreatment severity on fungal cellulase production using SCB as the inducing substrate. Sequential screening was used to select an efficient *A. ustus* strain as the producer organism. Experiments showed that NaOH was a superior method for pretreating SCB for cellulase production by this fungus, with 2.7-fold higher cellulase activity than hydrothermal pretreatment. Substrate treated with conditions corresponding to 3 different severity factors (SF1.32, SF1.79, and SF3.64) ranging from low to high were used for cellulase production. Results showed that the intermediate SF (1.79) produced the highest cellulase titres while the lowest and highest SFs had significantly lower titres. Characterization of the substrates using chemical composition, SEM, and FTIR revealed that extremes (low and high)

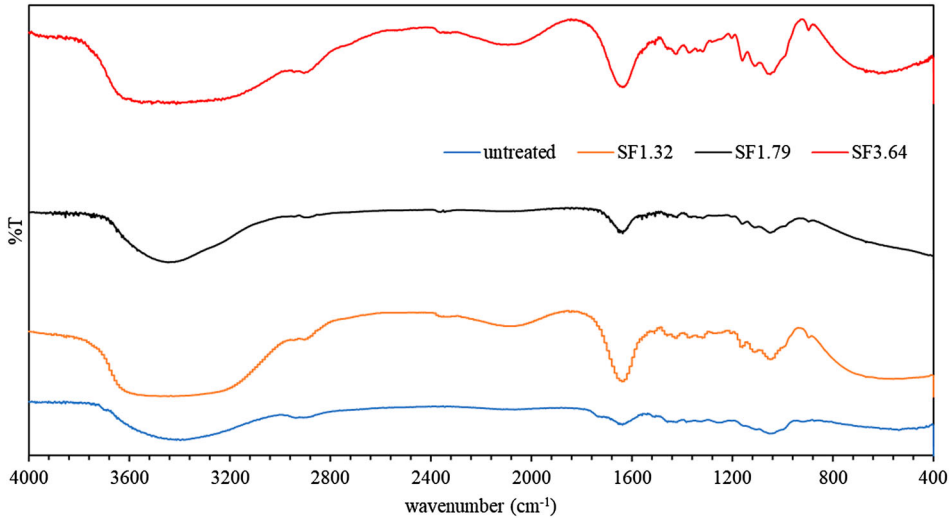


Figure 5. FTIR spectra of untreated SCB and SCB pretreated at different SF.

of severity produced SCB with unfavourable characteristics for cellulase production. Meanwhile, moderate severity supported higher cellulase activities. Preservation of fibre integrity and higher cellulose content seen with SF1.79 correlated with higher enzyme production while extremely high severity (SF3.64) caused fibre damage and low enzyme titres.

These findings have revealed that extremely harsh treatments, which may be beneficial for other biorefining applications, should be avoided when using SCB as substrate for cellulase production. The use of a quantitative parameter such as SF has allowed an objective and systematic evaluation of the impact of different severities on cellulase production rather than the arbitrary way this has been done in the past. To be able to generalize the findings from this study, these experiments need to be repeated with other substrate and pretreatment types. Furthermore, more insights can be gained regarding the mechanisms by testing a wider range of SFs than was used in this study.

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