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The Effects of Alkaline Pretreatment on Agricultural Biomasses (Corn Cob and Sweet Sorghum Bagasse) and Their Hydrolysis by a Termite-Derived Enzyme Cocktail

Mpho. S. Mafa [1,2], Samkelo Malgas [1], Abhishek Bhattacharya [1,3], Konanani Rashamuse [4] and Brett I. Pletschke [1,*]

1 Enzyme Science Programme (ESP), Department of Biochemistry and Microbiology, Rhodes University, Grahamstown 6140, South Africa; mafams@ufs.ac.za (M.S.M.); samkelomalgas@yahoo.com (S.M.); abhishek.bhattacharya@biochemistry.lu.se (A.B.)
2 Department of Plant Sciences, University of the Free State P.O. Box 339, Bloemfontein 9300, South Africa
3 Department of Biochemistry and Structural Biology, Lund University, 22100 Lund, Sweden
4 CSIR Biosciences, Building 18, Meiring Naudé Road, Brummeria, PO Box 395, Pretoria 0001, South Africa; Krashamuse@gmail.com
* Correspondence: b.pletschke@ru.ac.za; Tel.: +27-46-603-8081

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Abstract: Sweet sorghum bagasse (SSB) and corncob (CC) have been identified as promising feedstocks for the production of second-generation biofuels and other value-added chemicals. In this study, lime ($Ca(OH)_2$) and NaOH pretreatment efficacy for decreasing recalcitrance from SSB and CC was investigated, and subsequently, the pretreated biomass was subjected to the hydrolytic action of an in-house formulated holocellulolytic enzyme cocktail (HEC-H). Compositional analysis revealed that SSB contained 29.34% lignin, 17.75% cellulose and 16.28% hemicellulose, while CC consisted of 22.51% lignin, 23.58% cellulose and 33.34% hemicellulose. Alkaline pretreatment was more effective in pretreating CC biomass compared to the SSB biomass. Both $Ca(OH)_2$ and NaOH pretreatment removed lignin from the CC biomass, while only NaOH removed lignin from the SSB biomass. Biomass compositional analysis revealed that these agricultural feedstocks differed in their chemical composition because the CC biomass contained mainly hemicellulose (33–35%), while SSB biomass consisted mainly of cellulose (17–24%). The alkaline pretreated SSB and CC samples were subjected to the hydrolytic action of the holocellulolytic enzyme cocktail, formulated with termite derived multifunctional enzymes (referred to as MFE-5E, MFE-5H and MFE-45) and exoglucanase (Exg-D). The HEC-H hydrolysed NaOH pretreated SSB and CC more effectively than $Ca(OH)_2$ pretreated feedstocks, revealing that NaOH was a more effective pretreatment. In conclusion, the HEC-H cocktail efficiently hydrolysed alkaline pretreated agricultural feedstocks, particularly those which are hemicellulose- and amorphous cellulose-rich, such as CC, making it attractive for use in the bioconversion process in the biorefinery industry.

Keywords: alkaline pretreatment; enzyme cocktail; enzymatic hydrolysis; glycosyl hydrolase; termite metagenome derived enzymes

1. Introduction

Agricultural feedstocks such as sweet sorghum bagasse (SSB) and corncob (CC) have been identified as promising alternatives for the production of second-generation biofuels and other value-added chemicals [1–3]. These feedstocks consist of polysaccharides (cellulose and hemicellulose)

that can be hydrolysed to simple monosaccharides which can be fermented to produce ethanol. The cellulose consists of glucose moieties linked by β-1,4-glycosidic bonds, which forms crystalline cellulose that is recalcitrant to enzymatic hydrolytic activity, and also amorphous cellulose that is easily hydrolysed by cellulases. Mnich et al. [4] reported that in Poaceae (Gramineae), such as sorghum or maize plants, hemicellulose is mostly composed of a type of xylan called glucuronoarabinoxylans (GAX). Generally, xylans are composed of a backbone of β-1,4-glycosidic bonds linking xylose moieties, which can be O-acetylated or substituted at O-2 by α-arabinose, α-glucuronic acid or α-methyl-glucuronic acid side-chains that give rise to arabinoxylan, glucuronoxylan and GAX, respectively [4,5]. The cellulose and hemicellulose are generally covered and cross-linked by lignin which impedes glycoside hydrolase (GHs) hydrolytic activity [4,6]. As a result, to achieve efficient feedstock hydrolysis, the lignin is removed from the feedstock through chemical, physical, or biological pretreatments.

Agricultural biomasses have been pretreated with various acids such as phosphoric acid, sulfuric acid and acetic acid [6,7]. The acid pretreatment of agricultural feedstocks effectively removes lignin, however, it also removes a high amount of hemicellulose. In addition, some acids such as sulfuric acid are not environmentally friendly or require higher temperature for long periods (140 °C for 6 h) for the pretreatment to be effective [2]. Biological pretreatments such as the use of fungi or bacteria to degrade lignin were introduced as alternative environmentally friendly pretreatment technologies [8]. For instance, Mishra and Jana [2] pretreated SSB biomass with *Coriolus versicolor* using solid-state fermentation (SSF) in a bioreactor. *C. versicolor* removed more than 40% (w/w) of lignin from the SSB biomass, but this process takes many days to remove high amounts of lignin. An effective pretreatment process of the biomass should remove lignin within a few hours and must achieve the following; (1) a reduction of the cellulose crystallinity, (2) an increase in the surface area of the biomass, (3) an increase in porosity, (4) a reduction in the degree of polymerisation of cellulose, and (5) a reduction in the particle size of the biomass [6,7]. All these properties associated with the pretreatment of feedstocks improve the access of the enzymes to the biomass and significantly increases the enzymatic hydrolytic activity.

Alkaline pretreatment is among the most established techniques for the removal or redistribution of lignin and mercerisation of the biomass [9–12]. Alkaline pretreatments are relatively affordable compared to other methods (like acid pretreatments) because they generally use mild reaction conditions, alkaline chemicals can be recovered, reused and have a high selectivity for separation of lignin [13]. According to Kim et al. [13], alkaline pretreatment technologies can be divided into: (1) sodium hydroxide pretreatment, (2) ammonia pretreatment, (3) aqueous ammonia pretreatment, (4) anhydrous ammonia pretreatment, and (5) lime ($Ca(OH)_2$) pretreatment. In addition, da Silva Neto et al. [14] achieved more than 60% lignin removal from the SSB biomass using 6% (*w*/*v*) hydrogen peroxide within 4 h and solubilised 32% of the hemicellulose in the same process. A study showed that the alkaline pretreatment increased the swelling of the biomass, removed lignin, and converted the crystalline cellulose I into cellulose II which is less crystalline [9]. The crystallinity of the biomass is generally studied with X-ray diffraction (XRD) or Fourier-transform infrared spectroscopy (FTIR) [11,14,15]. These techniques show the changes of the biomass from a crystalline structure to a more amorphous one after pre-treatment, which is generally associated with improvements in the GH enzyme hydrolytic activity on the biomass. However, a few studies have investigated the performance of the activity of the GH enzymes on the biomass that was pretreated with $Ca(OH)_2$ and sodium hydroxide (NaOH/mercerised), and have determined which of the two pre-treatments is more effective at improving biomass saccharification.

A better understanding of lignocellulose digestion by termite-metagenome derived enzymes may help to uncover and eventually overcome challenges in the conversion of lignified plant cell walls into simple sugars [16]. The termite gut microflora acts as an important reservoir of novel GHs that are able to efficiently hydrolyse lignocellulosic biomass [16,17]. Functional metagenomic screening for hemicellulases in the termite, *Pseudacanthotermes militaris*, revealed a large number of hemicellulose degrading enzymes [18]. These enzymes included arabinofuranosidases, xylosidases and endo-xylanases. In addition, the metagenomic approach has been used previously to mine various

GHs and feruloyl esterase enzyme genes from bacterial symbionts in the hindgut of the *Trinervitermes trinervoides* termite species [19,20]. These studies demonstrated that termites and their symbionts possess hemicellulase enzymes which could be used to hydrolyse the hemicellulosic fraction of biomass in a synergistic manner.

In this study, we aimed to remove lignin from the SSB and CC biomasses by pretreating them with NaOH and $Ca(OH)_2$. Subsequently, the efficacy of each pretreatment on the biomass was evaluated with compositional analyses, morphological change assessments using a scanning electron microscope (SEM), and biomass chemical changes were analysed using FITR. Furthermore, the alkaline pretreated biomass was subjected to enzymatic hydrolysis using an in-house formulated holocellulolytic enzyme cocktail (HEC-H) consisting of multifunctional enzymes (MFEs) and an exoglucanase (Exg-D) derived from bacterial symbionts in the hindgut of *Trinervitermes trinervoides* (Sjöstedt).

2. Materials and Methods

2.1. Materials

The four GH enzymes [purified Exo-glucanase (Exg-D), MFE-5E, MFE-5H, and MFE-45] used in the current study were derived from hindgut bacterial symbionts of a termite (*T. trinervoides*) metagenome identified and isolated by Rashamuse et al. [20]. The model substrates; carboxymethylcellulose (CMC), Avicel PH101, locust bean gum and the *p*-nitrophenyl (*p*NP) based substrates (*p*NP-α-L-arabinofuranoside (A), *p*NP-β-D-glucopyranoside (G), *p*NP-β-D-mannopyranoside (M), *p*NP-β-D-cellobioside (C) and *p*NP-β-D-xylopyranoside (X)) were purchased from Sigma-Aldrich, South Africa. Beechwood xylan, soluble wheat flour xylan, insoluble wheat flour arabinoxylan and xyloglucan were purchased from Megazyme, Ireland. All chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Lignocellulosic Biomass Pretreatment

The 5 g of dried and pulverised (>2 mm particles) sweet sorghum bagasse (SSB) and corncob (CC) samples were chemically pretreated with alkali (2 g of $Ca(OH)_2$ and 1.25 g of NaOH) according to protocols described by Panagiotopoulos et al. [21] and Beukes and Pletschke [10], respectively. After pretreatment, SSB and CC samples were filtered and washed with Milli-Q water until the filtrates had a pH of 7.0. The samples were air dried and stored in an airtight container until use.

2.3. Biomass Composition and Scanning Electron Microscope (SEM) Analysis

Biomass composition of untreated, $Ca(OH)_2$ and NaOH pretreated SSB and CC samples were determined according to protocols by the National Renewable Energy Lab (NREL) [22] at the Wood Sciences Faculty at Stellenbosch University, South Africa.

To determine the effect of each pretreatment ($Ca(OH)_2$ and NaOH) on biomass morphology, the dried SSB and CC samples were mounted on scanning electron microscope (SEM) stubs using double sided graphite tape, sputter coated with gold using a Balzers Union sputtering device. The gold coated SSB and CC samples were viewed under a Tescan Vega scanning electron microscope at 20 kV. Digital images were captured using the Vega imaging system.

2.4. Fourier-Transform Infrared Spectroscopy Analysis

The functional group analysis of the untreated or pretreated SSB and CC biomass samples was analysed with Fourier Transform Infrared (FTIR) Spectroscopy. The FTIR spectra of Avicel (cellulose control), untreated, $Ca(OH)_2$ and NaOH pretreated SSB and CC samples were recorded at room temperature using an UATR-FTIR instrument (PerkinElmer, USA). All FTIR spectra were collected at a spectrum resolution of 4 cm^{-1}, with 4 co-added scans per sample over the range of 4000 to 650 cm^{-1}. The Perkin-Elmer software (Spectrum version. 6.3.5) was used to perform spectra normalisation, baseline corrections, and peak integration. The spectra of the Avicel PH101 microcrystalline cellulose,

untreated, pretreated SSB and CC samples were presented as absorbance values, and each value represented the means of four scans.

2.5. Formulation of the Holoenzyme Cocktail (HEC)

The substrate specificities of the MFE-5E, MFE-5H, and MFE-45 enzymes were performed according to Mafa et al. [23], and the released total reducing sugars were measured according to a modified 3,5-dinitrosalicylic acid (DNS) method [24]. An amount of 1% (*w*/*v*) beechwood xylan, wheat arabinoxylan, Avicel, xyloglucan, locust bean gum and CMC were used as substrates. For *p*NP based substrate specificity assays, 2 mM *p*NP-A, *p*NP-G, *p*NP-M, *p*NP-C and *p*NP-X were used. Four GH enzymes; exo-glucanase (Exg-D), MFE-5E, MFE-5H and MFE-45, derived from the termite bacterial hindgut metagenome were used to formulate the holocellulolytic enzyme cocktail HEC-H (60% MFE-5H, 20% MFE-5E, 10% MFE-45 and 10% Exg-D). HEC-H was then supplemented with 10% protein loading of each of the following enzymes, *Aspergillus niger* β-glucosidase (Novozyme 188) and *Selenomonas ruminantium* xylosidase, SXA, during the hydrolysis of pretreated feedstocks. The reactions were initiated by adding the HEC-H and auxiliary enzymes to 2% (*w*/*v*) of untreated, $Ca(OH)_2$ and NaOH pretreated SSB or CC biomass samples suspended in 50 mM sodium citrate buffer at pH 5.5. The reaction was carried out by incubating the samples at 37 °C in an incubation room. The release of the total reducing sugars was measured using the DNS assays.

2.6. Data Analysis

One-way analysis of variance (ANOVA) was used to analyse and detect significant increases in activity exhibited by the HEC-H. All pairwise comparison procedures were conducted using the data analysis feature in Microsoft Excel 3.

3. Results

3.1. Biomass Composition and SEM Analysis of Pre-Treated Feedstocks

Lignin in lignocellulosic biomass is an established GH enzyme inhibitor as it results in non-productive binding and enzyme stalling. Thus, it is important to pretreat the agricultural feedstocks before the application of GH enzymes for effective hydrolysis. In the current study, the untreated CC biomass composition showed that its lignin, cellulose and hemicellulose contents were about 22.51%, 23.58% and 33.34%, respectively (see Table 1). The $Ca(OH)_2$ pretreated CC composition showed that lignin content was significantly ($p < 0.01$) reduced by 7.7%, while cellulose content significantly ($p < 0.01$) increased by 3.76% compared to the untreated biomass. The NaOH pretreated CC lignin content was reduced by about 13.01%. Interestingly, when compared to the untreated biomass, the cellulose and hemicellulose contents were significantly ($p < 0.01$) increased by 9.74% and 1.69%, respectively. These results demonstrate that alkaline pretreatment effectively removed/modified lignin from the CC samples. The removal of lignin resulted in the increased cellulose and hemicellulose content of the CC feedstock. These results suggested that the carbohydrates were more exposed for hydrolytic activity because the pretreatments had removed significant amounts of lignin from these samples.

In addition, untreated SSB lignin, cellulose and hemicellulose contents were 29.34%, 17.75% and 16.28%, respectively (Table 1). The $Ca(OH)_2$ pretreated SSB lignin content was not significantly reduced compared to that of the untreated biomass. The hemicellulose content was reduced from 16.28 to 11.54% and the cellulose content significantly ($p < 0.01$) increased from 17.75% to 19.60% after the SSB was pretreated with $Ca(OH)_2$ (Table 1). The NaOH pretreated SSB led to an 18.45% reduction in the amount of lignin and a 6.06% increase in cellulose content compared to the untreated biomass. The NaOH pretreated SSB had a hemicellulose content that was significantly ($p < 0.01$) reduced from 16.28% to 13.05% compared to the untreated biomass. Also, the SSB pretreated with NaOH demonstrated that the pretreatment was effective in removing lignin and exposing the hemicellulose and cellulose.

Table 1. Alkaline pretreated feedstock compositional analysis.

Biomass Pre-Treatment	Biomass Composition (%)		
Corncob (CC)	**Lignin**	**Cellulose**	**Hemicellulose**
Untreated	22.51 ± 0.19	23.58 ± 0.47	33.34 ± 0.56
$Ca(OH)_2$	14.81 ± 0.10 #	27.34 ± 0.33 #	33.49 ± 0.11
NaOH	9.50 ± 0.04 #	33.32 ± 0.41 #	35.03 ± 0.63 *
Sweet sorghum bagasse (SSB)			
Untreated	29.34 ± 0.042	17.75 ± 0.36	16.28 ± 0.46
$Ca(OH)_2$	29.08 ± 0.092	19.60 ± 0.1 #	11.54 ± 0.47 *
NaOH	10.88 ± 0.031 #	23.81 ± 0.22 #	13.05 ± 0.25 #

Values represent the means ± SD and $n = 3$. * represents p-value < 0.05 and # represents p-value < 0.01.

SEM analysis demonstrated that $Ca(OH)_2$ and NaOH pretreatment resulted in different pretreatment severities in the CC and SSB feedstocks. Figure 1 shows that both untreated CC and SSB biomasses were covered with lignin, which formed a thick smooth whitish layer. After $Ca(OH)_2$ pretreatment, both biomasses had a morphology which showed that the lignin had broken down and condensed into droplets and tiny sheets. The hydrolysis of the lignin created a larger biomass surface area which was ideal for enzymatic activity. Table 1 shows that the amounts of the lignin for untreated SSB and $Ca(OH)_2$ pretreated were similar. SEM analysis also revealed that lignin was not totally removed, but rather modified to form droplets and sheets on the surface of the biomass. The SEM results for $Ca(OH)_2$ pretreatment also showed similar effects on CC biomass. However, the NaOH pretreatment was more effective in removing the lignin from CC and SSB biomass. Roughly more than 90% of lignin was removed from the biomass, leaving cellulose and hemicellulose threads exposed with pores on their surfaces (Figure 1). There was a high correlation between the biomass composition (Table 1) and the SEM results, which suggested that the NaOH pretreatment was more effective at removing lignin from the biomass compared to $Ca(OH)_2$ pretreatment.

3.2. FTIR Analysis of Pretreated Feedstock

FTIR is generally used to assess chemical functional group changes in biomass and this information is used to compare the structural changes of the biomass before and after chemical pretreatments. The alkaline pretreated CC and SSB biomasses were analysed with FTIR to validate the compositional analysis and SEM results. Figure 2 demonstrates that the alkaline pretreated CC and SSB biomasses possessed more cellulose II than crystalline cellulose I, which is an indication of reduced biomass crystallinity. The positive control for cellulose Iβ (Avicel PH101 microcrystalline cellulose) showed high intensity peaks at 1428.66, 1160.54 and 1104.67 cm^{-1}. These peaks represent the presence of predominately crystalline cellulose Iβ and small quantities of cellulose II in the biomass. Similar peaks (1428.06, 1157.46 and 1111.00 cm^{-1}) were present in the untreated SSB biomass, which suggest that there was a presence of crystalline cellulose I and cellulose II in this sample. However, only one peak was present in the $Ca(OH)_2$ (1158.11 cm^{-1}) and NaOH (1157.23 cm^{-1}) pretreated SSB biomass (Figure 2). These results demonstrated that after pretreatment, the SSB biomass had a reduced content of crystalline cellulose Iβ by producing cellulose II. In addition, the $Ca(OH)_2$ pretreatment shifted the 1428.66 cm^{-1} peak to 1410.45 cm^{-1}, while NaOH pretreatment shifted the 1428.66 cm^{-1} peak to 1421.90 cm^{-1}. This shift is characteristic to the formation of cellulose II and amorphous cellulose, and the disappearance of the crystalline cellulose I in the biomass. Figure 2B showed that the similar spectra were identified in the untreated and alkaline pretreated CC biomass, suggesting that alkaline pretreatment removed lignin and changed the CC biomass crystallinity in a similar fashion to the SSB biomass.

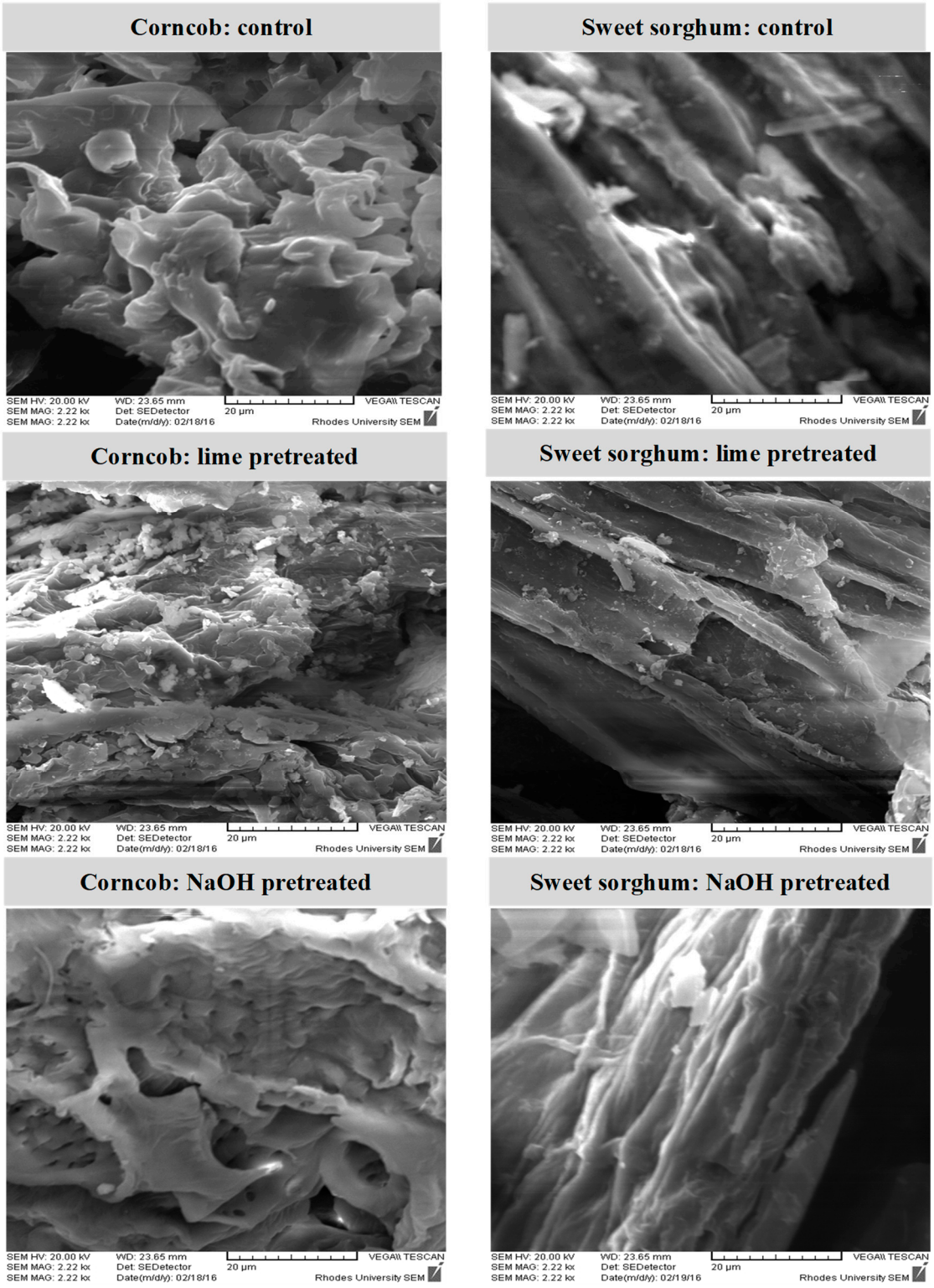

Figure 1. Topological analysis of pretreated sweet sorghum (SSB) and corncob (CC) feedstocks using scanning electron microscopy (SEM). The captions above the images indicate the control (untreated), type of treatment and the biomass in each SEM image. The scale bar was 20 µm and the magnification was 2.22 kx.

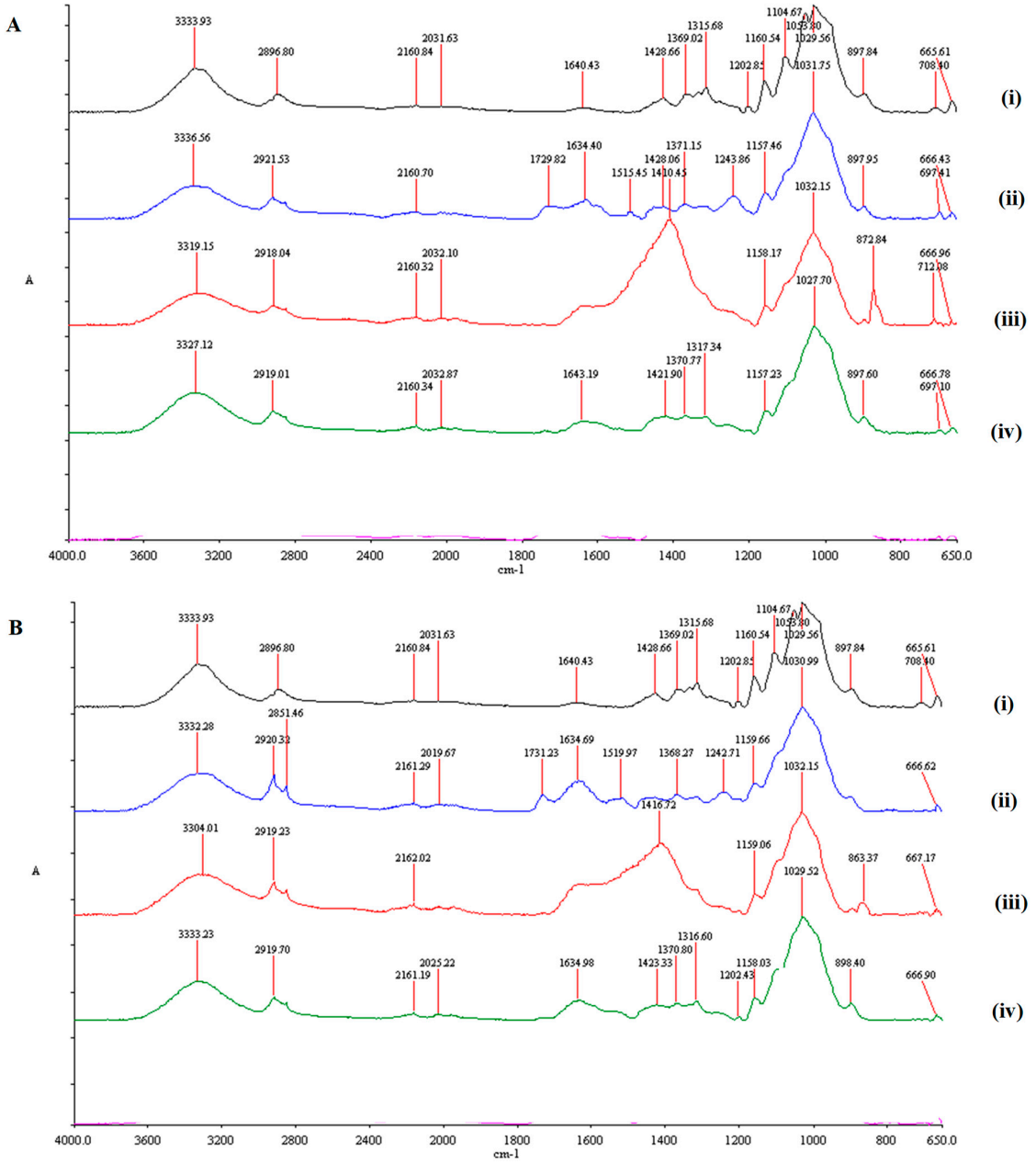

Figure 2. Comparative FTIR in the spectra range 4000–650 cm^{-1} corresponding to Avicel PH101 (i), untreated (ii), $Ca(OH)_2$ pretreated (iii) or sodium hydroxide pretreated biomass. (**A**) represents the SSB biomass and (**B**) represents the corncob biomass. Keys: (**A**) in the *y*-axis represents arbitrary units.

The peaks at 1515.45 cm^{-1} and 1519.97 cm^{-1} in the untreated SSB and CC biomasses, respectively, represent the C≡C vibration of the aromatic rings of the lignin. In contrast, these bands were absent in the IR spectrum of the positive control (Avicel) and NaOH pretreated SSB and CC biomasses (Figure 2). These results supported the biomass composition analysis and SEM findings which revealed that NaOH pretreatment removed the lignin from the biomass. However, the spectrum of the $Ca(OH)_2$ pretreated SSB and CC biomass samples had a broad and higher intensity peak starting from 1640 cm^{-1}, reaching a maximum at 1410 cm^{-1} and ending at 1370 cm^{-1} (Figure 2). This observation suggested that lignin was not completely removed by the $Ca(OH)_2$ pretreatment but was re-distributed as revealed by SEM (Figure 1). In addition, the untreated and the alkaline pretreated SSB and CC biomasses displayed one peak between 1033 and 1027, which represented the hemicellulose. These results revealed that the hemicellulose was still present in the SSB and CC after alkaline pretreatment.

3.3. Substrate Specificity

The enzyme substrate specificity results showed that MFE-5E was a multifunctional enzyme that displayed 17.189, 13.471, 7.483 and 6.831 U/mg protein specific activity on CMC, wheat flour xylan, beechwood xylan and xyloglucan, respectively. These findings demonstrate that MFE-5E had a higher propensity to hydrolyse amorphous cellulose followed by various xylan substrates and xyloglucan (Table 2). In addition, MFE-5H exhibited the highest specific activity on xyloglucan (84.975 U/mg protein), followed by wheat flour xylan, beechwood xylan and CMC with 67.365, 52.015 and 28.584 U/mg protein, respectively. Both MFE-5E and MFE-5H were classified under GH family 5 (with accession numbers AMO13175 and AMO13178, respectively), which consists of some of the enzymes displaying diverse activities on various carbohydrate substrates (http://www.cazy.org/GH5.html). This explains the broad range of MFE-5E and MFE-5H activity on various carbohydrate substrates. The results in Table 2 also shows that the MFE-45 displayed highest specific activity on xyloglucan (12.86 U/mg protein). MFE-45 showed similar activity during hydrolysis of beechwood xylan and CMC, which were 4.55 and 4.75 U/mg protein, respectively. MFE-45 is reported to belong to GH family 45 and its accession number is AMO13193. According to the CAZy Database (http://www.cazy.org/GH45_bacteria.html), enzymes in family 45 possess endoglucanase activity, xyloglucanase activity but not xylanase activity, which suggests that MFE-45 possesses a novel xylanase activity. The MFEs did not show any activity on Avicel, and the mannan substrate, locus bean gum, and several *p*-nitrophenyl-substrates (Table 2). However, MFEs presented in this study were true multifunctional enzymes that displayed specific activities on various substrates with their backbones linked by β-1,4-glycosic linkages, and possessed different sidechains.

Table 2. Substrate specificity of enzymes on polymeric and *p*-nitrophenyl substrates.

Substrates	Enzyme Specific Activity (U/mg Protein)		
	MFE-5E	**MFE-5H**	**MFE-45**
CMC	17.189 ± 0.049	28.584 ± 0.025	4.75 ± 0.053
Avicel	N/A	N/A	N/A
Beechwood xylan	7.483 ± 0.013	52.015 ± 0.071	4.55 ± 0.013
Wheat flour xylan	13.471 ± 0.161	67.365 ± 0.116	#
Xyloglucan	6.831 ± 0.008	84.975 ± 0.012	12.86 ± 0.19
Locus bean gum	N/A	N/A	N/A
*p*NP-A	N/A	N/A	NA
*p*NP-G	N/A	N/A	N/A
*p*NP-X	N/A	N/A	N/A
*p*NP-C	N/A	N/A	N/A
*p*NP-M	N/A	N/A	N/A

Values represent means ± SD and $n = 3$; N/A means there was no activity; # - means not tested on this substrate; pNP-A, G, -X, -C and M represents *p*-nitrophenyl-arabinofuranoside, -glucopyranoside, -xylopyranoside, -cellobioside and –mannopyranoside, respectively; Concentrations of polymeric and *p*-nitrophenyl substrates were 2% and 4 mM, respectively; U represents μmol/min.

3.4. Holocellulolytic Enzyme Cocktail Formulation and Its Application on Pretreated Biomass

The holocellulolytic enzyme cocktail (HEC-H) used to assess the effectiveness of the alkaline pretreatments in lowering biomass recalcitrance was formulated using a 60%: 20%: 10%: 10% combination of the MFE-5E, MFE-5H, MFE-45 and exoglucanase (Exg-D) enzymes. Exg-D was previously characterised by Mafa et al. [23] and its biochemical properties demonstrated that it can effectively be used in synergy with MFEs.

HEC-H hydrolysed the alkaline pretreated SSB and CC substrates better than their untreated biomasses (i.e., the controls) (Figure 3A). Also, HEC-H exhibited the highest activity on the CC biomass compared to SSB biomass. Higher activity (approximately 0.72 μmol/mL) was observed when HEC-H hydrolysed $Ca(OH)_2$ pretreated CC compared to SSB biomass, while an approximately 1.14 μmol/mL higher activity was recorded when the HEC-H hydrolysed NaOH-pretreated CC compared to SSB

biomass. We propose that the HEC-H hydrolysed the alkaline pretreated CC better than the SSB due to differences in their biomass composition; CC had a higher hemicellulose content compared to SSB. Furthermore, our results also demonstrated that the alkaline pretreatment removed lignin from the biomass and improved the activity of the HEC-H, while there was no significant difference between the CC and SSB biomass controls (Figure 3A).

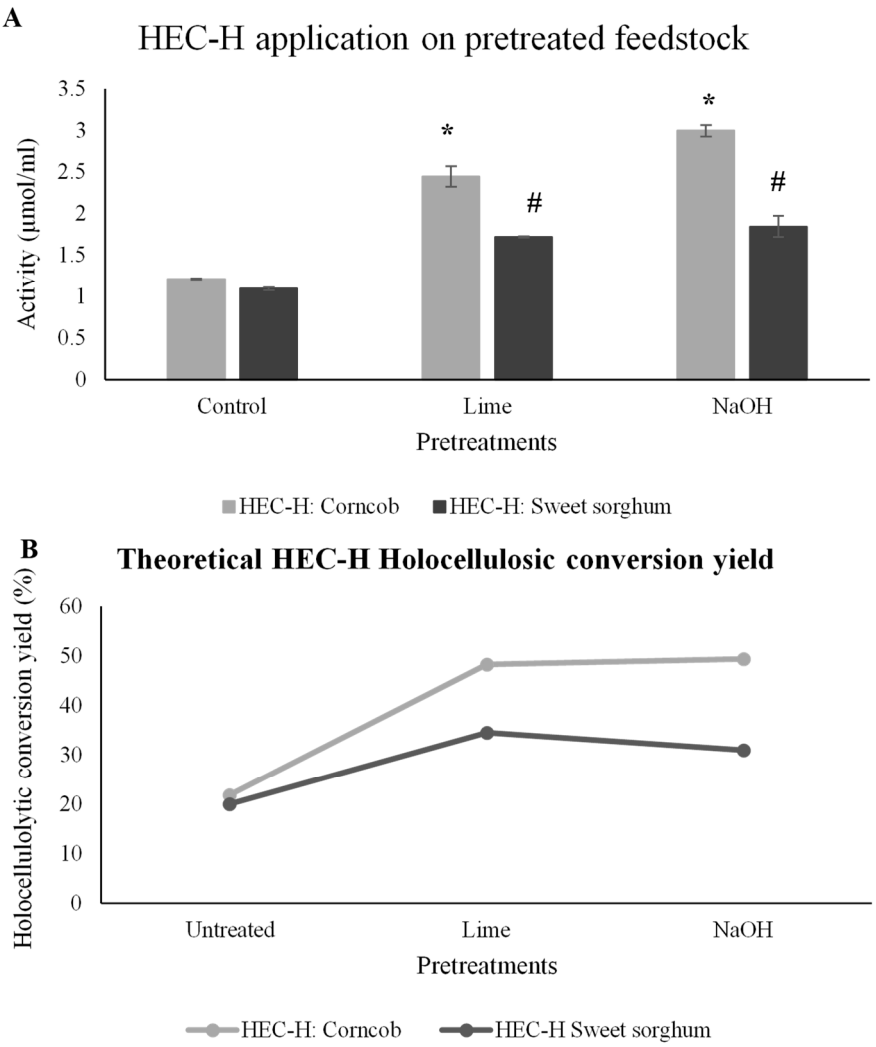

Figure 3. Application of the holocellulolytic enzyme cocktail (HEC-H) formulated with multifunctional enzymes (MFEs) and exoglucanase (Exg-D) derived from a termite bacterial hind-gut metagenome on pretreated sweet sorghum bagasse (SSB) and corncob (CC). (**A**) Activity in µmol/min, and (**B**) holocellulose conversion yields (total reducing sugars produced by HEC-H divided by the sum of cellulose and hemicellulose content) of SSB and CC samples. * Represents p-values < 0.01 and # represents p-values < 0.05. All experiments were performed in triplicate and the values represent means ± SD.

Figure 3B shows the theoretical holocellulosic hydrolytic yields of the HEC-H activity on the SSB and CC samples. The results suggest that the conversion rates of the $Ca(OH)_2$ pretreated CC samples was 48.30%, while the conversion rate of the NaOH pretreated CC was about 49.46%. The HEC-H only converted about 21.91% of the untreated CC samples, which illustrated that the enzyme cocktail displayed about 2.25-fold higher efficiency on the alkali pretreated biomass. In contrast, the HEC-H displayed an approximate 1.67-fold increase in the theoretical conversion rate of alkali pretreated SSB samples, compared to the untreated SSB (control). We suggest that the enzyme cocktail efficiency was

higher on CC compared to SSB samples, because of the higher holocellulosic content in CC biomass (see Table 1).

4. Discussion

The SSB and CC biomass samples were pretreated before they were hydrolysed with the holocellulolytic enzyme cocktail formulated with MFEs and Exg-D to assess their hydrolysability. El-Naggar et al. [7] argued that one of the factors that hinders lignocellulosic hydrolysis are biomass structural or chemical factors, which include biomass crystallinity, cellulose degree of polymerisation, porosity, accessible surface area, particle size, lignin, hemicellulose and acetyl-group content. In addition, Álvarez et al. [25] reported that chemical pretreatments were essential to changes in the biomass composition. The pretreatment of the biomass results in the removal of lignin, which makes the biomass less recalcitrant to enzyme hydrolysis [6,25]. The current study demonstrated that NaOH pretreatment of the CC and SSB samples significantly removed the lignin that led to an increase in relative cellulose, hemicellulose and biomass porosity (Table 1; Figure 1). However, $Ca(OH)_2$ pretreatment did not remove all the lignin as shown in Table 1 and Figure 1. Beukes and Pletschke [10] also argued that the $Ca(OH)_2$ pretreatment of the sugar cane bagasse modified the lignin and did not remove most of it from the biomass. Alkaline pretreatments induce a saponification reaction, where free hydroxide ions break ester bonds that exist between the inner molecules of lignocellulose, which connect hemicellulose and other components (such as between lignin and other hemicelluloses) which results in an increase in the pore structures of lignocellulose due to disappearance of the connecting bonds [26].

The FTIR results validated the morphological and chemical changes of the biomass after pretreatment. FTIR results demonstrated that crystalline cellulose Iβ peaks were absent in the spectrum of the $Ca(OH)_2$ and NaOH pretreated SSB and CC biomass. The 1730 cm^{-1} peak corresponded to C-O stretching vibration for the acetyl and ester linkages in lignin and hemicellulose, while the 1250 cm^{-1} peak corresponded to C–O out of plane stretching due to the aryl group in lignin [27]. The presence of 1430, 1162 and 1111 cm^{-1} peaks indicates a prevalence of crystalline cellulose II [15]. The alkaline pretreated biomass showed improved cellulose II, hemicellulose and amorphous content. However, the SEM, chemical composition and FITR results revealed that the NaOH pretreatment was more effective in removing lignin from the biomass than $Ca(OH)_2$ pretreatment.

The MFEs displayed activity on the CMC, various xylan and xyloglucan substrates as shown in Table 2. Malgas et al. [5] reported that xylan is a complex substrate with different side chains that can hinder enzyme optimal function, if it possess a catalytic active site that is not suited to the side chains. Xyloglucan is also a complex substrate with various side chains as described by Fry et al. [28] and Rashmi and Siddalingamurthy [29]. These diverse and complex properties of the CMC, various xylan and xyloglucan substrates demonstrate that the MFE-5E, MFE-5H and MFE-45 were indeed multifucntional enzymes capable of hydrolysing the β-1,4-glucosidic bonds of the backbone chain. Several multifunctional (or promiscuous) enzymes have been reported previously and were defined as enzymes that play multiple physiological roles in a cell, or enzymes that display catalytic activities on a range of substrates which are structurally and chemically different [30–32]. In addition, mulifunctional/promiscous enzymes change their hydrolytic activities under different reaction conditions, which include various solvents, extreme temperature, various pHs and a range of substrates specificities. Aspeborg et al. [33] emphasised the idea that GH family 5 contains some of the multifunctional enzymes, which could explain the observed multifunctional nature of MFE-5E and MFE-5H.

HEC-H was applied to the alkaline pretreated biomass and the untreated biomass was used as a benchmark for hydrolysis improvement due to pretreatment. It was evident from Figure 3 that HEC-H demonstrated higher activity on pretreated biomass compared to the untreated biomass. In addition, the HEC-H performed much better on the CC biomass compared to the SSB biomass. The specific activity demonstrated that the MFEs used to formulate the HEC-H were highly active on the hemicellulose substrate and amorphous cellulose. These findings explain why the theoretical holocellulosic conversion

yields for alkali pretreated biomass were between 35% and 50%. Takada et al. [3] also demonstrated that the chemical composition of CC biomass consisted mostly of hemicellulose. However, SSB biomass after alkaline pretreatment consisted mainly of cellulose as the major component. We believe that the HEC-H is the first enzyme cocktail formulated using GHs derived from a termite hindgut metagenome for the effective hydrolysis of hemicellulose and amorphous cellulosic components of agricultural feedstocks. Other studies have used two or three enzymes derived from termites, i.e., Feng et al. [34] reported on the synergy formed by two β-glucosidase (CfGlu1C and CfGlu1B) enzymes from a lower termite (*Coptotermes formosanus*). CfGlu1C and CfGlu 1B displayed synergy on lactose hydrolysis and significantly increased the rate of hydrolysis. The GH7 enzyme, endoglucanase and β-glucosidase from *Reticulitermes flavipes* worker termites also displayed synergism on pine sawdust [35]. The synergism between these enzymes significantly increased the amounts of glucose released during the hydrolysis of the pine sawdust.

5. Conclusions

Alkaline pretreatment was more effective in pretreating CC biomass compared to the SSB biomass. Both $Ca(OH)_2$ and NaOH pretreatment removed lignin from the CC biomass, while only NaOH removed lignin from the SSB biomass. Biomass compositional analysis revealed that these agricultural feedstocks differed in their chemical composition because the CC biomass contained hemicellulose (33–35%) as its major component, compared to SSB biomass that consisted mainly of cellulose (17–24%). Specific enzyme activity revealed that the termite metagenome derived enzymes MFE-5E, MFE-5H and MFE-45 were multifunctional enzymes, which hydrolysed substrates associated with hemicellulose (various xylan and xyloglucan) and amorphous cellulose (CMC). These observations explain why the HEC-H formulated with MFEs effectively hydrolysed the CC biomass preferentially to SSB biomass. We propose that the HEC-H cocktail can be used for the hydrolysis of hemicellulose- and amorphous cellulose-rich agricultural feedstocks during the bioconversion process in the biorefinery industry.

Author Contributions: M.S.M. carried out all the experiments and performed data analysis. K.R. purified and provided the termite derived enzymes. M.S.M., S.M. and A.B. participated in the experimental design of synergy studies and synergy data analysis. M.S.M., S.M. and A.B. designed the pretreatment and data analysis. M.S.M. and B.I.P. conceptualised the study and participated in its design coordination. M.S.M. drafted the manuscript. B.I.P. and K.R. supervised and co-supervised the study, respectively. M.S.M. prepared the manuscript while S.M., K.R. and B.I.P. contributed in editing and final preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

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