Effects of Pretreatment of Single and Mixed Lignocellulosic Substrates on Production of Endoglucanase by *Bacillus aerius* S5.2

Mushafau Adebayo Oke,[a,b] Mofoluwake M. Ishola,[c,d] Mohammad J. Taherzadeh,[c] Mohamad Suffian Mohamad Annuar,[a] and Khanom Simarani [a,*]

A mixed substrate (MS) comprising oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH) was evaluated for endoglucanase production by *Bacillus aerius* S5.2. Effects of sulphuric acid, sodium hydroxide, *N*-methylmorpholine-*N*-oxide (NMMO), and hydrothermal pretreatments on endoglucanase production were investigated. Endoglucanase production by *B. aerius* on the untreated (0.677 U/mL) and pretreated MS (0.305 – 0.630 U/mL) was generally similar, except that the acid (0.305 U/mL) and hydrothermal (0.549 U/mL) pretreatments that were more severe consequently produced significantly lower titres. Alkali pretreatment supported the highest enzyme production (0.630 U/mL) among all pretreatments that were studied. When endoglucanase production on the alkali-pretreated MS and single substrates (SS) was compared, alkali-pretreated EFB produced a titre (0.655 U/mL) similar to the MS, and this was significantly higher than titres recorded on OPF (0.504 U/mL) and RH (0.525 U/mL). Lower enzyme production was found to be consistent with higher pretreatment severity and greater removal of amorphous regions in all the pretreatments. Furthermore, combining the SS showed no adverse effects on endoglucanase production.

Keywords: Bacillus aerius; Endoglucanase; Mixed lignocellulosic substrate; Pretreatment

*Contact information: a: Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; b: Department of Microbiology, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Nigeria; c: Swedish Centre for Resource Recovery, University of Borås, Sweden; d: Department of Chemical and Polymer Engineering, Faculty of Engineering, Lagos State University, Nigeria; *Corresponding author: hanom_ss@um.edu.my*

INTRODUCTION

Lignocellulosic agricultural wastes such as oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH) are generated in large amounts annually from the vibrant oil palm sector and the agricultural industry in Malaysia and other countries in the region. Up to 20.7 million metric tons of such residues are produced yearly in Malaysia and Indonesia (Ishola *et al.* 2014). The disposal of these wastes through open burning or by dumping on landfills often contributes to environmental pollution. Utilisation of these wastes for the production of value-added products such as commercial enzymes would be a double-pronged measure aimed at wealth creation and ensuring environmental sustainability.

Cellulose is the most abundant polysaccharide component of lignocellulosic materials (Maki *et al.* 2009; Isikgor and Becer 2015). It can be hydrolysed enzymatically

into its glucose monomers with cellulases such as endoglucanase, exoglucanase, and β-glucosidase, a group of hydrolytic enzymes that act synergistically. Endoglucanases are particularly important because they initiate cellulose hydrolysis, and their action on amorphous regions of cellulose is a rate-limiting step of cellulose hydrolysis (Malherbe and Cloete 2002). Fungal cultures have been widely used for commercial production of cellulases because of the high titres they produce. However, bacterial cellulases have gained increased interest because of their ease of genetic manipulation, high growth rate, expression of cellulase in highly efficient enzyme complexes called cellulosomes, and the stability of their cells and enzymes under challenging bioprocessing conditions (Maki *et al*. 2009).

The high cost of cellulase is one of the major bottlenecks of lignocellulose utilisation. This is partly due to the expensive nature of the synthetic substrates/inducers (such as lactose, Solka floc, Avicel, and carboxymethyl cellulose) used in commercial cellulase production. The use of less expensive substrates has therefore been suggested as a means of reducing the cost of cellulase production (Klein-Marcuschamer *et al*. 2012).

The use of a single lignocellulosic biomass type as a substrate has been investigated for microbial cellulase production (Bigelow and Wyman 2002; Kshirsagar *et al*. 2016). However, the use of mixed lignocellulosic substrates has been less explored. Feedstock supply challenges arising from logistic problems, seasonal availability, unstable weather, and other competing uses of the material would, in the long run, make the dependence on a single feedstock for bioprocessing unsustainable. The use of mixed substrates has potential benefits, such as elimination of the need for nutrient supplementation with expensive additives during downstream fermentations, combination of favourable characteristics of several feedstocks, and cost reduction (Yang *et al*. 2015). Thus, the use of mixed lignocellulosics for cellulase production could be a viable option for reducing the cost of the enzyme and ultimately reducing the cost of lignocellulosic bioprocessing. While mixed lignocellulosic substrates have been investigated for fermentable sugar and ethanol production (Moutta *et al*. 2013; Elliston *et al*. 2015; Yang *et al*. 2015), the use of such substrates for bacterial cellulase production has been less reported (Oke *et al*. 2016).

Pretreatment is an important step during the bioconversion of lignocellulosic biomass. This is done to open up the lignocellulosic structure and to improve cellulose digestibility (Zhao *et al*. 2012; Ishola *et al*. 2014). For cellulase production, pretreatment processes are required to alter the substrate's physicochemical characteristics to aid microbial utilisation and enzyme induction (Brijwani and Vadlani 2011). For example, dilute acid causes hemicellulose removal while in addition to that, hydrothermal pretreatment effects partial lignin depolymerization, reduction of cellulose crystallinity, and increased specific surface area (Zhao *et al*. 2012). Alkali pretreatment causes swelling of cellulose fibrils, removal of lignin, and increased internal surface area (Zhang *et al*. 2012; Zhao *et al*. 2012). *N*-methylmorpholine-*N*-oxide (NMMO) increases the porosity of the biomass surface microstructure and reduces the crystallinity (Shafiei *et al*. 2014). In adopting the use of mixed substrates for cellulase production, and other fermentation processes, it is important to establish whether the combination of substrates would have any deleterious effects on the process. This is because of the diverse characteristics of the individual substrates and the uncertainty regarding the effects of such mixtures on the microorganism.

The objective of this study was to compare the performance of four pretreatment methods in obtaining a substrate with improved chemical and physical characteristics for endoglucanase production by *B. aerius* S5.2. Changes in the composition, functional groups, crystallinity, and microstructure of pretreated and untreated mixed (MS) and single substrates (SS) were studied to understand the effect of each pretreatment on the observed endoglucanase production pattern. This allowed for better comprehension of the relationship between substrate physicochemical properties and subsequent endoglucanase synthesis. Endoglucanase production on MS from the best pretreatment was also compared with its production on each SS to ascertain the effectiveness of combining the substrates.

EXPERIMENTAL

Bacterial Strain

Bacillus aerius S5.2 was previously isolated from decomposing EFB residues in an oil palm plantation at Kuala Selangor, Malaysia. It was identified after 16S rDNA sequencing and sequence search in the National Center for Biotechnology Information (NCBI) database. It had 99% similarity with *Bacillus aerius* 24K. The sequence was deposited with GenBank with accession number KP178216. This strain has been deposited at the Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia, under the accession number UPMC 1179.

Substrates

Fresh OPF samples were collected from the Malaysian Palm Oil Mill Board (MPOB), Bangi, Malaysia. The leaflets were removed and petioles were cut into smaller chips and sun-dried. The petioles alone were used in this work. Dried and shredded EFB fibers were obtained from the Biorefinery Complex, University Putra Malaysia, Serdang, Malaysia. RH was collected from a paddy field in the state of Kedah, Malaysia. The samples were separately ground to smaller sizes using a Rapid granulator (GK 205-K, Terramar, Hamburg, Germany). The ground fibers were then sieved using a laboratory sieve (Endecotts Ltd, London, United Kingdom) to obtain particle sizes of 300 to 425 µm. The samples were kept dry in airtight containers until they were ready for use.

Substrate Pretreatments

The three single substrates (SSs) used in this study, EFB, OPF, and RH, were mixed in a 1:1:1 ratio, and the resulting mixture (MS) was used as a substrate in the pretreatment studies. MS was subjected to dilute acid, dilute alkali, hydrothermal, and organic solvent pretreatments with 1% (v/v) H_2SO_4, 1% (v/v) NaOH, distilled water, and 85% (w/w) *N*-methylmorpholine-*N*-oxide (NMMO), respectively, as pretreatment solvents. Besides the unique effects of each of these pretreatment methods on lignocellulose mentioned earlier, they also represent a fairly diverse range of physicochemical pretreatments commonly applied for rice and oil palm residues (Imman *et al*. 2013; Purwandari *et al*. 2013; Ang *et al*. 2013). MS was suspended in the respective solvents of each pretreatment method in 500 mL bottles to obtain a solid loading of 10% (w/v) on a dry weight basis. The suspension was heated in an autoclave at 121 °C, 103.4 kPa for 1 h. After cooling, the liquid fraction

was separated from the slurry by vacuum filtration. The solid fraction was washed with deionised water until neutral pH was reached. For the NMMO pretreatment, 150 mL of hot distilled water was added as an anti-solvent to recover the dissolved materials before separation (Kabir *et al.* 2014). Washing of the solids was repeated until a clear filtrate was obtained. The washed pretreated solids were freeze-dried (Freezone 7670530, Labconco, Kansas City, MO) and kept at 4 °C until they were ready for use.

Endoglucanase Production on MS from Different Pretreatments

B. aerius S5.2 was cultivated in nutrient broth, and the culture was allowed to reach late log phase (12 h). An aliquot (containing approximately 10^7 cfu/mL) from this culture was used as the inoculum in the experiments. The modified medium of Dickerman and Starr (1951) with 2% (w/v) of respective pretreated MS as a carbon source was used for endoglucanase production. Medium pH was adjusted to 7.0 with 1.0 M NaOH or 1.0 M HCl. Fifty millilitres of medium in a 250 mL Erlenmeyer flask was inoculated with 10% (v/v) of inoculum. Each flask was incubated at 30 °C and at 170 rpm agitation speed for 72 h. The experiments were conducted in triplicate for each pretreatment method. Culture samples were collected at 12 h intervals and were centrifuged at 6000 rpm at 4 °C for 10 min. The cell-free supernatant obtained was used as the crude enzyme in the enzyme assay.

Endoglucanase Production on Single Substrates

Each SS was subsequently pretreated using the pretreatment method that supported the highest endoglucanase production by *B. aerius* S5.2 on MS. The pretreatment conditions (substrate loading, temperature, residence time, *etc*.) and fermentation conditions used for MS were applied for SS. Culture samples were collected at 12 h intervals and analysed to determine endoglucanase activity.

Analytical Methods

All substrates (raw and pretreated) in this study were used on a dry weight basis after determination of the total solids of each material. Total solids were determined by monitoring the difference in oven-dry weight of each material after drying to constant weight at 105 °C (Sluiter *et al.* 2008a). Cellulose, hemicellulose, and lignin in the pretreated substrates were determined using National Renewable Energy Laboratory (NREL) protocols (Sluiter *et al.* 2008b). The biomass (300 mg) was hydrolysed with 3 mL of 72% H_2SO_4 at 30 °C for 60 min. The acid was then diluted to 4% by addition of deionised water, and the sample was heated at 121 °C for 60 min in an autoclave for a second hydrolysis. The sample was then vacuum-filtered. Sugars in the filtrate were analysed by high-performance liquid chromatography (HPLC) (Waters 2695, Waters Corporation, Milford, USA). A lead based column (Aminex HPX-87P, Bio-Rad, Hercules, USA) was used at 85 °C and 0.6 mL/min with ultrapure water as the eluent. Detection was conducted using a refractive index (RI) detector (Waters 2414). The monomer sugar concentrations were used in calculating the amount of cellulose and hemicellulose in the biomass. The amount of acid-soluble lignin in the filtrate was determined using a UV spectrophotometer (Libra S60, Biochrom, England) at 320 nm and with an ε value of 30 L/(g.cm). Acid-insoluble lignin content was determined gravimetrically after heating the solid residue in a muffle furnace at 575 °C for 24 h and deducting the ash content.

The substrates were further characterised using field-emission scanning electron microscopy (FESEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FT-IR). Effects of pretreatment on the surface morphology of the substrates were observed with a scanning electron microscope (JSM-7001F, JEOL, Tokyo, Japan). Images were acquired at 5 to 15 kV and at various magnifications. For the MS samples, images of the different portions were repeatedly taken to ensure that each SS was captured. The single substrates were identified in the mixtures by making comparisons with micrographs of pure SS samples. Changes in the crystallinity of the samples were determined using a PANalytical Empyrean Multipurpose X-ray diffractometer (PANalytical BV, Netherlands). Scans were taken at 4 s per step from 2θ = 5° to 60° with a step size of 0.03°. The relative degree of crystallinity (*CrI*) of the samples was calculated according to the method of Segal *et al.* (1959), using the equation,

$$CrI = (I_{002} - I_{am})/I_{002} * 100 \quad (1)$$

Here, I_{002} is the maximum intensity of the 002 lattice diffraction around 2θ = 22.8° (corresponding to the crystalline region) and I_{am} is the intensity of diffraction around 2θ = 18° (corresponding to the amorphous region). FT-IR analysis was conducted using a Perkin-Elmer FTIR spectrum-400 spectrometer (Perkin-Elmer Inc., Wellesley, MA), and the spectra were obtained in the range of 500 to 4000 cm^{-1}.

The endoglucanase assay was performed with slight modification of the method reported by Zhang *et al.* (2009). The reaction mixture contained 200 µL of the crude enzyme preparation with 200 µL of 2% CMC (medium viscosity) in 0.05 M phosphate buffer (pH 7.0). The mixture was incubated at 50 °C for 30 min. The reaction was stopped by the addition of 800 µL of dinitrosalicylic acid (DNS) reagent, followed by boiling in a water bath for 5 min. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per min from the substrate.

Statistical Analysis

All statistical analyses were carried out using IBM SPSS Statistics, Version 22 (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Effects of Pretreatments on Chemical Composition of MS

The chemical compositions of the untreated and pretreated MS samples are presented in Table 1. Compared with the untreated MS, all pretreated MS samples had significantly ($P < 0.05$) higher cellulose and hemicellulose compositions, with the exception of the acid-pretreated MS, which had a significantly ($P < 0.05$) lower amount of hemicellulose (6.88%). This could be due to the high amount of other components present in the untreated mixture. The amount of other components in the untreated single substrates varied between 19% and 25%; this explains the high value obtained for the mixture. These other components may include protein and extractives (Sluiter *et al.* 2008b), but they were not individually analysed in this study. Acid-pretreated MS also had the highest composition of cellulose (48.65%) and lignin (30.87%). This can be attributed to the

removal of a greater portion of hemicellulose by the acid, which caused an increase in the proportion of the other two components in the sample (Zhao *et al.* 2012). There was no significant difference ($P > 0.05$) in the chemical composition among the alkali-, hydrothermal-, or NMMO-pretreated substrates. This observation suggested that the acid treatment had a stronger effect than the rest, despite the fact that similar mild conditions (*viz.* 1% (v/v/) solvent concentration, 121 °C, and 103.4 kPa) were applied in all the pretreatments investigated.

Table 1. Chemical Composition of MS after Various Pretreatments

Treatment	Chemical composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Others
Untreated MS	31.97 ± 0.30^{a}	15.07 ± 0.24^{b}	20.88 ± 0.10^{a}	4.70 ± 0.19^{a}	27.38 ± 1.63^{a}
Acid	48.65 ± 0.39^{c}	6.88 ± 0.97^{a}	30.87 ± 1.45^{b}	5.62 ± 3.22^{a}	7.98 ± 1.90^{b}
Alkali	37.84 ± 1.62^{b}	19.04 ± 0.87^{c}	23.36 ± 0.52^{a}	3.78 ± 1.27^{a}	15.98 ± 3.18^{b}
Hydrothermal	38.31 ± 0.56^{b}	19.80 ± 0.47^{c}	23.73 ± 1.86^{a}	2.25 ± 1.99^{a}	15.91 ± 3.27^{b}
NMMO	40.20 ± 1.35^{b}	20.07 ± 0.57^{c}	22.29 ± 0.27^{a}	1.83 ± 2.65^{a}	15.60 ± 3.95^{b}

Data are expressed on a dry weight basis. $^{a-c}$ Values represent means of at least two replicates ± standard deviation (SD). Values within the same column and having the same superscript letters are not significantly different ($p > 0.05$).

Effects of Pretreatments on Structural Characteristics of MS and SS

FESEM

Because of the heterogeneous nature of mixtures, it was difficult to monitor changes to individual components following pretreatments because of the irregular size, structure, and distribution of the diverse components (Moutta *et al.* 2013). To overcome this, images of each SS were taken separately before capturing those of the MS. When images of the MS were captured, repeated shots were taken so that each SS appeared in the MS micrographs. The untreated SS samples had relatively unruffled microstructures (Figs. 1a through d), with EFB showing intact microfibrils with embedded silica deposits. Untreated OPF had a fairly intact mesh-like inner surface structure and a smooth outer fibrillar surface. Similarly, untreated RH had a well-ordered surface with embedded silica bodies. However, RH in all the pretreated MS samples (Figs. 1e, f, g, and h) seemed to have undergone very little change compared with the untreated RH. This may have been due to the natural recalcitrance of the RH biomass as a result of the abundant silica bodies present. The surface of the EFB component of MS from all the pretreatments was altered, with exposed microfibrils and silica bodies. Exposure of the microfibrils was more evident in the acid (Figs. 1e, i, and m) and NMMO-pretreated MS samples (Figs. 1h, l, and p), while removal of silica bodies was more pronounced in the NMMO-pretreated MS. Disruption of the EFB component was least obvious in the hydrothermally-pretreated MS (Figs. 1g, k, and o). Distortion of the smooth OPF component's outer surface could be observed in the acid-pretreated MS, while the disruption of the mesh-like network of the OPF was most

obvious in the alkali- (Figs. 1f, j, and n) and hydrothermal-pretreated samples. Generally, there were not many differences among MS from all the pretreatments with respect to their microstructure. This is consistent with the chemical composition data, where all the MS except the acid-pretreated had similar composition, and the observation may be due to the relatively mild conditions applied in the pretreatments. The higher severity of the acid treatment was indicated by its relatively higher solubilisation of hemicellulose.

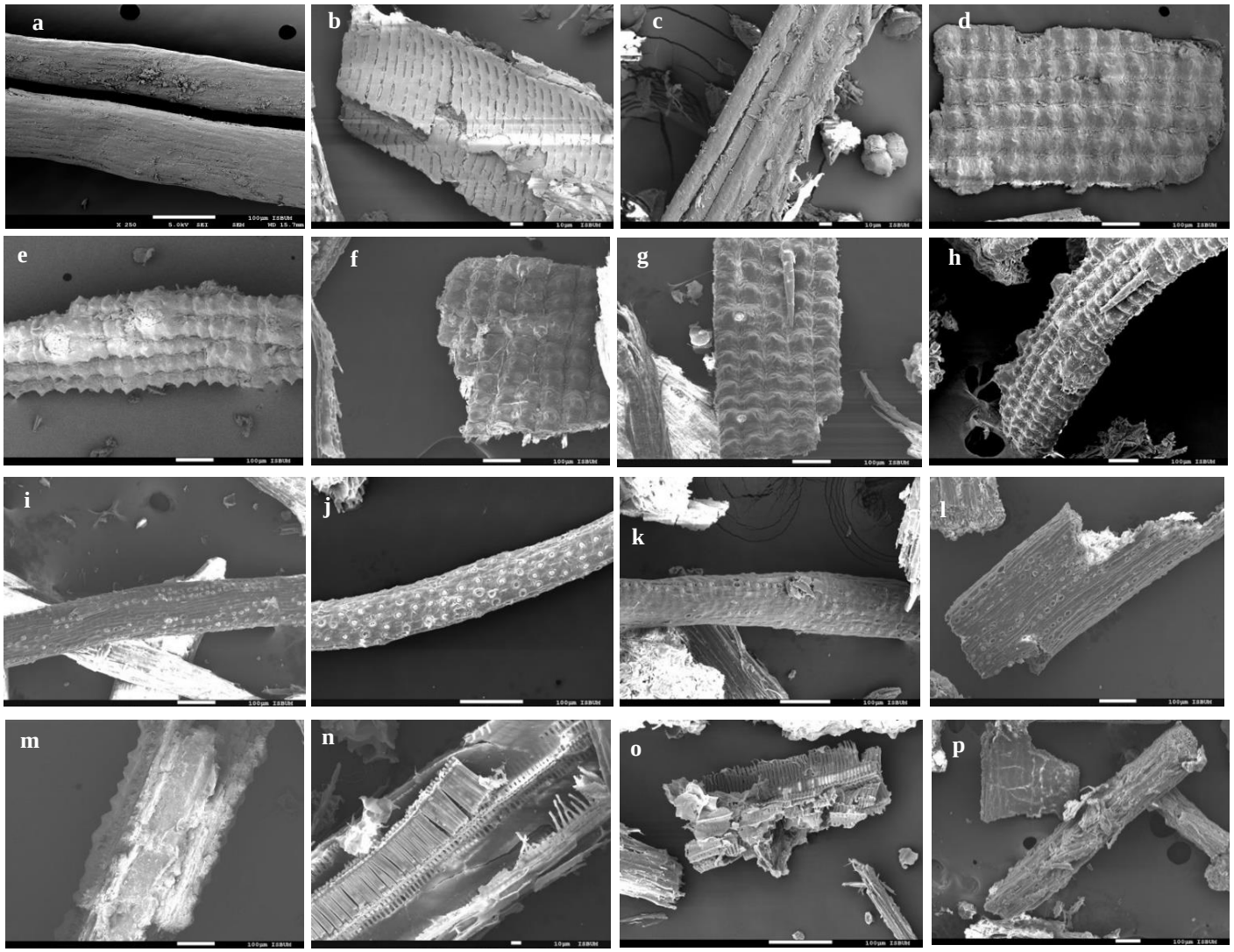

Fig. 1. Micrographs of untreated SS and individual components of pretreated MS samples. Untreated samples: a- EFB x 250; b- OPF x 500 (inner surface); c- OPF x 500 (outer surface); d- RH x 150. Pretreated MS samples: acid (e- RH x 150, i- EFB x 150, m- OPF x 150); alkali (f- RH x 150, j- EFB x 250, n- OPF x 400); hydrothermal (g- RH x 150, k- EFB x 200, o- OPF x 250); NMMO (h- RH x 120, l- EFB x 150, p- OPF x 100). SS were identified in the MS micrographs by comparing MS images with those of pure untreated SS.

Effects of Pretreatments on Endoglucanase Production on MS

The profile of endoglucanase production by *B. aerius* S5.2 on the MS from the various pretreatments and the untreated MS is shown in Fig. 2. A sharp rise in enzyme production was observed between 6 and 12 h on the untreated substrate and the pretreated substrates, except for the acid-pretreated substrate, which experienced some lag in enzyme production. Maximum enzyme production was reached on all the substrates at 48 h, except for the hydrothermally-pretreated MS (60 h). Interestingly, the untreated MS supported the highest endoglucanase production (0.677 U/mL), although this difference was not significant ($P > 0.05$) when compared with the alkali-pretreated and NMMO-pretreated MS. Next to it was the alkali-pretreated MS, which gave a maximum enzyme titre of 0.630 U/mL. The NMMO-pretreated MS and the hydrothermally pretreated MS had similar enzyme production profiles, but enzyme titres reached their peak value on NMMO-pretreated MS (0.557 U/mL at 48 h) faster than for the hydrothermally-pretreated MS (0.549 U/mL at 60 h). There was no significant difference ($P > 0.05$) in the enzyme production on the alkali-, NMMO-, and hydrothermally-pretreated MS samples. Enzyme production on the untreated MS was significantly higher ($P < 0.05$) than on the hydrothermally-pretreated and acid-pretreated MS samples. The acid-pretreated MS produced the least amount of enzyme, with a maximum titre of 0.305 U/mL, which was significantly lower ($P < 0.05$) than what was obtained on all other substrates.

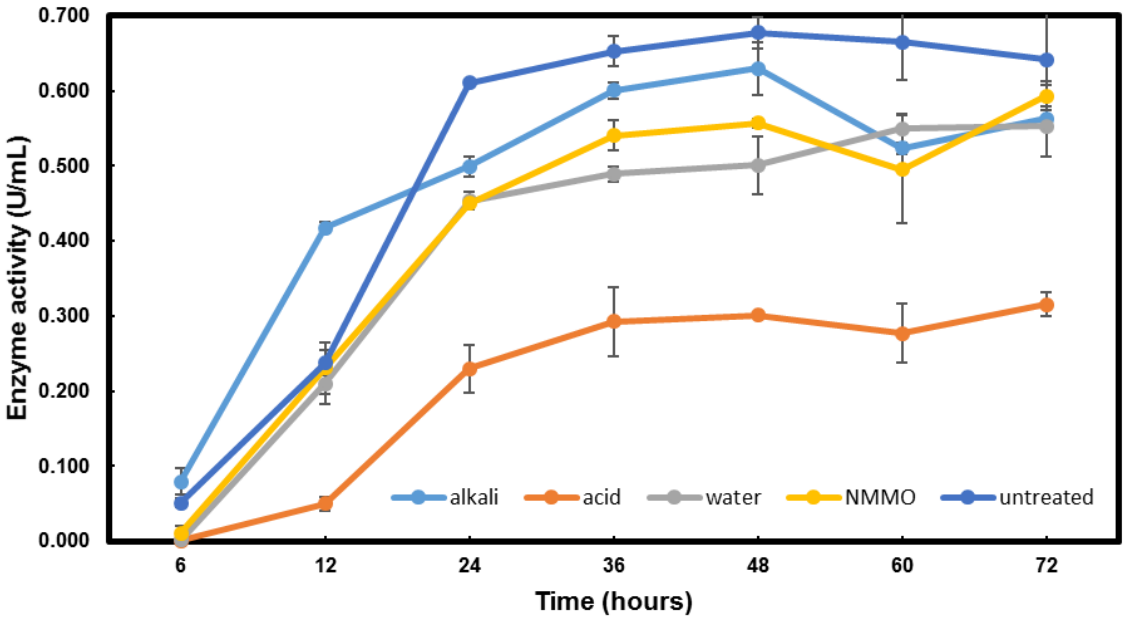

Fig. 2. Endoglucanase production by *B. aerius* S5.2 on pretreated and untreated MS samples

Several reasons could be speculated for the higher enzyme production recorded on the untreated MS in this study (Fig. 2). Firstly, the milling process used in obtaining the small particle sizes is a form of pretreatment itself. Milling brings about increased surface area, reduced crystallinity, and provides greater accessibility (Zhao *et al.* 2012). These

characteristics may have been altered in the pretreated substrates because of the negative effects of the treatments.

Olsson *et al.* (2003) reported that the removal of some parts of pectin and hemicellulose from sugar beet pulp as a result of pretreatment caused a lag in the growth of *Trichoderma reesei* as compared with the untreated substrate. This is an indication that in some instances, pretreatment could make a substrate less accessible and less suitable for microbial growth and utilisation when compared with the untreated one. Acid treatment can also cause lignin to condense on the surface of crystalline cellulose (Li *et al.* 2010), thereby limiting substrate accessibility for cellulase induction. As the untreated MS used was unwashed, the presence of free sugars on the surface of the untreated MS could also have led to the higher endoglucanase production. It was found that un-inoculated fermentation media containing the untreated MS had reducing sugar concentrations of 0.63 mg/mL, while those of the pretreated substrates were between 0.01 and 0.06 mg/mL. Cellulase production is normally induced by the presence of soluble cellulose derivatives and other low-molecular weight carbohydrates, such as cellobiose, xylose, sophorose, and lactose (James and Ming 1991). Furthermore, the removal of these substances and other water-soluble micronutrients from the MS during the pretreatments might have made the substrate less favourable for the organism's metabolism (Basu and Ghose 1960). Higher enzyme production on untreated substrates has been previously reported (Rodriguez-Zuniga *et al*. 2014; Sharma *et al*. 2015).

The acid-pretreated MS gave the least enzyme production despite its higher cellulose content (Fig. 2). It also had the highest amount of lignin and the lowest amount of hemicellulose compared with the other pretreated MS samples (Table 1). Although the extent of cellulase production/induction is dependent on the accessibility and exposure of cellulose in the substrate, previous studies have shown that the amount of cellulose is not the sole determinant of cellulase production in microbial fermentation.

The substrate's physicochemical and structural characteristics also influence cellulase production (Umikalsom *et al.* 1997; Brijwani and Vadlani 2011). Bigelow and Wyman (2002) reported that increasing cellulose levels of hot water-pretreated bagasse had little effect on cellulase production by *T. reesei* C30. This trend was not observed with similar concentrations of Solka floc, which is almost entirely composed of cellulose. The authors therefore suggested that other inhibitory effects inherent in the pretreated substrates were responsible for this observation. Similarly, Sharma *et al.* (2015) found no direct relationship between cellulose content and cellulase production by *Penicillium janthinellum* EMS-UV-8 on wheat straw samples that had been subjected to varying degrees of pretreatments. However, the significantly higher ($P < 0.05$) lignin content (Table 1) appears to be a major reason for the low endoglucanase production recorded on the acid-pretreated MS in this study.

Acid pretreatment is known to preferentially solubilise hemicellulose and less ordered forms of cellulose, thereby leaving a lignin-rich residue behind (Zhao *et al*. 2012). It has been well established from previous studies that lignin plays an inhibitory role towards cellulose accessibility. The effects of lignin on microbial cellulase production can be summarised as follows: (1) inhibition of microbial growth and cellulase production (Bigelow and Wyman 2002), (2) irreversible adsorption and cellulase loss (Bigelow and Wyman 2002), and (3) limiting exposure of cellulose, thereby reducing availability for

enzyme induction (Zhang *et al*. 2012). Lower cellulase production on acid-pretreated substrates as compared with other pretreatment methods have been reported previously (Zhang *et al.* 2012; Salihu *et al.* 2015).

Despite the similarity in the chemical composition of the alkali-, hydrothermal-, and NMMO-pretreated MS samples (Table 1), alkali-pretreated MS supported significantly higher ($P < 0.05$) endoglucanase production than the hydrothermally pretreated MS, but had a similar enzyme titre to the NMMO-pretreated MS. This can be attributed to the unique effect of alkali and NMMO on lignocellulose, which altered the characteristics of the substrate in a more favourable manner than the hydrothermal pretreatment. Alkali pretreatment causes the swelling of cellulose fibrils and increased internal surface area, thereby making the cellulose accessible for enzyme induction (Zhang *et al*. 2012). NMMO causes a reduction in surface lignin, reduced crystallinity, and increased porosity of the substrate microstructure (Shafiei *et al*. 2014). These effects might not have been as pronounced in the hydrothermal pretreatment applied in this study.

Chemical Composition of Alkali-Pretreated SS

Because the alkali-pretreated MS supported the highest endoglucanase production by *B. aerius* S5.2, the same pretreatment was subsequently applied on each SS. The pretreated SS were used as a carbon source for endoglucanase production by the strain. This was done to ascertain whether combining the SS is more favourable than using them separately for enzyme production. Results of the compositional analysis are presented in Table 2. Alkali-pretreated EFB had the highest cellulose and hemicellulose contents, while alkali-pretreated RH had the highest lignin content and the least amount of hemicellulose.

Table 2. Composition of Alkali-Pretreated Single Substrates

Substrate	Chemical composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Others
EFB	32.83 ± 0.08^{a}	21.97 ± 2.42^{a}	15.21 ± 0.52^{b}	0.72 ± 0.15^{a}	29.28 ± 1.80^{b}
OPF	26.49 ± 0.88^{c}	17.76 ± 0.44^{a}	13.60 ± 0.60^{b}	-	42.15 ± 1.04^{a}
RH	28.49 ± 0.73^{b}	16.65 ± 2.62^{a}	23.01 ± 3.39^{a}	0.48 ± 0.16^{a}	28.75 ± 0.70^{b}

Data are expressed on a dry weight basis. $^{a-c}$ Values represent means of at least two replicates ± standard deviation (SD). Values within the same column and having the same superscript letters are not significantly different ($p > 0.05$).

Structural Analysis of Alkali-Pretreated SS

FESEM

As observed after the MS pretreatments, alkali-pretreated RH (Fig. 3b) showed very few changes compared with its untreated counterpart (Fig. 1d). The EFB was visibly altered by alkali pretreatment, as can be seen in the exposed microfibrils and dislodged silica bodies (Fig. 3a). Disruption of the surface of the alkali-pretreated OPF fibers could also be seen (Fig. 3c). These changes may play an important role in the uptake and utilisation of the substrate by the organism and its subsequent use for endoglucanase production.

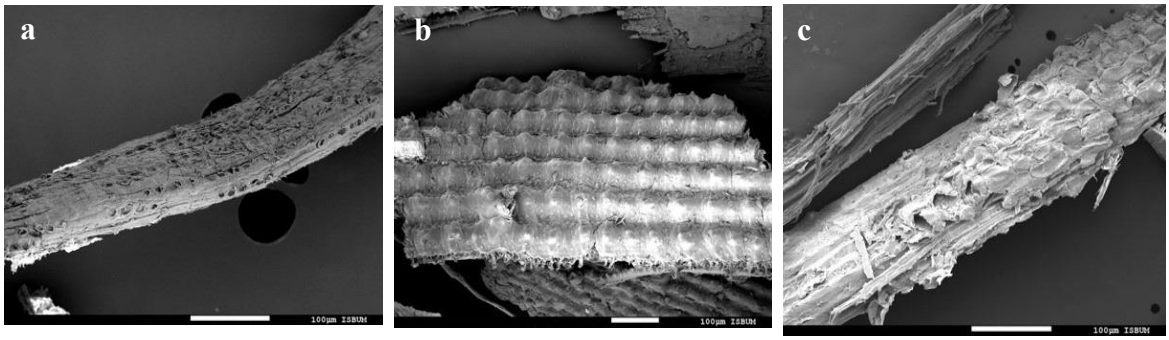

Fig. 3. FESEM micrographs of alkali-pretreated SS samples: (a) EFB x 250; (b) RH x 150; (c) OPF x 250

FTIR

The extent of deviation of each pretreated SS from its untreated form was determined using the difference in intensities at frequencies of prominent band in its spectrum. These are presented in Table 3 along with the band assignments. Comparison of the FTIR spectra shows that OPF had the most significant deviation from its untreated form (Fig. 4). It had the highest changes in band intensities at 1032 cm^{-1} and near 2920 to 2900 cm^{-1}. This showed greater degradation of the major lignocellulose components in alkali-pretreated OPF.

The highest change in band intensity at 3336 cm^{-1} was seen in the alkali-pretreated EFB (Fig. 4). This suggests that a higher amount of delignification occurred in the EFB because of the increased presence of the OH groups associated with cellulose. The spectrum of the alkali-pretreated RH was very similar to that of the untreated sample, except for the reduced band intensity at 1032 cm^{-1} (Fig. 4). This was consistent with the FESEM analysis, which showed that very little changes occurred in the pretreated RH.

Table 3. Band Intensity Changes in FTIR Spectra of Alkali-Pretreated SS

Wavenumber (cm^{-1})	Band assignment	Source component	Difference in band intensity			Ref.
			EFB	OPF	RH	
1033 – 1030	C–O, and C–C, and C–O–C stretching	Cellulose, hemicellulose, lignin	0.1986	0.3978	0.1644	(Sills and Gossett 2012)
2920 – 2900	Methylene C-H stretching	Cellulose	0.0545	0.0992	0.0293	(Hsu *et al.* 2010)
3336 – 3330	O–H stretching of hydrogen bonds	Cellulose	0.2325	0.2072	0.033	(Hsu *et al*. 2010)

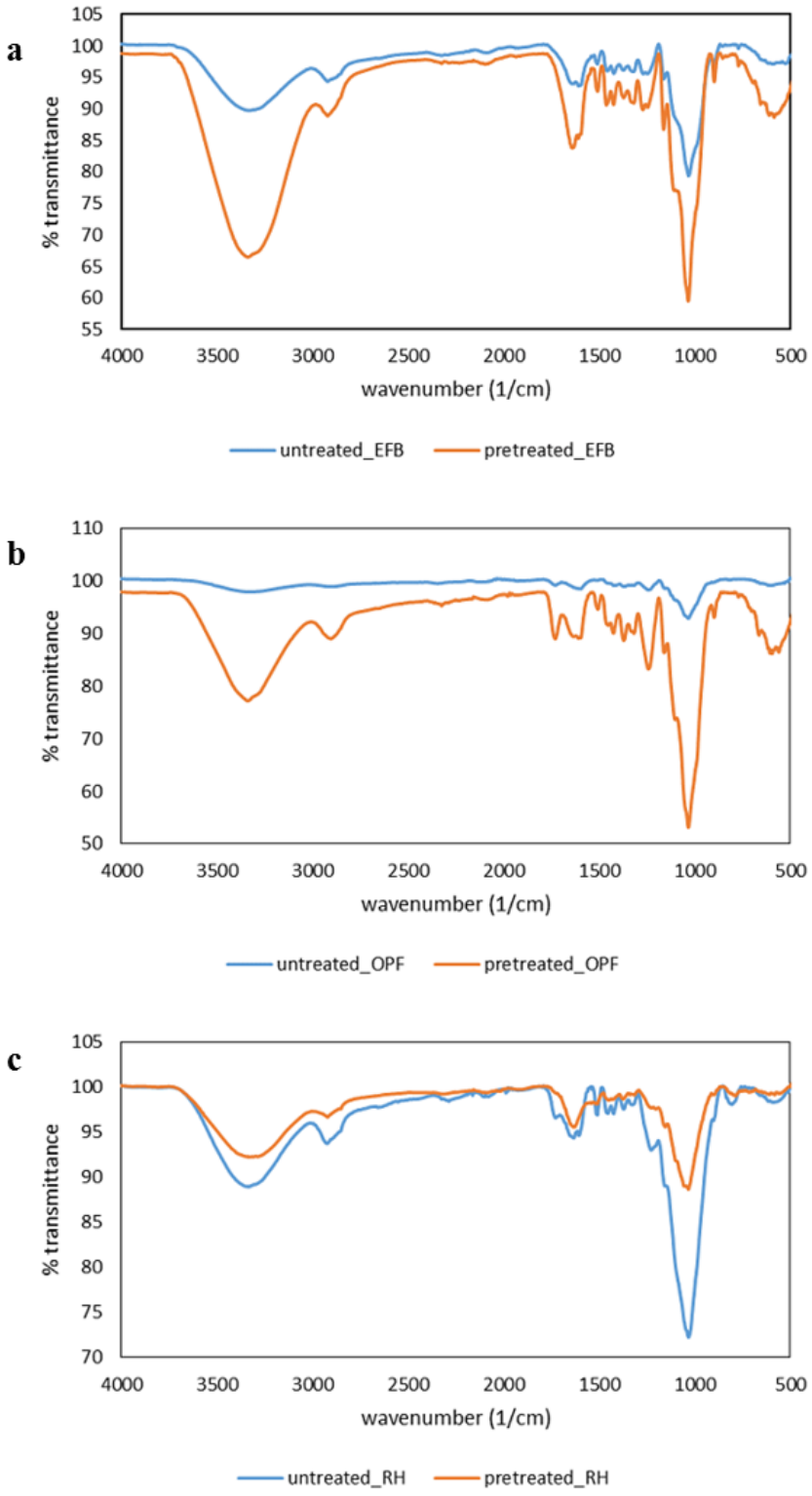

Fig. 4. FTIR spectra of alkali-pretreated SS samples: (a) EFB, (b) OPF, and (c) RH

XRD

Based on the XRD data (Fig. 5), the *CrI* values of the alkali-pretreated SS used in this study were 44.61, 50.08, and 41.10 for EFB, OPF, and RH, respectively. The high *CrI* value of the OPF was due to the severity of the pretreatment on this substrate as compared with EFB and RH. Higher *CrI* values are consistent with decreased abundance of amorphous portions of biomass (amorphous cellulose, hemicellulose, and lignin) following their removal during pretreatment (Rodriguez-Zuniga *et al.* 2014). This is supported by the chemical composition of the substrates (Table 2), which showed that samples with higher *CrI* had lower amorphous components (EFB- 37.18%, OPF- 31.36%, and RH- 39.66%) *viz*. hemicellulose and lignin.

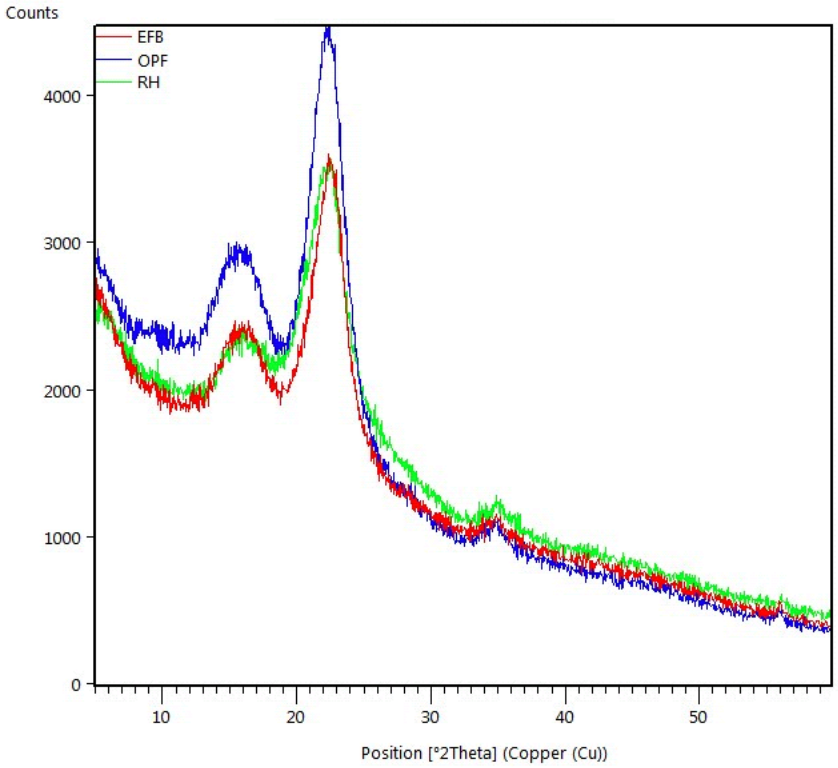

Fig. 5. XRD diffraction patterns of alkali-pretreated EFB, OPF, and RH

Endoglucanase Production on MS Compared to SS

The endoglucanase production profile of *B. aerius* S5.2 on the alkali-pretreated SS is presented in Fig 6. Endoglucanase production data on the alkali-pretreated MS are included for comparison. There was a sharp rise in enzyme production on the SS, similar to what was observed in the MS. Enzyme production peaked at 48 h on all the substrates. The enzyme titre was highest on alkali-pretreated EFB, but was not significantly higher ($P > 0.05$) than that recorded on alkali-pretreated MS. The enzyme production profile on the two substrates was very similar. Although enzyme production was considerably higher on alkali-pretreated RH in the first 36 h of fermentation than was obtained on alkali-pretreated OPF, there was no significant difference ($P > 0.05$) in their maximum enzyme titres at 48 h.

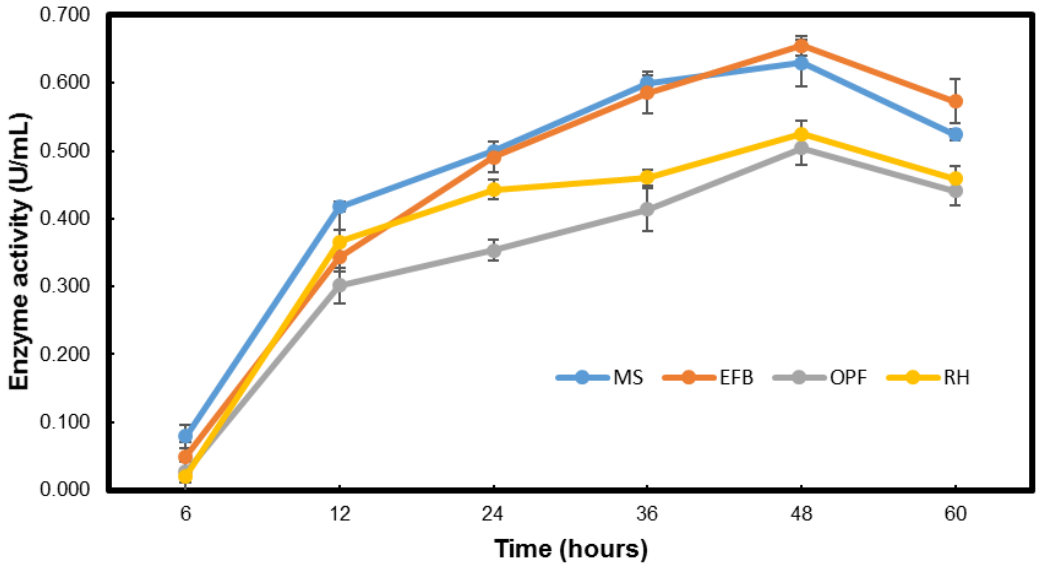

Fig. 6. Endoglucanase production by *B. aerius* S5.2 on alkali-pretreated single and mixed substrates

The greater severity of alkali pretreatment on OPF as indicated by FESEM (Fig. 3), FTIR (Fig. 4, Table 3), and XRD (Fig. 5) data may have caused the low enzyme production recorded on this substrate. Severe pretreatments erode amorphous portions of biomass, thereby rendering the substrate unfavourable for microbial uptake and enzyme production. In a recent study, Sharma *et al*. (2015) reported that higher cellulase production was recorded with increasing amorphous nature of the substrate when *Penicillium janthinellum* EMS-UV-8 was grown on wheat straw subjected to varying levels of pretreatment severity. The FESEM and FTIR data for RH showed that it underwent very little change after pretreatment, as the silica were still intact (Figs. 1 and 3). Retention of silica bodies in this substrate may have made it unfavourable for the organism. Silica bodies have been reported to prevent bacterial attachment to plant biomass and are also inhibitory to cellulolytic microorganisms (Bae *et al*. 1997).

Thus, it seems that EFB had a greater contribution to the substrate features that made the mixture more favourable for endoglucanase production than the other SS. This assumption is further strengthened by the observed similarity in enzyme production on the MS and EFB. Previous studies have shown that individual components vary in their contribution to enzyme production on the mixture, with some favouring enzyme production more than others. Jecu (2000) reported that wheat straw (WS) used singly or in higher proportion supported higher endoglucanase production than sole wheat bran (WB) or WS:WB mixtures with higher WB proportions. Similar findings have also been reported for RS:WB mixtures (Sherief *et al.* 2010). Therefore, it may be necessary to optimise the ratios of the mixture components used to achieve significantly higher enzyme production on the MS. Although these studies used fungi and solid substrate fermentations, it is generally known that cellulase production is inducible and substrate-dependent in most

microorganisms (Lynd *et al*. 2002). The higher cellulose and hemicellulose content was also influential in making EFB more favourable for enzyme production, as hemicellulose content is known to influence cellulase production (Basu and Ghose 1960).

Findings from this study thus show that combining the SS did not have any deleterious effect on endoglucanase production because the enzyme titre obtained on the MS was higher than that obtained on most of the SS (Fig. 6). The difference in enzyme production between the MS and EFB was also not significant ($P > 0.05$). Hence, combining all the SS together under a single pretreatment was more favourable for endoglucanase production than using pretreated SS separately. These results are in agreement with the findings of Olsson *et al*. (2003), who reported that higher levels of endoglucanase, endoxylanase, and polygalacturonase were obtained on mixtures of cellulose and pretreated sugar beet pulp than on single substrates when *T. reesei* Rut C-30 was used. It is however necessary to optimise MS pretreatment conditions to obtain enzyme titres higher than those on the SS or at least comparable to any of the SS. This would ensure that the use of MS would be economically advantageous.

CONCLUSIONS

1. It can be inferred from the results that the relatively more severe pretreatments were unfavourable for endoglucanase production by *B. aerius* S5.2. Thus, with respect to *B. aerius* endoglucanase production, thermochemical pretreatment of lignocellulosic substrates might not be necessary, particularly for small particle sizes (300 to 425 µm).
2. Optimisation of mixture proportions using statistical tools (*e.g.*, mixture designs) could further enhance endoglucanase titres.
3. Unique characteristics of the single substrates should be considered before selecting them as mixture components. The ones with more favourable features for the intended application should be available in higher proportion in such mixtures.

ACKNOWLEDGMENTS

This research was funded by University of Malaya under research grants RG048-11BIO, FP020-2013A and PG114-2013B. The authors wish to express gratitude to Mr. Behzad Satari of the Swedish Centre for Resource Recovery, University of Boras, Sweden, for his kind assistance during the compositional analyses experiments.

REFERENCES CITED

Ang, T. N., Ngoh, G. C., and Chua, A. S. M. (2013). "Comparative study of various pretreatment reagents on rice husk and structural changes assessment of the optimized pretreated rice husk," *Bioresource Technology* 135, 116-119. DOI: 10.1016/j.biortech.2012.09.045

Bae, H. D., McAllister, T. A., Kokko, E. G., Leggett, F. L., Yanke, L. J., Jakober, K. D., Ha, J. K., Shin, H. T., and Cheng, K. J. (1997). "Effect of silica on the colonization of rice straw by ruminal bacteria," *Animal Feed Science and Technology* 65, 165-181. DOI: 10.1016/S0377-8401(96)01093-0

Basu, S. N., and Ghose, S. N. (1960). "The production of cellulase by fungi on mixed cellulosic substrates," *Canadian Journal of Microbiology* 6(3), 265-282. DOI: 10.1139/m60-031

Bigelow, M., and Wyman, C. E. (2002). "Cellulase production on bagasse pretreated with hot water," *Applied Biochemistry and Biotechnology* 98(1), 921-934. DOI: 10.1385/abab:98-100:1-9:921

Brijwani, K., and Vadlani, P. V. (2011). "Cellulolytic enzymes production *via* solid-state fermentation: Effect of pretreatment methods on physicochemical characteristics of substrate," *Enzyme Research* 2011, 10. DOI: 10.4061/2011/860134

Dickerman, J., and Starr, T. (1951). "A medium for the isolation of pure cultures of cellulolytic bacteria," *Journal of Bacteriology* 62(1), 133-134.

Elliston, A., Wilson, D. R., Wellner, N., Collins, S. R., Roberts, I. N., and Waldron, K. W. (2015). "Effect of steam explosion on waste copier paper alone and in a mixed lignocellulosic substrate on saccharification and fermentation." *Bioresource Technology* 187, 136-43. DOI: 10.1016/j.biortech.2015.03.089

Hsu, T.-C., Guo, G.-L., Chen, W.-H., and Hwang, W.-S. (2010). "Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis," *Bioresource Technology* 101(13), 4907-4913. DOI: 10.1016/j.biortech.2009.10.009

Imman, S., Arnthong, J., Burapatana, V., Laosiripojana, N., and Champreda, V. (2013). "Autohydrolysis of tropical agricultural residues by compressed liquid hot water pretreatment," *Applied Biochemistry and Biotechnology* 170(8), 1982-1995. DOI: 10.1007/s12010-013-0320-1

Ishola, M. M., Isroi, and Taherzadeh, M. J. (2014). "Effect of fungal and phosphoric acid pretreatment on ethanol production from oil palm empty fruit bunches (OPEFB)," *Bioresource Technology* 165, 9-12. DOI: 10.1016/j.biortech.2014.02.053

Isikgor, F. H., and Becer, C. R. (2015). "Lignocellulosic biomass: A sustainable platform for the production of bio-based chemicals and polymers," *Polymer Chemistry* 6(25), 4497-4559. DOI: 10.1039/C5PY00263J

James, C. L., and Ming, S. (1991). "Bacterial cellulases: Regulation of synthesis," in: *Enzymes in Biomass Conversion*, G. F. Leatham and M. E. Himmel (eds.), American Chemical Society, Washington, DC, pp. 331-348. DOI: 10.1021/bk-1991-0460.ch025

Jecu, L. (2000). "Solid state fermentation of agricultural wastes for endoglucanase production," *Industrial Crops and Products* 11(1), 1-5. DOI: 10.1016/S0926-6690(99)00022-9

Kabir, M. M., Niklasson, C., Taherzadeh, M. J., and Horvath, I. S. (2014). "Biogas production from lignocelluloses by N-methylmorpholine-N-oxide (NMMO) pretreatment: Effects of recovery and reuse of NMMO," *Bioresource Technology* 161, 446-450. DOI: 10.1016/j.biortech.2014.03.107

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., and Blanch, H. W. (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels," *Biotechnology and Bioengineering* 109(4), 1083-1087. DOI: 10.1002/bit.24370

Kshirsagar, S. D., Saratale, G. D., Saratale, R. G., Govindwar, S. P., and Oh, M. K. (2016). "An isolated *Amycolatopsis* sp. GDS for cellulase and xylanase production using agricultural waste biomass," *Journal of Applied Microbiology* 120(1), 112-125. DOI: 10.1111/jam.12988

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M., Vogel, K. P., Simmons, B. A., and Singh, S. (2010). "Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification," *Bioresource Technology* 101(13), 4900-4906. DOI: 10.1016/j.biortech.2009.10.066

Lynd, L. R., Weimer, P. J., Zyl, W. H. v., and Pretorius, I. S. (2002). "Microbial cellulose utilization: Fundamentals and biotechnology," *Microbiology and Molecular Biology Reviews* 66(3), 506-577. DOI: 10.1128/mmbr.66.3.506-577.2002

Maki, M., Leung, K. T., and Qin, W. (2009). "The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass," *International Journal of Biological Sciences* 5(5), 500-516. DOI: 10.7150/ijbs.5.500

Malherbe, S., and Cloete, T. E. (2002). "Lignocellulose biodegradation: Fundamentals and applications," *Reviews in Environmental Science and Biotechnology* 1(2), 105-114. DOI: 10.1023/A:1020858910646

Moutta, R. D. O., Silva, M., Reis Corrales, R., Santos Cerullo, M., and Ferreira-Leitão, V. (2013). "Comparative response and structural characterization of sugarcane bagasse, straw and bagasse-straw 1:1 mixtures subjected to hydrothermal pretreatment and enzymatic conversion," *Journal of Microbial and Biochemical Technology* S12, 1-8. DOI: 10.4172/1948-5948.S12-005

Oke, M. A., Annuar, M. S. M., and Simarani, K. (2016). "Enhanced endoglucanase production by *Bacillus aerius* on mixed lignocellulosic substrates," *BioResources* 11(3), 5854-5869. DOI: 10.15376/biores.11.3.5854-5869

Olsson, L., Christensen, T. M. I. E., Hansen, K. P., and Palmqvist, E. A. (2003). "Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30," *Enzyme and Microbial Technology* 33(5), 612-619. DOI: 10.1016/S0141-0229(03)00181-9

Purwandari, F. A., Sanjaya, A. P., Millati, R., Cahyanto, M. N., Horvath, I. S., Niklasson, C., and Taherzadeh, M. J. (2013). "Pretreatment of oil palm empty fruit bunch (OPEFB) by N-methylmorpholine-N-oxide (NMMO) for biogas production: Structural changes and digestion improvement," *Bioresource Technology* 128, 461-466. DOI: 10.1016/j.biortech.2012.10.088

Rodriguez-Zuniga, U. F., Bertucci Neto, V., Couri, S., Crestana, S., and Farinas, C. S. (2014). "Use of spectroscopic and imaging techniques to evaluate pretreated sugarcane bagasse as a substrate for cellulase production under solid-state

fermentation," *Appllied Biochemistry and Biotechnology* 172(5), 2348-2362. DOI: 10.1007/s12010-013-0678-0

Salihu, A., Abbas, O., Sallau, A., and Alam, M. Z. (2015). "Agricultural residues for cellulolytic enzyme production by *Aspergillus niger*: Effects of pretreatment," *3 Biotech* 5(6), 1101-1106. DOI: 10.1007/s13205-015-0294-5

Segal, L., Creely, J. J., Martin, A. E., and Conrad, C. M. (1959). "An empirical method for estimating the degree of crystallinity of native cellulose using the X-Ray diffractometer," *Textile Research Journal* 29(10), 786-794. DOI: 10.1177/004051755902901003

Shafiei, M., Karimi, K., Zilouei, H., and Taherzadeh, M. J. (2014). "Enhanced ethanol and biogas production from pinewood by NMMO pretreatment and detailed biomass analysis," *BioMed Research International* 2014, 469378, 10 pgs. DOI: 10.1155/2014/469378

Sharma, B., Agrawal, R., Singhania, R. R., Satlewal, A., Mathur, A., Tuli, D., and Adsul, M. (2015). "Untreated wheat straw: Potential source for diverse cellulolytic enzyme secretion by *Penicillium janthinellum* EMS-UV-8 mutant," *Bioresource Technology* 196, 518-524. DOI: 10.1016/j.biortech.2015.08.012

Sherief, A. A., El-Tanash, A. B., and Atia, N. (2010). "Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran," *Research Journal of Microbiology* 5(3), 199-211. DOI: 10.3923/jm.2010.199.211

Sills, D. L., and Gossett, J. M. (2012). "Using FTIR spectroscopy to model alkaline pretreatment and enzymatic saccharification of six lignocellulosic biomasses," *Biotechnology and Bioengineering* 109(4), 894-903. DOI: 10.1002/bit.24376

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Wolfe, J. (2008a). *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples* (NREL/TP-510-42621), National Renewable Energy Laboratory, Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008b). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO.

Umikalsom, M. S., Ariff, A. B., Zulkifli, H. S., Tong, C. C., Hassan, M. A., and Karim, M. I. A. (1997). "The treatment of oil palm empty fruit bunch fibre for subsequent use as substrate for cellulase production by *Chaetomium globosum* Kunze," *Bioresource Technology* 62(1–2), 1-9. DOI: 10.1016/S0960-8524(97)00132-6

Yang, M., Kuittinen, S., Zhang, J., Vepsäläinen, J., Keinänen, M., and Pappinen, A. (2015). "Co-fermentation of hemicellulose and starch from barley straw and grain for efficient pentoses utilization in acetone–butanol–ethanol production," *Bioresource Technology* 179, 128-135. DOI: 10.1016/j.biortech.2014.12.005

Zhang, Y. H. P., Hong, J., and Ye, X. (2009). "Cellulase assays," in: *Biofuels*, J. R. Mielenz (ed.), Humana Press, New York, NY, pp. 213-231. DOI: 10.1007/978-1-60761-214-8_14

Zhang, L., Liu, Y., Niu, X., Liu, Y., and Liao, W. (2012). "Effects of acid and alkali treated lignocellulosic materials on cellulase/xylanase production by *Trichoderma reesei* Rut C-30 and corresponding enzymatic hydrolysis," *Biomass and Bioenergy* 37, 16-24. DOI: 10.1016/j.biombioe.2011.12.044

Zhao, X., Zhang, L., and Liu, D. (2012). "Biomass recalcitrance. Part II: Fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose," *Biofuels, Bioproducts and Biorefining* 6(5), 561-579. DOI: 10.1002/bbb.1350

Article submitted: April 14, 2016; Peer review completed: May 31, 2016; Revised version received and accepted: June 10, 2016; Published: June 28, 2016.
DOI: 10.15376/biores.11.3.6708-6726