Carbohydrate Polymer Technologies and Applications 7 (2024) 100426

Contents lists available at ScienceDirect

Carbohydrate Polymer Technologies and Applications

journal homepage: www.sciencedirect.com/journal/carbohydrate-polymer-technologies-and-applications

Peroxidase application reduces microcrystalline cellulose recalcitrance towards cellulase hydrolysis in model cellulose substrates and rooibos biomass

Mamosela Marriam Mohotloane [a], Orbett Alexander [b], Vanthini Nelson Adoons [c], Brett Ivan Pletschke [d], Mpho Stephen Mafa [a,*]

[a] Carbohydrates and Enzymology Laboratory (CHEM-LAB), Department of Plant Sciences, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa
[b] Department of Chemistry, University of the Western Cape, Bellville, Cape Town 7535, South Africa
[c] Department of Physics, University of the Free State, QwaQwa Campus, Private Bag X 13, Phuthaditjhaba 9866, South Africa
[d] Enzyme Science Programme (ESP), Department of Biochemistry and Microbiology, Rhodes University, Makhanda 6140, South Africa

ARTICLE INFO

Keywords:
Cellulose
Microcrystalline cellulose
Horseradish peroxidase
Biomass pretreatment
Endoglucanase
Enzyme cocktail

ABSTRACT

We have identified a HRP enzyme with microcrystalline cellulose activity, which has not yet been explored. The current study investigated the effect of HRP pretreatment on the microcrystalline cellulose substrates, Avicel and filter paper. SEM findings showed that HRP pretreatment catalysed the para-microcrystalline regions of Avicel, cracking and opening the pores on the surface. On filter paper, HRP removed the para-microcrystalline regions exposing fibres. Crystallinity index (CrI) analysis confirmed that HRP increased the CrI of Avicel from 49 % to 54.19 % and filter paper from 42 % to 47 %. The cellulose crystallite sizes increased from 45 to 47 nm at the 002 lattices in Avicel, suggesting a reduction of crystalline cellulose. In addition, endoglucanase displayed 1.15-fold increased activity on HRP-pretreated Avicel, confirming reduced crystalline cellulose. These findings showed that HRP pretreatment changed the structural and chemical properties of Avicel, i.e., loosening crystalline cellulose to make the substrate accessible to enzymes during hydrolysis. Finally, these findings were supported by rooibos microcrystalline cellulose modification post-HRP pretreatment, resulting in a 95 % yield of soluble sugars at 25 mg enzyme cocktail/g biomass.

1. Introduction

Second-generation biofuel (2G) is produced from raw materials such as agricultural residues that can be utilised without affecting food supply (Kumar et al., 2020). However, it is challenging to produce biofuel from agricultural residues due to its complex lignocellulose structure, which interferes with the saccharification processes or combined saccharification and fermentation processes (Bagewadi et al., 2017). The lignocellulosic biomass is composed of major components such as cellulose, hemicelluloses, and lignin. The cellulose and hemicellulose components are made up of fermentable sugars, while lignin is a complex polymer made up of phenolic compounds. Lignin is well known for hindering CAZyme activities by forming a barrier that inhibits lignocellulosic biomass degradation through enzymatic application (Mnich et al., 2020). The hindrance of CAZyme activities directly influences the production of simple sugars, which can be fermented to produce biofuel (Bagewadi et al., 2017).

The removal of lignin from the biomass is helpful because it improves the substrate accessibility for holocellulolytic enzymes and subsequently increases the production of soluble sugars, which can be fermented to produce bioethanol and other value-added products (VAP) (van Dyk & Pletschke, 2012; Zhu & Pan, 2022). Several chemical pretreatment methods have been developed for the removal of lignin. Most chemical pretreatment methods such as acids or alkaline pretreatment successfully remove lignin from the biomass. However, their disadvantages include expensive costs, corrosion, harm to the environment, and acid-pretreatment produces inhibitory compounds that affect the production of fermentable sugars (Harmsen et al., 2010; Limayem & Ricke, 2012, Badiei et al., 2014, Behera et al., 2014, Bagewadi et al., 2017). Physical and chemical methods such as evaporation, solvent extraction, ion exchange, and activated charcoal adsorption can be deployed to remove the toxic compounds; however, these processes are expensive

* Corresponding author.
E-mail address: mafams@ufs.ac.za (M.S. Mafa).

https://doi.org/10.1016/j.carpta.2024.100426

Available online 22 January 2024

and only add to the production costs (Bagewadi et al., 2017). To align with Sustainable Development Goal number 12 regarding the production of green chemicals that have less negative effect on the environment, several studies have used enzymatic or microbial methods to delignify biomass.

The use of biological pretreatments, such as ligninase, peroxidase, laccase, and carbohydrate esterase enzymes or microbes, is increasing and several researchers argue that it can be a preferable alternative to chemical pretreatment (Behera et al., 2014, Kong et al., 2017, Mohotloane et al., 2023). Biological pretreatments have attractive features, i.e. they are sustainable, environmentally friendly without producing inhibitory compounds, require low energy, and are highly selective, resulting in high yields of sugar products (Bagewadi et al., 2017, Wagner et al., 2018, Sharma et al., 2019, Devi et al., 2022). The current disadvantages of this method include a slow reaction process if microbes (fungi) are used for pretreatment and growth conditions require careful optimization (Menon & Rao, 2012, Behera et al., 2014; Hernández-Chaverri et al., 2021). However, ligninase enzymes successfully reduced the lignin content from the biomass, particularly lignin peroxidase (LiP: EC1.11.1.14), versatile peroxidase (VP: EC 1.11.1.16), manganese peroxidase (MnP: EC 1.11.1.13), and laccase (Lac: EC 1.10.3.2) (Kersten et al., 1990, Behera et al., 2014, Manavalan et al., 2015, Kong et al., 2017, Kumar et al., 2020, Mohotloane et al., 2023).

Peroxidases are enzymes known to contain a heme group and are used in the catalysis of organic or inorganic compounds (Lavery et al., 2010, Pandey et al., 2017, Veitch, 2004). They have various applications, which include the treatment of wastewater by removing phenols and dyes, or the detoxification of foodstuffs and industrial effluents by removing peroxide (Lavery et al., 2010). Additionally, the previous study demonstrated that HRP removed lignin from rooibos biomass and changed the structural properties of the rooibos biomass after pretreatment (Mohotloane et al., 2023). This study proposed HRP pretreatment decrystallized microcrystalline cellulose regions in rooibos samples, resulting in a higher subsequent production of soluble sugars. These findings generated the following questions: can HRP pre-treatment remove lignin and decrystallize cellulose of other woody biomasses? Given that it decrystallized the microcrystalline cellulose of rooibos, does it have cellulose activity? and if so, which mechanism does it employ to catalyse cellulose? In the current study we attempted to answer these questions using crystalline cellulose model substrates, Avicel and filter paper.

Previous studies claimed that crystalline cellulose is recalcitrant to enzymatic activity, and that it generally inhibits endoglucanase activity (van Dyk & Pletschke, 2012, Mafa et al., 2021). In contrast, para-crystalline are easily degraded by endoglucanases. Chemical pretreatments have been used to reduce the recalcitrance of microcrystalline cellulose by exposing the amorphous cellulose or regenerated para-crystalline cellulose, resulting in increased enzyme activity (Tian et al., 2018, Mafa et al., 2020a). The physicochemical changes of the biomass can be measured using current technologies, which include scanning electron microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray diffraction (XRD) (Zhou et al., 2007, Khan et al., 2018, Ling et al., 2019, Yu et al., 2020).

Ling et al. (2019) used various techniques, including SEM, FTIR, and XRD to show that ball milling has an effect on cotton cellulose by breaking the crystalline cellulose while exposing or regenerating the amorphous cellulose. Mohotloane et al. (2023) used SEM to show the removal of lignin and topological surface changes in rooibos samples after HRP pretreatment. The current study aims to elucidate how HRP pretreatment changes the physicochemical properties of microcrystalline cellulose substrates (Avicel and filter paper) and subsequently enhances the hydrolytic activities of the enzymes.

2. Materials and methods

2.1. Materials

Rooibos (*Aspalathus linearis*) samples were supplied by Rooibos Limited (Clanwilliam, Western Cape, South Africa). The horseradish root *(Armoracia rusticana)* was provided by Mr. Barry Newton and Mr. John Parr from Yaxham farm situated in Tweespruit, Free State, South Africa. The commercial cellulase and xylanase enzymes, such as xylanase from *Aspergillus oryza*, endoglucanase1 (EG1) from *Aspergillus niger*, endoglucanase2 (EG2) from *Aspergillus* sp., and β-glucosidase from *Aspergillus niger,* and commercial substrate Avicel were all purchased from Sigma (Johannesburg, South Africa). The filter paper was purchased from Whatman International Limited (Springfield Mill, Maidstone, Kent, England). All the analytical chemicals used in the study were purchased from Sigma (Johannesburg, South Africa) unless stated differently.

2.2. Effect of HRP on cellulose model substrates

2.2.1. Avicel and filter paper pretreatment with HRP

Filter paper was crushed using liquid nitrogen and an industrial blender to produce a biomass similar to cotton wool. The cellulose model substrates were pretreated with HRP to assess its effect on the cellulose region of the biomass. An amount of 2.5 g of Avicel and filter paper was dissolved in 50 mM sodium citrate buffer pH 4.5, and the reaction conditions were similar to the ones described by Mohotloane et al. (2023). Briefly, the HRP concentration was kept at 0.5 mg/mL and H_2O_2 at 0.5 % (v/v); both HRP and H_2O_2 were added to the pretreatment solution three times within 6 h intervals, and the reactions lasted for 24 h. HRP pretreated samples were dried, crushed, and stored in an airtight container for later use.

2.2.2. HPR pretreated cellulose substrate FTIR analysis

FTIR spectroscopy was used to determine the chemical (functional group) changes of the HRP pretreated Avicel and filter paper samples. Peaks' functional groups were assigned, FTIR spectra were overlaid, and an absorbance mode was used to compare the changes between the control and pretreated samples, according to Mafa et al. (2020a).

2.2.3. XRD analysis of Avicel and filter paper

X-ray diffractograms were recorded with a Bruker D8 Advance diffractometer. All XRD measurements were performed using the reflectance technique. The incident X-ray radiation was the Cu Kα (λ = 1.54 Å) characteristic X-ray passing through a nickel (Ni) filter with a power of 40 kV and 40 mA. Both the air-scattering prevention slit and the divergence slit were 1°. The width of the detection slit was 0.6 mm. Counts were collected over a range of angles between 10^o and 80^o, with an increment of 0.01945° for 1 h. The X-ray source was a copper (Cu) target bombarded with electrons. All samples were fixed on the sample holder. Each crystalline peak was determined by the XRD deconvolution (curve fitting) method. Fig. S1 shows the curve fitting of the Avicel control, which was used to measure the crystallinity index. In addition, the crystallinity index (CrI) was calculated using the Segal method by determining the ratio between the maximum peak height subtracting the minimum peak height (I_{002}–I_{AM}) and total maximum peak height (I_{002}) (Park et al., 2010; Segal et al., 1959).

$$CrI\% = \frac{(I002 - IAM)}{I002} x100 \quad (1)$$

Where CrI% is the percentage crystallinity index, I_{002} is the maximum peak height at $2\theta = 22.8°$ and I_{AM} is the minimum peak height at $2\theta = 18.5°$

The crystallite sizes of all samples were calculated based on the Scherrer Eq. (2) (Scherrer, 1918):

$$D = \frac{k\lambda}{\beta\cos\theta} \quad (2)$$

where k is a constant whose value ranges between 0.9–0.99, λ is the incident an X-ray wavelength, β is full width at half maximum (FWHM) of diffraction peaks in radians and θ is the Bragg angle in degrees. The value of k used in Eq. (2) was 0.9.

The d-spacing values were calculated using Bragg's Eq. (3):

$$n\lambda = 2d\sin\theta \quad (3)$$

where n is an integer, λ is the wavelength of incident wavelength, d is the spacing between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes.

2.2.4. Topological studies of cellulose model substrates using SEM

SEM was used to determine the effect of HRP pretreatment on the topology of the microcrystalline cellulose model substrates Avicel and filter paper. Completely dry samples were mounted on aluminium pin stubs using double-sided carbon tape and coated with gold (2 μm) for conductivity using a Bio-Rad sputter coater (United Kingdom). Specimens were imaged at 5 kV using a JEOL JSM IT-200 SEM (Kyoto, Japan). The samples were analyzed with different magnifications ranging between 1000 and 10000x and photos were taken with a built-in camera to capture the results.

2.2.5. The effect of HRP pretreatment on Avicel and filter paper

Enzyme activity assays were conducted using EG1 (from *A. niger*) and EG2 (from *Aspergillus* sp.). The HRP pretreated Avicel and filter paper, and the untreated control were used as substrates. Each reaction consisted of about 1 % (w/v) substrate (pretreated Avicel and filter paper dissolved in 50 mM sodium phosphate buffer pH 5) added to 0.1 mg/mL of EG1 and EG2 enzymes. All the reactions were incubated at 37°C for about 0.5,1, 1.5, 2, and 24 h. After the completion of the reactions, samples were centrifuged at 5 000 x *g*, and the total reducing sugars were quantified using the DNS method (Miller, 1959).

2.3. Improvement of the holocellulolytic enzyme cocktail

2.3.1. Formulation of the holocellulolytic enzyme cocktail

The most effective β-glucosidase concentration was determined using 1 % (w/v) of pretreated Avicel and filter paper in 50 mM sodium citrate buffer pH 5, and the enzyme-loading combinations were 95, 90, 85, 80, 75, 70 % for EG1, and 5, 10, 15, 20, 25, 30 % for β-glucosidase, respectively. The enzyme concentration for all the combinations was kept constant at 2.5 μg/mL and incubation occurred for 24 h at 37°C. After the completion of the reactions, the total reducing sugars were detected using the DNS method.

The most effective concentration of β-glucosidase (i.e.,10 %) was used in combination with EG1 and xylanase to formulate the holocellulolytic enzyme cocktail. The reaction consisted of 1 % (w/v) HRP pretreated rooibos biomass in 50 mM sodium citrate buffer pH,5 and the enzyme loading combinations between EG1, and xylanase were 100, 75, 50, 25, 0 %, respectively. The enzyme combinations were kept at 2.5 μg/mL. The DNS method was used to measure the total reducing sugars produced.

2.3.2. HRP treated rooibos degradation with the holocellulolytic enzyme cocktail

The most effective holocellulolytic enzyme combination (50 %:50 %, EG1: xylanase) and (100 %:0 %, EG1: xylanase) were used to determine the required concentration (mg/g) to produce a high sugar yield percentage from HRP-treated biomass. The yield was determined using 1 % (w/v) HRP pretreated rooibos biomass in 50 mM sodium citrate buffer (pH 5) and using varying enzyme concentrations of 0.25, 0.5, 0.75, and 1 mg/mL, respectively. The reactions were incubated for 24 h at 37°C. All experiments were performed in triplicate. After the completion of the reactions, reducing sugars were detected using the DNS method.

2.4. Statistical analysis

The significant difference between HRP treated and untreated samples during enzymatic hydrolysis were analyzed with one-way ANOVA with alpha value set at 0.05.

3. Results

3.1. Determination of the topology using SEM

The previous findings showed that HRP pretreatment not only removed lignin from fermented rooibos biomass, but that it also decreased the cellulose crystallinity. For a comprehensive understanding of this phenomenon, further studies were conducted to determine the effect of HRP pretreatment on the crystalline cellulose model substrates (Avicel and filter paper). SEM topological analysis showed that the Avicel control samples had a smooth surface layer of a fibre-like texture that was visible under 6000x magnification (Fig. 1). However, the HRP-treated Avicel displayed a rough surface showing cracks and pores, with thick pieces of material that were seemingly being removed. The filter paper control had a smooth fibre-like surface, with a few thread-like structures visible on the surface layer. Interestingly, the HRP-treated filter paper exhibited a rough surface, showing the exposed microcrystalline cellulose fibres with pores between some fibres. These results show that the HRP had cellulase activity on the para-crystalline cellulose region, but it could not hydrolyse the microcrystalline cellulose. The paracrystalline cellulose regions are referred to as cellulose II, while the crystalline cellulose was mostly cellulose I_β.

3.2. FTIR analysis of the HRP pretreated cellulose model substrate

FTIR analysis was used to determine the changes in the chemical functional groups of the untreated or pretreated Avicel and filter paper samples. The FTIR results confirmed the SEM findings by showing that

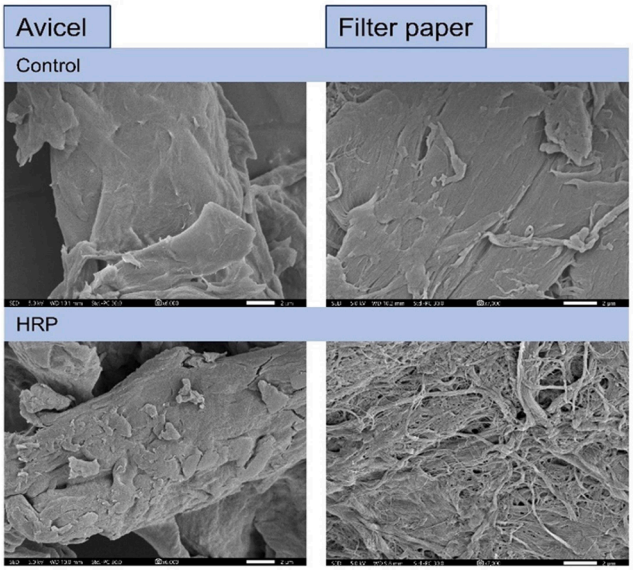

Fig. 1. SEM topological analysis of Avicel and filter paper. The substrates pretreated with HRP (Avicel, bottom left and filter paper, bottom right images) were compared to the controls (Avicel, top left and filter paper, top right images). Avicel samples were analyzed at 6000-x magnification, while the filter paper samples were analyzed at 7000-x magnification; scale bar for all images is 2 μm.

HRP pretreatment modified the cellulose model substrates. The Avicel control showed a lower peak at 3330.82 cm^{-1} compared to the pretreated samples (Fig. 2A). This peak corresponded to the presence of OH group in the sample, which was broader in the microcrystalline cellulose-containing samples, because of the inter- and intra- hydrogen bonds that formed between the fibres containing OH groups. Interestingly, the same pattern was observed for the filter paper samples at peak 3330.82 cm^{-1}. The HRP pretreated samples displayed a significantly higher peak compared to the control (Fig. 2B). Similar findings were observed at peak 1029.91 cm^{-1}, which represented the glycosidic bonds. The Avicel control had a lower peak, while the pretreated Avicel had a slight increase compared to the control at peak 1029.91 cm^{-1}. The HRP pretreated filter paper showed a substantial increase, compared to the control at the same peak. Thus, the SEM and FTIR findings suggest that the pretreatment modified the structural and chemical properties of the two well-known cellulose model substrates.

3.3. Crystallinity analysis of the HRP pretreated cellulose substrates

X-Ray diffraction (XRD) further supported the SEM and FTIR findings by showing that HRP pretreatment modified the crystallinity of the cellulose model substrates. Avicel and filter paper XRD diffractogram intensities showed clear differences between the HRP-treated and control. The pretreatment increased the peak intensity at the 101 and 002 lattice plane in the Avicel, compared to the control samples. The two lattice planes corresponded to cellulose II and cellulose Iβ, respectively. In the filter paper, the intensity values at the $10\bar{1}$ lattice plane were similar for the HRP treated samples and controls, but there was a significant increase in the intensities of the pretreated samples, compared to the controls at the 002 lattice plane (Fig. 3A and B).

In addition, the peak height method showed that HRP pretreatment resulted in an increased crystallinity index (CrI) of about 54.19 % and 47 % in Avicel and filter paper, respectively, compared to a CrI of about 48.89 and 41.89 % in the respective control samples (Table 1). It is worth noting that the crystallinity index determined with the deconvolution method showed a 1 % change for the CrI of the Avicel samples. The control displayed a CrI of 71 %, while the HRP pretreatment sample had a CrI of about 72 %. The filter paper showed no change between the control and HRP pretreated samples, which had a CrI of about 93 % for both samples.

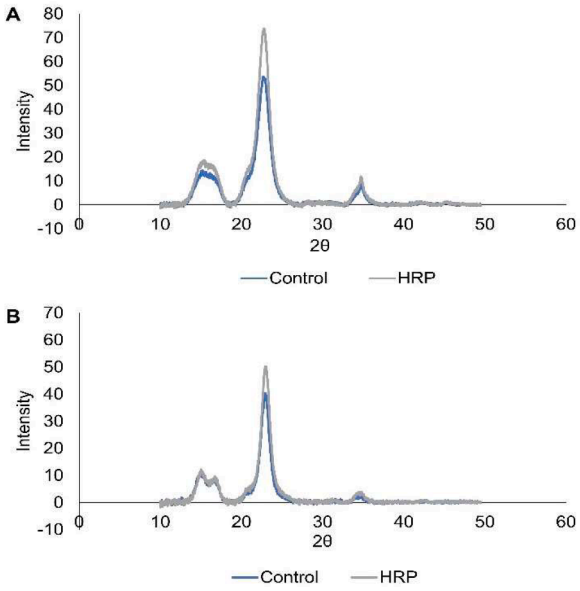

Fig. 3. XRD diffractogram of the HRP-pretreatment cellulose model substrate Avicel (A) and filter paper (B). The spectra were overlaid to compare the differences between HRP-treated samples and the controls.

The determination of the crystallinity is not limited to the crystallinity index; there are other indicators to consider when determining cellulose crystallinity, such as crystallite sizes. The crystallite size was determined using the Scherrer's equation and the results showed that the HRP pretreatment has an effect on the crystallite sizes (Table 1). The HRP pretreated Avicel had reduced crystallite sizes (39.6 and 55.5 nm) at the 101 and 040 lattice planes compared to 40.6 and 58.1 nm in the controls. In contrast, the crystallite sizes of pretreated Avicel at the 021 and 002 lattice planes increased to 58.8 and 47.3 nm, compared to 57.5 and 45.7 nm in the controls. Interestingly, the crystallite size at peak $10\bar{1}$

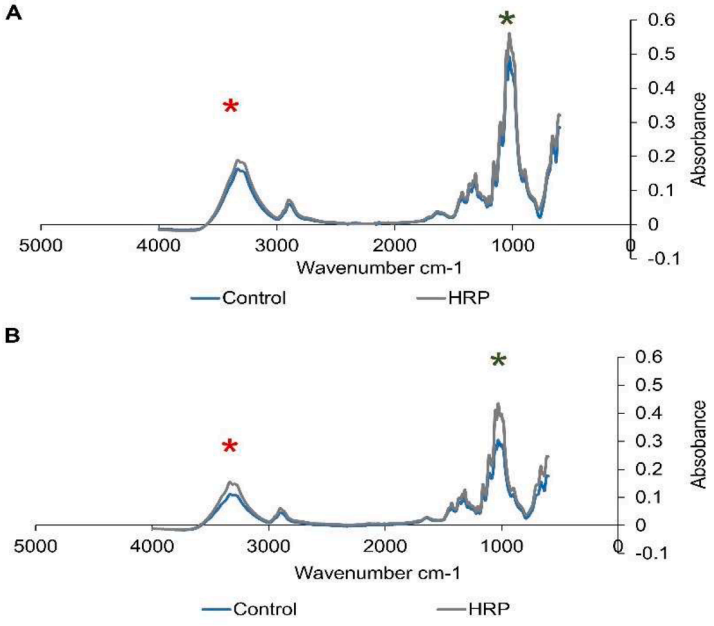

Fig. 2. FTIR analysis of horseradish peroxidase (HRP) pretreated cellulose model substrates. The HRP treated Avicel (A) and filter paper (B) samples were compared to the controls by overlaying the spectra using the absorbance mode. Two regions of interest, the OH group, and glycosidic bonds regions, are represented by a red and green asterisk, respectively.

Table 1
Mean crystallite size (C.S) calculated, computed (deconvolution) crystallinity indexing, and peak height crystallinity indexing for Avicel and filter paper samples.

Miller Indices Sample	101 C.S (Å)	$10\bar{1}$ C.S (Å)	021 C.S (Å)	002 C.S (Å)	040 C.S (Å)	Peak height [CrI (%)]	Deconvolution [CrI (%)]
Avicel (Control)	40.6	53.5	57.5	45.7	58.1	48.89	71
Avicel (HRP)	39.6	53.5	58.8	47.3	55.5	54.19	72
F. Paper (Control)	51.8	66.1	49.4	60.7	63.4	41.89	93
F. Paper (HRP)	55.9	66.1	53.1	59.3	62.9	47.00	93

1 nm is equal to 1 Å

showed no change in both the control and HRP pretreated Avicel and filter paper.

Additionally, the crystallite sizes at 002 and 040 lattices decreased to 59.3 and 62.9 nm in the HRP pretreated filter paper compared to 60.7 and 63.4 nm in the control, respectively. In contrast, the crystallite sizes at 101 and 021 lattices increased to 55.9 and 53.1 nm in HRP pretreated filter paper compared to 51.8 and 49.4 nm in the controls. The modification of the crystallite sizes further supports the hypothesis that the HRP pretreatment modified the physicochemical properties of the cellulose model substrates. The decreased d-spacing at the 101 and 002 lattice planes in the HRP pretreated filter paper samples (compared to the control) confirmed the change in the structure of the biomass at the molecular level (Table 2).

3.4. Endoglucanase activity on HRP treated cellulose model substrates

The SEM, FTIR, and XRD findings indicated that the HRP pretreatment modified the structural and chemical properties of microcrystalline cellulose model substrates. To test if these modifications were essential for higher enzymatic activity, endoglucanase 1 (EG1: from *A. niger*) and endoglucanase 2 (EG2: from *Aspergillus* sp.) were used to hydrolyse HRP pretreat Avicel and filter paper. The findings showed that EG1 and EG2 activities significantly increased in the HRP pretreated Avicel and filter paper samples. EG1 showed a steadily increasing activity over time in both the control and the HRP pretreated Avicel samples (Fig. 4A). The highest activity for this enzyme was recorded at 24 h incubation, with an increase of 10 % activity in the HRP pretreated Avicel, compared to the control. The second endoglucanase, EG2, showed little to no change over time with a sharp increase at 24 h incubation in both the control and HRP pretreated Avicel samples (Fig. 4B). EG2 had an increase of 20 % activity in the HRP pretreated Avicel compared to the control. In contrast, the endoglucanase activities (EG1 and EG2) were comparable between HRP treated filter paper and the controls. The findings suggest that the filter paper was more crystalline compared to the Avicel (Fig. 4C and D).

3.5. Holocellulolytic enzyme formulation

After confirming the most effective β-glucosidase enzyme loading in combination with the EG1 (Fig. S2), we attempted to formulate the holocellulolytic enzyme cocktail that could improve the yield of soluble sugars from the HRP pretreated complex substrate (rooibos biomass). The results in Fig. 5 showed that the most effective combinations were 50 % EG1/50 % xylanase, and 100 % EG1/0 % xylanase, which displayed 1.4-fold and 1.5-fold higher specific activities, respectively, compared to their controls. In addition, the HRP-pretreated fermented rooibos was hydrolysed better than the controls, suggesting that β-glucosidase and EG1 or xylanase acted synergistically on the pretreated substrate.

3.6. Producing soluble sugars from HRP treated biomass

Quantification of the total amount of soluble sugars was conducted using 1 % (w/v) of the HRP-pretreated rooibos biomass. A 50 % EG1/50 % xylanase combination, and 100 % EG1 (mixed with 10 % β-glucosidase enzyme loading) were used in the production of soluble sugars, as they displayed high activity in the holocellulolytic enzyme formulation (Fig. 5). Interestingly, the results indicated that the soluble sugar yield decreased as the enzyme loading concentration increased (Fig. 6). The substrate hydrolysis with 100 % EG1 (i.e. with no xylanase) showed a sharp decrease in the yield (Fig. 6), while 50 % EG1 and 50 % xylanase showed a steady decline until 75 mg/g biomass. Above 75 mg/g biomass concentration, the 100 % EG1 enzyme rapidly lost activity and there was, consequently, a decline in yield at 100 mg/g. However, the 50 % EG1 and 50 % xylanase combinations only lost 11 % in total reducing sugars yield at the same concentration. The 25 mg enzyme loading /g biomass was the effective concentration required to achieve 99 % and 96 % of total reducing sugar production (Fig. 6).

4. Discussion

Agricultural biomass pretreatment is vital for removing or modifying lignin, reducing crystalline cellulose, increasing enzymes accessibility to the biomass, and enhancing hydrolysis (Van Dyk & Pletschke, 2012, Mafa et al., 2020b). Therefore, higher cellulases activity on the cellulose model substrates or lignocellulose is generally attributed to structural and chemical modification, which increases enzyme accessibility to the biomass (Van Dyk & Pletschke, 2012). The previous study demonstrated that HRP pretreatment removed lignin and significantly decreased the crystallinity of rooibos biomass (Mohotloane et al., 2023). However, this study did not address the mode or mechanisms of actions used by HRP to reduce the crystallinity of the cellulose. Hence, the current study

Table 2
Position of maximum (2θ), *d*-spacing, and full width at half maximum intensity (FWHM) for Avicel and filter paper samples

Miller Indices	101		$10\bar{1}$		021		002		040	
Sample	2θ	FWHM	2θ	FWHM	2θ	FWHM	2θ	FWHM	2θ	FWHM
Avicel (Control)	15.135	1.972	16.753	1.499	20.724	1.404	22.751	1.772	34.665	1.433
d-spacing (nm)	0.584		0.528		0.428		0.390		0.258	
Avicel (HRP)	15.122	2.024	16.776	1.501	20.738	1.373	22.756	1.713	34.632	1.499
d-spacing (nm)	0.585		0.528		0.427		0.390		0.258	
F. Paper (Control)	15.034	1.545	16.726	1.215	21.138	1.634	22.933	1.335	34.469	1.311
d-spacing (nm)	0,589		0,529		0,420		0,387		0,259	
F. Paper (HRP)	15.087	1.433	16.731	1.215	21.105	1.521	22.993	1.366	34.565	1.322
d-spacing (nm)	0.587		0.529		0.421		0.386		0.259	

Note: C.S = Crystal size, FWHM = Full Width Half Maximum, 2θ = 2Theta

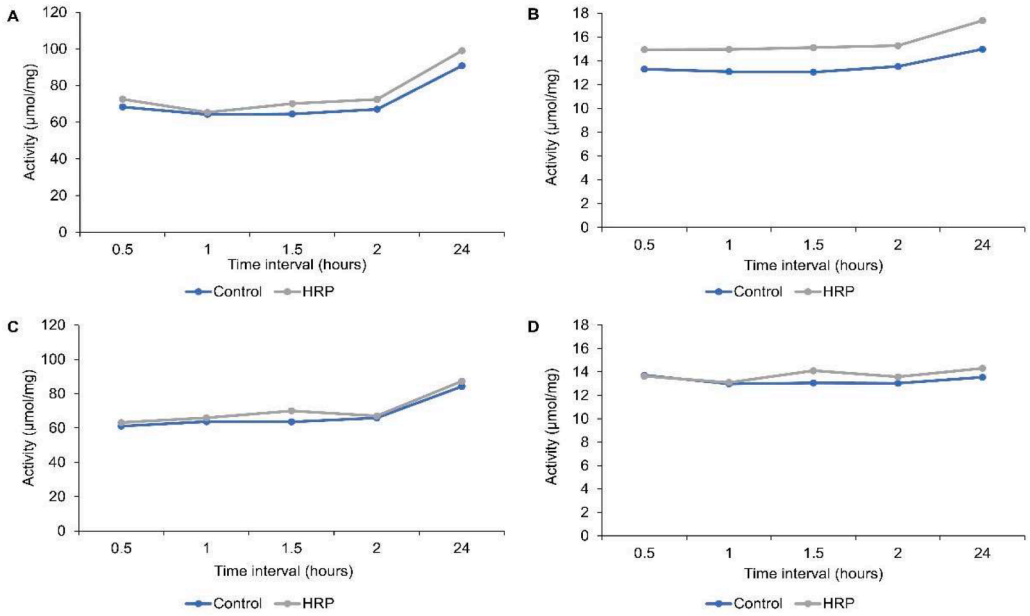

Fig. 4. The endoglucanase-1 (EG1) and endoglucanase-2 (EG2) activity assays on HRP pretreated microcrystalline cellulose substrates (Avicel and filter paper). A and B represent EG1 and EG2 activities acting on Avicel, respectively, while C and D represent EG1 and EG2 activities on pretreated filter paper, respectively. Controls were used to confirm the effect of HRP treatment on the cellulose model substrates. The values represent the means ± standard deviation. Activity is equivalent to total reducing sugars produced EG during substrate hydrolysis (in umol/mg). One-way ANOVA ($p < 0.05$) was used to test for significant differences between HRP-treated and untreated Avicel and filter paper.

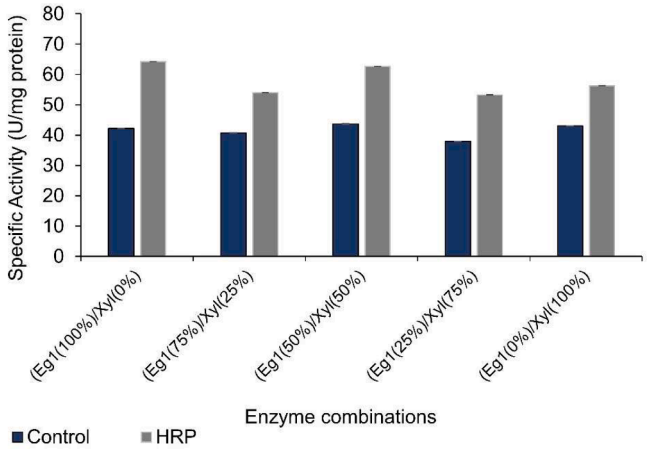

Fig. 5. Formulations of the holocellulolytic enzyme cocktail using different combinations of endoglucanase 1 (EG1) and xylanase on fermented rooibos substrate. β-glucosidase was dosed at 10 % enzyme load relative to the concentrations of EG1 and xylanase. The values represent the means ± standard deviation. The U represent units, which is equal to µmol.h^{-1}. One-way ANOVA ($p < 0.05$) was used to test for significant differences between HRP-treated and untreated Avicel and filter paper.

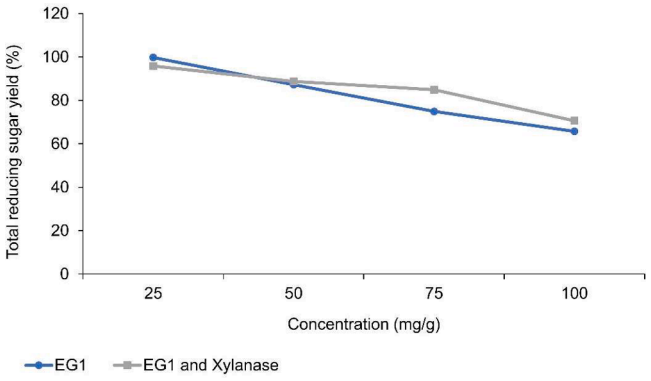

Fig. 6. The application of the formulated cellulase and holocellulolytic enzyme cocktails using the most effective combinations, 100 % endoglucanase 1 (EG1) (A), and 50 % endoglucanase 1 (EG1)/50 % Xylanase (B) on HRP pretreated fermented rooibos substrate. The values represent the means ± standard deviation.

investigated the effects of HRP pretreatment on crystalline cellulose region of the biomass by using the cellulose model substrates, Avicel and filter paper.

The HRP pretreatment of Avicel and filter paper improved EG1 activity during hydrolysis compared to the control. Additionally, the EG2 showed increased activity on the HRP pretreatment cellulose model substrates. The EGs showed a much higher activity on the Avicel compared to filter paper; suggesting that the pretreatment made Avicel less crystalline. Mohotloane et al. (2023) demonstrated that EG1 is a multifunctional enzyme because it was able to hydrolyse both cellulosic and hemicellulosic substrates. However, EG2 is a true endoglucanase that showed activity only on the amorphous cellulose substrate (CMC). Min et al. (2022) demonstrated that MnP displayed activity on the cellulose substrates or acted in a synergistic fashion with cellulases. Other studies showed lytic polysaccharide monooxygenases (LPMOs) act on the recalcitrant microcrystalline cellulose, increasing biomass accessibility for the cellulases enzymes (Levasseur et al., 2013, Chang et al., 2022). In addition, LPMO application increased endoglucanase activity by minimizing the recalcitrant nature of microcrystalline cellulose (Monclaro & Ferreira Filho, 2017). These observations support the findings of the current study, suggesting HRP pretreatment of microcrystalline cellulose (Avicel) reduced its recalcitrance to towards endoglucanase.

SEM analysis showed that the HRP pretreatment structurally modified the crystalline cellulose substrates. The pretreated Avicel developed a rough surface layer, showing cracks and pores compared to the smooth surface observed in the control sample. The cracks and pores indicate that the HRP pretreatment increased substrate accessibility, which

resulted in higher EGs hydrolytic activity. Similarly, HRP pretreatment removed the paracrystalline cellulose from the filter paper samples, resulting in more exposed microcrystalline cellulose fibres compared to the control sample, which had a smooth surface. Min et al. (2022) also demonstrated that Avicel pretreatment with MnP had peeling effects on the surface of biomass that exposed cracks on the biomass. These observations support our findings, except that the filter paper results suggested that HRP pretreatment acted mostly on cellulose II (para-crystalline cellulose), exposing cellulose I_β (microcrystalline cellulose).

The FTIR peak reduction at 3000–3500 cm^{-1} implied that the crystalline region of cellulose was modified. The peak is assigned to OH groups and for microcrystalline cellulose samples a broad peak indicated the presence of inter- and intra- hydrogen bonds between the fibres. Some studies that showed decreased IR absorbance values due to biomass pretreatment in this region (3000-3500 cm^{-1}) are associated with the regenerated amorphous/para-crystalline cellulose, which resulted in increased cellulase activity (Mafa et al., 2020b, Imai et al., 2023). Ling et al. (2019) showed that the mechanical treatment of ball milling decreased the absorbance values at 3000-3500 cm^{-1} associated with cellulose Iα and Iβ, suggesting a modification of the crystalline structures, resulting in higher amorphous or paracrystalline region. In contrast, the HRP pretreatment increased the peak at 3330.82 cm^{-1} in Avicel and filter paper. These observations supports our findings, which demonstrated HRP reduced the microcrystalline cellulose in the Avicel samples.

The SEM and FTIR findings were confirmed by the XRD results, which showed that HRP pretreatment increased CrI in Avicel and filter paper. The commonly used peak height method by Segal et al. (1959) showed about a 5 % increase in CrI of the pretreated samples, compared to the controls of Avicel and filter paper. Although peak height is the most commonly used method for determining CrI, a few studies caution against using CrI as a measure of biomass crystallinity, because it does not account for changes in the peak morphology or peak area, crystallite sizes, and d-spacing (Park et al., 2010, Ju et al., 2015, Nam et al., 2016). Bommarius et al. (2008) indicated that an increase in CrI during Avicel pretreatment with ammonia, alkaline, and organo-solvent did not reduce cellulase activity. These observations and increased CrI of Avicel and filter paper post-HRP treatment suggested a need to determine crystallite size and d-spacing to understand the effect of HRP pretreatment at the molecular level.

The findings showed a significant change at the molecular level after the HRP pretreatment of Avicel and filter paper. For Avicel there was a 1 nm decrease in the crystallite size and an increase of 0.001 nm d-spacing at the 101 lattice plane. These results suggest that HRP pretreatment acts on the cellulose crystallites and breaks down the intermolecular hydrogen bonds, leading to increased distance between the crystallites (Imai et al., 2023). In addition, crystallite size at the 002 lattice-plane increased by about 1.6 nm, but there was no change in the d-spacing between the crystallites. These crystallinity changes at the molecular level can explain the increased EGs activity on HRP pretreated Avicel samples.

For HRP pretreated filter paper, there was a significant (4 nm) increase in the crystallite size and 0.002 nm d-spacing at the 101 lattice plane. However, at the 002 lattices there was about 1 nm decrease in the crystallite size and 0.001 nm in d-spacing. The 101 lattice plane corresponds to the presence of cellulose II, while 002 represents cellulose I allomorphs. The increased crystallite size of cellulose II suggested that the HRP enzyme shows higher activity on the cellulose II allomorph and exposes crystalline cellulose I_β. These imply that there was an increase in the microcrystalline fibres in filter paper samples, leading to less endoglucanase biomass accessibility and EGs activity. In addition, several studies showed that samples which have large crystallites display two distinct peaks at 101 and $10\bar{1}$ lattice planes, while samples with small crystallites have a broad peak combining the two peaks of 101 and $10\bar{1}$ (Garvey et al., 2005, Ju et al., 2015, Ling et al., 2017). Ju et al. (2015) also argued that the increment in d-spacing and crystallite sizes showed that the biomass crystals were loosening while the decrease in d-spacing and crystallite size indicated a more compared biomass. These observations confirmed that HRP pretreatment was essential for loosening cellulose in Avicel samples, while it removed paracrystalline cellulose and exposed crystalline fibres of cellulose in filter paper.

After determining the effects of HRP pretreatment on the cellulose model substrates, we formulated an enzyme cocktail by including 10 % (v/v) β-glucosidase. β-glucosidase is known as a cellulase enzyme that plays a role in degrading cellobiose, which is known to inhibit endoglucanase and exoglucanase activities (Zhang & Zhang, 2013). The previously formulated enzyme cocktail produced 10 % total reducing sugars in the HRP pretreated rooibos (Mohotloane et al., 2023), meaning that there was a need to formulate an enzyme cocktail with improved sugar production in the current study. The holocellulolytic enzyme cocktail resulted in two effective combinations that were used at varying enzyme concentrations to quantify the percentage yield produced per gram of biomass. The enzyme cocktails effectivity produced over 95 % total reducing sugars at a lower concentration of 25 mg/g biomass, validating the efficacy of the HRP pretreatment in structurally and chemically modifying the biomass. Mafa et al. (2020b) demonstrated that NaOH pretreatment effectivity removed lignin from corncob and sweet sorghum bagasse, which improved the hydrolytic activity of the formulated enzyme cocktail obtained from termite metagenome. Similarly, Malgas et al. (2017) formulated a holocellulolytic enzyme with superior hydrolytic efficiency on pretreated hardwoods, which resulted in high sugar conversion and yield.

5. Conclusion

Horseradish peroxidase (HRP) modifies the structural and chemical properties of microcrystalline cellulose. SEM analysis showed that the HRP had a peeling effect on Avicel, exposing cracks and pores, while it removed the para-crystalline cellulose from filter paper, exposing the crystalline fibres (cellulose I). In addition, the FTIR and XRD analysis confirmed the HRP pretreatment's ability to increase d-spacing and crystallite sizes at the 101 and 002 lattice planes in the Avicel substrates, which is associated with reduced crystallinity. Furthermore, the decreased cellulose crystallinity improved the activity of EGs on the Avicel. These findings explain the HRP's ability to remove lignin and decreases the crystallinity in the pretreated rooibos samples. The physicochemical changes in rooibos led to higher biomass conversion rate yielding about 95 % soluble sugars per 25 mg enzyme loading/g biomass.

CRediT authorship contribution statement

Mamosela Marriam Mohotloane: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Orbett Alexander:** Writing – review & editing, Visualization, Validation, Software, Methodology, Formal analysis. **Vanthini Nelson Adoons:** Writing – review & editing, Visualization, Software, Methodology, Data curation. **Brett Ivan Pletschke:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Mpho Stephen Mafa:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this work.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by the University of the Free State, Department of Plant Science and Centre for Graduate Support. This research was also partially supported by the South African National Research Foundation through a Thuthuka grant awarded to Dr. Mpho S Mafa.

Funding

Ms. MMM received a MSc bursary from the University of the Free State Centre for Graduate Support, MSM received an NRF-Thuthuka grant (TTK2204102938).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2024.100426.

References

Badiei, M., Asim, N., Jahim, J. M., & Sopian, K. (2014). Comparison of chemical pretreatment methods for cellulosic biomass. *APCBEE Procedia, 9*, 170–174.

Bagewadi, Z. K., Mulla, S. I., & Ninnekar, H. Z. (2017). Optimization of laccase production and its application in delignification of biomass. *International Journal of Recycling of Organic Waste in Agriculture, 6*, 351–365.

Behera, S., Arora, R., Nandhagopal, N., & Kumar, S. (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews, 36*, 91–106.

Bommarius, A. S., Katona, A., Cheben, S. E., Patel, A. S., Ragauskas, A. J., Knudson, K., & Pu, Y. (2008). Cellulase kinetics as a function of cellulose pretreatment. *Metabolic Engineering, 10*(6), 370–381.

Chang, H., Gacias Amengual, N., Botz, A., Schwaiger, L., Kracher, D., Scheiblbrandner, S., Csarman, F., & Ludwig, R. (2022). Investigating lytic polysaccharide monooxygenase-assisted wood cell wall degradation with microsensors. *Nature Communications, 13*(1), 6258.

Devi, A., Bajar, S., Kour, H., Kothari, R., Pant, D., & Singh, A. (2022). Lignocellulosic biomass valorization for bioethanol production: A circular bioeconomy approach. *Bioenergy Research, 15*(4), 1820–1841.

Garvey, C. J., Parker, I. H., & Simon, G. P. (2005). On the interpretation of X-ray diffraction powder patterns in terms of the nanostructure of cellulose I fibres. *Macromolecular Chemistry and Physics, 206*(15), 1568–1575.

Harmsen, P. F. H., Huijgen, W., Bermudez, L., & Bakker, R (2010). Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. In *Proceedings of the Wageningen UR food & biobased research (Report /Wageningen UR, Food & Biobased Research 1184)*. ISBN: 9789085857570.

Hernández-Chaverri, R. A., Buenrostro-Figueroa, J. J., & Prado-Barragán, L. A. (2021). Biomass: Biorefinery as a model to boost the bioeconomy in Costa Rica, a review. *Agronomía Mesoamericana, 32*(3), 1047–1070.

Imai, T., Naruse, M., Horikawa, Y., Yaoi, K., Miyazaki, K., & Sugiyama, J. (2023). Disturbance of the hydrogen bonding in cellulose by bacterial expansin. *Cellulose, 30*(13), 8423–8438.

Ju, X., Bowden, M., Brown, E. E., & Zhang, X. (2015). An improved X-ray diffraction method for cellulose crystallinity measurement. *Carbohydrate Polymers, 123*, 476–481.

Kersten, P. J., Kalyanaraman, B., Hammel, K. E., Reinhammar, B., & Kirk, T. K. (1990). Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochemical Journal, 268*(2), 475–480.

Khan, S. A., Khan, S. B., Khan, L. U., Farooq, A., Akhtar, K., & Asiri, A. M. (2018). Fourier transform infrared spectroscopy: Fundamentals and application in functional groups and nanomaterials characterization. Sharma, S. (eds). *Handbook of Materials Characterization* (pp. 317–344). Cham: Springer. https://doi.org/10.1007/978-3-319-92955-2_9.

Kong, W., Fu, X., Wang, L., Alhujaily, A., Zhang, J., Ma, F., Zhang, X., & Yu, H. (2017). A novel and efficient fungal delignification strategy based on versatile peroxidase for lignocellulose bioconversion. *Biotechnology for Biofuels, 10*(1), 1–15.

Kumar, B., Bhardwaj, N., Agrawal, K., Chaturvedi, V., & Verma, P. (2020). Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Processing Technology, 199*, Article 106244.

Lavery, C. B., MacInnis, M. C., MacDonald, M. J., Williams, J. B., Spencer, C. A., Burke, A. A., Irwin, D. J., & D'Cunha, G. B (2010). Purification of peroxidase from horseradish (Armoracia rusticana) roots. *Journal of Agricultural and Food Chemistry, 58*(15), 8471–8476.

Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M., & Henrissat, B. (2013). Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnology for Biofuels, 6*(1), 1–14.

Limayem, A., & Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science, 38*(4), 449–467.

Ling, Z., Chen, S., Zhang, X., & Xu, F. (2017). Exploring crystalline-structural variations of cellulose during alkaline pretreatment for enhanced enzymatic hydrolysis. *Bioresource Technology, 224*, 611–617.

Ling, Z., Wang, T., Makarem, M., Santiago Cintrón, M., Cheng, H. N., Kang, X., Bacher, M., Potthast, A., Rosenau, T., King, H., & Delhom, C. D. (2019). Effects of ball milling on the structure of cotton cellulose. *Cellulose, 26*, 305–328.

Mafa, M. S., Malgas, S., Bhattacharya, A., Rashamuse, K., & Pletschke, B. I. (2020b). The effects of alkaline pretreatment on agricultural biomasses (corn cob and sweet sorghum bagasse) and their hydrolysis by a termite-derived enzyme cocktail. *Agronomy, 10*(8), 1211.

Mafa, M. S., Malgas, S., Rashamuse, K., & Pletschke, B. I. (2020a). Delineating functional properties of a cello-oligosaccharide and β-glucan specific cellobiohydrolase (GH5_38): Its synergism with Cel6A and Cel7A for β-(1, 3)-(1, 4)-glucan degradation. *Carbohydrate Research, 495*, Article 108081.

Mafa, M. S., Pletschke, B. I., & Malgas, S. (2021). Defining the frontiers of synergism between cellulolytic enzymes for improved hydrolysis of lignocellulosic feedstocks. *Catalysts, 11*(11), 1343.

Malgas, S., Chandra, R., Van Dyk, J. S., Saddler, J. N., & Pletschke, B. I. (2017). Formulation of an optimized synergistic enzyme cocktail, HoloMix, for effective degradation of various pre-treated hardwoods. *Bioresource Technology, 245*, 52–65.

Manavalan, T., Manavalan, A., & Heese, K. (2015). Characterization of lignocellulolytic enzymes from white-rot fungi. *Current Microbiology, 70*, 485–498.

Menon, V., & Rao, M. (2012). Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science, 38*(4), 522–550.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426–428.

Min, K., Kim, Y. H., Kim, J., Kim, Y., Gong, G., & Um, Y. (2022). Effect of manganese peroxidase on the decomposition of cellulosic components: Direct cellulolytic activity and synergistic effect with cellulase. *Bioresource Technology, 343*, Article 126138.

Mnich, E., Bjarnholt, N., Eudes, A., Harholt, J., Holland, C., Jørgensen, B., Larsen, F. H., Liu, M., Manat, R., Meyer, A. S., & Mikkelsen, J. D. (2020). Phenolic cross-links: Building and de-constructing the plant cell wall. *Natural Product Reports, 37*(7), 919–961.

Mohotloane, M. M., Alexander, O., Pletschke, B. I., & Mafa, M. S. (2023). Horseradish peroxidase delignification of fermented rooibos modifies biomass structural and chemical properties and improves holocellulolytic enzyme cocktail efficacy. *Biologia, 78*, 1943–1959.

Monclaro, A. V., & Ferreira Filho, E. X. (2017). Fungal lytic polysaccharide monooxygenases from family AA9: Recent developments and application in lignocellulose breakdown. *International Journal of Biological Macromolecules, 102*, 771–778.

Nam, S., French, A. D., Condon, B. D., & Concha, M. (2016). Segal crystallinity index revisited by the simulation of X-ray diffraction patterns of cotton cellulose Iβ and cellulose II. *Carbohydrate Polymers, 135*, 1–9.

Pandey, V. P., Awasthi, M., Singh, S., Tiwari, S., & Dwivedi, U. N. (2017). A comprehensive review on function and application of plant peroxidases. *Biochemistry and Analytical Biochemistry, 6*(1), 308.

Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., & Johnson, D. K. (2010). Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels, 3*, 1–10.

Scherrer, P. (1918). Bestimmung der grosse und inneren struktur von kolloidteilchen mittels rontgenstrahlen. *Nach Ges Wiss Gottingen, 2*, 8–100.

Segal, L. G. J. M. A., Creely, J. J., Martin Jr, A. E., & 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.

Sharma, H. K., Xu, C., & Qin, W. (2019). Biological pretreatment of lignocellulosic biomass for biofuels and bioproducts: An overview. *Waste and Biomass Valorization, 10*, 235–251.

Tian, S. Q., Zhao, R. Y., & Chen, Z. C. (2018). Review of the pretreatment and bioconversion of lignocellulosic biomass from wheat straw materials. *Renewable and Sustainable Energy Reviews, 91*, 483–489.

Van Dyk, J. S., & Pletschke, B. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—factors affecting enzymes, conversion and synergy. *Biotechnology Advances, 30*(6), 1458–1480.

Veitch, N. C. (2004). Horseradish peroxidase: A modern view of a classic enzyme. *Phytochemistry, 65*(3), 249–259.

Wagner, A. O., Lackner, N., Mutschlechner, M., Prem, E. M., Markt, R., & Illmer, P. (2018). Biological pretreatment strategies for second-generation lignocellulosic resources to enhance biogas production. *Energies, 11*(7), 1797.

Yu, S., Liu, Z., Xu, N., Chen, J., & Gao, Y. (2020). Influencing factors for determining the crystallinity of native cellulose by X-ray diffraction. *Analytical Sciences, 36*(8), 947–951.

Zhang, X. Z., & Zhang, Y. H. P. (2013). Cellulases: characteristics, sources, production, and applications. In S.-T. Yang, H. A. El-Enshasy, & N. Thongchul (Eds.),

Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers (pp. 131–146). John Wiley & Sons, Inc.

Zhou, W., Apkarian, R., Wang, Z. L., & Joy, D. (2007). Fundamentals of scanning electron microscopy (SEM). *Scanning Microscopy for Nanotechnology: Techniques and Applications*, 1–40.

Zhu, J. Y., & Pan, X. (2022). Efficient sugar production from plant biomass: Current status, challenges, and future directions. *Renewable and Sustainable Energy Reviews, 164*, Article 112583.