Cellulase stimulation during biodegradation of lignocellulosic residues at increased biomass loading

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Abstract

Microbial degradation of lignocellulosic biomass is primarily affected by the composition and structure of biomass, as well as enzyme activities that are influenced by the presence of in-process degradation products. This study focuses on the latter, and demonstrates that cellulase activity of *Neurospora discreta* is stimulated in the presence of in-process soluble lignin degradation products. Two types of biomass, cocopeat and sugarcane bagasse, with contrasting lignin content and cellulose structure were tested at two biomass loadings each. At the higher biomass loading, cocopeat showed the highest amount of hydrolyzed cellulose and cellulase activity, despite its low cellulose content and recalcitrant cellulose structure. A strong positive correlation was revealed between the amount of in-process degraded lignin and cellulase activity, indicating a stimulatory effect on cellulase, which contradicts most previous literature. Furthermore, the causal relationship between the amount of degraded lignin and cellulase activity was established in a model system of commercial cellulase and standard soluble lignin. This work could pave the way for using biomass loading as a process lever to enhance cellulose hydrolysis in microbial conversion of lignocellulosic biomass.

Keywords: Cellulase stimulation; cellulose hydrolysis; cocopeat; lignin degradation products; *Neurospora discreta*, sugarcane bagasse.

1.0 Introduction

Production of bioethanol from lignocellulosic biomass residues is a sustainable solution to the growing energy crisis, as well as for utilization of abundantly available agricultural residues (Kim and Dale 2004; Sukumaran et al. 2010; Sarkar et al. 2012). The process involves pre-treatment of biomass to remove lignin, hydrolysis of cellulose and hemicellulose, and subsequent fermentation of the resulting sugars to produce ethanol (Limayem and Ricke 2012). Existing pre-treatment strategies include chemical and thermochemical methods that require the use of strong chemicals or high energy inputs. Microbial delignification is a promising, environment-friendly alternative as it operates at much milder conditions compared to other techniques (Arora et al. 2016; Pamidipati and Ahmed 2017).

Ethanol yields depend not only on the amount of cellulose present in the biomass, but also on the structure of cellulose which determines its 'degradability' (Yoshida et al. 2008; Poletto, Ornaghi Júnior, and Zattera 2014; Jeoh et al. 2007) . Increased cellulose crystallinity is associated with recalcitrance as crystalline cellulose structures are more difficult to break down compared to amorphous structures (Pérez et al. 2002). Apart from the biomass used, the activity of cellulolytic enzymes (cellulases) also has a direct bearing on ethanol yields as it affects the amount of glucose produced from cellulose. Efforts have been directed towards increasing cellulase activity using techniques such as mutagenesis, co-culture techniques (Paramjeet, Manasa, and Korrapati 2018; Fang et al. 2013), addition of cofactors (Obeng, Budiman, and Ongkudon 2017; Saito et al. 2003) as well as ultrasound (Luzzi et al. 2017) and microwave based pre-treatment technologies (Mondal, Roy, and Gupta 2004).

Cellulase activity is also affected by the presence of lignin degradation products generated during the pre-treatment step. Several studies have reported inhibition of cellulases by lignin

degradation products generated during thermal or chemical pre-treatment of biomass (Tejirian and Xu 2011; Qin et al. 2016; Sineiro et al. 1997). A few reports also exist on the stimulatory effect of model lignin monomers on purified cellulase at specific concentrations (Zhao and Chen 2014; Tsujiyama, Sumida, and Ueno 2001). However, to our knowledge, no reports exist on the effects of *in-process* degradation products produced during microbial degradation of lignocellulosic biomass on cellulase activity.

In our previous publication, we reported the use of a locally isolated fungus, *Neurospora discreta*, which resulted in greater de-lignification of biomass compared to traditionally used white rot fungi (Pamidipati and Ahmed 2017). Neurospora also has the enzymatic machinery to hydrolyse cellulose (Lynd et al. 2002) and to produce ethanol (Cardona and Sánchez 2007), which makes it a promising candidate for complete bioprocessing of lignocellulosic residues. The objective of the present study was to evaluate the effect of lignin degradation products generated during biodegradation of lignocellulosic residues by *N. discreta* on its cellulase activity and the resulting cellulose hydrolysis. Two lignocellulosic residues: cocopeat and sugarcane bagasse were selected for this study owing to the large contrast in the amount of lignin, as well as the amount and crystallinity of cellulose present in each. Each biomass was tested at two loadings in order to influence the amount of degraded lignin released due to fungal action.

2.0 Materials and methods

2.1. Lignocellulosic biomass substrates

Sugarcane bagasse and cocopeat were obtained from local vendors. The substrates were thoroughly washed and dried to remove the moisture content. Sugarcane bagasse was chopped and sieved to maintain a particle size between 0.3mm and 1mm.

2.2. Microorganism

Neurospora discreta which is a locally isolated fungal strain (Pamidipati and Ahmed 2017) was used for this study. The strain was stored at 2-4^{o}C on potato dextrose agar (PDA) slant and was regularly sub-cultured on PDA plates by incubating them at 28^{o}C for 2-4 days.

2.3. Fermentation studies

Submerged fermentations were set up in Erlenmeyer flasks, using sub-cultures of *N. discreta*. Vogel's minimal medium with 0.5% sucrose was used in all cases. Two loadings were tested for each biomass: 1g of biomass in 100 ml medium (10 g/l) and 5g of biomass in 150 ml medium (~33 g/l). The additional medium in the 5g loading was added to ensure submerged conditions. It is to be noted that the additional medium does not impact the amount of lignocellulosic biomass available to the cells. 0.5% sucrose was added to the media to initiate cell growth. The flasks containing the biomass and medium were autoclaved at 121^{o}C for 20 minutes and then cooled to room temperature before inoculation. For inoculation, a spore suspension was prepared by gently scraping the fungal cells from sub-cultured PDA plates and adding them to a known quantity of sterile minimal media which was then filtered through double layered muslin cloth (Pamidipati and Ahmed 2017). To each flask, 1 ml of the spore suspension with a spore count of approximately 10^{7}cells ml^{-1} was added aspetically. The flasks were mixed well and incubated at 30 ^{o}C for 30 days. At the end of the fermentation, the

contents of each flask were filtered. The solid fractions were thoroughly washed, filtered and dried till a constant weight was achieved. Lignin and cellulose content were analyzed in the solid substrates. Liquid fractions were centrifuged at 84,448 (g) for 20 minutes and the clear supernatant samples were analyzed for lignin degradation products and cellulase activity.

2.4. Lignin estimation

The lignin content in the solid biomass samples was measured using standard Klason's method (Sluiter et al. 2008) before and after fungal treatment. The amount of degraded lignin was calculated from the difference between initial and final values and the degree of deligification was calculated as follows:

$$Degree\ of\ delignification\ (\%) = 100\ X \left[\frac{Initial\ lignin\ content(g) - Final\ lignin\ content\ (g)}{Initial\ lignin\ content(g)}\right]$$

2.5. Cellulose estimation

The cellulose content in the solid fraction was analyzed using the Updegraff method detailed in the paper (Bauer and Ibáñez 2014). However, instead of the anthrone assay, a glucose oxidase assay kit from Sigma-Aldrich (GAGO 20) was used to determine the glucose content, to avoid overestimation by the anthone reagent due to the presence of other sugars released from the biomass. The pH of the samples was adjusted to around 6.5 using suitable concentrations of NaOH solution before using the glucose oxidase assay to ensure uniform conditions. Glucose content in the samples was calculated using a calibration curve prepared using the glucose standard provided with the kit. An anhydro-correction factor of 0.9 was used for calculating the concentration of cellulose from corresponding monomeric (glucose) sugars

(Sluiter et al. 2008). The amount of cellulose hydrolyzed was calculated from the difference between initial and final cellulose content. The degree of cellulose hydrolysis was calculated as follows:

$$Degree\ of\ cellulose\ hydrolysis\ (\%) = 100\ X \left[\frac{Initial\ cellulose\ content(g) - Final\ cellulose\ content\ (g)}{Initial\ cellulose\ content(g)}\right]$$

2.6. Fourier transform infrared (FT-IR) spectroscopy for cellulose crystallinity

FT-IR spectroscopy of the two biomass substrates was carried out using pellets prepared with 1 mg of substrate and 100 mg of KBr on FT/IR 4200 spectrophotometer (JASCO Make) in the absorbance mode in the range of 400 to 4000 cm^{-1}(Pamidipati and Ahmed 2017). The baseline was corrected between 800 to 3500 cm^{-1} and the absorbance values at 897, 1430, 1372, 2900, 3400 and 1320 cm^{-1} were noted to calculate crystallinity indices of cellulose. Lateral Order Index (LOI) was calculated by taking the ratio of absorbance at 1430 and 897 (A_{1430}/A_{897}) and Hydrogen Bond Intensity (HBI) was calculated as ratio of A_{3400}/A_{1320}. (Fan et al. 2012; Poletto et al. 2014).

2.7. Reversed phase high-performance liquid chromatography (RP-HPLC)

The soluble products of lignin degradation were analyzed in the liquid fraction obtained at the end of fermentation on a C-18 column using acetonitrile- water solvent system using RP-HPLC as detailed in our previous paper (Pamidipati and Ahmed 2017).

2.8. Cellulase activity

Samples collected on days 10 and 20 of the fermentation were centrifuged and the supernatants were sparged with CO_2 to adjust the pH to between 6 and 6.5 in order to maintain uniform pH conditions. The cellulase activity in these samples was measured using a modified NREL protocol (Adney and Baker 2008). Briefly, to 50 mg of cellulose powder (insoluble α-cellulose, Himedia, GRM-126), 1ml of 50mM pH 4.8 citrate buffer was added and the mixture was equilibrated to 37ºC in glass test tubes. To this mixture, 0.5 ml of the sample supernatant was added and incubated for 3 hrs at 37 ºC. At the end of 3 hrs, the sample was centrifuged and the supernatant was analyzed for glucose content using the glucose oxidase enzyme assay described in section 2.5. Cellulase activity was calculated in terms of the amount of glucose produced per unit volume per unit time and reported as U/l. While the original protocol uses the Dinitrosalicylic assay (DNSA) for estimating the glucose produced, in a fermentation study this could lead to an overestimation due to the non-specific binding of the DNS reagent to other sugars released from the biomass. Therefore, a more specific glucose oxidase assay was used here which would provide a more accurate estimation of cellulase activity.

2.9. Study on model system of commercial cellulase and standard soluble lignin

Pure cellulase from *Trichoderma reesei* (Sigma aldrich, C2730) was used for studying the effect of varying concentrations of soluble standard lignin (Sigma-Aldrich, 471003) on cellulase activity. The concentration range of standard lignin tested was 0.5-4 g/l. Cellulase activity was measured using the standard NREL protocol described above. As there was no possibility of interference from other sugars in this experiment, glucose content was measured using Dinitrosalicylic assay (DNSA) method (Miller 1959) as per the original protocol.

3.0 Results and discussion

3.1. Biomass composition and cellulose crystallinity

Table 1 shows the differences in sugarcane bagasse and cocopeat in terms of the percentage of lignin and cellulose, measured using Klason's and Updegraff methods respectively, as well as the crystallinity indices of cellulose calculated using the absorbances from the FT-IR spectra (figure 1) as described above. As per literature, sugarcane bagasse consists of approximately 43-50% cellulose and 18-25% each of hemicellulose and lignin (A. Pandey et al. 2000; Limayem and Ricke 2012). Cocopeat consists of 21-36% cellulose and 48-54% lignin. (Israel et al. 2011; Shashirekha and Rajarathnam 2007). The data on biomass composition is consistent with the values reported and indicates that the two biomass substrates are significantly different in terms of the composition.

Table 1 Biomass composition and cellulose crystallinity

Sample	Biomass composition		Cellulose Crystallinity indices	
	Lignin (%)	Cellulose (%)	A_{1430}/A_{897} (LOI)	A_{3400}/A_{1320} (HBI)
Sugarcane bagasse (n=3)	21.00±0.14	49.00±0.05	2.06±0.34	22.11± 2.64
Cocopeat (n=3)	46.0±1.3	35.0±0.1	7.71±0.42	34.21± 0.22

Figure 1 shows the FT-IR spectra of cocopeat and sugarcane bagasse and indicates the peaks that were used to calculate the crystallinity indices. Crystallinity index is a parameter that is commonly used to describe the degree of crystallinity of polymers and can be measured using FT-IR spectroscopy (Poletto, Ornaghi Júnior, and Zattera 2014; Fan, Dai, and Huang 2012; Sun et al. 2004). Two types of indices are used to describe the crystallinity of cellulose. The ratio of absorbances at 1430-1420 cm^{-1} (associated with crystalline cellulose) and 897cm^{-1}

(corresponding to amorphous cellulose) is called Lateral Order Index (LOI) (Poletto, Ornaghi Júnior, and Zattera 2014; Fan, Dai, and Huang 2012). Higher the LOI, greater the crystallinity of cellulose. Another parameter that is used to determine the intermolecular regularity and crystallinity of cellulose is called Hydrogen Bond Intensity (HBI), which is determined by the ratio of absorbances at 3400 and 1320 cm^{-1} (Poletto, Ornaghi Júnior, and Zattera 2014). Both indices were found to be significantly higher in cocopeat (Table 1), indicating that the cellulose in cocopeat is more crystalline compared to that in sugarcane bagasse, making it more difficult to degrade (Poletto, Ornaghi Júnior, and Zattera 2014).

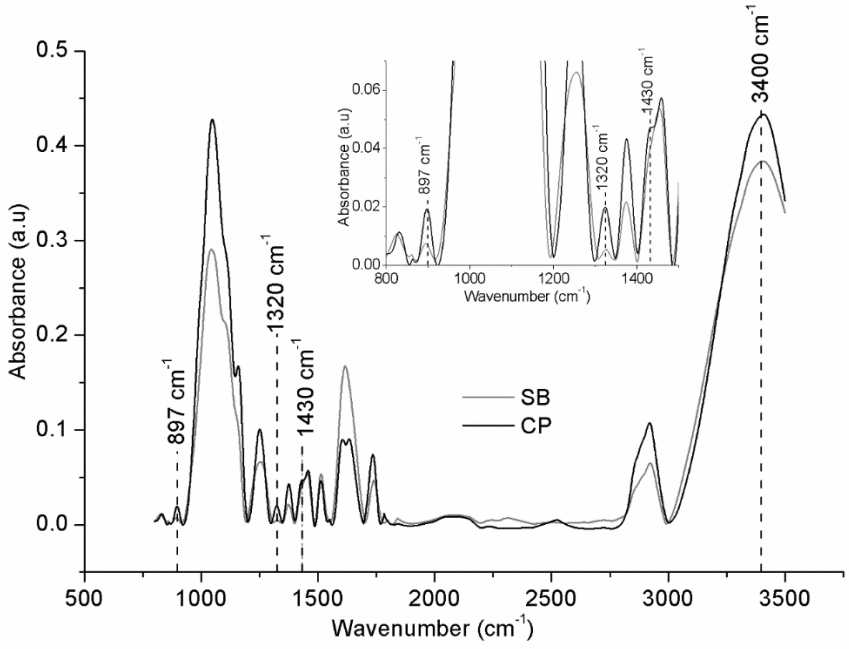

Figure 1. FT-IR spectra of cocopeat (CP) and sugarcane bagasse (SB) samples. The inset shows a zoomed portion of the spectra containing the smaller peaks.

3.2. Analysis of lignin degradation products

Lignin degradation products released into the fermentation broth during biomass degradation were analyzed at the end of the fermentation using RP-HPLC. Figure 2 shows the RP-HPLC

chromatograms of the liquid supernatant samples of cocopeat and sugarcane bagasse at different biomass loadings. All samples show a major peak at a retention time of 2.9 ±0.5, which matches with the peak of the standard lignin as discussed in our previous paper (Pamidipati and Ahmed 2017), indicating the production of soluble degradation products from lignin. Control samples prepared by soaking the biomass in the medium without any cells were also run using the same method, and did not show any peaks. For each biomass type, the area under the curve, which represents the concentration of degraded lignin released into the medium, increased nearly four-fold with increase in biomass loading. Due to the higher initial lignin content in cocopeat, the amount of lignin degraded was higher in cocopeat compared to bagasse. The largest peak was seen in the higher biomass loading of cocopeat indicating the highest amount of lignin degradation products released into the medium, followed by the higher loading condition of sugarcane bagasse.

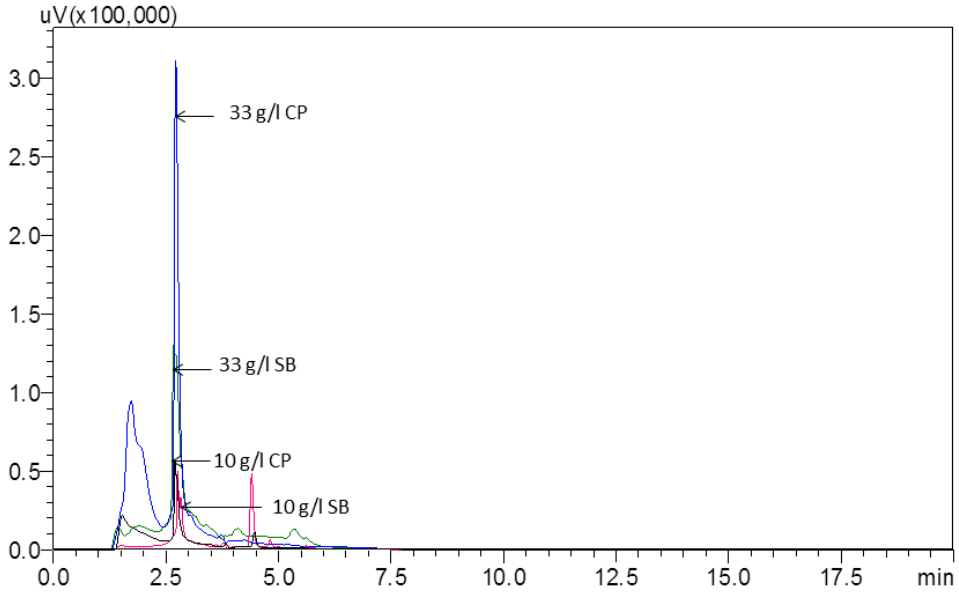

Figure 2 RP-HPLC chromatograms of liquid supernatant samples of sugarcane bagasse (SB), cocopeat (CP) at both biomass loadings.

3.3. Effect of degraded lignin on cellulase activity and cellulose hydrolysis

The amount of degraded lignin estimated using Klason's lignin protocol was higher in cocopeat compared to sugarcane bagasse, and increased with biomass loading for both cases, which was also seen in the RP-HPLC chromatograms. While the degree of delignification was lower in cocopeat (inset of figure 3), the higher initial lignin content in cocopeat (table 1) resulted in higher amount of lignin being released into the medium. However, despite the lower cellulose content and higher crystallinity indices in cocopeat, the amount of cellulose hydrolyzed was nearly 40% higher in cocopeat at the 10 g/l loading and 27% higher at the 33 g/l loading compared to sugarcane bagasse. Similarly, the cellulase activities were also higher in cocopeat samples at each biomass loading. Furthermore, the cellulase activities and cellulose hydrolysis also increased with increase in biomass loading for each biomass type.

Across all four conditions, cellulase activities and cellulose hydrolysis increased with increase in the amount of lignin degradation products, indicating a stimulatory effect of these products on cellulase, as shown in figure 3 (top). A strong positive correlation (Pearson's correlation coefficient >0.99) was seen between the degraded lignin and cellulase activity. The degree of cellulose hydrolysis also increased with increase in biomass loading and was the highest for the higher loading of cocopeat.

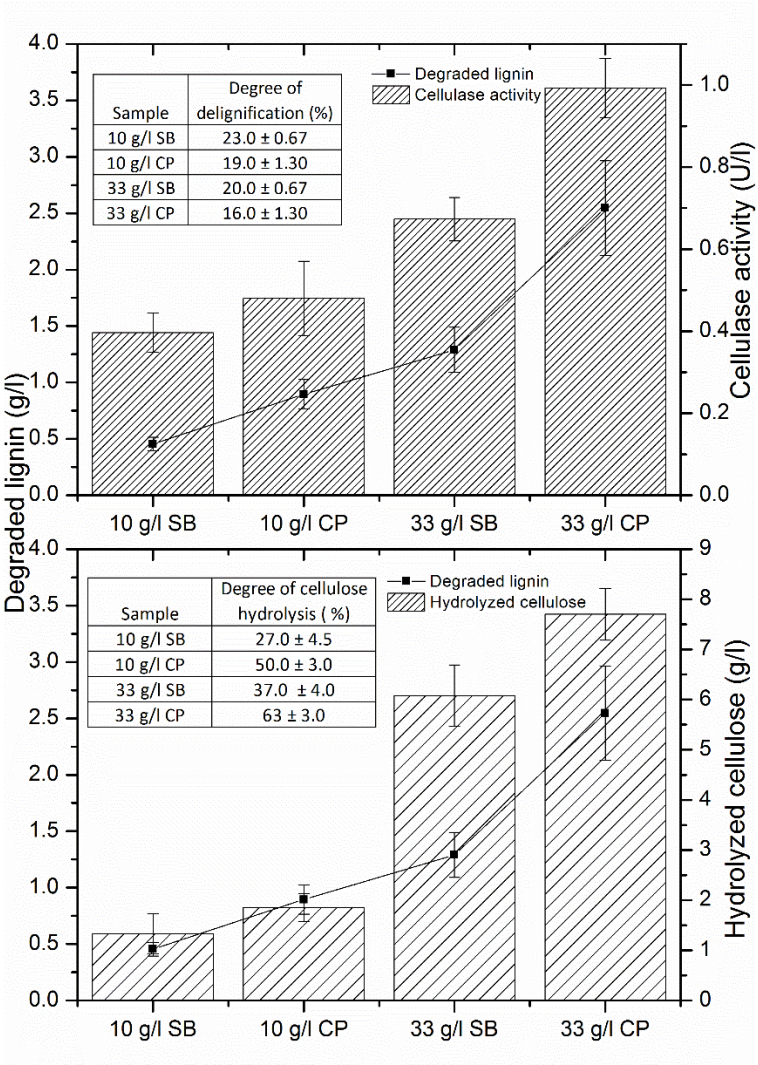

Figure 3 Degraded lignin and cellulase activity in sugarcane bagasse (SB) and cocopeat (CP) samples (top). Degraded lignin and hydrolyzed cellulose content in sugarcane bagasse (SB) and cocopeat (CP) samples (bottom). The insets show the degrees of delignification and cellulose hydrolysis.

Several studies have reported that the phenolic compounds released during lignin degradation inhibit cellulase activity (Ximenes et al. 2010; Qin et al. 2016; Tejirian and Xu 2011). However these lignin-derived compounds were generated using physical or chemical pretreatment

methods such as steam explosion, acid or alkali treatment techniques. Other researchers have reported a concentration-dependent stimulatory effect of certain lignin monomers on cellulase activity (Müller, Trösch, and Kulbe 1988; Paul et al. 2003; Zhao and Chen 2014; Tsujiyama, Sumida, and Ueno 2001). It was hypothesized by Zhao et al., that lignin-derived phenolic compounds at a certain concentration increase the hydrophobicity of cellulase surface just enough to cause better enzyme-substrate connections and lead to a stimulatory effect (Zhao and Chen 2014). Other studies have indicated a suppression of non-productive binding of cellulase to lignin in the presence of certain concentrations of lignosulfonates (Wang, Lan and Zhu 2013) or organosolv lignin at certain pH conditions (Lai et al. 2018). The nature of these phenolic compounds also plays a very important role in its action on cellulases (Zhao and Chen 2014). However, most of these studies were carried out using synthetic phenolic compounds and not with in-process degraded lignin generated during microbial degradation of biomass.

The stimulation of cellulase by lignin degradation products seen in the present study indicates that biomass loading can be used as a process lever to influence the amounts of lignin degradation products released, which would in turn influence the cellulase activity and cellulose hydrolysis. This also implies that the influence of in-process intermediates on enzyme activities could help overcome the inherent recalcitrance of the biomass, such as in the case of cocopeat.

3.4. Effect of standard lignin on commercial cellulase activity

While a correlation between degraded lignin and cellulase activity is seen in the fermentation studies, in order to use biomass loading as a process lever, it is important to establish a causal relationship betwen the two factors. To this end, a model system of commercial cellulase

and standard soluble sulfonated lignin was used. Figure 4 shows the effect of varying concentrations of standard soluble lignin on the activity of pure cellulase. The standard lignin was chosen based on its similarity to the lignin degradation products released by the action of *N. discreta*, which was shown in our previous paper using RP-HPLC (Pamidipati and Ahmed 2017). The concentration range of standard lignin was fixed based on the soluble lignin concentrations seen in the actual fermentation samples from the present study. An increase in cellulase activity was seen with increasing concentrations of standard lignin beyond 1 g/l, which further confirmed the concentration-dependent stimulation of cellulase seen in actual fermentation set-up with increased amounts of degraded lignin in this concentration range. This study not only confirmed the stimulatory effect of soluble lignin on cellulase, but also showed that the stimulation is not specific to the cellulase secreted by *N. discreta* alone.

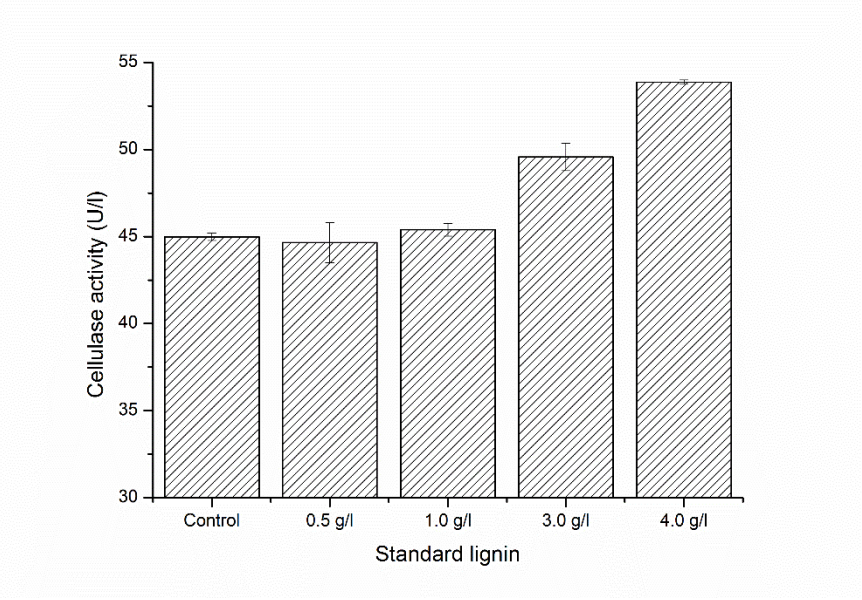

Figure 4 Effect of varying concentrations of standard soluble lignin on commercial cellulase activity. The control sample was run in the absence of lignin.

4.0 Conclusion

Despite the lower cellulose concentrations and higher crystallinity indices of cellulose in cocopeat, an increase in biomass loading resulted in higher cellulose hydrolysis owing to the stimulatory effect of degraded lignin on cellulase. Biomass loading can therefore be used as a simple and cost effective process lever to enhance cellulase activity and cellulose hydrolysis in lignocellulosic residues. Moroever, this study paves the way for utilization of relatively recalcitrant residues such as cocopeat with high lignin content, for production of biofuels. This work has also demonstrated a general principle that increase in lignin concentrations increases cellulase activity. Further investigation on the nature of lignin degradation products released during microbial degradation of lignin and their individual impact on cellulase activity could provide additional insights into the process.

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6.0 Disclosure statement

The authors report no conflict of interest.

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