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Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass

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The polysaccharide monoxygenase enzyme AA9 (formerly known as GH61) was shown to interact synergistically with cellulases to enhance the enzymatic hydrolysis of a range of "commercially-relevant" pretreated and "model" cellulosic substrates. Although an exogenous source of reducing power was required when AA9 was added with cellulases to a "pure" cellulosic substrate, it was not required when added to pretreated lignocellulosic substrates. It appears that the non-cellulosic components such as soluble components, lignin, and possibly xylan, can all act as AA9 reducing cofactor. Of the various substrate characteristics that influenced the efficacy of the enzyme mixture, the relative amount of accessible crystalline cellulose, assessed by the specific cellulose binding module (CBM), appeared to be the most critical. Cellulases and AA9 acted synergistically when hydrolysing cellulose I but it did not occur during the hydrolysis of cellulose II and III.

Introduction

Although considerable progress has been made in reducing the cost of cellulase enzyme mixtures and in better elucidating the synergistic interaction of the individual components, relatively high protein loadings are still required to achieve effective cellulose hydrolysis [1]. Recent work has shown [2, 3] that accessory enzymes and/or disrupting proteins, such as xylanases, lytic polysaccharide monooxygenases (LPMOs, e.g. AA9 formerly known as GH61) and swollenin can all significantly enhance the hydrolytic performance of cellulase enzyme mixtures over a range of cellulosic substrates. For example, the addition of a polysaccharide monoxygenase AA9 was shown to significantly enhance the hydrolytic potential of a cellulase mixture during the enzymatic hydrolysis of dilute-acid pretreated corn stover [3]. Unlike the canonical cellulase enzymes which have been shown to cleave cellulose by a hydrolytic mechanism involving the conserved carboxylic acid residues within either channel or cleft shape substrate loading sites, AA9 is thought to cleave cellulose chains by an oxidative mechanism at the proteins planar active site which contains a divalent metal ion [4-7].

As well as needing the divalent metal ion within the active site for effective action the AA9 enzyme also requires the presence of redox-active cofactors with metal reducing capacity to potentiate its activity, such as the compounds provided by cellobiodehydrogenase (CDH), synthetic small molecule reductants (eg. gallate or ascorbate), or some "still unidentified" compounds that are typically found in pretreated lignocellulosic biomass [8-13]. However, the source of these cofactors within different type of lignocellulosic biomass and their influence on cellulases-AA9 synergistic cooperation still remain unknown.

The exact mechanism of "cellulase synergism" has been extensively discussed ever since the "C_1-C_x" hypothesis was first proposed by Reese in 1950 [14]. Cellulose hydrolysis was thought to be initiated by a non-hydrolytic chain-separating enzyme "C_1" which caused the cellulose to swell, facilitating subsequent attack by the hydrolytic "C_x" enzyme mixture. Although the "C1" concept is attractive, it has proven difficult to ascertain the existence of such a swelling factor. Alternatively, the recently identified AA9 has been shown to significantly increase the accessibility of cellulases to cellulose by the oxidative cleave/disruption of crystalline cellulose region [6, 8]. However, despite this this potential, the mechanism of the synergistic cooperation between cellulase enzymes (hydrolytic cellulose cleavage) and AA9 (oxidative cellulose cleavage), especially on lignocellulosic biomass, has remained elusive.

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Typically, a thermochemical pretreatment step is firstly required to open up the lignocellulosic materials for the subsequent enzymatic hydrolysis [15, 16]. Under pretreatment conditions which have been shown to result in good overall carbohydrate recovery (that is, hemicelluloses and cellulose) while producing a cellulose-rich substrates amenable to enzymatic hydrolysis, the cellulose component of the pretreated substrates is always found in association with residual hemicellulose and lignin. In addition, depending on the pretreatment strategy applied and the nature of the starting biomass, the cellulose itself in the pretreated substrate also exhibits different physical characteristics. Previous work has shown that the extent and degree of enzyme synergism is significantly influenced by the nature of the cellulosic substrate including the overall accessibility of the cellulose, its crystallinity, degree of polymerization, the nature of the cellulose allomorphs and the type and the amount/distribution of residual lignin and hemicellulose [17-19].

In the work reported here we assessed the extent of synergistic cooperation between a highly active *Thermoascus aurantiacus* AA9 and *Trichoderma reesei* cellulases during hydrolysis of a range of "commercially-relevant" pretreated lignocellulosic and "model" cellulosic substrates. We also hoped to determine if the non-cellulosic components within the pretreated lignocellulosic substrates (i.e. soluble compounds, lignin fragments, hemicellulose derivatives, etc.) might have a high enough reducing capacities to act as cofactors for AA9 and assess those substrate characteristics that most influenced the extent and nature of the synergism.

Results

It is increasingly apparent that, as well as the nature of the cellulose itself, both the residual hemicellulose and lignin are also two of the most influential barriers that limit effective enzymatic hydrolysis of cellulose when low enzyme loadings are used to deconstruct pretreated agricultural, hardwood and softwood residues [15, 20]. To try to better assess how the nature of the substrate might influence the synergistic deconstructive cooperation between cellulases and AA9, a range of lignocellulosic substrates including corn stover, poplar and lodgepole pine, (representative of agricultural, hardwood, and softwood substrates), were steam or organosolv pretreated at compromise conditions [21]. These compromise pretreatment conditions provided good hemicellulose recovery while allowing effective enzymatic hydrolysis, at moderate enzyme loading, of the cellulose-rich, water insoluble component. As had been previously observed, steam pretreatment primarily solubilize the hemicellulose component and generated substrates with relatively high residual lignin content (Table S1), while the organosolv pretreatment predominately solubilized the lignin and hemicellulose and provided pretreated substrates with high cellulose content and lower amounts of residual lignin and hemicellulose [15]. Dissolving pulp (DSP), which is a relatively pure cellulosic substrate (94% cellulose), was derived from hardwood poplar and it was used as a substrate control.

To assess the possible synergistic interaction of AA9 and cellulases, 5% of the protein present in the original cellulase mixture was replaced by an equivalent amount of AA9 protein based on mass. Earlier work had shown that adding increased amounts of AA9, at least under the conditions tested, did not further improve the overall hydrolytic performance of the enzyme mixture. A total enzyme loading of 20 mg per g cellulose was used to ensure that about 70-80% of the cellulose present in the range of pretreated substrates could be hydrolysed within 48 hrs [22]. It was apparent that the presence of AA9 resulted in significant enhancement of the hydrolysis yields for all of the pretreated substrates (Fig. 1). However, the extent to which the hydrolysis yields could be enhanced, i.e. the AA9 boosting effect, was highly substrate specific (Fig. 1), indicating that AA9 catalytic performance and its cooperative interaction was likely to have been influenced by the nature of the substrate. The greatest boosting effect resulting from AA9 addition to the cellulase mixture was observed with the organosolv pretreated substrates. The organosolv pretreated corn stover (OPCS) showed a 25% increase in cellulose hydrolysis after 48 hrs while the steam pretreated corn stover (SPCS) showed a 14% increase. For those substrates pretreated in the same way (either organosolv or steam), the addition of AA9 had a greater boosting effect on the corn stover followed by hardwood and, to a lesser extent, softwoods.

In order to assess whether the differences in the extent of cellulose hydrolysis improvement by AA9 was caused by its limited activity due to the amount of a redox cofactor within the pretreated biomass, an exogenous synthetic reducing agent (gallate) was added to the reaction mixture prior to the addition of enzymes. No further increase in hydrolysis yields was observed for any of the pretreated substrates (Fig. 1). This was in contrast to the results obtained with the relatively pure cellulose (isolated from pretreated lignocellulosic substrates after extensive delignification and xylan removal) where the AA9 boosting effect was only observed when gallate was also added with the AA9 and cellulase enzymes (Fig. S1). Therefore, it was likely that non-cellulosic material present in the pretreated substrates was able to act as a reducing agent/cofactor for the AA9.

To try to determine the likely nature of the biomass derived cofactor that enhanced the boosting effect of LPMO, various substrate fractions/components (soluble substrate compounds, primarily lignin and hemicellulose derived) were systematically evaluated. Previous work had shown that soluble compounds derived from pretreated substrates were able to enhance AA9 activity due to their metal reducing capacity [6]. The substrate-derived soluble compounds contributed a clear boosting effect to the action of the AA9 when they were added to the cellulose enriched dissolving pulp (DSP), with this boosting effect correlating well with their reducing capacity (Fig. 2). However, this correlation was not repeated when the AA9 was added to the pretreated lignocellulosic substrates. For example, although the soluble fraction from the steam pretreated corn stover (SPCS) had a significantly higher Fe^{3+} reducing capacity (~2.9) as

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compared to the fraction from the organosolv pretreated corn stover (OPCS) (~0.4), after AA9 addition, the extent of cellulose hydrolysis was increased to a greater extent with the OPCS substrate (~27%) than with the SPCS substrate (~14%) (Fig. 2).

To assess if the residual lignin in the pretreated substrate was acting as an AA9 cofactor, steam pretreated softwood (SPLP), which is essentially a cellolignin (Table S1), was delignified to varying extents, and these partially delignified substrates were used as substrates to determine the influence of AA9 addition on enzymatic hydrolysis. In order to avoid any possible background contamination of the reducing capacity which might be derived from the commercial cellulase enzyme preparation used in the previous experiments, a reconstituted enzyme mixture (MIX), consisting of the purified major cellulase monocomponents including exo-glucanase (Cel7A), endo-glucanase (Cel5A), and β-glucosidase (GH3 βG) were used instead. Although the reconstituted cellulases mixture (MIX) had a lower hydrolytic performance than the original Celluclast enzyme mixture, the overall hydrolytic potential was still good enough to determine if lignin could act as a reducing cofactor for AA9. The beneficial effect of adding an exogenous reducing agent to enhance AA9 activity was only observed with the completely delignified SPLP substrate (Fig. 3). Although there appeared to be no clear correlation between the amount of residual lignin present and the extent of AA9 boosting, it seems that when lignin is present, the addition of a synthetic co-factor such as gallate was no longer required.

We next assessed the possible influence of hemicellulose by selectively removing the lignin (using sodium chlorite) and soluble components (by extensive washing) from the pretreated substrates, to produce substrates containing either only cellulose or cellulose and hemicellulose (holocellulose). In the absence of a synthetic reducing agent, the AA9 boosting effect was only observed on substrates containing some residual hemicellulose, such as delignified hardwood (dl-SPP and dl-OPP) and agricultural residues (dl-SPCS and dl-OPCS) (Fig. S2A), suggesting that the residual hemicellulose could also serve as an AA9 cofactor. When we assess this possibility by adding birchwood xylan to the dissolving pulp (DSP), it did result in a significant boosting effect (Fig. S2B).

Although it appeared that the pretreated biomass-derived soluble compounds, lignin and possibly the residual xylan could all act as AA9 cofactors, their metal-reducing capability did not seem to be the determining factor that provided the boosting the effect for AA9. Even a 12% residual lignin content (already less than the typical lignin content often present in the pretreated lignocellulosic biomass as shown in Table. S1) could provide enough reducing power to result in a maximum AA9 boosting effect (Fig. 3).

As it was possible that the observed AA9 boosting effect was influenced by other substrate properties such as fiber characteristics (fiber width and length), cellulose accessibility, the degree of polymerization (DP) of the cellulose and cellulose crystallinity (CrI), each of these substrate characteristics were assessed (Table. S2), to see if there was any relationship with the AA9 boosting effect. Earlier work [9, 10] has shown that AA9 cleaves the chains within the crystalline cellulose regions, suggesting that it increases the binding sites of the substrate to the cellulase enzymes. Although we anticipated that there might have been a correlation between cellulose crystallinity and the addition of AA9 increasing cellulose hydrolysis, this did not seem to be the case for all of pretreated substrates. In contrast, a good relationship was observed between cellulose accessibility, as determined by the Simons' Stain (SS) technique, and the AA9 boosting effect (Fig. S3; Table. S2). In the same way that the hydrolytic cellulases must first access the substrate it seems that AA9 must also first gain access to the cellulose to perform its oxidative cleavage. Thus, it is likely that the AA9-cellulases interaction occurs on the accessible cellulose area of the pretreated lignocellulosics substrates while other biomass components provide the reducing capacity to potentiate AA9 activity.

In order to further elucidate the specific characteristics of the accessible cellulose surface areas, two cellulose binding modules (CBMs), one with a planar binding face that preferentially adsorbs to crystalline cellulose (CBM2a) and the other with a cleft-shaped binding site that preferentially adsorbs to amorphous cellulose (CBM44), were used to determine the specific crystalline and amorphous regions within the accessible cellulose [23]. As shown earlier, the ratio between CBM2a and CBM44 provided a good indicator for the relative amount of accessible crystalline cellulose to amorphous cellulose within the substrates. Interestingly, a strong correlation was evident between the CBM2a:CBM44 adsorption ratio and the extent of AA9 boosting effect on the hydrolysis of the pretreated lignocellulosic substrates (Fig. 4). Other substrate properties that were also evaluated such as crystallinity index, average initial fiber length and width and the degree of polymerization of the cellulose did not show any correlation with the extent of the AA9 boosting effect.

To try to confirm that the major substrate property governing the AA9 boosting effect was the accessible specific surface area of the cellulose, we next assessed the extent of AA9-cellulase interaction when using "model/pure" cellulose when the reaction was supplemented with exogenous reducing agents. The cellulose present in the steam and organosolv pretreated lignocellulosic substrates used so far was cellulose I [15]. However, during the different pretreatments, the cellulose I within the lignocellulosic feedstock can be altered to cellulose II or cellulose III, which have different cellulose crystallinity and accessibility properties [24, 25] and have been shown to be more easily hydrolyzed than cellulose I [26]. Therefore, in

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addition to various cellulose I model substrates (CNC, Avicel, DSP), which ranged from high to low accessible specific surface area of the cellulose, we also used other cellulose alloforms such as cellulose II (PASC, mercerized cellulose) and cellulose III, which were produced from Avicel and cotton linters, respectively, using the procedure described in the material and methods section. As anticipated, the supplementation of AA9 to the cellulase mixture resulted in the greatest increase in cellulose hydrolysis when added to the highly crystalline cellulosic substrates such as cellulose nanocrystals (CNC) (Fig. 5A). An increase in cellulose hydrolysis was also observed on those substrates that had a moderate level of crystallinity such as Avicel and dissolving pulp (DSP). The highly amorphous substrates, such as cellulose II and Cellulose III, showed little increase in the extent of hydrolysis when supplemented with AA9 (Fig. 5A). Interestingly, no clear correlation was observed between the ability of AA9 adsorption to the cellulosic substrates and its ability to enhance cellulose hydrolysis (Fig. 5B). However, as was observed for the pretreated lignocellulosic substrates (Fig. 4), a good relationship was also observed between CBM2a:CBM44 adsorption and the extent of AA9 boosting (Fig. 5C) for the various "model" cellulosic substrates. It appears that, provided that there is enough reducing capacity available to potentiate AA9 activity, the relative amount of accessible crystalline cellulose area is the determining factor when maximising the synergistic cooperation between AA9 and cellulase enzymes.

Discussion

Traditionally a cellulase enzyme mixture was thought to consist of hydrolases that act cooperatively and synergistically at the ends (exo-glucanases) and within (endo-glucanases) cellulose chains, subsequently releasing soluble cellodextrins, mostly in the form of cellobiose [18, 27], which is then further hydrolyzed to glucose by β-glucosidases. A more recently reported enzyme group that has been shown to contribute significantly to the hydrolytic potential of a "cellulase mixture" are oxidative components that act cooperatively with hydrolases [28, 29]. The oxidative cleavage of glycosidic bonds in cellulose occurs upon attack of reactive oxygen species (ROS) [28]. In the late 90's, it was shown that in brown-rot fungi, which are some the most effective microorganisms that degrade cellulose in nature, the formation of ROS is mediated by low molecular weight fungal reductants [30]. Over the last few years, a new class of oxidative enzyme (recently classified as AA9) has been found in many cellulolytic microorganisms, including various species of industrial interest such as *T. reesei* [3]. Although these oxidative systems have been shown to release only low levels of monomeric sugars *in vitro*, the combined action of the oxidative and hydrolytic systems has been shown to result in greater levels of sugar release [3, 9, 31]. While various LPMOs have been identified, characterized and shown to act synergistically with hydrolases during enzymatic hydrolysis of a range of cellulosic substrates [3, 5, 9, 10], the extent of the observed synergistic cooperation between these two systems has been found to range from very low to high. In this study, we investigated the influence of various substrate properties on the cooperative action between oxidative (represented by AA9) and hydrolytic (represented by cellulase enzymes) systems with the overall goal of better understanding the basis of this synergistic interaction.

It was apparent that AA9 and cellulase enzymes cooperated synergistically to deconstruct the cellulosic fraction over a broad range of pretreated lignocellulosic substrates as evidenced by the substantial increase in glucose yields. However, the extent of enhancement was highly substrate dependent. Recently, it has been shown that the AA9 "boosting effect" during hydrolysis of "pure" cellulose by cellulases depends on the amount of exogenous reducing cofactor, such as gallate, added to the reaction mixture [6, 12]. However, contrary to the hydrolysis of pure cellulosic substrates, a synthetic reducing factor was not required by AA9 to synergistically interact with cellulase enzymes during hydrolysis of pretreated lignocellulosic substrates (Fig. 1). Therefore, we initially thought that the different extents of enhancement in sugar yields by AA9 during hydrolysis of pretreated lignocellulosic substrates by cellulases could be associated with reducing agents present within pretreated biomass. Interestingly, despite the fact that many biomass-derived non-cellulosic compounds, namely lignin, hemicellulose, and soluble low molecular weight compounds, were able to act as AA9 co-factors, no correlation was found between the metal reducing capacity of these reducing agents and the different extents of hydrolysis enhancement promoted by AA9. It appears that the cofactor requirement of AA9 was satisfied even when low amount of lignin (12%) were present in the pretreated biomass (Fig. 3). Thus, it is unlikely that the amount/type of redox-active co-factors within pretreated biomass is the key factor governing the extent of AA9 boosting effect.

The anti-oxidant activity observed with lignin and the soluble biomass-derived compounds is likely derived from lignin-derived phenolic fragments. Several lignin-related phenol derivatives have been shown to display high polyvalent metals (e.g. ferric iron) reduction activity [40]. In addition, low molecular weight lignin fragments have also been suggested to function in mediating/assisting in the breakdown of holocellulose to monomeric sugars. They do this by potentiating the biochemical oxidative degradative system that also acts synergistically with various hydrolases to efficiently deconstruct biomass during brown-rot decay [42, 43]. The hemicellulose's apparent ability to act as LPMO cofactor is likely derived from related lignin-carbohydrate complexes (LCC). When the amount of accessible crystalline cellulose and accessible amorphous cellulose were assessed, using a recently developed assay that makes use of two substructure-specific cellulose-binding CBMs [23], a linear correlation ($R^2 > 0.94$) was observed between the AA9 "boosting effects" on cellulose hydrolysis and the ratio of accessible crystalline to accessible amorphous cellulose. This assay involves using CBM2a as a probe

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for accessible crystalline regions of the cellulose, and CBM44 as a probe for the more amorphous regions. Contrary to the CrI techniques, this assay only quantifies changes at the surface of the accessible cellulose, rather than the bulk cellulose. The correlation between increases in the ratio of adsorbed CBM2a:CBM44 on the various substrates with the increased boosting effect of AA9. This suggested that AA9 synergizes with cellulases by creating more reactive sites for cellulases on the originally recalcitrant crystalline regions. While this hypothesis might help explain the beneficial effects during initial stages of hydrolysis (the CBMs were applied to unhydrolyzed substrates), it does not help explain how this initial CBM adsorption profile on the unhydrolyzed substrates is able to predict the boosting efficiency of AA9 during the longer time points of hydrolysis. One possible explanation is that the enzyme mediated deconstruction may proceed in an "onion peeling" fashion, where the layers at the cellulose surface (containing both amorphous and crystalline regions) are systematically hydrolyzed. Thus, as hydrolysis proceeds, the removal of the outermost layers of the cellulose uncovers buried layers that have a similar surface morphology (relative amounts of crystalline and amorphous cellulose) as the previous layer. This model has previously been suggested for pure/model cellulosic substrates [32, 33] and pretreated lignocellulosic biomass.

Interestingly, a significant amount of the AA9 was absorbed onto the highly accessible/amorphous Cellulose II substrates such as the mercerized cellulose and PASC (Fig. 5B), even though the AA9 was anticipated to bind preferentially to the crystalline regions of the cellulose. As it had been shown previously that AA9 only recognises a few adjacent ordered cellulose chains as being "crystalline" [5, 34], the observed high binding capacity of Cellulose II was likely due to the increased exposure of small microcrystalline substructures within the overall disorganized/amorphous cellulose structure [23]. The higher amount of CBM2a and CBM44 absorption (Fig S4) also seemed to support this possibility. It appears that the disruption of these microcrystalline regions by AA9 did not influence the hydrolytic performance of cellulase enzymes, likely because of the large number of readily accessible, reactive sites that already exist within the cellulose II substrates. The existence of microcrystalline substructures in the "amorphous" cellulose could also explain why, unlike the related bacterial CBM33 proteins (CBP21 and CelS2) that cleave only highly ordered/crystalline substrates [31, 35], AA9 has been shown to cleave amorphous cellulose (PASC), releasing various native and oxidized cello-oligosaccharide products [6, 10, 11].

Conclusions

Over the past few years the lytic polysaccharide monooxygenase (LPMOs) family of enzymes, which utilize an oxidative rather than a hydrolytic mode of action, have been shown to contribute significantly to polysaccharide degradation. In the work described here, one of the LPMOs, known as AA9, was shown to cooperate synergistically with cellulases in more effectively hydrolysing a range of lignocellulosic substrates. We also showed that AA9 could utilise various biomass components, such as biomass derived soluble compounds and lignin, to potentiate the action of these enzymes. Although the activated AA9 cooperated synergistically with the cellulases and significantly increased cellulose deconstruction, the extent of improvement was shown to be highly dependent on the relative amounts of accessible crystalline to amorphous cellulose within the substrates. It was also apparent that the nature of the cellulose allomorph also influenced the beneficial, synergistic AA9-cellulase interaction as the addition of AA9 did not seem to enhance cellulose deconstruction on the already highly accessible cellulose II and cellulose III.

Materials and Methods

Cellulosic and lignocellulosic substrates. SPCS, SPP, SPLP, OPCS, OPP, and OPLP were produced at relatively compromised conditions as described in our previous studies [21]. SPLPs with various lignin content were prepared by partially delignification of steam pretreated lodgepole pine as described in [36]. Cellulose NanoCrystals (CNC's) were prepared by H_2SO_4 hydrolysis, dissolving pulp (DSP), cellulose III were kind gifts from Forest Products Laboratory - USDA Forest Service, MSU Biomass Conversion Research Laboratory, respectively. Avicel was purchased from Sigma. PASC and Mercerized cellulose were produced from Avicel according to [37]. Dissolving pulp (DSP) was kind gift from Forest Products Laboratory - USDA Forest Service.

Delignification and hemicellulose removal treatments. The complete delignification of the pretreated substrates was conducted with sodium chlorite according to the procedure in the Pulp and Paper Technical Association of Canada's (PAPTAC) Useful methods G10.U. The removal of residual hemicelluloses after delignification was executed as described in the TAPPI standard method T203 cm-99. Briefly, 15 g (dry weight base) never-dried delignified substrates were resuspended in enough deionized water and 50% (w/v) NaOH to give a final volume of 750 ml and a final NaOH concentration of 17.5% (w/v). The pulp slurry was stirred for 30 min and then the deionized water was added to dilute the NaOH concentration to 9.5% (w/v). The diluted pulp slurry was stirred for a further 30 min and filtered *in vacuo* and rinsed 3 times with 9.5% (w/v) NaOH followed by deionized water until the pH of the filtrate was neutral.

Substrates physiochemical characteristics. The chemical composition of the substrates water insoluble fraction after steam and organosolv pretreatment were determined using the modified Klason lignin method derived from the TAPPI standard method T222 om-88 as previously described [36, 45]. The pretreatment conditions and chemical composition of the pretreated substrates are shown in Tab. S1. Cellulose crystallinity (CrI) and cellulose degree

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of polymerization (DP) were measured by X-ray diffraction (XRD) (Bruker TOPAS 4.2) and gel permeation chromatography (GPC) (Agilent 1100 HPLC system), respectively, as described in [38] and [39], [45]. Substrate's external surface area was estimated by the gross fiber characteristics were analyzed by a fibre quality analyzer (FQA) (LDA02; OpTest Equipment, Inc., Hawkesbury, ON, Canada) [39]. The settings on the FQA were adjusted to measure particles down to 0.07 mm, and the average fibre length/width and percent of fines were analyzed as described previously [2]. Specific cellulose accessibility was determined by using the Simons' staining (SS) technique according to the modified procedure [2] and by assessing the relative amount of accessible crystalline cellulose (CBM2a) and amorphous cellulose (CBM44) as described in [23].

Enzymatic hydrolysis. Celluclast (cellulase mixture), Novozym 188 (β-glucosidase), and AA9 enzymes were generously provided by Novozymes. Cel7A, Cel5A, GH3 BG were purified from these commercial enzyme stocks as described earlier [20]. The purity of the enzymes was confirmed by SDS-PAGE and Liquid chromatography–mass spectrometry/mass spectrometry (LCMS/MS). The hydrolysis experiments were carried out at 2% (w/v) solids loading in sodium acetate buffer (50 mM, pH 4.8), 50 °C, 150 rpm in a bench top hybridization incubator (combi - H12). In the case of gallate supplementation, a concentration of 10 mM was used, according to [7, 9-11]. During hydrolysis, the cellulases were partially replaced with AA9 enzymes (5%) to avoid an increase in total protein loading. Hydrolysis samples (500 µl) were taken at certain time points and the supernatants were separated and collected after centrifugation at 16000 g for 10 min. The samples were stored at −20°C for further analyses. All of the experiments were performed in duplicate or triplicate and the mean values and error bars arereported.

Reducing capacity analysis. The soluble substrate components were prepared by incubating 2% of pretreated substrates with 50 mM acetate buffer in a rotating incubator overnight at 50 °C. The soluble components were then separated from the solid substrates after centrifugation at 16000 g for 30 min. Assays for reduction of the Fe^{3+} ions within the soluble components were carried out using 1.5 ml centrifuge tubes containing 0.4 ml sodium acetate buffer at pH 5.0 (20 mM), 0.375 ml substrate soluble component and 0.025 ml freshly prepared $FeCl_3$ (20 mM) at room temperature for 30 min. An 0.2 ml amount of ferrozine sodium salt 1% (w/v) was added to the tubes to make up a final volume of 1 ml. The absorption of Fe^{2+}/ferrozine complexation was assessed on a UV/Visible spectrophotometer at 562 nm ($\varepsilon_{562\ nm} = 27{,}900\ M^{-1}\ cm^{-1}$) [40].

Sugar and protein assay. The chemical composition of the various steam pretreated lignocellulosic substrates after Klason treatment were determined using high performance anion exchange chromatography (Dionex DX-3000, Sunnyvale, CA) as described earlier [2].The glucose concentration present in the hydrolysate was determined by the Glucose Oxidase Assay [20]. Glucan conversions (%) of the pretreated substrates were calculated from the original glucan content as a percentage of the theoretical glucan available in the original substrate. The total protein content was measured by the Ninhydrin assay using bovine serum albumin (BSA) as the protein standard [41].

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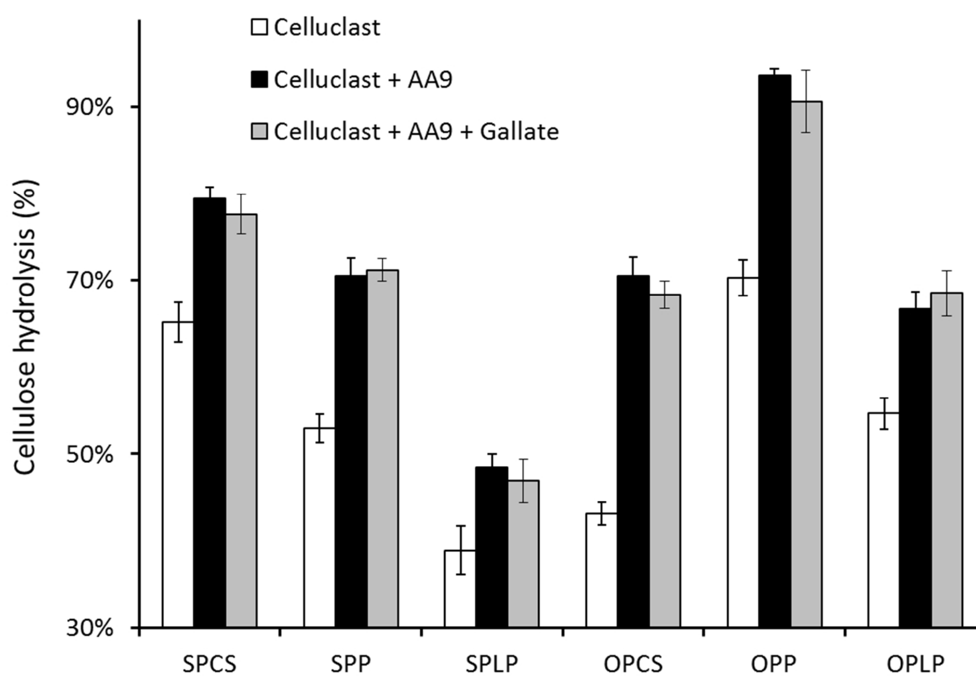

Fig. 1 The percentage increase after 48h hydrolysis of steam pretreated (SP) and organosolv pretreated (OP) corn stover (CS), poplar (P) and lodgepole pine (LP) with-and-without the addition of AA9 and gallate. 178x128mm (150 x 150 DPI)

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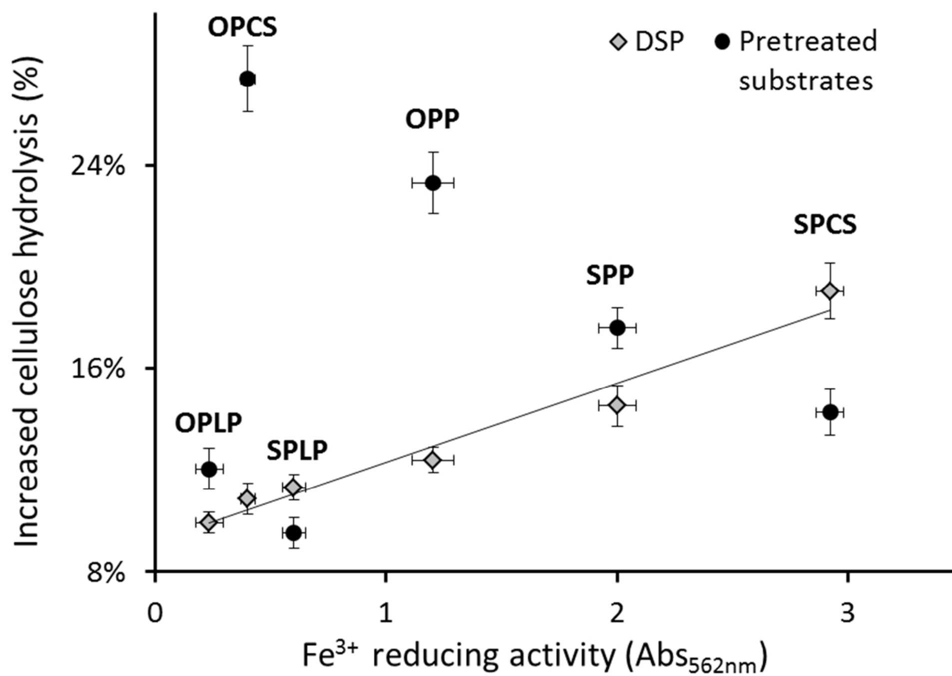

Fig. 2 To assess if there is a linear relationship between the Fe3+ reducing activity of the soluble compounds derived from steam pretreated (SP) and organosolv pretreated (OP) corn stover (CS), poplar (P), lodgepole pine (LP) when they are added back to each of these substrates and dissolving pulp (DSP) and the percentage increase in 48 h cellulose hydrolysis when supplemented with AA9.
154x108mm (150 x 150 DPI)

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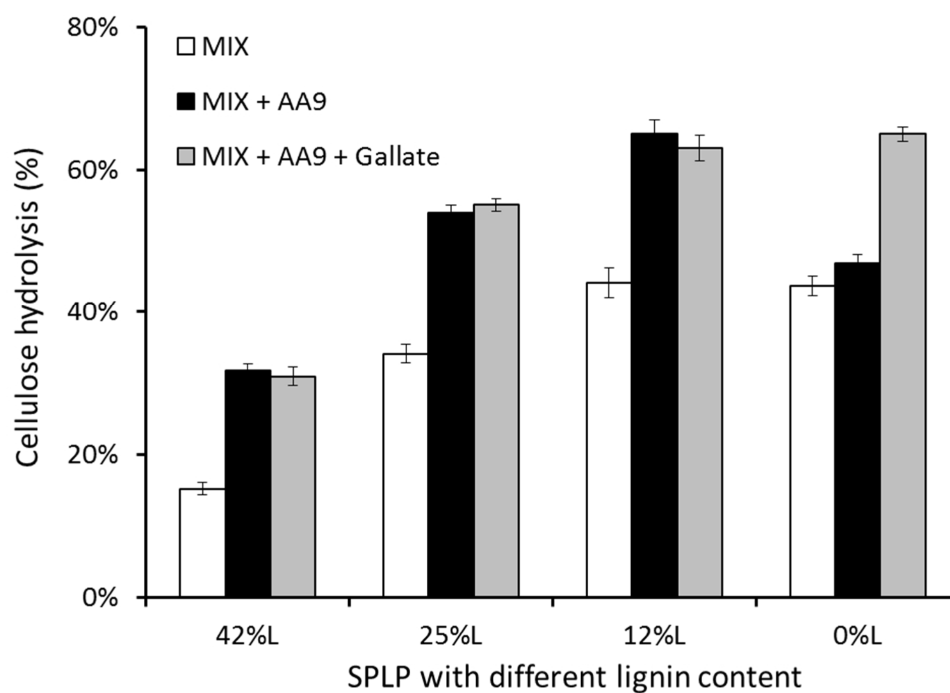

Fig. 3 The percentage increase in cellulose hydrolysis, after 48h, of increasingly delignified steam pretreated lodgepole pine (SPLP) by the reconstituted cellulase mixture (MIX, Cel7A, Cel5A, and GH3 βG) with-and-without the addition of AA9 and gallate.
166x119mm (150 x 150 DPI)

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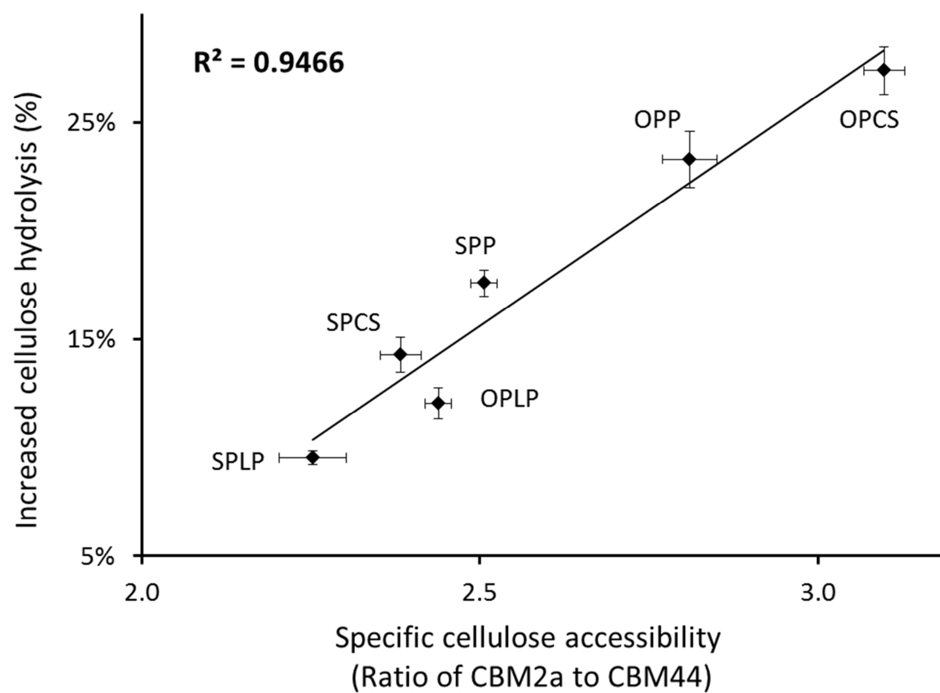

Fig. 4 To assess if there is a linear relationship between the percentage increase after 48h hydrolysis of steam pretreated (SP) and organosolv pretreated (OP) corn stover (CS), poplar (P) and lodgepole pine (LP) with the ratio of accessible crystalline cellulose (as indicated by CBM2a adsorption) to accessible amorphous cellulose (as indicated by CBM44 adsorption) of these substrates.
CBM2a/CBM44: cellulose binding module family 2a/44.

174x128mm (150 x 150 DPI)

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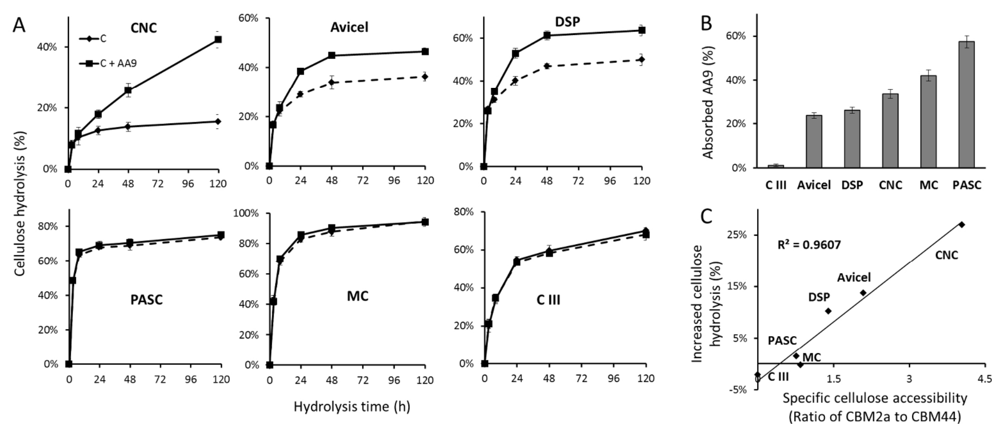

Fig. 5 (A) The time course of hydrolysis of various “model” cellulosic substrates with the addition of gallate by cellulase enzymes with-and-without AA9. (B) AA9 adsorption on various cellulosic substrates. (C) To assess if there is a linear relationship between the AA9 increased cellulose hydrolysis and the ratio of accessible crystalline cellulose (as indicated by CBM2a adsorption) to accessible amorphous cellulose (as indicated by CBM44 adsorption) of these substrates.
CNC: cellulose nanocrystals; DSP: dissolving pulp; PASC: phosphoric acid swollen cellulose; MC: mercerized cellulose; C III: cellulose III; CBM2a/CBM44: cellulose binding module family 2a/44.

364x157mm (150 x 150 DPI)

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