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Jean-Lou Reyre, Sacha Grisel, Alexandra Lechar, Antoine Margeot, Simon Arragain, et al.. Implementation of CDH in LPMO-Containing Minimal Cellulase Cocktails to Tune Bioproduct Production from Lignocellulosic Biomass. ACS Sustainable Chemistry & Engineering, 2024, 12 (21), pp.8128-8138. 10.1021/acssuschemeng.4c00851 . hal-04577990

HAL Id: hal-04577990

<https://ifp.hal.science/hal-04577990v1>

Submitted on 16 May 2024

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Implementation of CDH in LPMO-containing minimal cellulases cocktails to tune bioproducts production from lignocellulosic biomass

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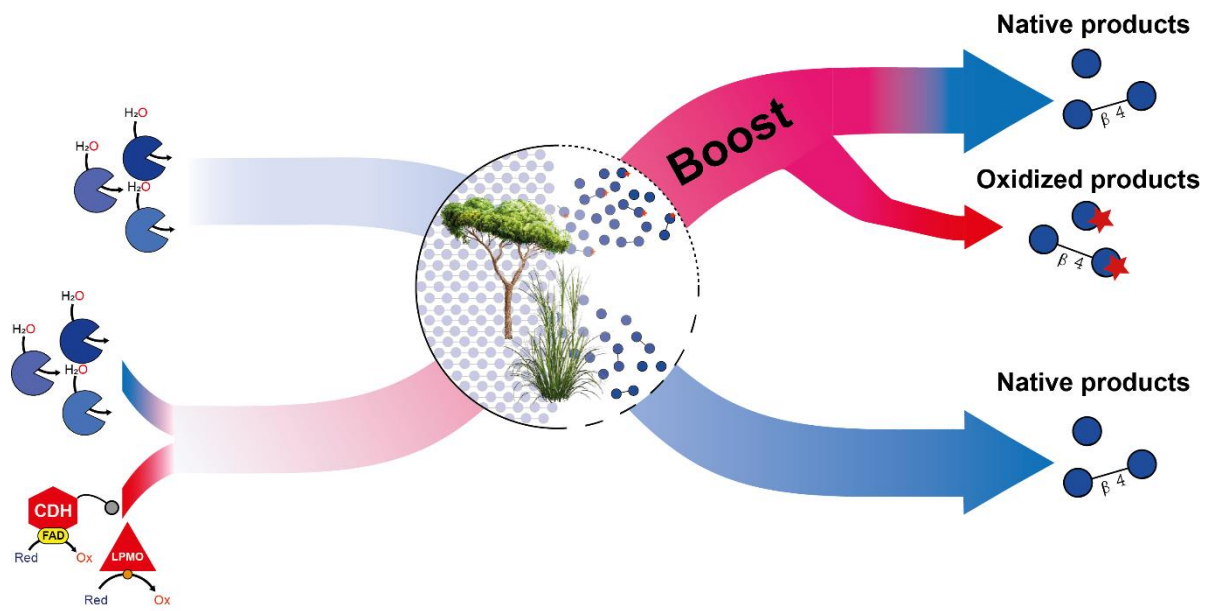
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Abstract

Plant biomass can be converted to bioproducts using fungal enzymatic cocktails that have been improved upon implementation of lytic polysaccharide monooxygenases (LPMOs), known to boost cellulases. However, due to the complexity of their oxidative catalysis, it is still difficult to properly control and sustain LPMOs activity. Here, we investigated whether the implementation of the natural enzymatic partner of LPMOs, i.e. cellobiose dehydrogenase (CDH), could be a promising solution. To this end, we reconstituted a minimal cocktail using the main cellulases from *Trichoderma reesei*, and evaluated the impact of the addition of two LPMOs and one CDH on the conversion of wheat straw, miscanthus, pine and poplar. Surprisingly, while the addition of LPMOs or LPMO/CDH showed little to no increase in glucose equivalent yields, the sole addition of CDH caused an increase of up to 27% (on miscanthus). Interestingly, CDH supplementation allowed to tune the stream of degradation products towards cellobionic acid while maintaining equivalent or increasing the overall conversion yields, in a biomass-dependent manner. Our study shows that extracting reasonable amounts of high value-added oxidized sugars from industrial biomass is feasible, opening thereby new perspectives for the use of CDH in industry.

Keywords: bioeconomy, *Trichoderma reesei*, cellulose, cellulases, CDH, lytic polysaccharide monooxygenase

TOC



Introduction

The production of second generation (2G) biofuels and bioproducts is a promising yet still challenging alternative to fossil-based fuels and chemicals. Plant carbohydrates, as products of photosynthesis, constitute an abundant and appealing renewable source of carbon for the bioindustries. However, the extraction of simple fermentable sugars from the complex plant cell wall (PCW) matrix remains a major hurdle, known as “biomass recalcitrance”, which has to be studied for each type of biomass (1). Since cellulose represents the most important source of glucose in PCW, various enzymatic cocktails have been designed in recent years to efficiently degrade this recalcitrant biopolymer (2–6). Due to biomass-dependent co-polymers content and structure (e.g. lignin, hemicelluloses), and pre-treatment strategies, it becomes increasingly clear that the one-for-all cocktails (such as Cellic CTec® series) need to evolve towards tailor-made cocktails adapted to different types of lignocellulosic biomass.

Today, a striking observation is that most cellulolytic cocktails used in industry are of fungal origin (6). This is in great part related to the well-documented efficiency of (some) filamentous fungi, notably wood decayers, to deconstruct PCW in nature. To do so, filamentous fungi secrete a wide array of carbohydrate active enzymes (CAZymes ; (7)). Notably, in addition to the long-studied glycoside hydrolases (GH), the last decade has seen the emergence of redox-active CAZymes as key players in biomass degradation (8), forming altogether what has been referred to as “enzyme interactome” (9). Among these, the discovery of lytic polysaccharide monooxygenases (LPMOs) has marked a turning point (10–12). LPMOs are monocopper oxidoreductases that perform the oxidative cleavage of glycosidic bonds at the surface of cellulose, thereby creating new chain ends accessible to cellulases and also reducing the cellulose crystallinity by modifying its structure, leading to an overall boost in the biomass depolymerization (13, 14). In addition, recent studies have also highlighted that the action of LPMOs increases the hydration of cellulose fibers, further reinforcing their partnership with hydrolases (15, 16). Because of these peculiar capacities, the boosting effect resulting from the addition of LPMOs in cellulolytic cocktails for biomass conversion purposes, first reported in the early 2010's (11, 12, 17, 18), has been extensively studied since then (19, 20).

To perform their reaction, LPMOs need first to be reduced from the Cu(II) resting

state to the Cu(I) active state. Then, upon reaction with H₂O₂ as co-substrate (21), LPMOs will oxidize the C1 and/or C4 carbon of scissile glycosidic bonds. The impacts of the nature, abundance, and mode of action of the reductant and co-substrate on LPMO catalysis are notoriously difficult to control (22). Furthermore, despite several reports on various redox enzymes competing or partnering with LPMOs (12, 23–27), only a few studies have evaluated the effect of such partners in the perspective of designing new cocktails (28, 29).

Remarkably, the effect of cellobiose dehydrogenase (CDH), described as a natural redox partner of LPMOs during wood decay by filamentous fungi (12, 30–33, 23, 34–38), has been little studied from such an application perspective (39, 40). These bimodular flavodehydrogenases are able to oxidize cellobiose into its corresponding lactone and sequentially transfer the two electrons resulting from this reaction to their heme-containing cytochrome (41). One of these electrons can then be further transferred to the LPMO Cu(II) atom to form the active, LPMO-Cu(I) state (35). Although less efficient than genuine oxidases, CDHs are also capable to use molecular oxygen as electron acceptor (42). Conveniently, when paired with LPMOs, it has been demonstrated that CDH could fulfill both functions of copper reduction (43, 44) and H₂O₂ supply (45).

In this study, we evaluated the effect of a CDH and two cellulose-active LPMOs from the auxiliary activity family 9 (AA9) on the biomass conversion efficiency of a tailor-made cellulases cocktail from *Trichoderma reesei*. To this end, we used oxidases from the coprophilous ascomycete *Podospora anserina*, viz. PaCDHB, the C1-oxidizing PaAA9E and the C1/C4-oxidizing PaAA9H. Using a design of experiment approach, in which neutral and oxidized products solubilized by different enzyme proportions were quantified, we evaluated the effect of the CDH on the release of soluble products from wheat straw and other recalcitrant lignocellulosic biomass of industrial interest: miscanthus, pine and poplar.

Results

AA9/CDH couples show negative to moderately positive effects on glucose release from wheat straw by a minimal *T. reesei* cellulases cocktail

The first step of this study was to design a minimal *T. reesei* cellulases cocktail. Inspired by data reported in the literature on the use of *T. reesei* enzymes for the deconstruction of various (ligno)cellulosic substrates (**Table 1**), we designed a cocktail including: two cellobiohydrolases (40% w/w of CBHI (also referred to as *TrCel7A*) and 40% w/w of CBHII (*TrCel6A*)), two endoglucanases (5% w/w of EGI (*TrCel7B*) and 5% w/w of EGII (*TrCel5A*)), and one β -glucosidase (10% w/w of *TrBGL1* (*TrCel3A*)). After purifying these enzymes from a secretome of *T. reesei* RUT-C30 strain, we assessed the efficiency of this cellulases minimal cocktail (henceforth called “*TrCC*”) on steam exploded wheat straw. In good agreement with previous results (46, 47) (**Table 1**), a glucose yield of 80% was obtained after 48 h of hydrolysis with an enzyme loading of 20 mg/g of dry matter (DM) (**Fig. S1A**). In order to supplement *TrCC* with an oxidative system, we selected two highly active cellulolytic AA9 LPMOs, *viz.* *PaAA9E* and *PaAA9H* (which both harbors a CBM1 domain) (23), and the CDHB from the same fungus, *PaCDHB*, previously demonstrated to act in synergy with the selected LPMOs (23). After heterologous production of each of these enzymes in *Pichia pastoris*, the activity of both LPMO/CDH couples was validated on phosphoric acid swollen cellulose (PASC) by identifying the typical C1 or C1/C4 soluble oxidized products, respectively released by the action of *PaAA9E* or *PaAA9H*. (**Fig. S1B**).

With both the hydrolytic and oxidative systems being separately operational (**Fig. S1**), the next goal was to assess their combined activity on lignocellulosic substrates. Using a design of experiment approach (MODDE software v12.0), we determined the best enzymes proportions to obtain the highest glucose yield from steam exploded wheat straw. To this end, the concentration of *PaCDHB*, AA9 LPMOs (*PaAA9E* or *PaAA9H*) and *TrCC* were set as variables, resulting in a total of 30 combinations (see **Table S1** for more details on the model). Figure 1 shows the resulting contour plot with the glucose yields obtained after 48 h of reaction. Maximum glucose yields of 34 %, 61 % and 82 % were obtained for *TrCC* loadings of 2, 11 and 20 mg/g of DM, respectively. Regardless of the assayed condition, both the C1-oxidizing *PaAA9E* and the C1/C4-

oxidizing *PaAA9H* did not appear to have any significant beneficial effect on glucose release. Surprisingly, in all tested conditions, the presence of *PaCDHB* clearly revealed to be detrimental: the higher the CDH content, the lower the glucose yield (**Fig. 1**). Since *TrBGL1* and *PaCDHB* both share the same substrate (e.g. cellobiose), and while noting that the CDH is poorly active on glucose, we think that potential substrates competition between these two enzymes may reduce the overall glucose production coming from the hydrolysis of cellobiose by *TrBGL1*, in favor of cellobiose oxidation by *PaCDHB*. Thus, when aiming at increasing glucose yields, this screening on wheat straw suggested that, if included at all, *PaCDHB* should be implemented at low loading levels in a *TrCC* cocktail complemented with redox-active CAZymes. We then decided to check whether similar results would still be observed on other industrially relevant steam exploded lignocellulosic biomass, i.e. miscanthus, pine and poplar (see **Table S2** for biomass pretreatment details and **Table S3** for biomass compositions).

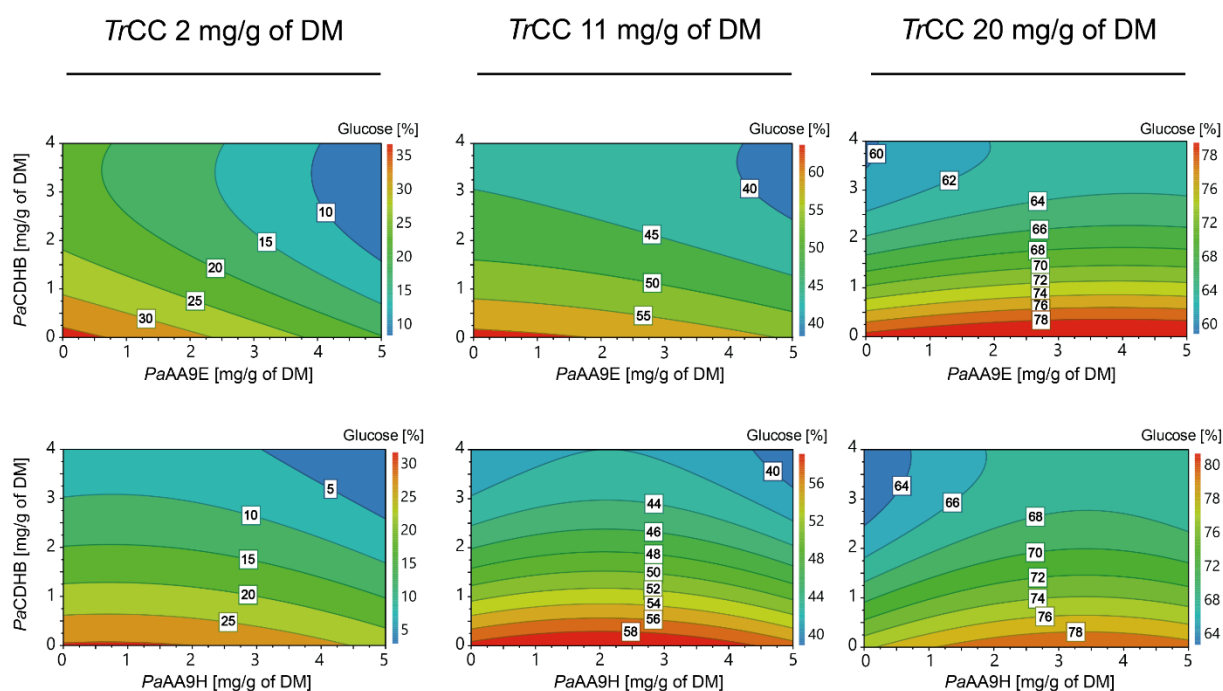


Fig.1. Contour plot of glucose yields obtained on steam-exploded wheat straw degraded by *TrCC* as a function of *PaAA9s* and *PaCDHB* loadings. Based on the design of experiment output (see **Table S1** for more details), we tested three *TrCC* loadings (2, 11 and 20 mg/g of DM, from left to right hand side) mixed with *PaCDHB* (0 to 4 mg/g of DM) and two different AA9s (0 to 5 mg/g of DM), namely *PaAAE* (top row) or *PaAA9H* (bottom row). The glucose yields obtained after 48 h are associated with a color gradient from blue (lowest yields) to red (highest yields) (note that the color scale is specific to each panel). All reactions were carried out with steam-exploded wheat straw (1% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm).

Conversion yields on miscanthus, pine and poplar

To evaluate the potential impact of the AA9/CDH couples on the soluble products released from miscanthus, pine and poplar, we set the enzymes loading of *TrCC* cocktail at 10 mg/g of DM, in the range of other previously reported cocktails (**Table 1**). As for the oxidative system, LPMO concentration was set at 3 mg/g of DM, and since the results obtained on wheat straw suggested a potential competition between cellobiose hydrolysis and oxidation (**Fig. 1**), we first added *PaCDHB* at a low concentration of 0.25 mg/g of DM. The released products were analyzed after 4, 24 and 48 h of reaction using a high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (**Fig. 2**). The analysis revealed the presence of four main products identified as glucose, cellobiose, and cellobionic acid (**Fig. S2**). Of note, when detected, gluconic acid was found in negligible amounts (< 0.1% glucose equivalents yields) and was therefore not considered in the following quantifications. Unsurprisingly, glucose stood out as the main released product in all conditions, with yields ranging between 24-27%, 15-19% and 7-8% on pine, miscanthus and poplar, respectively. However, supplementation of *TrCC* with oxidative enzymes did not yield any major improvement in glucose yields, regardless of the biomass (**Fig. 2**). Interestingly when looking at the release of soluble cellobionic acid, some substrate-specific observations could be made. While glucose yields appeared higher on pine (27%) than on miscanthus (19%), the opposite trend was observed when considering cellobionic acid (up to 0.6% on pine and 1.6% on miscanthus). On poplar, no significant release of cellobionic acid could be detected. While the exact reasons for such differences in products yields are complicated to determine, one could speculate on the presence of potential biomass-dependent enzyme inhibitors (such as xylotriose, 3,5-dimethoxy-4-hydroxybenzaldehyde or 4-hydroxy-3-methoxy-cinnamaldehyde) (48–50) and/or different accessibility of the cellulose chains. In general, these assays suggest that (i) *PaCDHB* efficiency seems to be biomass-dependent, (ii) the release of cellobionic acid appears to be LPMO-independent (see **Fig. 2** insets) and mainly due to the action of *PaCDHB*.

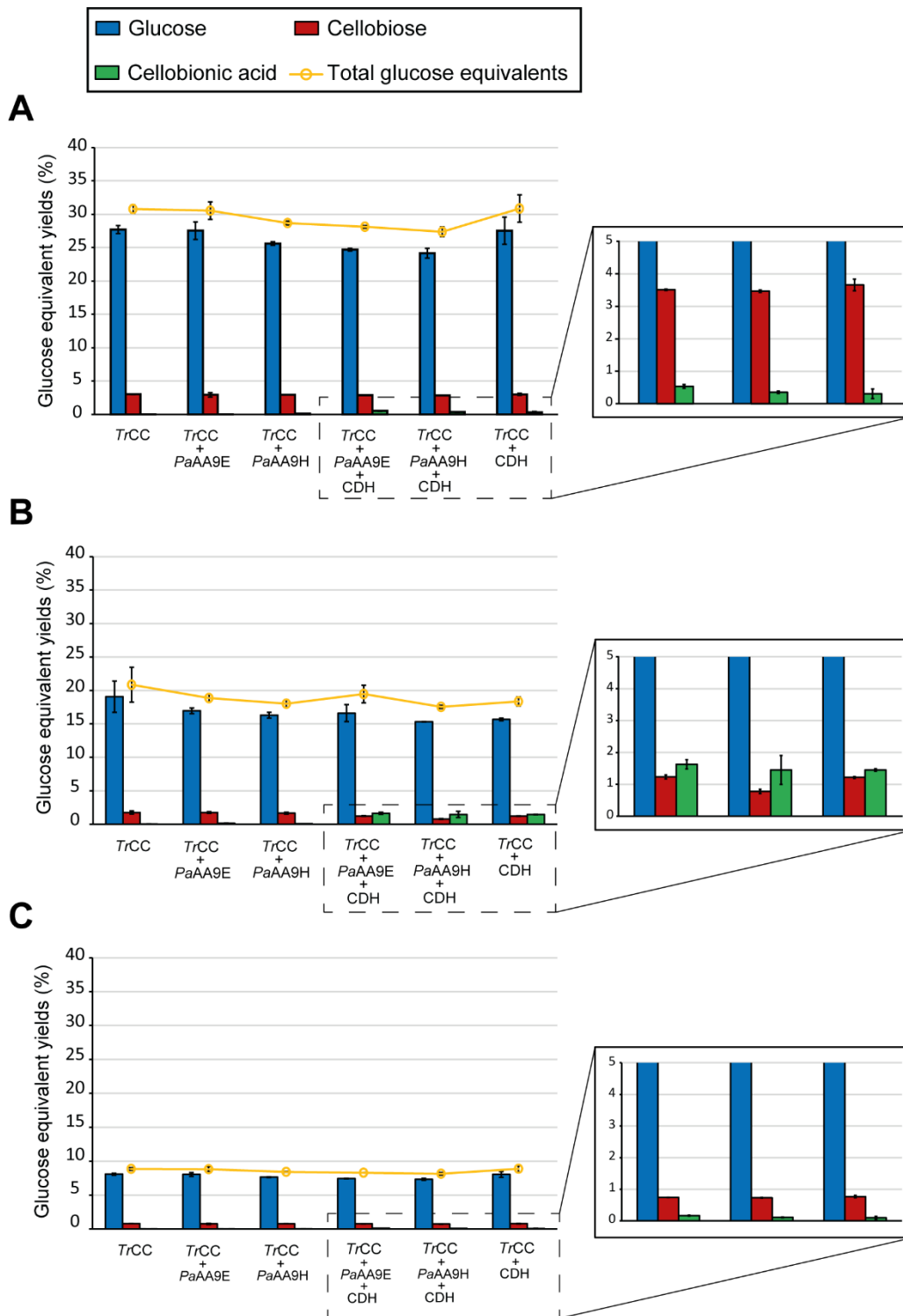


Fig. 2. Total soluble products released from (A) pine, (B) miscanthus and (C) poplar after 48 h of reaction in the presence of *TrCC* supplemented with oxidative enzymes. The graphics show the released soluble products and the corresponding total glucose equivalent yields (one cellobiose or cellobionic acid molecules account for two glucose equivalents) released from pine (A) miscanthus (B) and poplar (C) by *TrCC* (10 mg/g of DM) upon the addition of *PaAA9E* or *PaAA9H* (3 mg/g of DM) and/or *PaCDHB* (0.25 mg/g of DM). All reactions were carried out with pretreated biomass (2% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm). Bars show average values and error bars represent the standard deviation (n = 3 independent biological replicates).

Impact of *Pa*CDHB on pine and miscanthus cellulose depolymerization

As shown above (**Fig. 2**), C1-oxidized celooligosaccharides released during biomass degradation by *Tr*CC cocktail spiked with oxidases appeared to originate mainly from *Pa*CDHB action. Therefore, we decided to further investigate the impact of the latter on cellulose depolymerization testing a range of *Pa*CDHB concentrations (from 0.5 to 6 mg/g of DM). In general, glucose yields remained slightly higher on pine (17%, **Fig. 3A**) than on miscanthus (maximum 14%, **Fig. 3B**) (note that poplar was left aside because of very low glucose yields and quasi-null CDH activity, **Fig. 2**). Strikingly, in the *Pa*CDHB concentration range of 0.5 to 3 mg/g of DM, the activity of *Pa*CDHB did not seem impacted by the complex biomass composition as the more CDH the more cellobionic acid was detected, resulting in a quasi linear dose-response ($R^2=0.98$) (**Fig. 3C**). Of note, the absolute amount of cellobionic acid appeared always higher (up to 12% glucose equivalent yield) in the reactions performed on miscanthus than on pine. Consistently, a time-course experiment showed that *Pa*CDHB operates at constant speed in reactions done on miscanthus, as shown by the very linear time-dependent release of cellobionic acid until the last analyzed time point (48 h), in contrast to pine on which the CDH reaction nearly stopped at 24 h (**Fig. S3**). As for the total glucose equivalent yields, while no clear differences between the tested conditions could be observed on pine, significant boosts were obtained on miscanthus upon addition of *Pa*CDHB at the two highest tested concentrations (3 and 6 mg of *Pa*CDHB/g of DM), allowing to obtain the best overall yields from this series of experiments (19%). This boost represented a relative increase in total glucose equivalent yields of 27 % when compared to the *Tr*CC alone condition(**Fig. 3D**). We speculate that this boosting effect may originate from the alleviation of cellulases inhibition by cellobiose upon oxidation of the latter by CDH as previously described (51, 40).

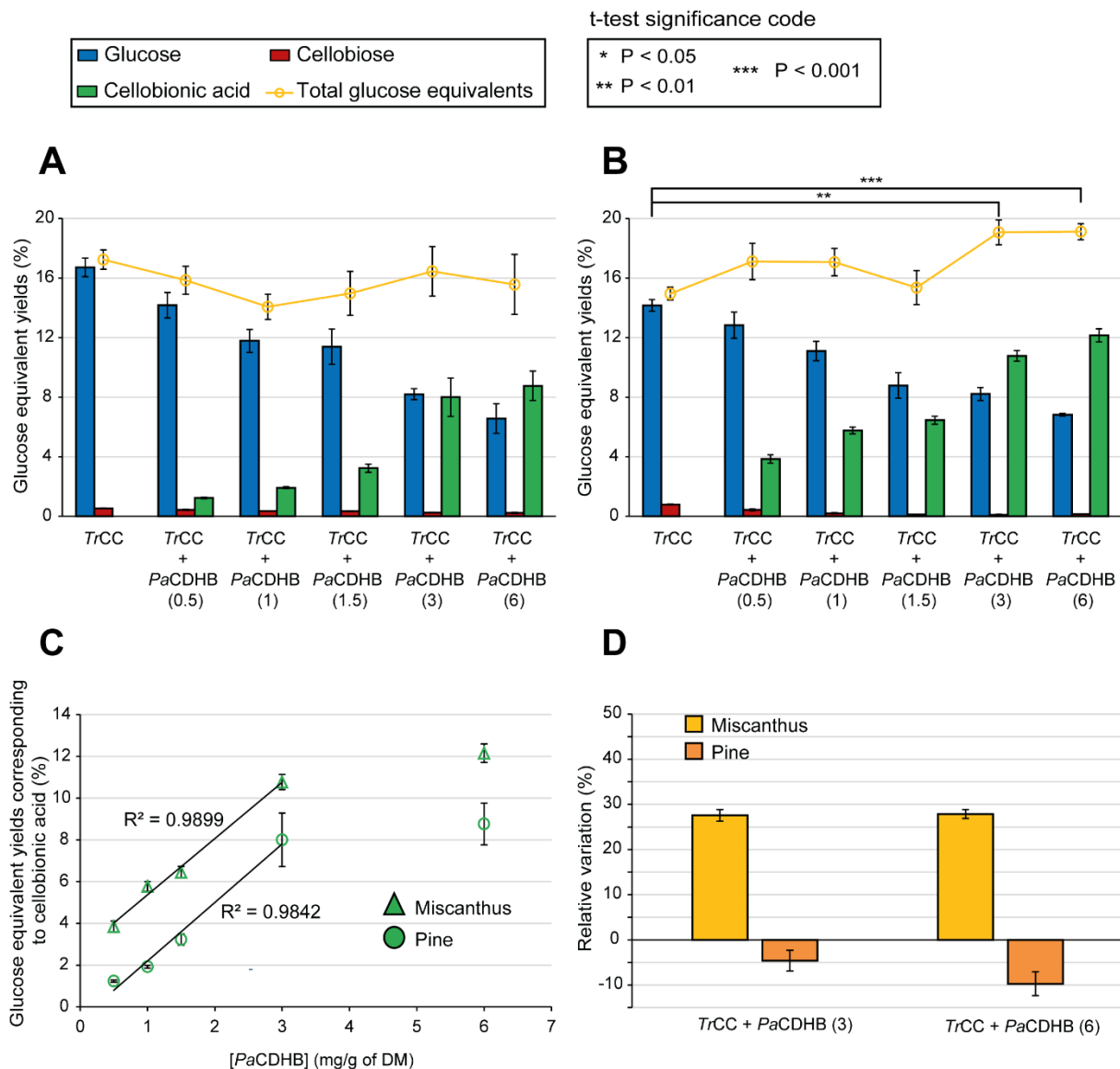


Fig. 3. Effect of *PaCDHB* on total products released by *TrCC* from pine (**A**) and miscanthus (**B**) after 48 h. The graphs show soluble products and the corresponding total glucose equivalent yields (one cellobiose or cellobionic acid molecules account for two glucose equivalents) released from pine (**A**) and miscanthus (**B**) by *TrCC* (10 mg/g of DM) with varying concentrations of *PaCDHB* (0 to 6 mg/g of DM). All reactions were carried out with pretreated biomass (2% w/v) in sodium acetate buffer (50 mM, pH 5.2), at 50°C under stirring (850 rpm). (**C**) Glucose equivalent yields corresponding to cellobionic acid as a function of *PaCDHB* concentration in reactions on pine (circles) or miscanthus (triangles). Trend curves and their R^2 are shown for conditions involving 0.5 to 3 mg of *PaCDHB*/g of DM. (**D**) Relative variation in glucose equivalent yields (vs *TrCC* condition) in the conditions (i.e. *TrCC* + *PaCDHB* at 3 or 6 mg/g of DM) where a significant boost was observed on miscanthus. The corresponding t-test is displayed in panel **B** (see significance code at the top of the figure). In all panels, bars show average values and error bars represent the standard deviation ($n = 4$ independent biological replicates).

Discussion

In the present study, our goal was to assess the impact of two AA9/CDH couples from the coprophilous ascomycete *P. anserina* on the overall depolymerizing power of a reconstituted minimal cocktail of *T. reesei* cellulases (*TrCC*) on four different lignocellulosic biomass of industrial relevance. Throughout the entire set of experiments presented in this study, the main products extracted from the biomass were glucose, cellobiose and cellobionic acid. While the presence of gluconic acid could have been expected from the hydrolysis of cellobionic acid by *TrBGL*, no evidence of such activity could be detected here, in line with the previously demonstrated very poor BGL activity of commercial cellulolytic cocktails on cellobionic acid (52) or from a purified BGL from *Phanerochaete chrysosporium* (*PcBGL*) (53). This poor activity may be attributed to an inhibitory effect of cellobionolactone on BGL, as suggested by Igarashi and colleagues, who have shown that the activity of *PcBGL* on cellobiose was reduced in the presence of cellobionic acid (53). Although the preferred substrate of *PcBGL* is not cellobiose but laminaribiose, a similar negative effect of cellobionic acid on *TrBGL* is plausible. Thus, in addition to a potential substrate competition between *TrBGL* and *PaCDHB*, the inhibitory effect of cellobionic acid itself on *TrBGL* activity could contribute to the observed decrease in glucose production concomitantly to the increase in cellobionic acid production.

By setting up reaction conditions where the LPMO would act in concert with a CDH partner, we expected to better control the overall activity of this oxidative system and potentially boost the yields of extracted sugars (**Fig. 4**). Surprisingly, on all the tested biomass, the addition of either *PaAA9E* or *PaAA9H* to the enzymatic reactions did not yield any significant improvement. The reasons for such lack of “boosting” effect can be manifold. Very recent studies have shown that decrystallization of the cellulose surface by LPMOs can have a positive effect on the cellulose surface water retention (16) and productive binding of CBHs (54), improving in turn the overall hydrolysis. It is possible that, under the biomass loadings used in our study ($\leq 2\%$ w/v), CBHs have sufficient productive binding sites, and therefore do not need the action of LPMOs. A non-mutually exclusive hypothesis to explain the lack of boosting effect could be related to the accessibility and nature of the substrate (that depends on the biomass and their pretreatment) or the production and fate of H_2O_2 in reactions. Notably, the lignin remaining after the pretreatment may contribute to redox side reactions that

consume or produce H₂O₂, and may also prevent LPMOs from accessing optimally to cellulose. Indeed, it has been shown that the hydrophobic properties of CBM1 (which is present in both *PaAA9E* and *PaAA9H*) contribute to the non-productive binding to lignin (55, 56). The biomass used in this study indeed contain high concentrations of remaining lignins (**Table S3**), which could explain the lack of boosting effect coming from the addition of the tested LPMOs. Therefore, although *PaAA9E* and *PaAA9H* are efficient cellulolytic AA9s (23), it appears clear that observing a high activity on somehow simple cellulosic substrates (such as PASC or Avicel) cannot be taken as a token of efficiency once mixed with cellulases on more complex biomass. Nevertheless, in a recent study Cai and colleagues have showed that an AA9 from *P. anserina* (enzyme ID not reported) could enhance the absolute glucose extraction yield on corncobs by up to 24% (using 8 mg/g of the industrial Cellic CTec3 cocktail and 4 mg/g of LPMO) (57). Although it is possible that the boost observed in this study directly originate from the action of the tested *PaAA9*, it is also likely that this LPMO could act as a H₂O₂ supplier for the AA9s potentially present in the Cellic CTec3 cocktail (3 AA9s are encoded in the genome of *T. reesei*). Noteworthy, a similar LPMO-LPMO interplay mediated by H₂O₂-producing activity has already been proposed between two LPMOs from the fungus *Myceliophthora thermophila*, viz. *MtAA16A* and *MtLPMO9s* (26). Ultimately, our results suggest that LPMO reduction, co-substrate feeding but also substrate availability should be finely regulated in industrial cellulolytic cocktails and adapted from one biomass to another to ensure optimal boosting effect conditions.

Primarily intended as a partner to fuel LPMOs reactions, the impact of *PaCDHB* alone on pine and miscanthus degradation appeared unexpectedly significant. With a maximum relative boosting effect on the total glucose equivalents yields of 27% on miscanthus (when applied at a concentration threshold ≥ 3 mg/g of DM), *PaCDHB* revealed itself as the only “boosting” oxidative enzyme tested. As a comparison, we carried out a meta-analysis of literature *positive* reports on LPMO boosting effects on glucose yields, measured on a broad range of types of biomass, which revealed a median boost value of 30% (**Fig. S4**). In our case, the boost observed in the soluble glucose equivalents yields mainly relied on the production of cellobionic acid (up to 12% on miscanthus).

On a side note, while the CDH-LPMO interplay has been clearly evidenced in several case studies, we underscore that such association is not systematic. For

instance, secretomic analysis of the basidiomycete *Laetisaria arvalis* grown on wheat straw showed that, while AA9s and CDHs are co-secreted at early stage of growth (day 3), the secretion level of AA9s completely drops at day 7 while CDHs continue to be increasingly co-secreted with cellobiohydrolases (33). On the basis of these observations, we speculate that the role of CDHs in PCW degradation may not be limited to its partnership with LPMOs.

In industry, oxidized products can be considered as platform sugars. For instance, cellobionic acid can be used as additive in the cosmetics and food industry (58). Additionally, considering its low inhibitory effect on cellulases (as compared to cellobiose) (59, 51), it was also investigated as potential substrate in biorefinery for the production of isobutanol (60). Gluconic acid, which could be produced from cellobionic acid, is also widely used in the food industry (61, 62) or as a building block for chemical synthesis (63). Hence, by modulating the concentration of BGL and CDH in the cellulolytic cocktails, it may be possible to tune the proportion of neutral and oxidized products depending on the desired bioprocessing route.

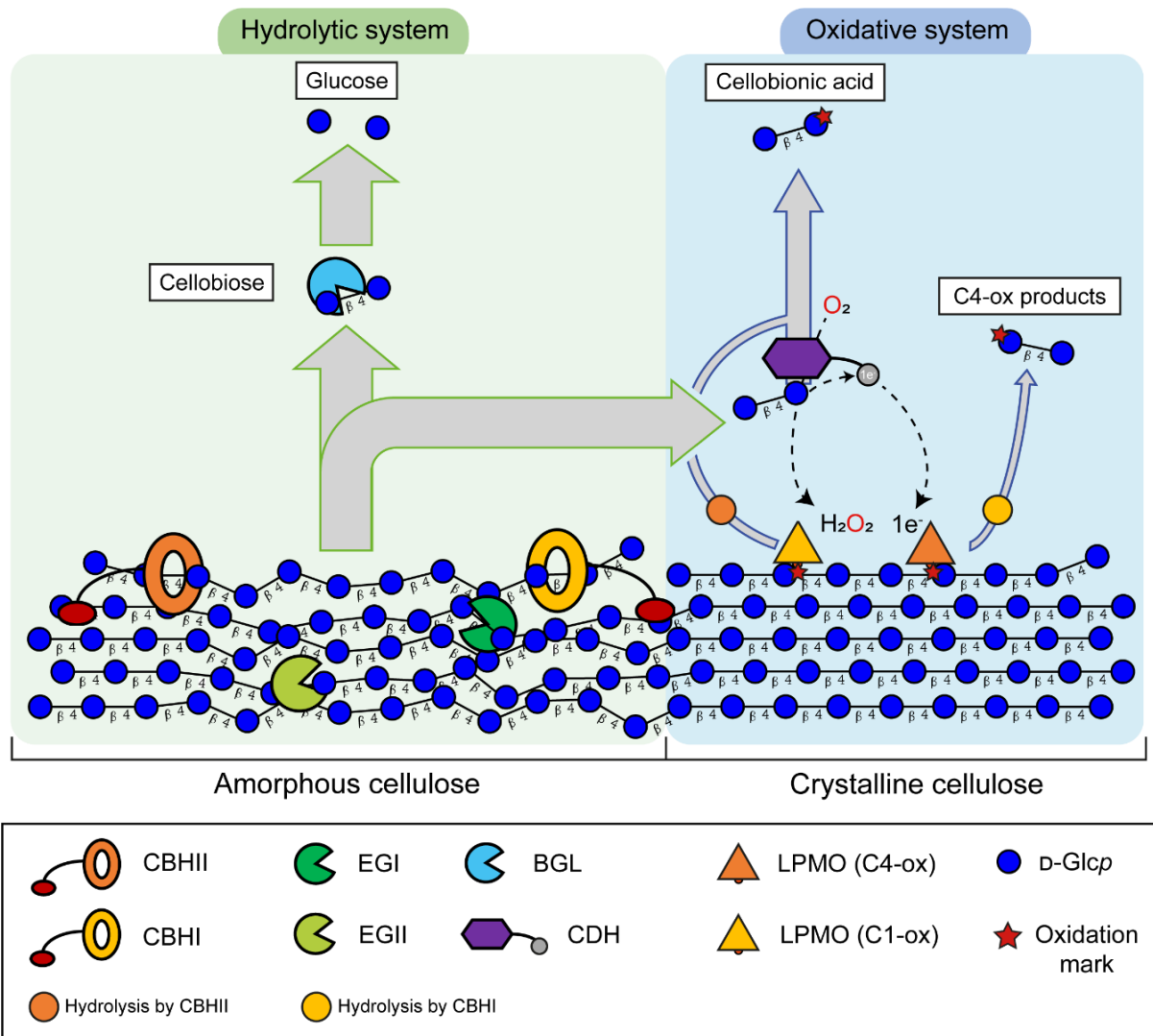


Fig. 4. Theoretical interplay between hydrolases and oxidative enzymes during cellulose degradation. The final products resulting from cellulose degradation by both the hydrolytic and oxidative systems are annotated on the figure. The possible hydrolytic and oxidative paths yielding final products are indicated by green or blue arrows, respectively. Some hydrolytic enzymes such as CBHs may also interact with oxidized products and are therefore also represented on the oxidative system panel. We underscore that, for the sake of clarity, this simplified scheme does not represent the potential action of LPMOs on amorphous regions of cellulose or of that of hydrolases on crystalline ones. BGL, β -glucosidase; CBH, cellobiohydrolase; CDH, cellobiose dehydrogenase; EG, endoglucanase; LPMO, lytic polysaccharide monoxygenase; D-Glcp, D-glucopyranose.

Table 1. Enzymes proportions in previously reported *Trichoderma reesei* minimal cellulases cocktails

| References | CBHI (TrCel7a) | CBHII (TrCel6a) | EGI (TrCel7b) | EGII (TrCel5a) | TrBG (TrCel3a or TrCel2a) | Other enzymes | Total enzymes loading | Substrates | Glucose yields (%) |
|--------------------------------------|-------------------|--------------------|------------------|-------------------|------------------------------------|------------------|---------------------------------|--|-----------------------|
| Rosgaard et al. 2007 (64) | 27% | 46% | 27% | - | - | - | | Acid impregnated + steam exploded straw | 56 |
| | 17% | 45% | 38% | - | - | - | 11 mg/g of dry matter | Water impregnated + steam exploded barley straw | 34 |
| | 20% | 43% | 37% | - | - | - | | Hot water extracted barley straw | 67 |
| Harris et al. 2010 (17) | 50.5% | 28% | 11.5% | - | - | 10% | 2.5 mg/g of cellulose | Acid-pretreated corn stover | 66 |
| Gao et al. 2010 (65)* | 27 - 30% | 17 - 20% | 29 - 35% | - | - | 11 - 31% | 7.5, 15 or 30 mg/g of glucan | AFEX corn stover | 52.6 - 80 |
| Banerjee et al. 2010 (66)** | 35 - 43% | 4% | 26 - 30% | - | 5 - 12% | 8 - 23% | | Corn stover | 40.7 - 58.2 |
| | 16 - 28% | 4% | 23 - 34% | - | 20 - 43% | 8 - 24% | 15 mg/g of glucan | Dried distillers' grains plus solubles | 22.6 - 29.8 |
| | 36 - 48% | 4% | 32 - 46% | - | 4% | 8 - 11% | | Miscanthus | 17.5 - 32.1 |
| | 44 - 47% | 4% | 36 - 37% | - | 4% | 8% | | Poplar | 9.8 - 13.8 |
| | 31 - 47% | 4% | 19 - 30% | - | 4 - 12% | 8 - 39% | | Switchgrass | 24.4 - 39.1 |
| | | | | | | | | | |
| Tejirian et al. 2011 (67) | 60% | 15% | 5% | 6% | - | 24% | 10,8 mg/g of substrate | Avicel | 77 |
| | | | | | | | 9.8 mg/g of cellulose | Acid-pretreated corn stover | 85 |
| Billard et al. 2012 (46) | 41.4% | 27.4% | 15.9% | 6.3% | 2.5 mg/g of enzyme mixture | 9% | 2.5 mg/g of substrate | Steam exploded wheat straw | 65.8 |
| Chylenski et al. 2017 (47) | 28% | 30% | 30% | - | - | 13% | 8 mg/g of substrate | Sulfite-pulped sugarcane Bagasse | 80.5 |
| | 28% | 17% | 48% | - | - | 7% | | Sulfite-pulped Norway spruce | 82.3 |
| Borisova et al 2022 (68) | 58.4% | 19.5% | - | 9,80% | - | 12.3% | 10 mg/g of substrate | Avicel | 30 |

Abbreviations: AFEX, ammonia fiber expansion; BG, β -glucosidase; CBH, cellobiose hydrolase; EG, endoglucanase.

* Glucose yields and enzymes proportion reported in Gao et al. vary depending on the total enzymes loading.

** Glucose yields and enzymes proportions reported in Banerjee et al. vary depending on the pretreatment of the biomass (AFEX, 0.25% NaOH or alkaline peroxide).

Conclusions

With the emergence of LPMOs as a must-have component of the new best performing enzymatic cocktails, biomass degradation products will tend to become more diversified, including neutral and oxidized sugars. Today, these oxidized soluble oligosaccharides are often overlooked in favour of the hydrolytic system products (namely glucose and cellobiose), used as substrates of various bioprocesses (**Fig. 4**). In the present study, the tested LPMOs did not prove efficient at boosting the conversion of the selected biomass, underpinning the complexity of implementing efficiently these enzymes in biorefinery processes and the need for further research. Nevertheless, our results show that the addition of CDH into industrial enzymatic cocktails could be a promising path to generate high value-added products from industrial lignocellulosic biomass, expanding thereby valorization routes to contribute to the success of the emerging bioeconomy.

Materials and methods

Materials

(Oligo)saccharide substrates were purchased from Megazyme (Wicklow, Ireland), ascorbic acid was purchased from Sigma-Aldrich (Saint-Louis, Missouri, United-states) and PASC was prepared as described in Wood (69).

Lignocellulosic substrates

Wheat straw, miscanthus, poplar and pine (*Pinus pinaster*) were obtained from IFP Energies Nouvelles (Rueil-Malmaison, France). Wheat straw, miscanthus and poplar were pre-treated as described in (70). In short, the lignocellulosic biomass was steam exploded under acidic conditions, washed in hot water to remove free products, and then dried at 55°C. After one week at room temperature, the biomass was shredded and sieved to a 0.8-mm maximal size. Pine was pre-treated differently. It was first dried at 90°C then shredded and sieved to 30 mm maximal size, further affined to 5 mm. Pine was finally steam exploded under acidic conditions and washed in hot water to remove free products. **Table S2** provides more details on the steam explosion process for each type of biomass. Compositional analysis of pretreated biomass was carried out following standardized procedures developed by the National Renewable Energy Laboratory (NREL/TP-510-42618) (See **Table S3** for composition of the biomass).

Production and purification of *P. anserina* AA9 LPMOs and CDH

PaAA9H (GenBank ID: CAP61476), *PaAA9E* (GenBank ID: CAP67740) and *PaCDHB* (GenBank ID: CAP61651) were recombinantly produced and purified as previously described (71, 23). Recombinant *P. pastoris* clones (strain X33) expressing *PaAA9E*, *PaAA9H* and *PaCDHB* were grown in 2 L of Buffered Glycerol- complex Medium (BMGY) media in flasks at 30°C in an orbital shaker (200 rpm) for 16 h to an OD600 of 2 to 6. Expression was induced by transferring cells into 400 mL of Buffered Methanol- complex Medium (BMMY) media at 20°C in an orbital shaker (200 rpm) for another 3 days. Each day, the medium was supplemented with 3% (v/v) methanol. The cells were harvested by centrifugation, and just before purification the pH of the supernatant was adjusted to 7.8 by addition of Tris-HCl buffer (1 M, pH 8) and was filtered on a 0.45 µm membrane (Millipore, Burlington, Massachusetts, USA). The filtered supernatants were then loaded onto a His-Trap Resin (GE Healthcare, Buc,

France) column (1.6 × 2.5 cm or 1.60 × 10 cm) equilibrated with buffer A, (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 10 mM), using an Äkta purifier 100 (GE Healthcare). (His)₆-tagged recombinant enzymes were eluted with buffer B (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 500 mM). Fractions containing recombinant enzymes were pooled, concentrated, and dialyzed against sodium acetate buffer (50 mM, pH 5.2).

The *T. reesei* cellulases were produced, as described in Poggi-Parodi et al. (72), in DASGIP bioreactors. *T. reesei* strain CL847 cultures were grown on plates of Potato Dextrose Agar (Difco Laboratories, USA) at 30°C. The cellulase production was performed in two steps. In the first step, a growth phase was conducted at 27°C in 400 mL of starting medium containing 35 g/L of glucose as carbon source during 30 h. The pH was regulated at 4.4 with 5.5 M ammonia. The air flow was adjusted at 0.5 vvm and initial stirring was set at 500 rpm. This parameter was gradually increased to maintain pO₂ above 40% oxygen saturation. The second step was done at 25°C during 170 h and when the glucose was depleted in the media, a fed-batch solution containing 250 g/L lactose was added at a rate of 1 g/h. At the end of the fermentation, the musts were filtered and sodium benzoate at 0.35% w/v were added to the recovered filtrates before storing them in a bottle at 4°C.

T. reesei cellulases were then purified as described in Billard et al. (46). Culture supernatants were salted out using a HiTrap desalting column (Biorad, Marnes-la-coquette, France) and equilibrated with 25 mM imidazole-HCl buffer (pH 7.5). Chromatofocusing was performed on an ÄKTA® FPLC (GE Healthcare, Chalfont St Giles, UK) using a Mono Q 5/50 GL column (GE Healthcare, Chalfont St Giles, UK) equilibrated with the initial buffer. Proteins bound (20 mg) under the initial conditions were eluted by a pH gradient (from 7.4 to 3.9) using PB74 Polybuffer (GE Healthcare) at a constant flow rate of 0.7 ml/min. Recovered fractions were analyzed by one dimensional gel electrophoresis indicating a purity of >95% with Image Lab Software.

LPMOs copper saturation and desalting

Prior to enzymatic assays, PaAA9E and PaAA9H were saturated with Cu(II)SO₄. To this end, the enzymes were incubated in sodium acetate buffer (50 mM, pH 5.2) with a 3-fold molar excess of Cu(II)SO₄ for 30 min at 4°C and excess copper was removed by passing the enzyme solution through a PD MidiTrap G-25 (GE Healthcare) desalting column pre-equilibrated with sodium acetate buffer (50 mM, pH 5.2), at room

temperature using gravity flow. Proteins concentration were determined by measuring the absorbance at 280 nm using a Nanodrop NB-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The proteins samples were stored at 4°C until further use.

Design of experiment assays on wheat straw

The MODDE Design of Experiments software version 12.0 (MKS Data Analytics Solutions, Umeå, Sweden) was used for experimental design and data analysis. A quadratic design with three mixture components was used in all experiments including *T. reesei* cellulases cocktail (2 to 20 mg/g of DM loading), *PaCDHB* (0 to 4 mg/g of DM loading) and *PaAA9E/PaAA9H* (0 to 5 mg/g of DM loading). The best subset of experiments from a candidate set was generated with MODDE by Full Fac (3 levels) design (the full list of factors is presented in **Table S1**). An optimal subset consisting of 30 individual experiments were thus obtained and performed for each AA9/*PaCDHB* couples.

Enzymatic reactions

Enzymatic hydrolysis reactions were carried out in 2 mL deep 96-wells plates (ThermoFischer, Waltham, USA), with 1% (w/v; i.e. 10 mg/mL) (for wheat straw) or 2% (for miscanthus, pine and poplar) of DM in sodium acetate buffer (50 mM, pH 5.2), with a total reaction volume of 500 µL. The reactions were incubated in an incubator (Infors, Massy, France) at 50°C with orbital shaking (850 rpm). For each time point, 5 µL of the supernatant was sampled and diluted 20 times in milliQ water before heating 5 minutes at 100°C to stop the reactions. The diluted supernatants were then immediately filtered on 96-wells 0.2 µm micro filter plates (Pall Life Sciences, New-York, USA) and stored at -20°C for further analysis.

For the AA9/*PaCDHB* reaction on PASC, reactions were performed at a final substrate loading of 0.2% in sodium acetate buffer (50 mM, pH 5.2) and incubated in a Thermomixer (30°C, 850 rpm). After 24 h of reactions, reactions were stopped by heating 5 minutes at 100°C and the soluble fractions were analyzed by high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). The system is equipped with a CarboPac-PA1 guard column (2 × 50 mm) and a CarboPac-PA1 column (2 × 250 mm) kept at 30 °C. Elution was carried out at a flow rate of 0.25 mL.min⁻¹ and 25 µL of sample was injected. The

solvents used were 100 mM NaOH (eluent A) and NaAc (1 M) in 100 mM NaOH (eluent B), and the following gradient was applied: 0 to 10 min, 0 to 10% B; 10 to 35 min, 10 to 35% B (linear gradient); 35 to 40 min, 30 to 100% B (curve 6); 40 to 41 min, 100 to 0% B; 41 to 50 min, 100% A. Integration was performed using the Chromeleon 7.2.10 chromatography data software.

Reaction products analysis

For the reactions on wheat straw, glucose concentration was measured as in (73). To perform the assay, 200 μ L of the glucose GOD-PAP reagent (Biolabo, Maizy, France) were added to 10 μ L of diluted reactions (2 times for the 4 and 8 h time points, 10 times for the 24 and 48 h time points) in 96-well microplates. The microplates were then left at ambient temperature for 30 min before reading the absorbance at 540 nm using a microplate spectrophotometer. Soluble glucose concentrations were evaluated using a standard curve, and yields were computed based on the initial amount of potential cellulosic glucose in each substrate, as follows: glucose yields (%) = extracted soluble glucose x 100 / theoretical total glucose content.

For the reactions on miscanthus, pine and poplar, the diluted supernatants were further analyzed by HPAEC-PAD, using glucose, gluconic acid and cellobiose as standards (Megazyme). Cellobionic acid standard were obtained by incubating 1 to 100 μ M of cellobiose (Megazyme) with 1 μ M of *Pa*CDHB for 24 h in a thermomixer (30°C, 850 RPM).

Acknowledgements

We thank Sacha Grisel and Mireille Haon from the 3PE protein production platform for performing the expression and purification of *P. anserina* enzymes. We thank all the BBF support staff (Chantal Parodi-Negri, Christophe Boyer).

Author contributions

J.-L.R. carried out most experiments. S.A. and A.L. performed the expression and purification of *T. reesei* enzymes. S.G. carried part of the HPAEC-PAD analysis. J.-L.R., A.M., S.A., J.-G.B. and B.B. designed the experiments and interpreted the data. A.M, S.A, J.-G.B. and B.B. conceptualized the study. A.M., S.A., J.-G.B. and B.B. supervised the work. All authors contributed to the writing of the manuscript, reviewed,

and approved the final version of the manuscript.

Funding

This work was supported by a grant overseen by the French National Research Agency (ANR) (SNOEBORD project, grant number ANR-21-CE43-0025).

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information: HPAEC-PAD chromatograms and time-course of enzymatic reaction products released from lignocellulosic biomass; Violin plot of best reported LPMO boost effects; design of experiments parameters; biomass pretreatment conditions; pretreated biomass compositions; abbreviations list; and supplementary references.

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Synopsis

The use of CDH in cellulolytic cocktails enables the shift in biomass-derived products towards high value-added oxidized oligosaccharides.