Novel bacterial enzymes for plant biomass degradation discovered by meta-omics approach

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Improved (hemi) cellulolytic enzyme cocktails for the conversion of lignocellulosic biomass into sugars.

Mukil Maruthamuthu, Maria Julia de Lima Brossi and Jan Dirk van Elsas

(Submitted)
Abstract
Enzymatic hydrolysis is a powerful approach for biomass conversion in the biorefinery industries, as it allows to produce fermentable sugars from complex polysaccharides. The goal of this study was to examine if the wheat straw conversion rate of a commercial enzyme preparation could be enhanced. Different combinations of (hemi)cellulolytic enzymes (denoted P1, P2, P5 and P6), next to secretomes from wheat straw degrader consortia, were tested with the commercial enzyme Celluclast® 1.5 L. Thus the efficiency of the resulting mixtures in releasing fermentable sugars from steam-exploded (SE) versus untreated wheat straw was assessed by using dinitro salicylic acid (DNS) colorimetry and high-performance anion exchange chromatography (HPAEC). The admixture of either of two secretomes did not yield any tangible enhancement of the wheat straw degradation activity. In contrast, addition of the purified enzymes P1, P5 and/or P6 significantly enhanced the release of sugars, in particular from SE wheat straw. In these systems, xylose, glucose and arabinose were released to greater extents (50, 5 and 35%, respectively) from this substrate. Moreover, the release of sugars from SE wheat straw was significantly higher than that from untreated wheat straw. Overall, combinations of the P1 with the P5 and/or P6 enzymes significantly enhanced the Celluclast-driven enzymatic hydrolysis of SE wheat straw.
Introduction

Lignocellulosic biomass (LCB) from agricultural waste is often proposed as a key source of energy and carbon that may serve multiple purposes, e.g. the production of biofuels or of value-added compounds [1]. For the conversion of LCB into the respective monomers, i.e. sugars, efficient enzymes are important, as they catalyze and so enhance the rate of substrate depolymerization [2,3]. Many microorganisms (bacteria and fungi) are capable of deconstructing the building blocks of LCB, i.e. cellulose, hemicellulose and pectin [4,5]. In contrast, lignin is known to be less easily decomposed. However, the use of whole microbial cells has limitations due to the often restricted availability of efficient enzymes and the scavenging, by the organisms, of the released sugars [6,7]. Nevertheless, microbial consortia that are adapted to growth on LCB constitute a highly-valued source of potentially novel enzymes [8–11]. If these offer enhancement of catalytic rates or product yields, they may subsequently be used in current cell-free LCB deconstruction approaches.

The production of efficient enzyme cocktails for LCB deconstruction will require an intricate understanding of the specific enzymes that can – jointly - deconstruct the polymeric matrix [7,12]. Most currently available commercial enzyme cocktails that are often produced on the basis of fungal (e.g. *Trichoderma reesii*)-excreted compounds are quite complex. Often, different proteins (more than 100) are present, with some of these being inefficient in the LCB degradation [13]. Moreover, the specific roles of the different components of such products in the decomposition are poorly understood [12]. Accordingly, little knowledge has been gained so far on the working of current enzyme cocktails, which are presumably often still sub-optimal [7,14]. To overcome this hurdle, such products might be enhanced using novel enzymes with proven catalytic ability or even whole secretomes of different (bacterial) origin [15].

Previous work has shown that the properties of each of the key enzymes and their relative abundances, next to potential enzyme-enzyme interactions, are key determinants of the activity of enzyme cocktails on LCB [16–21]. For instance, six fungal enzymes (with different loading concentrations) were used to promote corn stover hydrolysis following an ammonia fiber expansion pretreatment [22]. Within this cocktail, an endoglucanase, denoted EG1, played a vital role in maximizing hydrolysis yields. Barr et al (2012) developed an enzyme mixture (containing β-xylosidases (βX), β-glucosidases (βG), celllobiohydrolases (CBH), endoglucanases (EG), endoxylanases (EX) and acetylxylan esterases (AEX)), which were subsequently found to effectively degrade pretreated poplar and switchgrass biomass [23]. On the other hand, testing of the *Trichoderma reesii* derived enzymes Cel7a (celllobiohydrolase CBH1), Cel6a (CBH2), Cel7b (endoglucanase EG1), Cel5a (endoglucanase EG1), Cel12a (endoglucanase EG3) and Xyn11a (xylanase) for their activity on hydrothermally-pretreated wheat straw revealed that hydrolysis was still quite incomplete and further enhancement was required [24]. When these
enzymes were applied on SE wheat straw, Cel7b, Cel5a and Xyn11a turned out to be the key hydrolytic enzymes that promoted the release of sugars [25]. Here, the SE pretreatment was a key asset, as it made the substrate more accessible to specific enzymes.

In the current study, we selected two secretomes from wheat-straw degrading (hemi)cellulolytic microbial consortia [26], next to four biochemically-characterized enzymes with (hemi) cellulase activity obtained from a recent study [27]. The latter consisted of one β-galactosidase (P1; GH2 family), one bifunctional β-xylosidase/α-arabinosidase (P2; GH3) and two α-glucosidases (P5 and P6). We examined the potential roles of all of these enzyme sources in the deconstruction of either untreated or SE wheat straw. The underlying hypothesis of this study was that the addition of efficient enzymes (either purified proteins or secretomes) to the commercially-available enzyme preparation Celluclast enhances the release of reducing sugars from the substrate, thus paving the way towards improved enzymatic hydrolysis strategies.

Materials and methods
Substrates
Wheat straw was collected from a local farm in Groningen, The Netherlands. The plant biomass raw material was transported to the laboratory (<24 h following sampling) at room temperature (T = 20 °C) for further processing. The raw material was air-dried at 50 °C for 24 h before grinding using a hammer mill, yielding pieces <1 mm. Part of the wheat straw was pretreated using steam explosion, whereas the remaining part was kept as non-treated (“raw”) wheat straw. The pretreatment was performed in a laboratory-scale steam explosion machine, at the University of Groningen, The Netherlands. Briefly, 30 g of the pre-dried and milled straw was soaked in 70 mL of sterile distilled water. The treatment was carried out at 180°C, with an incubation time of 15 min before explosion. The pretreated material was dried at 50°C for 24h. A total amount of 300 g of material was produced in various steam explosion rounds. Thus, two different substrates were used in the analysis: non-treated wheat straw (NTWS) and steam-exploded wheat straw (SEWS).

Secretomes
In this study we tested two different secretomes. Extractions of extracellular protein fractions (secretomes) from the microbial consortia (10 enrichments of soil microbial community in wheat straw as unique carbon source) were performed as described in Jiménez et al. (2014a). This source consortium was then bred further, for up to 6 days, on either NTWS and SEWS (1%, as carbon and energy sources) in MSM medium [7 g/l Na₂HPO₄; 2 g/l K₂HPO₄; 1 g/l (NH₄)SO₄; 0.1 g/l Ca(NO₃)₂; 0.2 g/l MgCl₂, pH 7.5] supplemented with either NTWS or SEWS (1%, as carbon and energy sources. Both consortia presented progressively increasing
cell densities, from the inoculum level (around 5 log cells/ml) to around 9 log bacterial cells/ml after 5-6 days. The resulting cultures were centrifuged (12,000xg, 10 min; Eppendorf centrifuge, Hamburg, 351 Germany) to remove cells, and the supernatants passed through 0.22-µm syringe filters (Whatman FP30/0.22, Little Chalfont, UK). This yielded two secretomes, denoted SNT (secretome of non-treated) and SSE (secretome of steam-explosion treated), respectively.

Selection of novel enzymes
Four novel enzymes were tested in this study, i.e. P1 (GH2 family, β-galactosidase), P2 (GH3 family, β-xylosidase/α-arabinosidase), and P5 and P6, both with α-glucosidase activity. All had been retrieved from a metagenomic library, as described previously [11]. The enzyme P1 had a size of 116 kDa and revealed considerable sequence identity with a similar protein from Enterobacter hormaechei (74 %), the P2 enzyme was 85 kDa and had 83% of sequence identity with a similar protein from Enterobacter mori. Moreover, the enzymes P5 (45 kDa) and P6 (26 kDa) revealed sequence identity with proteins from Enterobacter cloacae (79 %) and Hyphomonas neptunium (65 %). In our study, we thus tested combinations of the secretomes (SNT and SSE) and the four enzymes with the commercially available enzyme preparation Celluclast 1.5L (Cellulases from Trichoderma reesei ATCC 26921; Sigma – Aldrich).

Production and purification of enzymes
Escherichia coli cells containing plasmid Origami2(DE3) pLysS (Novagen, Amsterdam, The Netherlands) loaded with genes for each of the four enzymes were grown in kanamycin (50ug/ml)- supplemented 2X-PY medium (2 ml; 16g Bacto-tryptone, 10g yeast extract, 10g NaCl/1L, pH 8.0) at 37°C (220 rpm, shaking incubator, overnight). Then, aliquots of the grown cultures were transferred to fresh 200mL 2xPY, setting an initial OD600 of 0.05, after which the cultures were incubated at 37°C (220 rpm) until they reached an OD600 of 0.5-0.6. Subsequently, the cultures were incubated for 1 h at 18°C (shaking at 220rpm), to induce expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.5M. Following this, the cultures were further grown at 18°C for 16-19 h, after which cells were harvested (3,000-4,000xg; 4°C, 15 min). The pellets were resuspended in 5 mL of lysis buffer (50mM HEPES, pH 8.0, 300mM NaCl, 50µL 1M DTT, 1 protease inhibitor mini tablet (Roche, Sigma-Aldrich Chemie B.V, Zwijndrecht, The Netherlands) and kept on ice for 15 min. Then, cells were disrupted using sonification with the following parameters (40 cycles – 6s ON/ 15s OFF – amplitude 6-10 µm. After disruption, the resulting cell lysates were centrifuged at 15,000g for 15 min at 4°C. The supernatants were removed and stored (and the pellets were discarded), and 10 µL supernatants checked for protein content with 12% SDS-PAGE. The preparation was then heated to 60°C for 15-20 min and centrifuged again at 15000xg to remove insoluble debris. Purification of the hist-
tagged proteins from the crude extracts was then carried out by gravity-flow chromatography through agarose. Thus, 600μl of Ni-NTA Agarose (Qiagen, Hilden, Germany) was added to 10 ml of lysis buffer. Incubation was for 5 min (shaking, 4°C), before the mixture was centrifuged for 5 min at 800g at 4°C. The supernatant was discarded and then 10 ml of equilibration buffer (50mM HEPES and 300mM NaCl) was added and incubated as above, before centrifugation. The crude extracts were added to the resin and incubated for 1 h as above, before being transferred to a gravity flow column and incubated at 4°C until the resin bed settled down. The cell-free lysates were removed by gravity flow and unbound proteins removed 3 times with 10 ml of wash buffer (50mM HEPES, 300mM NaCl, 20mM Imidazole). The bound enzyme was then eluted with 3 ml of elution buffer (50mM HEPES, 300mM NaCl, 400mM Imidazole). The resulting preparations were then concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Amsterdam, The Netherlands). Finally, the protein concentrations were determined using the Quick Start Bradford protein 1x dye reagent (Biorad, Veenendaal, The Netherlands) with bovine serum albumin (BSA) as a standard [28] using the Infinite M200®spectrophotometer (Tecan, Männedorf, Switzerland) at 595 nm. The purity of the preparations was analyzed using 12% SDS-PAGE [29].

The secretomes collected from the degrader consortia, and the Celluclast preparation were found to contain sugars. In order to remove these, we used Amicon® Ultra-15 Centrifugal Filter Devices (Millipore, Amsterdam, The Netherlands) on 12 ml of both. Following a spin at 5000xg for 60 min, solutes were recovered from the resulting preparations and stored at -20°C and the protein concentrations were determined.

Enzymatic activity assays

Enzymatic activity micro-assays were carried out to evaluate the effects of the selected (hemi) cellulolytic enzyme mixes. Three experiments (1, 2 and 3) were performed, in accordance with critical choices made along the work. The first experiment had 13 enzyme combinations, the second one 10 and the third one 5 (in each case, combining novel enzymes and/or secretomes with Celluclast). Thus, we analyzed the effect of: (1) treated versus non-treated wheat straw (2) the use of two secretomes, and effect of their origin, and (3) four selected enzymes with different activities. All treatments consisted of three replicates and were controlled with two negative controls (each substrate plus buffer). The independent variables were P1, P2, P5 and P6 (four levels), secretomes SNT and SSE (two levels), substrates NTWS and SEWS (two levels) and the enzymatic cocktail Celluclast 1.5L. The protein concentrations were set at 1 mg of proteins g substrate⁻¹ for all proteins, secretomes and the Celluclast. The reaction mixtures contained 20 mg of each substrate plus the enzyme combinations (Table 1) in sodium phosphate buffer (0.1 M, pH 6.0) in a final volume of 1.5ml. With respect to the experimental
design, ‘-’ corresponds to the absence of the enzyme/secretome and ‘+’ to its presence. All assays were done at 50°C (24h, shaking at 250rpm). After incubation, the mixtures were centrifuged (12000xg for 15 min at 4°C), and the supernatants collected for subsequent analyses. The amount of reducing sugars in the supernatants was measured according to the dinitro salicylic acid (DNS) colorimetric [30] method, and in specific cases by high pH anion exchange chromatography. All data are presented as total sugars released (mg/mL).

Analysis of released sugars by high pH anion exchange chromatography (HPAEC)
Samples from the third experiment were subjected to analysis by HPAEC on a Dionex ICS-3000 system (Thermo Scientific) equipped with a CarboPac PA-1 guard column (2x50 mm) and a CarboPac PA-1 column (2x250 mm). An internal pulsed amperometric detector with an Au working electrode and an Ag/AgCl pH reference electrode was used for detection. The system was run with 25 mM sodium hydroxide (NaOH) for 3 min, followed by a gradient up to 85 mM NaOH at 17 min and subsequently a gradient to 100 mM NaOH and 30 mM of sodium acetate at 22 min and finally a gradient of [100 mM NaOH] and 210 mM sodium acetate at 30 min. The flow rate of the system was 0.25 ml/min. All chromatograms were analyzed using Chromeleon 6.8 Chromatography data system software (Thermo Scientific).

To interpret the data, calibration curves encompassing arabinose, galactose, glucose (Sigma-Aldrich Chemie B.V. Zwijndrecht, Netherlands), xylose (Janssen chimica, Beerse, Belgium), xylobiose, cellobiose and xylotriose (Megazyme Inc, Illinois, USA) were made. Concentrations of the used standards ranged from 50 µM to 1 mM. Standards and samples had an injection volume of 5 µl. The calibration curves were used to quantify the amount of released saccharides from the wheat straw. The peaks at the end of the chromatogram (≤40%) probably represent decorated oligosaccharides (e.g. acetyl groups, ferulyl groups and glucoronic acid). We did not further take these oligosaccharides into account.

Statistical treatment of the data
All experiments were performed in triplicate. To assess the differences between treatments, we used permutational multivariate analysis of variance (PerMANOVA) [31]. Different factors were addressed in each experiment, and full permutation of the raw data was done with Monte-Carlo tests (accounting for type III error), where the fixed effects sum to zero with 9999 permutations. For the first experiment, the factors were the pre-treatment of the wheat straw (untreated versus steam explosion), the presence/absence of Celluclast, and the addition of secretomes and the addition of enzymes. For the second experiment, the factors were each enzyme separately. For all three experiments, statistical comparisons were also performed using one-way ANOVA (Tukey’s test) to
compare pairwise differences between treatments.

**Results**

**Steam explosion of wheat straw – effects**

Steam explosion of milled (average particle dimension < 1 mm) dry wheat straw was performed in a laboratory-scale steam explosion system, using an in-house made pressure device. We first experimentally tested the effects of different steam explosion regimes, including variations in the time at maximal temperature (5, 10 and 15 min) and temperature (170, 175 and 180°C). Taking the data together, a regime of 15 min at 180°C turned out to produce optimal results (as evidenced using direct microscopy of the treated versus untreated materials, Figure 1a). Thus, this regime was selected for the further steam explosion processing. We produced a total of 300g of SE wheat straw, of which the structure was clearly modified as compared to the control (Figure 1a). In addition, the liquid remaining after steam treatment (after filtering) had a raised amount of released sugars (1.2 mg/ml), as compared to the liquid from untreated wheat straw (NTWS; 0.2mg/ml) (Figure 1b).

**Metasecretomes produced from two biodegrader consortia – yield and purity**

The filtered supernatants of the NTWS and SEWS consortia were concentrated using Amicon centrifugal devices, giving 0.3 and 0.1 µg of protein/µl, respectively. As shown in a previous study [32] the presence of enzymes belonging to glycosyl hydrolase families GH3, GH10, GH43, GH51, GH67 and GH95 was predicted (Figure 2), thus the existence of (hemi)cellulolytic and other carbohydrate-active proteins in the liquid was plausible.

**Selection of four enzymes - yield and purity**

The enzymes encoded P1, P2, P5 and P6 were all successfully produced from the freshly-grown *E. coli* cultures. For all enzymes, we obtained maximally (approximately 80%) pure enzyme preparations after his-tag purification. The protein concentrations were: P1 (0.4 µg/µl), P2 (0.7 µg/µl), P5 (0.5 µg/µl) and P6 (0.7 µg/µl). Using enzymatic activity assays with βNP-beta-D-galactopyranoside (βNPGal), βNP-beta-D-xylanopyranoside (βNPXyl), βNP -alpha-L-
arabinofuranoside and ρNP-alpha-D-glucopyranoside (ρNPGlu), we confirmed the biochemical activities of the four enzymes as ( thermoalkaliphilic) β-galactosidase (P1), β-xylosidase and α-arabinosidase (P2), and α-glucosidase (P5 and P6) (data not shown).

Figure 2. a) Schematic representation of the methodology used in this study b) Taxonomic assignment of detected proteins in the metagenome database, and functional assignment of proteins detected in metasecretome.

Steam-exploded versus untreated wheat straw – enzymatic hydrolysis
Whereas Celluclast served as the control, the additional effects of different combinations of enzymes P1, P2, P5 and P6, and of secretomes SNT and SSE, were evaluated on both NTWS and SEWS. As extra controls, i) buffer, substrate (NTWS and/or SEWS) and sodium azide, and ii) only buffer, were used. Thus, initially 13 combinations of enzymes (Table 1) were used (Figure 3a; 3b) and released sugars measured. An overall statistical analysis of the data revealed that significantly higher amounts of sugars were released, across all enzyme preparations, from SEWS than from NTWS (P<0.05). First, Celluclast alone (treatment 8; Table 1) released significantly (p=0.001) higher amounts of reducing sugars from SEWS (2.0mg/ml) than from NTWS (0.7mg/ml). With respect to the Celluclast modifications, the addition of either of the two secretomes (Table 1) to Celluclast slightly but insignificantly enhanced the amount of released sugars in
comparison to the Celluclast alone (Figure 3a; 3b; p>0.05). Remarkably, the addition to Celluclast of the enzymes P1, P2, P5 and P6 (treatment 9, Table 1) strongly and significantly (p=0.018) enhanced the hydrolytic activity on SEWS, with the released sugars amounting to 3.42 mg/ml as compared to 1.86 mg/ml in the Celluclast-only treatment (Figure 3b). Thus, the degradation of SEWS was greatly aided by the supplementation of the four-enzyme mix to the Celluclast, significantly enhancing the release of sugars. Given this strong effect of the four enzymes (P1, P2, P5 and P6), in particular on SEWS, we further analyzed the potential role of each of these enzymes with Celluclast.

Table 1. Experiment 1: Different combinations of four enzymes, two secretomes, and the commercial enzyme mix Celluclast

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<th>Substrate (a)</th>
<th>Substrate (b)</th>
<th>Sodium azide</th>
<th>Secretome 1/20µg</th>
<th>Protein 1/20µg</th>
<th>Protein 2/20µg</th>
<th>Protein 5/20µg</th>
<th>Protein 6/20µg</th>
<th>Celluclast/20µg</th>
<th>Buffer (final volume)</th>
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<td>10 µL</td>
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<td>Upto 1.5 mL</td>
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<tr>
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<td>+</td>
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Selection of optimal enzyme mixture for release of sugars from SEWS

Based on the results obtained from the initial experiment, we selected the SEWS substrate and (different combinations of) each of the four enzymes (P1, P2, P5 and P6) for further analyses of their potential as Celluclast enhancers. Table 2 lists the experimental conditions, whereas the data from this second experiment are shown in Figure 4a. The addition of the P1 preparation to the Celluclast significantly (p=0.05) enhanced the release of sugars, i.e. a ~20% increase was found compared to the Celluclast alone treatment (3.90 mg/ml versus 3.27 mg/ml). In contrast, none of the other individual enzymes, i.e. P2, P5 and P6, significantly enhanced the activity of Celluclast. Remarkably, the combination of enzyme P1 with both P5 and P6 significantly (p=0.001) enhanced the Celluclast activity (Table 2: Treatment 8) by ~22%, with respect to the amount of released sugars (Figure 4a). Specifically, 3.98 mg/ml was released versus 3.27 mg/ml in the control. We thus concluded that a synergistic action of enzymes P1 with P5 and/or P6 was at the basis of the activity enhancement, and selected these three enzymes to further unravel their roles as Celluclast enhancers (Table 3). In the resulting experiment, the three-enzyme mix (P1+P5+P6) added to Celluclast (~3.7
mg/ml of released sugars) again revealed a significant (roughly 25%) increase over the Celluclast-alone treatment (~ 2.95 mg/ml of released sugars). Remarkably, the P1+P5 and P1+P6 mixes added to the Celluclast produced even more sugars (~ 4.45 mg/ml) than the Celluclast-alone (Figure 4b), amounting, in both cases, to 50 and 51% increases. These increases were highly significant (p=0.0001).

![Enzyme cocktail microassay 1 (NTWS - 24 hours)](image)

**Figure 3.** Experiment 1, Dinitro salicylic acid (DNS) colorimetric analysis a) with NTWS b) with SEWS

**Analysis of released sugars by HPAED**

We then analyzed the nature of the released sugars as a result of the treatments, with a focus on the SEWS. The Celluclast-only treatment (Treatment1; Table 3) was found to release considerable amounts of arabinose (0.3 µg), glucose (1.1 µg) and xylose (0.5 µg), but no cellobiose and xylotriose. In contrast, treatments 2 (P1+P5+P6+Celluclast; Table 3), 3 (P1+P5+Celluclast; Table 3), 4 (P1+P6+Celluclast; Table 3) and 5 (P5+P6+Celluclast; Table 3) revealed the presence of increased levels of the former three sugars, next to other ones (i.e. Oligosaccharides and xylobiose) (Figure 5). Moreover, the release of xylose and arabinose was significantly higher in treatments 2 to 5 than in the control treatment 1. In particular, treatment 3 produced higher amounts of xylose (2.1 µg)
and 1.5 µg of arabinose versus 0.5 µg and 0.3 µg in the control treatment 1. In addition, the presence of glucose (1.25, 1.21 and 1.28 µg) was found to be statistically raised (p<0.01) in treatments 3 to 5 when compared to control treatment 1 (1.1 µg). This suggested that, whereas the Celluclast likely cleaved primarily cellulose moieties, the additional enzymes worked mainly as (hemi)cellulases, thus increasing the conversion rates. Overall, the HPAEC data (Figure 5) confirmed that the addition of the enzyme P1 preparation together with P5 and P6 significantly enhanced the Celluclast activity by enhancing the release of the monosaccharides xylose, glucose and arabinose from the (hemi)cellulose matrix.

Discussion

Given the fact that current enzyme cocktails are as-yet suboptimal on LCB, an enhancement of the hydrolysis rates of such cocktails is desirable [33,34]. In this regard, the development of hemicellulases for the enzymatic hydrolysis of plant biomass is not as advanced as that of cellulases, because current commercial preparations have been primarily used on pretreated biomass from which the hemicellulose part was partially removed before saccharification [35]. Moreover, current fungal-derived cellulases tend to have only weak hemicellulolytic activity and so are not adequate for the complete conversion of LCB when it comes to the hemicellulose moiety. Therefore, the development of low-cost commercial hemicellulases that work synergistically with cellulases is a prime goal of current research [36]. We here tested two supplementary enzyme sources [four (hemi)cellulolytic enzymes (P1, P2, P5 and P6), and the secretomes of NTWS and SEWT bred microbial consortia], in conjunction with the commercially available Celluclast 1.5L, to increase the (untreated versus SE) wheat straw degradation efficiency [37]. The SE pre-treatment process was thought to unlock the different (cellulose, hemicellulose, lignin) moieties constituting the wheat straw fibers, thus facilitating enzyme access [38]. Indeed, our steam-exploded material had clear signs of structural modifications, as predicted [25]. Therefore, we presumed that SE was highly important as a physical mechanism that disrupted bonds in some of the fibers, thus allowing (hemi)cellulose fractions to detach from the rigid parts of cellulose and lignin. Our subsequent sugar release data clearly indicated that the SEWS, as compared to the NTWS, had enhanced susceptibility to enzymatic attack, enabling the release of significantly higher amounts of sugars (Figure 3a). As optimal degradation of LCB depends on the synergistic action of different enzymes [36] that can be found in lignocellulose-grown microbial consortia, we included two metasecretomes produced from microbial consortia grown on wheat straw (NTWS and SEWS). As hemicellulolytic enzymes, i.e. endo-1,4-beta-xylanases (GH10), beta-xylosidases (GH43), alpha-L-arabinofuranosidases (GH43 or GH51) and alpha-L-fucosidases (GH95), may critically aid cellulases in the degradation of wheat straw [11,32], secretomes were selected which predictably
had (hemi)cellulolytic enzymes of families GH43, GH16, GH3, GH51, GH10, GH20, GH95, GH12 and GH67 [32]. However, none of the two secretomes was a clear Celluclast enhancer, although SNT and SSE did show some minor effects.

![Enzyme cocktail microassay 2 (SEWS - 24 hours)](image)

![Enzyme cocktail microassay 3 (SEWS - 24 hours)](image)

Figure 4. Experiment 2 and 3, Dinitro salicylic acid (DNS) colorimetric analysis a) Enzymes (P1, P2, P5 and P6) cocktail assay with SEWS b) Enzymes (P1, P5 and P6) cocktail assay with SEWS

Table 2. Enzyme cocktail microassay 2: Different combinations of purified enzymes and commercial enzyme celluclast

<table>
<thead>
<tr>
<th>No</th>
<th>Substrate</th>
<th>Sodium azide</th>
<th>Protein 1/20µg</th>
<th>Protein 2/20µg</th>
<th>Protein 5/20µg</th>
<th>Protein 6/20µg</th>
<th>Celluclast/20µg</th>
<th>Buffer (final volume)</th>
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<tbody>
<tr>
<td>1</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>2</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>3</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>4</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>5</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>6</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>7</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>8</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>9</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
<tr>
<td>10</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Upto 1.5 mL</td>
</tr>
</tbody>
</table>
Table 3. Enzyme cocktail microassay 3: Different combinations of selected three purified enzymes and commercial enzyme cellulast

<table>
<thead>
<tr>
<th>No</th>
<th>Substrate</th>
<th>Sodium azide</th>
<th>Protein 1/20µg</th>
<th>Protein 5/20µg</th>
<th>Protein 6/20µg</th>
<th>Celluclast/20µg</th>
<th>Buffer (final volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Upto 1,5 mL</td>
</tr>
<tr>
<td>2</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Upto 1,5 mL</td>
</tr>
<tr>
<td>3</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Upto 1,5 mL</td>
</tr>
<tr>
<td>4</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Upto 1,5 mL</td>
</tr>
<tr>
<td>5</td>
<td>20 mg SEWS</td>
<td>10 uL</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Upto 1,5 mL</td>
</tr>
</tbody>
</table>

Secretomes of biodegrader microbial consortia are complex mixtures of several secreted proteins, including a suite of different glycosyl hydrolases. However, other microbial proteins may act as antagonists or degraders of the secreted enzymes, and so the actual activities of key glycosyl hydrolases may have been hampered. Given that the combination of secretome with Celluclast induced the conversion rate of SEWS up to 12%, increasing the concentration of particular secretome proteins in the Celluclast might increase the LCB degradative activity. Another limiting factor may have been the adsorption of the enzymes to the SEWS-exposed lignin and cellulose moieties [39]. The catalytic functions of the enzymes involved are controlled by structural conformation [40], and conformational modifications may come about as a result of the binding of small molecules, such as amino acids or nucleotides. For example “allosteric inhibition”, i.e. the binding of such a regulatory molecule, by changing the conformation of the protein structure, can swap the shape of the catalytic site and affect catalytic activity [40,41]. Such factors, although unstudied, may have reduced the activities of the secretomes.

Collectively, our findings highlight that key gains in enzymatic activity of a commercial enzyme preparation like Celluclast can be obtained by an educated guess with respect to additional enzymatic activities that may be required. Thus, the P1, P5 and P6 enzymes, when added to Celluclast, significantly increased the hydrolytic activity on SE wheat straw. Importantly, enzyme P1, which belongs to glycosyl hydrolase family 2 (GH2), was predicted to exert β-galactosidase activity, and P5 (and P6) were predicted to have α-glucosidase activities [42]. Interestingly, the β-galactosidase activity of P1, in concert with the Celluclast, appeared to spur the release of extra glucose, but not of galactose. It might be that the raw wheat straw has very low levels (e.g. 1-2%) of galactose, or that the prior steam explosion had removed it [43]. A previous study showed that Celluclast constitutes an effective enzyme mixture for cellulose and hemicellulose degradation, exhibiting synergistic interaction that fosters the release of arabinose and xylose from soluble...
wheat arabinoxylan [44]. Here, we hypothesized that the steam explosion treatment of the wheat straw reduced the traffic of cellulose moieties; thus Celluclast would easily attack the 1-4 linkage of D-glucose, releasing glucose. In addition, Celluclast may have interacted with the hemicellulose moiety, e.g. the xylan, on the b-1-4 link of D-xylose (main chain), thus releasing xylose as well as arabinose from the xyloglucan backbone (side chain). The additive effect of enzyme P1 (β-galactosidase) might be explained by its entering the hemicellulose moiety, in which side chains of xylose-glucose (of xyloglucan) were broken, increasing the release of xylose and glucose. Along with it, enzymes P5 and P6 (α-glucosidases) might have cleaved the region of 1→4 linked α-glucose residues to release α-glucose molecules. Therefore, the above findings indicate that our (hemi)cellulases assisted the Celluclast activity, enabling the release of enhanced sugars by cleaving hemicellulose parts. On the other hand, the untreated wheat straw consists of 19 to 21% xylan, and so (next to glucose) we recovered more xylose (and arabinose) in the combination of P1 with P5 or P6 (Figure 5).

Figure 5. HPAEC analysis a) chromatogram of released sugars b) amount of released sugars

Conclusion
In this study, we searched for enzymes that would be able to significantly enhance
the activity of the commercial enzyme mixture Celluclast on untreated versus steam-exploded wheat straw. Clearly, three enzymes denoted P1 (β-galactosidase, Tm 50°C, pH- 8.0, highly stable for 120 min), P5 (α-glucosidase, Tm 50°C, pH-10.0, highly stable for 120 min), and P6 (α-glucosidase, Tm 40°C, pH-10.0, highly stable for 120 min), significantly enhanced the degradation of SE wheat straw under the conditions (50 C; 24 h) used here. Thus the addition of the enzyme mixes P1+P5 and/or P1+P6 to the Celluclast enhanced the release of xylose (303%), arabinose (343%) and glucose (13%) from SE wheat straw, as compared to Celluclast alone.

Authors' contributions
MM designed, constructed the experiments and drafted the manuscript. MJL Brossi did enzyme cocktails assay. JDvE conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References


