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Characterisation of Specific Activities and Hydrolytic Properties of Cell-Wall-Degrading Enzymes Produced by *Trichoderma reesei* Rut C30 on Different Carbon Sources

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Abstract Conversion of lignocellulosic substrates is limited by several factors, in terms of both the enzymes and the substrates. Better understanding of the hydrolysis mechanisms and the factors determining their performance is crucial for commercial lignocelluloses-based processes. Enzymes produced on various carbon sources (Solka Floc 200, lactose and steam-pre-treated corn stover) by *Trichoderma reesei* Rut C30 were characterised by their enzyme profile and hydrolytic performance. The results showed that there was a clear correlation between the secreted amount of xylanase and mannanase enzymes and that their production was induced by the presence of xylan in the carbon source. Co-secretion of α-arabinosidase and α-galactosidase was also observed. Secretion of β-glucosidase was found to be clearly dependent on the composition of the carbon source, and in the case of lactose, 2-fold higher specific activity was observed compared to Solka Floc and steam-pre-treated corn stover. Hydrolysis experiments showed a clear connection between glucan and xylan conversion and highlighted the importance of β-glucosidase and xylanase activities. When hydrolysis was performed using additional purified β-glucosidase and xylanase, the addition of β-glucosidase was found to significantly improve both the xylan and glucan conversion.

Keywords *Trichoderma reesei* Rut C30 · Cellulase fermentation · Enzymatic hydrolysis · Accessory enzymes · Hemicellulases · β-Glucosidase

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Introduction

Cellulases are carbohydrate-hydrolysing enzymes that are of substantial industrial interest. Currently, however, the high market price of cellulases is one of the drawbacks in the attempt to commercialise the lignocellulosic ethanol process [1]. In addition to genetic and protein engineering, which aim to tailor enzyme properties and increase productivity, enzyme production on lignocelluloses as a carbon source is still an option for decreasing the enzyme cost. In addition to second-generation ethanol processes, cellulases are important in other industrial fields such as the food, brewery, textile, laundry, animal feed and pulp and paper industries [2].

The main components of lignocelluloses are cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer of glucose. Hemicelluloses are branched heteropolymers built up from C6 and C5 sugars. The backbone of xylan is built up of xylopyranose units linked with β-1,4 linkages, while the backbone of (galacto)glucomannan is built up of glucose and mannose units, linked by β-1,4 bonds [3]. Depending on the hemicellulose sources, the xylan backbone can be substituted with arabinose, glucuronic acid or acetic acid and glucomannan with galactose and acetic acid. The composition and structure of hemicelluloses depends on both the raw material and the processing conditions used. For efficient conversion, pre-treatment is necessary prior to utilisation in order to increase the accessibility of the enzymes [4]. One of the most studied pre-treatment methods is acid-catalysed steam explosion, where hemicellulose is partly hydrolysed and solubilised [5].

The most extensively studied cellulose-secreting microorganism is the filamentous fungus *Trichoderma reesei*, which is an anamorph of the pantropical ascomycete *Hypocrea jecorina* [6]. The term cellulase represents a broad group of enzymes with various specificities acting synergistically to hydrolyse cellulose [7]. Cellobiohydrolases (EC 3.2.1.91, Cel7A and Cel6A) attack the reducing and non-reducing ends of the cellulose chain, respectively, mainly releasing cellobiose. Endoglucanases (EC 3.3.1.4, the five major endoglucanases are Cel7B, Cel5A, Cel12A, Cel61A and Cel45A) randomly attack the amorphous regions of the cellulose chains, hydrolysing them into cello-oligomers. β-Glucosidase (EC 3.2.1.21, from glycosyl hydrolase families 1 and 3) acts on the cellobiose and cleaves it into glucose monomers. β-Glucosidase plays an important role in this synergistic action [8], as cellobiose is a strong inhibitor of cellobiohydrolases.

In addition to cellulases, a number of hemicellulases are needed for a complete hydrolysis of lignocellulosic substrates. Xylanases (EC 3.2.1.8) and mannanases (EC 3.2.1.78) are endo enzymes, hydrolysing the backbone of xylans or mannans, respectively. β-Xylosidases (EC 3.2.1.37) and β-mannosidases (EC 3.2.1.25) act on the solubilised xylo- or glucomannan-oligomers, respectively. Other debranching activities are performed by α-arabinosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), α-galactosidases (EC 3.2.1.22), ferrulic acid esterases (EC 3.1.1.73) and acetyl xylan esterases (EC 3.1.1.72) [9]. Most of these activities have also been characterised in *T. reesei* culture broths [3, 10].

In addition to the natural inducer cellulose, several small molecules such as sophorose [11], lactose, cellobiose and synthetic compounds (thiols or esters of disaccharides) have been found to be effective inducers [12]. Various lignocellulosic substrates have been investigated at the laboratory scale as inducers and carbon sources for production of cellulases [13]. The ratio of the enzymes secreted by the fungus is clearly dependent on the carbon source [10]. The *T. reesei* mutant Rut C30 is a parental strain of many presently used commercial strains [14]. It has been shown that Rut C30 is less sensitive to glucose repression than other mutants or the wild type, as it carries a mutant form of the *cre1* gene, which conveys glucose repression [15].

Sun and Cheng [16] have identified three main challenges in enzymatic hydrolysis: (1) substrate concentration, (2) hydrolytic performance of cellulases and (3) end product inhibition. However, end product inhibition can be reduced by simultaneous saccharification and fermentation process configuration. The effect of cellulases may be enhanced by applying an enzyme cocktail properly tailored to the substrate used [17]. Supplementation of cellulases with accessory enzymes (hemicellulases) seems to be beneficial. These enzymes can also enhance the action of cellulases, as already demonstrated in some cases [7, 17, 18]. β-Glucosidase can improve cellulose hydrolysis by decreasing end product inhibition, while xylanases or other hemicellulases can increase the accessibility of cellulose for cellulases [17].

The aim of the present work was to characterise fermentation broths produced by *T. reesei* Rut C30 on various carbon sources, namely Solka Floc 200, steam-pre-treated corn stover (SPCS) and lactose, by comparing the enzyme composition to the reference commercial enzyme Celluclast 1.5L. The enzyme pattern was investigated by different activity assays. Hydrolytic properties of the culture filtrates were evaluated by hydrolysis of Solka Floc 200, Avicel, SPCS and steam-pre-treated spruce substrates. The results have led to further understanding of the hydrolytic mechanisms and the factors determining the performance of different mixtures of *T. reesei* enzymes.

Materials and Methods

Substrates and Carbon Sources

Three different carbon sources were used in enzyme production: Solka Floc 200 (SF; International Fiber, New York, NY, USA), lactose-1-hydrate (Spektrum-3D, Hungary) and SPCS. SF is a commercially available delignified pine fibre product, manufactured from milled wood by several extraction steps. It has a significant xylan content [19], and it is commonly used as a model carbon source for cellulase production [1, 3, 13, 19]. Corn stover was steam-pre-treated at ENEA, Italy in a flow-through reactor at 190 °C for 5 min.

Enzymatic hydrolysis with the produced enzymes was performed on Solka Floc, SPCS, Avicel (pure microcrystalline cellulose, Serva, Heidelberg, Germany) and steam-pre-treated spruce (SPS). Spruce chips were pre-treated at Lund University, Department of Chemical Engineering, in a batch reactor after 20 min impregnation with 2.5% SO_2, at 210 °C for 5 min. Structural carbohydrates and lignin content of the substrates are presented in Table 1. Compositions were analysed using the NREL protocol, with minor modifications [20].

Microorganism and Inoculum Preparation

Freeze-dried conidia of *T. reesei* Rut C30 (ATCC56765) were obtained from the American Type Culture Collection. The stock culture of the strain Rut C30 was maintained on agar slants containing 50 g/L malt extract, 5 g/L glucose, 1 g/L proteose peptone and 20 g/L bacto agar. After 14 days of incubation at 30 °C, the greenish conidia were suspended in 5 mL sterile water, and 1.5 mL of this suspension was transferred to a 750-mL Erlenmeyer flask containing 150 mL sterile modified Mandels' medium [21]. The pH was adjusted to 5.6–5.8. Concentration of nutrients was 0.4 g/L urea, 1.9 g/L $(NH_4)_2SO_4$, 2.7 g/L KH_2PO_4, 0.5 g/L $CaCl_2{\cdot}2\ H_2O$, 0.8 g/L $MgSO_4{\cdot}7\ H_2O$, 0.3 g/L yeast extract, 1.0 g/L proteose peptone and 10.0 g/L lactose. The medium was supplemented with the following trace

Table 1 Composition of cellulosic carbon sources and hydrolysis substrates on percentage of the dry matter (DM).

	SF	SPCS	SPS
Glucan	75.9	34.2	46.0
Xylan	11.5	16.6	<0.1
Arabinan	<0.1	0.3	<0.1
Mannan	1.4	n.d.	<0.1
Galactan	<0.1	n.d.	<0.1
Acid insoluble lignin	0.2	20.7	n.d.
Ash	0.3	12.0	n.d.
Total determined compounds	89.3	83.7	46.0

Composition analysis was performed in triplicates, mean values are presented

n.d. not determined

elements: 6.6 mg/L $FeSO_4 \cdot 7\ H_2O$, 2.1 mg/L $MnSO_4 \cdot H_2O$, 1.9 mg/L $ZnSO_4 \cdot 7\ H_2O$, 26.7 mg/L $CoCl_2$. The shake flasks were incubated at 30 °C for 3 days on an orbital shaker (250 rpm).

Enzyme Production in Shake Flasks

An aliquot of 15 mL of 3-day-old inoculum culture was used to initiate growth in a 750-mL Erlenmeyer flask containing 150 mL of the modified Mandels' medium, as described above. To maintain a constant pH (5.8) in the flasks, a 0.1 M Tris base–maleic acid buffer system was applied [1, 22]. After inoculation, the flasks were incubated for 8 days at 28 °C and 250 rpm.

Purification of Enzymes

Purified β-glucosidase and Xyn11A (xylanase II) were used during the hydrolysis experiments when indicated. *Aspergillus* β-glucosidase (Mw 130 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 388 IU/mL activity) was purified from a commercial preparation (Novozym 188, Novozymes A/S, Bagsvaerd, Denmark) by a two-step chromatographic procedure, using columns and chromatography media available from GE Healthcare. The preparation was first desalted and equilibrated with 0.02 mM sodium citrate buffer (pH 3.5) using a Sephadex G-25C column. After that, the sample containing 35 g of protein was loaded in two identical runs on a SP Sepharose FF column (450 mL), equilibrated with 0.02 M sodium citrate and eluted using a gradient of 0–0.25 M sodium chloride. Protein fractions with high β-glucosidase activity that eluted in 0.12–0.18 M NaCl were pooled, concentrated by ultrafiltration (10,000 nominal MW cut-off), equilibrated with 0.02 mM sodium acetate buffer (pH 5.0) using a Sephadex G-25C and loaded (1.78 g protein) on a DEAE Sepharose FF column (390 mL) equilibrated by 0.02 mM sodium acetate buffer at pH 5.0. The fractions containing high β-glucosidase activity that eluted in a NaCl gradient at 0.16–0.18 M salt were combined and concentrated by ultrafiltration as above. In total, 0.70 g protein was recovered with an activity yield of 40% in the overall purification procedure.

Xyn11A (xylanase II), with a molecular weight of 23 kDa and an activity of 1860 IU/mL (in the purified fraction), was purified from *T. reesei* culture broth according to Tenkanen et

al. [23], omitting the last gel filtration step. Purification of *T. reesei* Cel7A (CBH I) and Cel6A (CBH II) used in the gel electrophoresis was carried out according to the method described previously [24].

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in a 5-mL reaction volume in test tubes with magnetic stirrers at an agitation speed of 400 rpm at 45 °C. Test tubes were plugged with plastic corks and sealed with parafilm to prevent evaporation. The substrate concentration was 10 g carbohydrate/L, and the pH was set to 5 using sodium citrate buffer. Enzyme was loaded at 10 filter paper units (FPU)/g carbohydrates. Four different enzymes were used: the commercial cellulase solution Celluclast 1.5L (Novozymes A/S, Bagsvaerd, Denmark) and *T. reesei* enzymes produced on the three different carbon sources. Samples were taken after 1, 2, 4, 8, 24 and 48 h. At each sampling time, three of the test tubes were placed into boiling water for 10 min and then centrifuged at 9,000 rpm. Supernatants were assayed for reducing sugars with the 2,4-dinitrosalycilic acid reagent [25].

In indicated experiments, β-glucosidase and xylanase activities were increased to equal the activities produced using lactose and SF (to 0.225 and 20.3 IU/mL) with the addition of purified enzymes.

In the hydrolysis samples taken after 0 and 48 h, one selected sample from the triplicate (based on released reducing sugars) was analysed for monomer sugars by high-performance liquid chromatography (HPLC), while in the case of the 8-h samples, soluble cello- and xylo-oligomers were measured in addition to monosaccharides. The applied HPLC system and method are described below.

Enzyme and Protein Assays

All enzyme activity assays were performed in 50 mM pH 5 sodium citrate buffer if not otherwise indicated. The filter paper activity (FPA) for the enzyme profile characterisation was measured according to the procedure recommended by IUPAC [26] and expressed as FPU.

Endoglucanase activity was assayed against hydroxy-ethyl cellulose (Fluka, Buchs, Switzerland) as recommended by IUPAC [26] according to the protocol of Bailey and Nevalainen [27].

Activities against 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma, St. Louis, MO, USA) were measured either with or without 50 mM cellobiose (Glu_2) [28, 29]. The activity obtained in the presence of cellobiose (MULac Glu_2) has generally been considered to be due to Cel7B (EG I). However, there may also be other enzyme components in the broths that act on MULac but are not inhibited by cellobiose. The activity obtained without cellobiose has been described as the combined activities of Cel7A (CBH I) and Cel7B (EG I), and the Cel7A (CBH I) activity was reported to be the agent that caused the difference between the activities on MULac and MULac (Glu_2). However, this consideration may not be reasonable due to the aforementioned reason. Activity against 4-methylumbelliferyl-β-D-cellotrioside ($MUGlu_3$, Sigma, St. Louis, MO, USA) was also measured. Cel5A (EG II) was reported to be the only known cellulase that acts on $MUGlu_3$; however, there might be other components in the broths that have this kind of activity. β-Glucosidase was inhibited with 1 M glucose addition in all cases when activity was assayed against 4-methylumbelliferyl derivatives. β-Glucosidase activity was measured using 1 mM 4-nitrophenyl-β-D-glucopyranoside (Merck, Hohenbrunn, Germany) as a substrate, according to Bailey and Nevalainen [27].

Xylanase [30] and mannanase [31] activities were measured on 1% xylan (birch glucuronoxylan, Roth, Karlsruhe, Germany) and 0.5% mannan (locust bean gum, Sigma, St. Louis, MO, USA) substrates, respectively. Xyloglucanase activity was assayed against 1% xyloglucan (Tamarind xyloglucan, Amyloid, Megazyme, Wicklow, Ireland) according to Benkő et al. [32]. α-Arabinosidase was measured using 2 mM 4-nitrophenyl-α-D-arabinofuranoside as a substrate in 50 mM pH 4 sodium citrate buffer [33]. β-Xylosidase activity was assayed using 5 mM 4-nitrophenyl-β-D-xylopyranoside substrate in 50 mM pH 4.5 sodium citrate buffer [34]. α-Galactosidase activity was detected using 1 mM 4-nitrophenyl-α-D-galactopyranoside [35]. Acetyl xylan esterase activity was measured against an acetylated xylo-oligomer (kindly supplied by Jürgen Puls, Hamburg, Germany), and the liberated acetic acid was assayed using an acetic acid test kit (Boehringer test, R-Biopharm AG, Darmstadt, Germany).

Protein content was assayed with the Coomassie Blue method [36] using BSA as standard.

Gel Electrophoresis

SDS-PAGE was run on 12% Bis–Tris pre-cast gels using the NuPAGE Bis–Tris Electrophoresis system (Invitrogen, Carlsbad, CA, USA). The gel was stained using colloidal Coomassie stain [37]. For the quantitative analysis of the culture supernatant samples, the gel was scanned and the picture was analysed with AlphaEaseFC™ software (Alpha Innotech Corporation, San Leonardo, CA, USA). The percentage of the individual protein components contributing to the total density was calculated from the integrated area of the peaks.

HPLC

Monosaccharides (glucose, xylose, galactose, mannose and arabinose) from the hydrolysates were determined with a Dionex ICS-3000 gradient HPLC system (Dinoex, Sunnyvale, CA, USA) using a CarboPac1 column with 0.3 mL/min eluent flow at 20 °C. Cello- and xylo-oligomers were detected using a 1 mL/min eluent flow at 30 °C. The solvents used were water, 100 mM NaOH, 300 mM Na-acetate-100 mM NaOH and 300 mM NaOH in a gradient with a flow rate of 1 mL/min. Standards were purchased from Fluka (monosaccharides), Serva (cellobiose) Seikagaku Kogyo (cello oligosaccharides) and Megazyme (xylo-oligomers).

Statistical Evaluation

Statistical evaluation of the results obtained was performed using Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA). Correlation analysis was performed at $p<0.05$.

Results and Discussion

According to the composition analysis data (Table 1), the content of glucose and xylose differed significantly depending on the substrates and carbon sources used. The glucan/xylan ratio in Solka Floc 200 was 6.6 (75.9% and 11.5%), whereas it was 2.05 (34.2% and 16.6%) in SPCS, and practically no xylan was detected in SPS. The high lignin content, though obvious, was not determined in this case. The composition of the pre-treated

materials is typical compared to the values reported elsewhere [3, 19] and they thus represent raw materials typically used in sugar platform biorefinery processes.

Enzyme Characteristics of the Produced Cellulase Mixtures

The cellulase activities measured in the culture filtrates after 8 days of fermentation and calculated as specific cellulase activities (based on their protein content) are presented in Fig. 1 and Table 2, respectively. The enzyme activity that is based on the protein content of the supernatant is referred to as specific activity. Enzymes produced on SF, lactose and SPCS are hereafter referred to as SF enzyme, lactose enzyme and SPCS enzyme, respectively. Protein content of the produced enzymes (Table 2) shows that SF and SPCS were much more efficient carbon sources for *T. reesei* as compared to lactose. The levels of secreted proteins were 2.5- to 2.8-fold higher on these two carbon sources than on lactose. However, the β-glucosidase/FPA ratio was highest (2.3) in the case of the lactose carbon source, which is favourable in enzymatic hydrolysis. *T. reesei* has previously been reported to have poor enzyme production in lactose-based minimal media [15]. The excretion of enzymes, however, is improved when supplemented with cellulosic substrates as additional inducers [38].

Enzymes used in this study were subjected to SDS-PAGE gel electrophoresis to detect the major differences in protein composition. In addition to the enzymes used (Celluclast, lactose, SF and SPCS enzymes), purified cellobiohydrolases (Cel6A and Cel7A) were loaded on the gel (Fig. 2). Quantitative analysis of the gel showed that cellobiohydrolase content (including both Cel6A and Cel7A) for Celluclast, SF, lactose and SPCS enzymes was found to be 55%, 52%, 60% and 54% of the total protein, respectively.

The specific cellulase activities were similar among the three enzymes produced. There was, however, a significant difference in specific cellulolytic power of the enzymes produced, as compared to Celluclast: the SF enzyme had 25%, the lactose enzyme had 31% and the SPCS enzyme had 45% higher specific filter paper activity than Celluclast. There was a slight difference in the overall endoglucanase activity (HEC). Thus, the SPCS

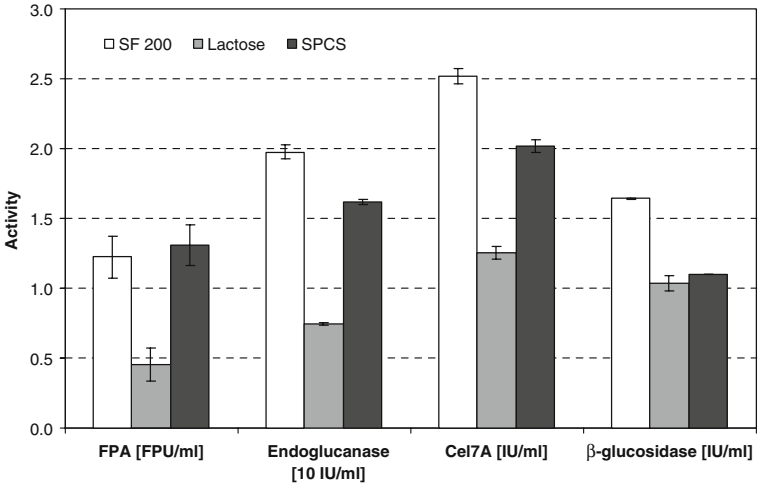

Fig. 1 Major (volumetric) cellulase activities in *T. reesei* Rut C30 culture broth after 8 days of fermentation. Mean values and standard deviation of triplicates are presented

Table 2 Protein content and specific cellulase activities in Celluclast 1.5L and enzymes produced on various carbon sources.

	Protein (mg/ml)	FPA (FPU/mg)	HEC (IU/mg)	MULac (Glu_2, IU/mg)	MULac (IU/mg)	$MUGlu_3$ (IU/mg)	BG (IU/mg)
Celluclast	39.17	1.07	21.63	0.86	3.04	1.23	0.69
SF	0.92	1.33	21.45	0.73	2.74	1.71	1.78
Lactose	0.33	1.40	22.72	0.64	3.83	1.68	3.17
SPCS	0.84	1.56	19.17	0.68	2.39	1.67	1.30

Mean values of triplicates are presented. Relative standard deviation was below 5% in all cases

SF Solka Floc, *SPCS* steam-pre-treated corn stover

enzyme had slightly lower specific endoglucanase activity compared to the others. The highest specific activity against MULac in the presence of cellobiose (probably due to Cel7B) was detected in Celluclast, and the produced enzymes were shown to be rather similar in terms of this aspect. The highest specific activity against MULac in the presence of cellobiose (which is expected to correspond to the Cel7B activity) was detected in Celluclast, and again, the produced enzymes proved to be rather similar in this respect. Activity against MULac, as inhibited by cellobiose (expected Cel7A activity), was the highest in the enzyme solution produced on lactose followed by Celluclast and enzymes produced on SF and SPCS. Celluclast had the lowest activity against $MUGlu_3$ (expected Cel5A activity), while the other three enzymes had similar activities.

The most important and significant difference can be found in the specific β-glucosidase (BG) activities. The activity was highest in the lactose enzyme, being 1.8- and 2.4-fold higher than in SF and SPCS enzymes, respectively, and 3.5-fold higher than the specific β-glucosidase activity of Celluclast. The β-glucosidase level has previously been described as a major factor in hydrolysis performed by cellulases [16]. This is also reflected in the lower filter paper activity of Celluclast.

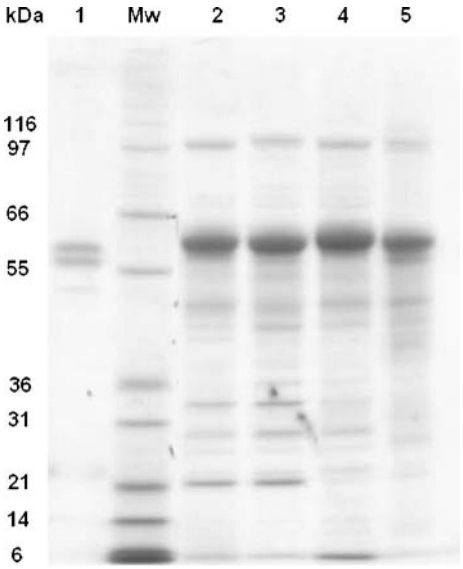

Fig. 2 SDS-PAGE analysis of the enzymes used, together with purified *T. reesei* Cel7A (CBH I) and Cel6A (CBH II). The samples are as follows: *1* purified *T. reesei* Cel7A and Cel6A, *2* SPCS enzyme, *3* SF enzyme, *4* lactose enzyme, *5* Celluclast 1.5L. The amounts of total protein loaded on the gel were 1.0 μg for both the purified Cel7A and Cel6A and varied between 3.0 and 3.5 μg for Celluclast and SPCS, SF and lactose enzymes. Staining was made with colloidal Coomassie Blue

Significant differences were observed in the hemicellulase activities (Fig. 3 and Table 3). The specific xylanase (XYL) activities of the enzymes produced on xylan-containing carbon sources were significantly higher than those produced on lactose or detected in Celluclast. Celluclast had the highest specific xyloglucanase (XGase) activity, more than 2-fold higher than in the enzymes produced in this work. Several enzymes have previously been reported to possess activity towards xyloglucan, including Cel7B (EG I), Cel12A (previously EG III) and Cel74A (xyloglucanase) [32]. The SF and SPCS enzymes had significantly higher (3- to 4-fold) mannanase (MAN) activities than Celluclast and the lactose enzymes. The SF enzyme had the highest specific acetyl xylan esterase (AXE) activity, followed by Celluclast and the SPCS enzyme. The lactose enzyme had the highest specific α-galactosidase (AG) activity, nearly 2-fold higher than the SPCS enzyme. In the case of Celluclast and SF enzyme, only a very low AG activity was detected. There were significant differences in β-xylosidase (BX) activities, as well. The highest BX activity was observed in the case of SF, followed by Celluclast. The other two enzymes (lactose and SPCS) had similar BX activities, at a quarter of the activity of the SF enzyme. In the case of α-arabinosidase (AA) activity, lactose and SPCS had the highest activity, which was 3- to 4-fold higher than the other two enzymes.

Induction of cellulase and hemicellulase secretion has already been investigated in detail both on the levels of transcription and secretion [3, 10, 14, 15, 39], and the most pronounced difference among the enzymes involved in cellulose degradation has been found in the case of β-glucosidase. Induction of cellulase secretion by lactose in particular has been investigated in detail [40, 41]. Here, it was found that *T. reesei* cannot take up lactose as it lacks lactose-permease and instead has to hydrolyse it by extracellular enzymes, then take it up as monomers [40]. Deletion of the galactokinase gene (*gal1*) from the Leroi pathway in *T. reesei* significantly decreased cellulase expression, which suggests that when lactose is used as a carbon source, cellulase expression is induced by an intermediate in the metabolic pathway of galactose [40]. The results suggest that the presence of galactose enhances the AG secretion of *T. reesei* (steam-pre-treated corn stover contains approx. 2–3% of galactan [42], and Solka Floc does not contain any [19]).

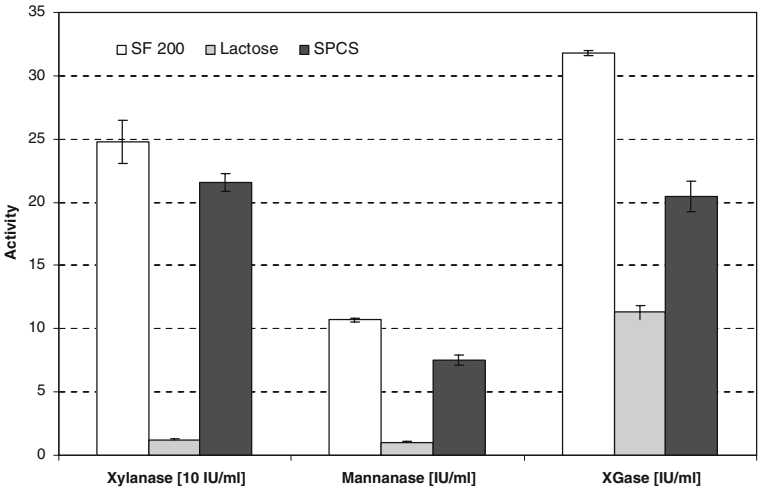

Fig. 3 Major (volumetric) hemicellulase activities in *T. reesei* Rut C30 culture filtrates after 8 days of fermentation. Mean values and standard deviation of triplicates are presented

Table 3 Specific hemicellulase activities (IU/mg) in Celluclast and enzymes produced on various carbon sources.

	XYL	XGase	MAN	AXE	AG	BX	AA
Celluclast	20.57	4.89	3.98	1.93	0.01	1.27	0.41
SF	269.42	2.07	11.61	2.88	0.09	2.09	0.53
Lactose	37.85	2.08	3.21	0.63	1.93	0.53	1.99
SPCS	255.63	1.46	8.89	1.66	0.99	0.48	1.43

Mean values of triplicates are presented. Relative standard deviation was below 5% in all cases

SF Solka Floc, *SPCS* steam-pre-treated corn stover

The secretion of α-arabinosidase showed a similar pattern to α-galactosidase activity. This correlation was found to be significant by statistical analysis (correlation coefficient was found to be 0.991). However, none of the carbon sources used contained a significant amount of arabinose. These results suggest that α-arabinosidase is co-secreted with α-galactosidase, as previously suggested by Juhász et al. [3].

Xylanase secretion was found to be dependent on the xylan content of the carbon source, which is in good agreement with previous results [3]. A low xylanase activity was also observed in the lactose-induced enzyme. Thus, the results suggest that there is constitutive xylanase production or that the xylanase activity is due to unspecific endoglucanases such as Cel7B, which are induced on lactose. As could be expected, the acetyl xylan esterase seemed to follow the same induction pattern as xylanases. The lactose enzyme had the lowest amount of protein produced among the tested enzymes.

On the other hand, β-xylosidase secretion did not correlate with the xylan content of the carbon source. The highest β-xylosidase activity was observed in case of SF enzyme, but it was poor in the case of SPCS and lactose enzymes.

Secretion of mannanase had a similar induction pattern as xylanase activity. This correlation was found to be significant by statistical analysis (correlation coefficient was calculated to be 0.963). In the case of carbon sources containing hemicellulose (SF and SPCS), 2- to 3-fold higher mannanase activities were observed compared to the lactose-induced enzyme or to Celluclast. However, the mannanase activity cannot be correlated to mannan content, as SPCS was reported to contain no mannan [42], and in SF, mannan comprises only 1.4%. It has been previously reported that the presence of monosaccharides represses the production of β-mannanase [15], and no expression of the mannanase encoding gene *man1* was observed when cultivating *T. reesei* on lactose. This can explain the low mannanase activity in the lactose-induced enzyme.

The results show that native biomass materials can be used successfully as inducers in cellulase production; however, in a large-scale process, insolubility and quality variation should be taken into consideration. The results also indicate that if these kinds of mixtures could be produced commercially and feasibly, improved hydrolysis efficiency as compared to traditional preparations based on *Trichoderma* strains could be obtained.

Enzymatic Hydrolysis

Four substrates (SF, SPCS, Avicel and SPS) were hydrolysed by the three on-site produced *T. reesei* enzymes and by Celluclast. The enzyme dosage was based on filter paper activity (10 FPU/g of carbohydrates). The other corresponding enzyme activities in the hydrolysis

are shown in Table 4 for comparison. The most significant differences can be observed in the cases of β-glucosidase and xylanase activities.

In the hydrolysis of Solka Floc, the SF and SPCS enzymes clearly performed best, based on reducing sugar released (Fig. 4a). In terms of glucan conversion after 48 h, the SF and lactose enzymes were found to be superior to Celluclast and SPCS enzyme (Table 5), while in xylan hydrolysis, the SPCS enzyme resulted in the highest conversion. As there was a significant difference between the performance of various enzymes after 8 h of hydrolysis (especially when steam-pre-treated corn stover was hydrolysed), these samples were analysed in more detail. Thus, during the early stage hydrolysis of Solka Floc (Figs. 5 and 6), cellobiose accumulation was observed, especially with enzymes poor in β-glucosidase activity. Analogously, xylobiose and xylotriose accumulation was observed with lactose and SPCS enzymes, which were deficient in β-xylosidase.

The SF enzyme released the highest amount of reducing sugars from the SPCS substrate after 8 h. This was due to the rapid xylan hydrolysis (62% conversion). However, at the end of the hydrolysis (after 48 h), the difference between the enzymes was within the experimental error (Fig. 4b). The lactose enzyme resulted in the highest glucan conversion (80%), while the highest xylan conversion (72%) was achieved using the SF enzyme (Table 5). Interestingly, the lactose enzyme could efficiently hydrolyse xylan to xylose from the SPCS substrate but not from Solka Floc. Significant xylobiose accumulation was observed by hydrolysis with the lactose enzyme at the beginning of the hydrolysis (Fig. 6), especially when using Solka Floc. It has previously been observed that substitution of the xylo-oligomer influences the action of the β-xylosidase on the substrate [43, 44]. In the case of the lactose enzyme, the low level of β-xylosidase may be an explanation for the poor xylan hydrolysis.

There were only minor differences observed in the release of reducing sugars, when Avicel was hydrolysed with the four enzymes (Fig. 4c). SF and lactose enzymes resulted in slightly higher glucan conversion (50–52%) compared to Celluclast and SPCS enzymes (44–45%; Table 5). At the beginning of the hydrolysis, the highest glucan conversion was achieved using the lactose enzyme, while with the other three enzymes, accumulation of cellobiose was more pronounced (Fig. 5). Avicel is microcrystalline cellulose; thus, cellobiohydrolases play the main role in the hydrolysis of this substrate. Obviously, there was no major difference in the overall cellobiohydrolase activity of the enzyme dosages used. The results also indicate that the FPA activity, which was the basis of dosing, corresponds well to hydrolysis performance on Avicel. The effect of β-glucosidase was less pronounced, probably due to the high crystallinity of the substrate. Thus, cellobiohydrolase obviously displayed as the limiting activity in the hydrolysis of Avicel.

Table 4 Calculated enzyme activities with FPA dosage of 0.5 FPU.

	Protein	HEC	MULac (Glu_2)	MULac	$MUGlu_3$	BG	XYL	MAN	AXE	AG	BX	AA
Celluclast	0.46	10.07	0.40	1.42	0.57	0.32	9.58	1.85	0.90	0.01	0.59	0.19
SF	0.38	8.07	0.28	1.03	0.64	0.67	101.35	4.37	1.08	0.03	0.79	0.20
Lactose	0.36	8.13	0.23	1.37	0.60	1.13	13.54	1.15	0.23	0.69	0.19	0.71
SPCS	0.32	6.16	0.22	0.77	0.54	0.42	82.15	2.86	0.53	0.32	0.15	0.46

Protein content and enzyme activities are presented as protein (mg) and as IU in the hydrolysis mixture, respectively. Hydrolysis was performed using 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C and pH 5

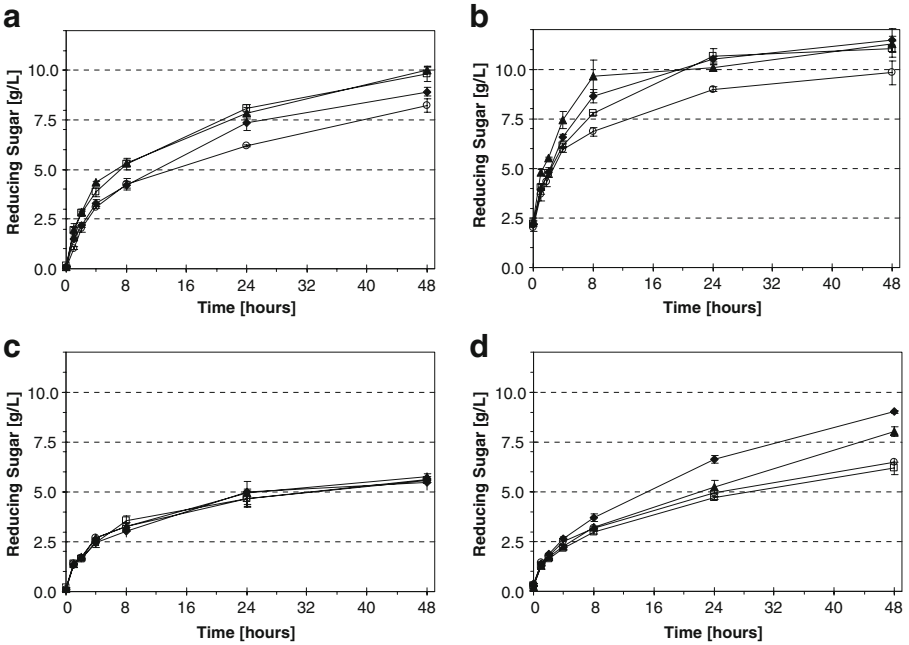

Fig. 4 Release of reducing sugars during hydrolysis of Solka Floc (**a**), steam-pre-treated corn stover (**b**), Avicel (**c**) and steam-pre-treated spruce (**d**). Enzymes were Celluclast (*circles*), SF enzyme (*triangles*), lactose enzyme (*diamonds*) and SPCS enzyme (*squares*). Mean values of triplicate experiments and standard deviations are presented. Hydrolysis was performed using a substrate concentration corresponding to 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C and pH 5

Steam-pre-treated spruce was the only lignocellulosic substrate used that does not contain any xylan and is therefore a suitable substrate to investigate the efficiency of cellulases alone. Even though cellulose is the main carbohydrate fraction in both SPS and Avicel, the enzymes performed in different ways. This may be due to the different crystallinity of the substrates and the presence of lignin in SPS. On steam-pre-treated spruce, the lactose enzyme performed most efficiently for all output measurements, i.e., in releasing reducing sugars (Fig. 4d) and in glucan conversion after both 8 and 48 h (Table 5 and Fig. 5). The SF enzyme performed slightly better than Celluclast and the SPCS

Table 5 Glucan and xylan conversions to glucose and xylose after 48-h hydrolysis.

	Substrate	Enzyme			
		Celluclast (%)	SF (%)	Lactose (%)	SPCS (%)
Glucan	Solka Floc	62.8	73.2	73.3	61.7
	Steam-pre-treated corn stover	55.9	75.1	80.6	65.9
	Avicel	44.3	50.4	51.8	45.4
	Steam-pre-treated spruce	32.7	48.2	59.8	31.6
Xylan	Solka Floc	62.2	74.6	30.2	88.2
	Steam-pre-treated corn stover	59.8	71.8	63.2	68.9

Hydrolysis was performed using 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C and pH 5

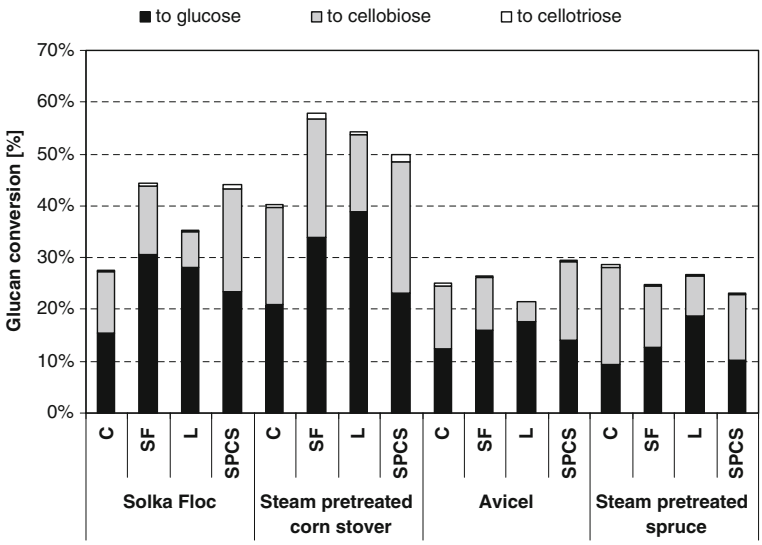

Fig. 5 Conversion of glucan (in Solka Floc, SPCS, Avicel and SPS) to glucose, cellobiose and cellotriose after 8-h hydrolysis with various enzymes (C, SF, L and SPCS). Hydrolysis was performed using 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C and pH 5

enzyme. It seems that the most important factor in the hydrolysis of SPS is the β-glucosidase activity present in the enzyme mixture. During the early stages of hydrolysis of SPS, there were minor differences between the enzymes that were reflected in cellobiose accumulation, which clearly depended on the β-glucosidase activity of the enzymes.

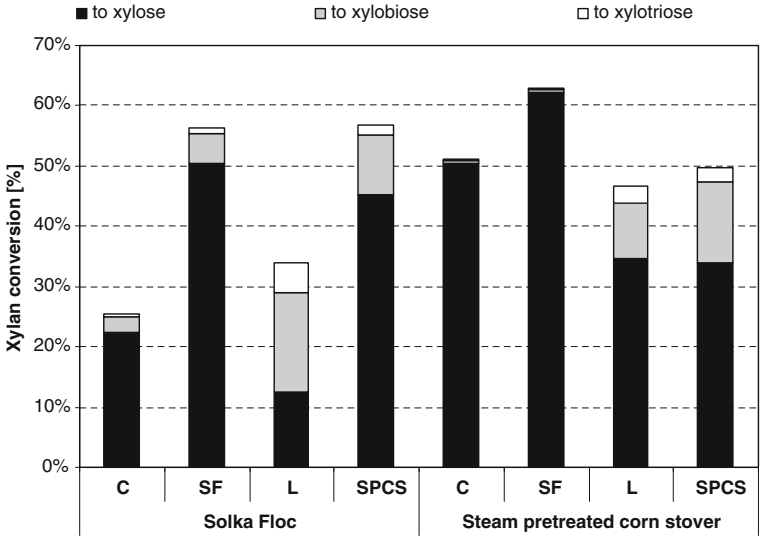

Fig. 6 Conversion of xylan (in Solka Floc and SPCS) to xylose, xylobiose and xylotriose after 8 h of hydrolysis with various enzymes (C, SF, L and SPCS). Hydrolysis was performed using 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C and pH 5

The correlation between xylan and glucan conversion after 8 h of hydrolysis is shown in Fig. 7. Xylobiose and cellobiose were also calculated as hydrolysis products. Statistical analysis proved that the correlation between these variables was significant at $p<0.05$ (correlation coefficient was found to be 0.80), showing the strong association between cellulose and hemicellulose.

Analysis of the correlation between conversions after 8 h of hydrolysis (to monomers and dimers) and the activities in the hydrolysis was also performed. It was observed that, in the case of glucan conversion, the only enzyme activity that significantly correlated with this conversion was that of β-glucosidase (correlation coefficient was found to be 0.91). This suggests that there were only minor differences between the cellulase activities when enzymes were loaded based on FPA. It also confirms that cellobiohydrolases are especially inhibited by cellobiose, leading to accumulation of the glucose dimer that result in a decrease of overall conversion. In the case of xylan conversion, xylanase and mannanase activities were found to correlate with the overall conversion (correlation coefficients were found to be 0.86 and 0.80, respectively). Clearly, as the substrates did not contain mannan, xylanase was the major activity affecting the conversion of xylan.

In the hydrolysis experiments, enzymes produced in our laboratory performed better than commercial Celluclast. An evident reason for this is the higher β-glucosidase activity (Table 3). Based on the hydrolysis results, high xylanase activity seems to be very important in substrates that contain xylan (see Table 3 and Fig. 4)

Enzymatic Hydrolysis with Enzymes Supplemented with Purified Xylanase and β-Glucosidase

Further hydrolysis experiments were performed to better understand the importance of xylanase and β-glucosidase levels during hydrolysis. Therefore, xylanase and

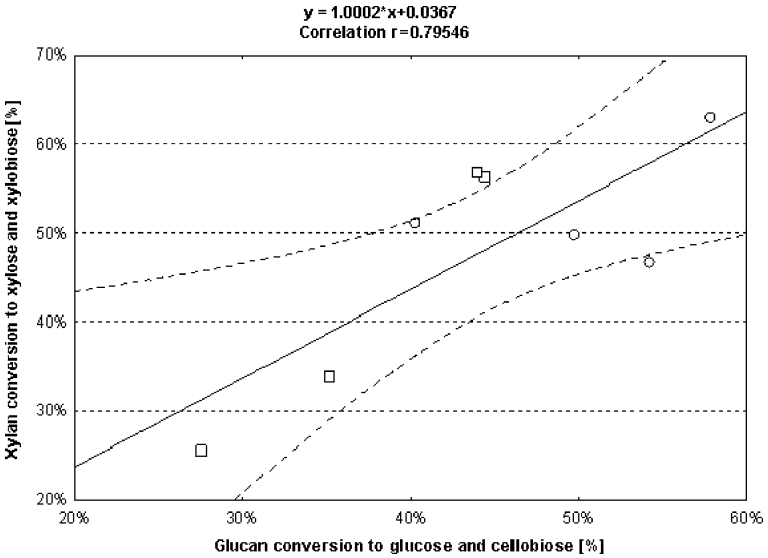

Fig. 7 Correlation between glucan and xylan conversion after 8-h hydrolysis of Solca Floc (*squares*) and steam-pre-treated corn stover (*circles*) substrates at 95% confidence interval. Hydrolysis was performed using 10 mg carbohydrate/mL and 10 FPU/g carbohydrates at 45 °C using pH 5

β-glucosidase activities were set to the level of the highest enzyme activity by addition of purified enzyme components. Addition of purified β-glucosidase to the level of the lactose enzyme increased the BG activity of Celluclast, SF and SPCS enzymes by 250%, 70% and 170%, respectively. Applying additional xylanase—enough to reach the level of SF enzyme—increased the xylanase activity of Celluclast and SPCS enzyme by 960% and 23%, respectively. Addition of β-glucosidase has notably increased the original filter paper activities (10 FPU/g carbohydrates in the first case) by 45–50% in the case of Celluclast and 5–15% in the case of SPCS enzyme, which is in good agreement with previous results [45]. Xylanase supplementation had no significant effect on the FPA. Whatman no. 1 filter paper, which is the substrate in the FPA assay, contains 96.1% glucan and trace amounts of mannan, but neither xylan nor arabinan.

Conversion of glucan and xylan in SPCS by Celluclast, SPCS and SF enzymes supplemented with the purified enzymes is shown in Fig. 8. When β-glucosidase was supplemented, both glucan and xylan conversion increased significantly compared to the references with the original enzymes. When purified xylanase was added, significant increase in the xylan conversion was observed in all cases. Expectedly, there was a less significant effect on the amount of released glucose. Notably, when both accessory enzymes were added to the hydrolysis, the conversion of glucan increased further, whereas xylan conversion was not improved from the level in which β-glucosidase or xylanase were supplemented alone.

The effect of β-glucosidase on xylan conversion can be explained by the inhibitory effect of cellobiose on xylanase activity. Leggio and Pickersgil [46] have shown that activity of an endoxylanase (XylA from *Pseudomonas fluorescens*) is inhibited by short xylo-oligomers, xylobiose and cellobiose. These results also suggest the existence of synergy between xylan and cellulose hydrolysis, as already shown in Fig. 7. This effect is possibly due to the structure of substrates and the close contact of xylan and cellulose in the fibre matrix, which would allow the improved hydrolysis of either one to help the hydrolysis of the other.

When steam-pre-treated spruce was hydrolysed, addition of β-glucosidase resulted in a significant increase in cellobiose hydrolysis, as well as in overall glucan conversion (Fig. 9). Xylanase addition had no significant effect on the glucan conversion but a significant amount of cellobiose was observed compared to the experiments with β-glucosidase addition. The results are well explained by the inhibiting effect of cellobiose on cellulases, resulting in decreased glucan conversion.

The performance of the traditional commercial preparation could be improved by boosting it with additional xylanase by using pre-treated substrates containing less residual xylan or by boosting with β-glucosidase. The deficiency of β-glucosidase in *T. reesei* broths is a well-known fact [3, 45]. The effect of β-glucosidase and xylanase seemed to almost completely explain the difference between the commercial preparation and the induced enzymes (Fig. 8), in spite of the fact that the loaded EG and MULac activities were lower (Table 4). However, differences in the profile of the minor side activities, like acetyl xylan esterase, which had significant differences, may still play a role in improved hydrolysis. The effect of these helper enzymes needs to be studied in more detail.

Conclusion

The need to produce large amounts of cellulases in full scale biomass-to-ethanol units at mill site motivates research to study the suitability and efficiency of real substrates for

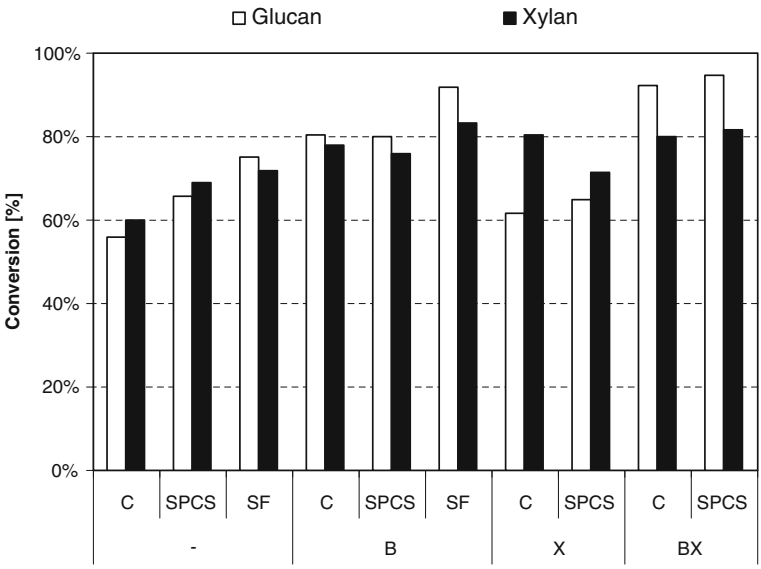

Fig. 8 Conversion of steam-pre-treated corn stover into glucose (*white*) and xylose (*black*) using Celluclast (*C*), SPCS and SF enzymes with supplementation of purified β-glucosidase (*B*) and/or xylanase (*X*) after 48-h hydrolysis. As reference, conversion data with the original enzymes (*dash*) are also presented. Hydrolysis was performed using 10 mg carbohydrate/mL at 45 °C using pH 5

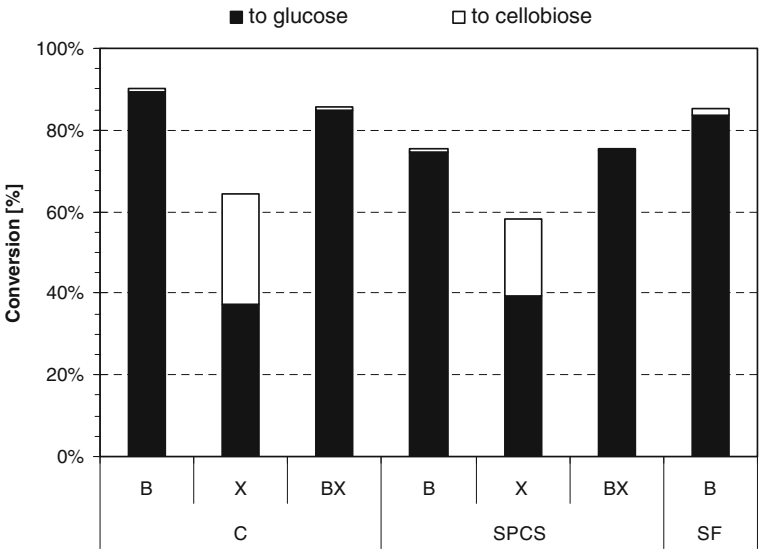

Fig. 9 Conversion of steam-pre-treated spruce into glucose and cellobiose after 48-h hydrolysis using Celluclast (*C*), SPCS and SF enzymes with supplementation of purified β-glucosidase (*B*) and/or xylanase (*X*). Hydrolysis was performed using 10 mg carbohydrate/mL at 45 °C using pH 5

enzyme production. Cell-wall-degrading enzymes of *T. reesei* Rut C30 were produced on three different carbon sources (Solka Floc, steam-pre-treated corn stover and lactose) and compared to commercial Celluclast 1.5L. The commercial preparation Celluclast has been most frequently used as reference enzyme in various studies on lignocellulose hydrolysis. It contains a fairly complete set of cellulases but is considered to be deficient in β-glucosidase. As shown also in this work, the major enzymes degrading crystalline cellulose, cellobiohydrolases, were the main enzymes in this preparation. The CBHs also represented the major proteins in the produced preparations, whereas there were more striking differences in the minor activities, obviously causing significant differences among the enzymes studied.

Enzyme characteristics were found to depend on the carbon source, especially in case of hemicellulases. All enzymes produced by *T. reesei* Rut C30 had higher specific filter paper activity than the commercial enzyme. The induction of cellulases by lactose has been studied previously, and lactose obviously induces a fairly efficient palet of cellulases, including β-glucosidase. In this work, lactose was also found to be an efficient inducer for cellulases, especially when evaluated on the basis of specific activities. The protein amount secreted on lactose was, however, lower than on other substrates. On the other hand, the induction of hemicellulases was poor on lactose. These characteristics were also reflected in the comparison of enzymes; the lactose-induced enzyme converted most efficiently cellulose fractions to glucose. When the carbon source contained hemicellulose, hemicellulases were induced and enzymes produced had especially high xylanolytic activities. These enzymes hydrolysed lignocellulosic substrates at higher extent than those induced on low hemicellulose containing substrates.

Interestingly, it could be thus demonstrated that the enzyme profiles produced on inducing carbon sources to be used as the substrate for hydrolysis were efficient in hydrolysis and even outperformed the commercial enzyme, Celluclast. The fungus obviously is able to express enzyme profiles based on the composition of the growth substrate, and the performance of these enzymes outweighs other enzymes when used for the hydrolysis of analogous substrates. This conclusion is especially valid when the lower amounts of β-glucosidase were compensated by supplementation of purified enzyme. Addition of β-glucosidase improved the release of both glucose and xylose, but xylanase addition did not significantly improve the release of glucose. These results also suggest synergistic effects of xylan and cellulose hydrolysis, due to the structure of substrates and the close contact of xylan and cellulose in the fibre matrix.

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References

1. Juhász, T., Szengyel, Z., Szijártó, N., & Réczey, K. (2004). *Applied Biochemistry and Biotechnology, 113*, 201–211.
2. Bhat, M. K. (2000). *Biotechnology Advances, 18*, 355–383.
3. Juhász, T., Szengyel, Z., Réczey, K., Siika-aho, M., & Viikari, L. (2005). *Process Biochemistry, 40*, 3519–3525.
4. Ramos, L. P. (2003). *Quimica Nova, 26*, 863–871.

5. Palmqvist, E., Hahn-Hägerdal, B., Galbe, M., Larsson, S., Stenberg, K., Szengyel, Z., et al. (1996). *Bioresource Technology, 58*, 171–179.
6. Kuhls, K., Lieckfeldt, E., Samuels, G. J., Kovacs, W., Meyer, W., Petrini, O., et al. (1996). *Proceedings of the National Academy of Sciences of the United States of America, 93*, 7755–7760.
7. Selig, M. J., Knoshaug, E. P., Adney, W. S., Himmel, M. E., & Decker, S. R. (2007). *Bioresource Technology, 99*, 4997–5005.
8. Wilson, C. A., McCrae, S. I., & Wood, T. M. (1994). *Journal of Biotechnology, 37*, 217–227.
9. Sørensen, H. R., Pedersen, S., & Meyer, A. S. (2007). *Enzyme and Microbial TechnologyEnzyme Microb Technol, 40*, 908–918.
10. Warzywoda, M., Larbra, E., & Pourquié, J. (1992). *Bioresource Technology, 39*, 125–130.
11. Sternberg, D., & Mandels, G. R. (1979). *Journal of Bacteriology, 139*, 761–769.
12. Bhat, M. K., & Bhat, S. (1997). *Biotechnology Advances, 15*, 583–620.
13. Réczey, K., Szengyel, Z., Eklund, R., & Zacchi, G. (1996). *Bioresource Technology, 57*, 25–30.
14. Olsson, L., Christensen, T. M. I. E., Hansen, K., & Palmquist, E. A. (2003). *Enzyme and Microbial Technology, 33*, 612–619.
15. Margolles-Clark, E., Ilmén, M., & Pentillä, M. (1997). *Journal of Biotechnology, 57*, 167–179.
16. Sun, Y., & Cheng, J. (2002). *Bioresource Technology, 83*, 1–11.
17. Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., et al. (2005). *Enzyme and Microbial Technology, 37*, 175–184.
18. Tabaka, M. G., Herpoël-Gimbert, I., Monod, F., Asther, M., & Sigoillot, J. C. (2006). *Enzyme and Microbial Technology, 39*, 897–902.
19. Wood, T. M. (1997). *Methods in Enzymology, 160*, 19–25.
20. Sluiter, A. (2006). *Determination of structural carbohydrates and lignin in biomass, laboratory analytical protocol*. Golden: National Renewable Energy Laboratory.
21. Mandels, M., & Weber, J. (1969). *Advances in Chemistry Series, 95*, 391–414.
22. Juhász, T., Egyházi, A., & Réczey, K. (2005). *Applied Biochemistry and Biotechnology, 121–124*, 243–254.
23. Tenkanen, M., Puls, J., & Poutanen, K. (1992). *Enzyme and Microbial Technology, 14*, 566–574.
24. Suurnäkki, A., Tenkanen, M., Siika-aho, M., Niku-Paavola, M.-L., Viikari, L., & Buchert, J. (2000). *Cellulose, 7*, 189–209.
25. Bernfeld, P. (1955). In S. P. Colowick & N. O. Kaplan (Eds.), *Methods in enzymology* (pp. 149–158). New York: Academic.
26. Ghose, T. K. (1987). *Pure and Applied Chemistry, 59*, 257–268.
27. Bailey, M. J., & Nevalainen, K. M. H. (1981). *Enzyme and Microbial Technology, 3*, 153–157.
28. van Tilbeurgh, H., Clayssens, M., & de Bruyne, C. K. (1982). *FEBS Letters, 149*, 152–156.
29. van Tilbeurgh, H., Loontiens, F. G., de Bruyne, C. K., & Clayssens, M. (1988). *Methods in Enzymology, 160*, 45–59.
30. Bailey, M. J., Biely, P., & Poutanen, K. (1992). *Journal of Biotechnology, 23*, 257–270.
31. Stålbrand, H., Siika-Aho, M., Tenkanen, M., & Viikari, L. (1993). *Journal of Biotechnology, 29*, 229–242.
32. Benkő, Z., Siika-aho, M., Viikari, L., & Réczey, K. (2008). *Enzyme and Microbial Technology, 43*, 109–114.
33. Poutanen, K., Rättö, M., Puls, J., & Viikari, L. (1987). *Journal of Biotechnology, 6*, 49–60.
34. Poutanen, K., & Puls, J. (1988). *Applied Microbiology and Biotechnology, 28*, 425–432.
35. Bailey, M., & Linko, M. (1990). *Journal of Biotechnology, 16*, 57–66.
36. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
37. Neuhoff, V., Arold, N., Taube, D., & Ehrhardt, W. (1988). *Electrophoresis, 9*, 255–262.
38. Benkő, Z., Drahos, E., Szengyel, Z., Puranen, T., Vehmaanperä, J., & Réczey, K. (2007). *Applied Biochemistry and Biotechnology, 136–140*, 195–204.
39. Martinez, D., et al. (2008). *Nature Biotechnology, 26*, 553–560.
40. Seiboth, B., Pakdaman, B. S., Hartl, L., & Kubicek, C. P. (2007). *Fungal Biological Reviews, 21*, 42–48.
41. Karaffa, L., Fekete, E., Gamauf, C., Szentirmai, A., Kubicek, C. P., & Seiboth, B. (2006). *Microbiology, 152*, 1507–1514.
42. Öhgren, K., Galbe, M., & Zacchi, G. (2005). *Applied Biochemistry and Biotechnology, 124*, 1055–1068.
43. Hermann, M. C., Vrsanska, M., Jurickova, M., Hirsch, J., Biely, P., & Kubicek, C. P. (1997). *Biochemical Journal, 321*, 375–381.
44. Tenkanen, M., Luonter, E., & Teleman, A. (1996). *FEBS Letters, 399*, 303–306.
45. Szengyel, Z., Zacchi, G., Varga, A., & Réczey, K. (2000). *Applied Biochemistry and Biotechnology, 84–86*, 679–691.
46. Leggioa, L. L., & Pickersgill, R. W. (1999). *Enzyme and Microbial Technology, 25*, 701–709.