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A homologous production system for *Trichoderma reesei* secreted proteins in a cellulase-free background

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Abstract Recent demands for the production of biofuels from lignocellulose led to an increased interest in engineered cellulases from *Trichoderma reesei* or other fungal sources. While the methods to generate such mutant cellulases on DNA level are straightforward, there is often a bottleneck in their production since a correct posttranslational processing of these enzymes is needed to obtain highly active enzymes. Their production and subsequent enzymatic analysis in the homologous host *T. reesei* is, however, often disturbed by the concomitant production of other endogenous cellulases. As a useful alternative, we tested the production of cellulases in *T. reesei* in a genetic background where cellulase formation has been impaired by deletion of the major cellulase transcriptional activator gene *xyr1*. Three cellulase genes (*cel7a*, *cel7b*, and *cel12a*) were expressed under the promoter regions of the two highly expressed genes *tef1* (encoding translation elongation factor 1-alpha) or *cdna1* (encoding the hypothetical protein Trire2:110879). When cultivated on D-glucose as carbon source, the Δ*xyr1* strain secreted all three cellulases into the medium. Related to the introduced gene copy number, the *cdna1* promoter appeared to be superior to the *tef1* promoter. No signs of proteolysis were detected, and the individual cellulases could be assayed over a background essentially free of other cellulases. Hence this system can be used as a vehicle for rapid and high-throughput testing of cellulase muteins in a homologous background.

Keywords Cellulase · Recombinant protein production · *Hypocrea jecorina* · *xyr1* · *cDNA1* · *tef1*

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Introduction

Lignocellulose, composed mainly of cellulose, hemicellulose, and lignin, is the most abundant renewable carbon source on earth and therefore an attractive resource to use it for the production of different chemical building blocks or biofuels. Due to the complex structure and the recalcitrance of lignocellulosic feedstocks, the conversion to their monomers is much more difficult to achieve than using, e.g., starch-based feedstocks (Himmel et al. 2007). As a result, the breakdown of the polymeric sugars, especially cellulose, requires large amounts of enzymes (Merino and Cherry 2007). Recent demands to reduce the production costs for biofuels from lignocellulosic plant matter led to a general renaissance in cellulase research (Carroll and Somerville 2009; Wilson 2009; Kumar et al. 2008). Fungal cellulases are attractive for enzymatic cellulose conversion as they are highly active and can be expressed at levels exceeding 100 g per liter in fungal hosts such as *Trichoderma reesei* (*Hypocrea jecorina*, Cherry and Fidantsef 2003; Kubicek et al. 2009).

To date, many important commercial enzyme preparations for biomass conversion of cellulose or hemicelluloses are from *T. reesei*. These *T. reesei* cellulase mixes include different types of cellulases including cellobiohydrolases (CEL7A and CEL6A), endoglucanases (e.g., CEL7B and CEL6B) and different β-glucosidases (e.g., CEL3A) which synergistically degrade cellulose (Henrissat et al. 1985).

While the economic production of cellulosic ethanol can be tackled by various approaches, protein engineering of cellulases to produce enzymes with improved catalytic activity (K_{cat}) or enzyme stability is a major issue. In addition, different hosts and environments are screened for novel cellulase activities (Zhang et al. 2006). The engineering of enzymes by rational design or directed evolution uses different methodologies to optimize existing enzymes by construction of mutant libraries followed by their testing to identify mutant proteins with improved properties. This involves, e.g., the mutagenesis of potential active center residues and their subsequent kinetic analysis and is based upon the identification of the invariant residues identified in the sequence-based CAZy family classification (http://www.cazy.org; Cantarel et al. 2009). While the methods to generate such mutant libraries are straightforward, there is a bottleneck in the testing and production of the mutant enzymes. Today, such muteins can be routinely expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*, but the functional requirement for modifications such as disulfide bridges or delicate *N*- and *O*-glycosylation present at, e.g., the linker domains of the cellulase proteins render most of these expression hosts inappropriate. Examples are the production of the cellobiohydrolase CEL7A in *P. pastoris* and *S. cerevisiae* which resulted in hyper- or overglycosylated enzymes with compromised activities (Boer et al. 2000; Godbole et al. 1999; Jeoh et al. 2008; Penttilä et al. 1988; Van Arsdell et al. 1987). The alternative—to produce these mutant enzymes in *T. reesei* itself—is hampered by the fact that traces of native cellulases are even produced on cellulase-noninducing carbon sources or during sporulation. The resulting protein is therefore contaminated with other enzyme activities which render the kinetic analysis prone to errors (Carle-Urioste et al. 1997).

A useful alternative would be the production of mutant cellulases in a host whose cellulase formation has been impaired. Cellulase induction in *T. reesei* is dependent on the function of the Zn_2Cys_6 transcriptional regulator XYR1, and knockout mutants in *xyr1* are cellulase negative (Stricker et al. 2006). In addition, these cellulases are even not produced during sporulation in such a Δ*xyr1* strain (Metz et al. 2011). However, it is not yet known whether XYR1 also influences other traits related to cellulase formation such as protein transport or secretion in *T. reesei*. For example a comparison of the transcriptomic response of an *Aspergillus oryzae* overproducer of the orthologous *xlnR* to an *xlnR* deletion strain did not only reveal changes in the expression pattern of various glycoside hydrolases and genes encoding for pentose catabolism but also changes in various transport processes (Noguchi et al. 2009).

In this paper, we therefore tested the hypothesis that a *xyr1*-knockout strain could be used as a production host for individual cellulase components under constitutive expression signals. Our data show that this strain is a useful vehicle for production of pure cellulases for kinetic analysis.

Materials and methods

Strains and culture conditions *T. reesei* QM9414 (ATCC 26921) and the Δ*xyr1* strain (Stricker et al. 2006) derived from it were used throughout this study. They were maintained on potato dextrose agar (PDA) plates at 28°C. For cellulase expression strains were grown for the indicated time in 250 mL medium in 1 L flasks at 28°C on a rotary shaker at 250 rpm (Multitron 2, Infors AG). A modified medium (Vaheri et al. 1979) containing 10 g/L carbon source, 1.4 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L peptone, 5 mM urea, 0.02% (*w*/*v*) Tween80, and 1/50 (*v*/*v*) of the trace element solution (0.25 g/L $FeSO_4 \cdot 7H_2O$, 0.08 g/L $MnSO_4 \cdot H_2O$, 0.07 g/L $ZnSO_4 \cdot 7H_2O$, 0.1 g/L $CoCl_2 \cdot 2H_2O$) was adjusted to pH 5.0 by citric acid and not further controlled.

Expression plasmid construction and fungal transformation Vectors for expression of *cel7a* (*cbh1*), *cel7b* (*egl1*), and *cel12a* (*egl3*) under the *tef1* (translation elongation factor 1 alpha) promoter were constructed by inserting the respective PCR amplified cellulase gene in pPtef1-hph (Akel et al. 2009) and named pPtef1-cel7a, -cel7b, or -cel12a. The final expression vector contains 740 bp of the promoter region the *T. reesei tef1*, followed by the coding and terminator region of the respective cellulase gene and the hygromycin B phosphotransferase (*hph*) expression cassette as selection marker. The three cellulase genes including their respective terminator region were amplified from genomic DNA of *T. reesei* QM9414 with the primer pairs Egl1_ClaI_F/Egl1_HindIII_R for *cel7b*, Egl3_ClaI_F/Egl3_HindIII_R for *cel12a* Cbh1_ClaI_F and Cbh1_SalI_R for *cel7a*, thereby introducing the denoted cloning sites (Table 1).

For expression under the *cdna1* promoter region, the *tef1* promoter region was removed by an XhoI/ClaI digest and the PCR amplified, XhoI/ClaI restricted *cdna1* promoter region was inserted resulting in pPcdna1-cel12a. For pPcdna1-cel7b construction, the *tef1* promoter region was removed from pPtef1-hph by an XhoI/HindIII digest followed by the insertion of the XhoI/ClaI restricted *cdna1* promoter region and the ClaI/HindIII restricted *cel7b* gene via ligation.

Fungal transformation was performed as described (Gruber et al. 1990) using uncut plasmid DNA for transformation. Selection media contained 50 μg/mL hygromycin B (Roth). For sporulation, the transformants were transferred to small PDA plates and purified by plating conidiospores onto PDA plates with 0.1% Triton X-100 as colony restrictor. Single

Table 1 Oligonucleotides for amplification of the individual cellulase genes and the *cdna1* promoter (with restriction sites underlined)

Target	Oligonucleotides	
cel7a	Cbh1_ClaI_F	5′-GTTATCGATTCCGGACTGCGCATCATGTATC-3′
	Cbh1_SalI_R	5′-CATGTCGACTTGTCTCCCTATGGGTCATTAC-3′
cel7b	Egl1_ClaI_F	5′-GTTATCGATTCTTAGTCCTTCTTGTTGTCCC-3′
	Egl1_HindIII_R	5′-GATAAGCTTGGTGGGAGAAGACTTTGGAC-3′
cel12a	Egl3_ClaI_F/	5′-GTTATCGATTAGCGTCGCAATGAAGTTCC-3′
	Egl3_HindIII_R	5′-GGAAAGCTTGCCGTGAGAATTGTAC-3′
P_{cdna1}	Cdna1_XhoI	5′-TGACTCGAGCAGACAATGATGGTAGCAGC-3′
	Cdna1_ClaI	5′-AGTATCGATGAGAGAAGTTGTTGGATTGATC-3′

colonies which showed regular growth were transferred to selective media. To test stability, the transformants were passaged three to four times to nonselective media before they were retested on selective media for growth and expression of the individual cellulases.

Molecular analysis of transformants Genomic DNA of the transformants was extracted using a phenol–chloroform method (Seiboth et al. 2002). Transformants were screened for the presence of the different cellulase expression cassettes by amplifying the expression cassette by PCR with one oligonucleotide specific for the promoter region of *tef1* or *cDNA1* and the second specific for the terminator region of the respective cellulase gene. Southern analysis was used to verify the integration of the construct and determine the approximate copy number of the integrated cellulase genes. For Southern blot analysis, genomic DNA (~15–20 μg) of QM9414, the Δ*xyr1* strain and the transformants were digested with BamHI or PstI for *cel7b*, and XhoI or PstI for *cel12a* copy number determination. Digested DNA was size fractionated by gel electrophoresis and transferred to a Biodyne B 0.45-μm membrane (Pall Corporation). DNA labeling, hybridization, and detection were performed according to the DIG High Primer DNA Labeling and Detection Starter Kit I protocols (Roche Applied Science). DIG-labeled probes were amplified with oligonucleotides Egl1_ClaI_F/Egl1_HindIII_R for *cel7b* and Egl3_ClaI_F/Egl3_HindIII_R for *cel12a*. The above-mentioned restriction enzymes cut the respective cellulase gene approximately in the middle of the coding region which results upon hybridization with the probe in two hybridizing bands of similar intensity. The copy number of integrated cellulase genes was hence determined by the number of additional fragments ($n-1$) to the endogenous cellulase gene copy.

SDS-PAGE Supernatants were loaded to SDS gel after EtOH precipitation as follows: 500 μL of culture supernatant was mixed with 1 mL 96% EtOH and stored o/n at −20°C. Following centrifugation at 13,000 rpm for 20 min at 4°C, the protein pellets were resuspended with 40 μL ddH_2O. Samples were run with constant current (15 mA/gel) on 12% denaturing SDS gel.

Cellulase activity assays *Cel7b* transformants were assayed using 4-methylumbelliferyl-β-D-cellobioside (MUC) and *cel7a* transformants using 4-methylumbelliferyl-β-D-lactoside (MULAC) as substrate. Substrates (MUC/MUCLAC) were dissolved in DMSO, and the activities were measured with 50 nmol substrate, 50 mM NaAc buffer (pH 5.0) and 50 μL supernatant in a reaction volume of 200 μL/well. Fluorescence was measured with a Gemini XS spectrofluorometer with 1 min intervals for 1 h at 40°C using ddH_2O as blank, with the following parameters: excitation 330 nm, emission 456 nm, and cutoff 455 nm. Initial linear release rate of fluorescence was used to calculate activities.

The carboxymethylcellulase assays for CEL12A were done in quadruplets. To remove interfering D-glucose in the culture filtrate, the extracellular protein was precipitated by the addition of 2 vol. of ethanol and then resuspended in ddH_2O. Reagents were preheated to 50°C and 96-well plates were covered with a plastic mat during incubations to prevent evaporation. Thirty-microliter supernatants were added to 96-well PCR plates and heated to 50°C. Thirty microliters 2% carboxymethylcellulose (CMC) in NaOAc Buffer (pH 4.8) was added to wells simultaneously, mixed quickly, and incubated for 30 min at 50°C. Sixty microliters DNS reagent was added to wells, mixed shortly, and incubated at 95°C for 5 min for color development. PCR plate was then placed on ice, and absorbances of 100 μL of mixtures were read at 550 nm after transferring them to flat bottomed 96-well microplate. CMCase activity was calculated as described previously (Ghose 1987). One unit catalyzes the liberation of 1 μmol glucose from sodium carboxymethyl cellulose per min. Specific activities are related to the total extracellular protein produced.

Total protein concentration and dry biomass determination Total protein concentrations in the culture supernatants were measured by the Bradford assay (Bradford 1976). For dry biomass determination, 20 mL of culture were

centrifuged at 5,000×*g* for 10 min, the pellet was resuspended with ddH_2O to get rid of salts and centrifuged again. The pellets were dried in an oven (70°C) to constant weight for 1 week.

Results

Cellulase expression vector construction

For the expression of cellulases in a Δ*xyr1* strain, we had to switch their endogenous promoter regions. We chose the promoters of two genes that were isolated during the screening for highly expressed genes during growth on D-glucose as carbon source—i.e., *tef1* (translation elongation factor 1-α-encoding; abbreviated as "t") and the uncharacterized "*cdna1*" (abbreviated as "c," Nakari et al. 1993; Nakari-Setälä and Penttilä 1995). With the help of the previously released *T. reesei* genome sequence, it was now possible to localize the complete *cDNA1* gene on scaffold 23:43726–44652 (http://genome.jgi-psf.org/Trire2/Trire2.home.html). In agreement with earlier results, a high number of ESTs are assigned to the *cDNA1* gene. BLASTP search of the translated nucleotide sequence showed that the protein is only moderately conserved showing a 39 to maximal 46% aa identity to other fungal hypothetical proteins from *Nectria haematococca*, *Gibberella zeae*, *Verticillium alboatrum*, *Magnaporthe grisea*, *Sclerotinia sclerotiorum*, and *Botryotinia fuckeliana*. Three cellulase genes were chosen for testing cellulase expression in the Δ*xyr1* background in *T. reesei*: one cellobiohydrolase encoded by *cel7a* (*cbh1*) and two endoglucanases encoded by *cel7b* (*egl1*) and *cel12a* (*egl3*). *cel7b* and *cel12a* were expressed under both promoters, whereas *cel7a* was tested only with the *tef1* promoter region. All three cellulases together with their terminator region were amplified by PCR and ligated downstream of the respective promoter region (Fig. 1)

Recombinant expression of three cellulases in the Δ*xyr1* strain

After transformation of the Δ*xyr1* strain with different expression plasmids and purification of putative transformants, the integration of the expression vector was tested by PCR and Southern analysis (data not shown). Strains with one additional copy of the expression cassette were chosen for further analysis and named according to the promoter region and cellulase expressed (e.g., *t-cel7b* for a transformant expressing *cel7b* under the *tef1* promoter region). Their growth rate on glucose was similar to the parental strain. Strains produced 2.5 g/L dry biomass after

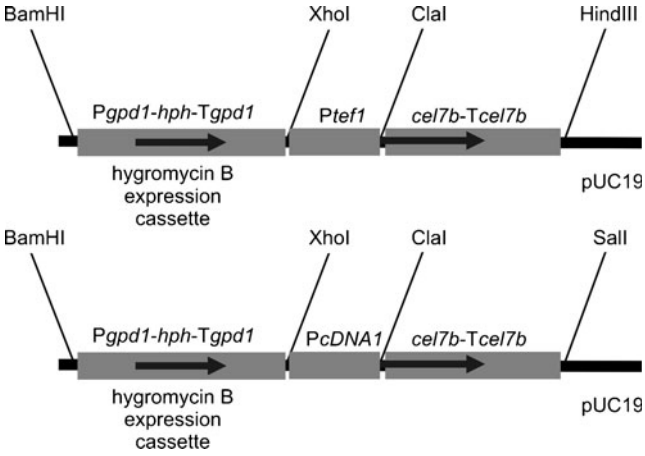

Fig. 1 Expression plasmids for cellulase production. Schematic presentation of the two cellulase expression plasmids used in this study. Both plasmids contain the hygromycin B expression cassette as fungal selection marker followed by either the *tef1* or the *cDNA1* promoter region and the coding and terminator region of the respective cellulase gene (e.g., the endoglucanase encoding gene *cel7b*)

20 h and reached a maximum of 5 g/L after 30 h of cultivation. To test for cellulase production in these strains, we followed the total extracellular protein production over time: all strains secreted protein into the medium until around 36 h of cultivation. Best transformants reached extracellular protein concentrations of more than 40 mg/L, which exceeds the Δ*xyr1* recipient strain which formed up to 18 mg/L protein as background (Fig. 2). To visualize cellulase expression, the proteins in the culture filtrate were separated by SDS-PAGE: Consistent with previous findings (e.g., Jeoh et al. 2008; Shoemaker et al. 1983; Sprey and Uelker 1992), CEL7B is detected as a broad band between 60 and 70 kDa (Fig. 2a), CEL12A is found around 25 kDa (Fig. 2b), and CEL7A, which is found around 65 kDa, was evident from a band around 70 kDa in the gels (Fig. 2c). No degradation products of the recombinant produced cellulases which would have been evident from a decrease of the cellulase band or an increase in bands with lower molecular mass with time were observed. In the Δ*xyr1* control background, only two faint protein bands appeared after 28 h of cultivation which were only visible when a higher volume of culture supernatant was used (Fig. 2a, c).

Quantification of cellulase expression in the Δ*xyr1* strain

To test whether the secreted cellulases were active, CEL7A and CEL7B enzymes in the culture filtrates were quantified using the specific fluorogenic substrates MULAC (van Tilbeurgh et al. 1982) or MUC (Claeyssens and Aerts 1992), respectively. The results, shown in Fig. 3a, indicate that only the CEL7B producing transformants show activity towards MUC, strain c-cel7b-6 thereby giving highest

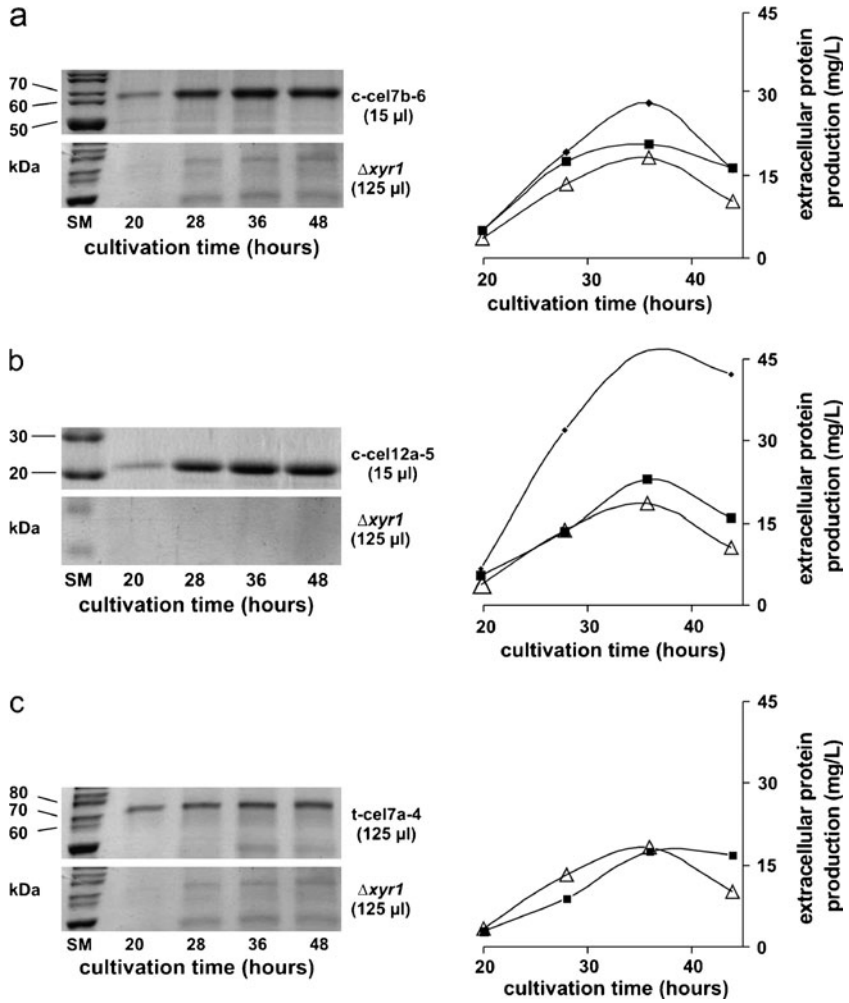

Fig. 2 Endoglucanase CEL7B, endoglucanase CEL12A, and cellobiohydrolase CEL7A production in the cellulase negative background of *T. reesei* Δ*xyr1* transformants. Expression profile of typical Δ*xyr1* transformants expressing CEL7B (**a**), CEL12A (**b**), or CEL7A (**c**) under the *tef1* (t) or *cdna1* (c) promoter regions as indicated. *Left* SDS-PAGE gel of the supernatant of selected transformants after Coomassie blue staining. The amount of supernatant loaded to each gel is indicated in parentheses. *Right* total extracellular protein concentration (in milligrams per liter) of selected transformants expressing the respective cellulase under the *cDNA1* (*filled diamond*) or *tef1* promoter region (*filled square*). Δ*xyr1* (*empty triangle*) is included as a negative control

values (dotted lines). Solid lines in Fig. 3a show the specific activities in micromoles/minute/milligram extracellular protein, illustrating that the specific activity was highest during the early time points (till around 28 h of growth). Thereafter, the specific activities decreased, consistent with the delayed secretion of the two faint proteins and other proteins expressed in the Δ*xyr1* background. Extra sugar addition (10 g/L) after 20 h of growth increased enzyme purity and yield (c-cel7b-6+).

CEL7A producing transformants were active on MULAC, and their volumetric and specific activities displayed the same time course as that of CEL7B (Fig. 3c). Activity of CEL12A was quantified by following the release of reducing sugars from CMC (Ghose 1987). The volumetric activities obtained confirmed the production seen from SDS-PAGE, and the specific activities were—as with CEL7A and CEL7B—also highest during the early cultivation periods (Fig. 3b). It is also evident from a comparison of the activities produced by the different strains that the *cdna1* promoter enabled higher protein production when using single-copy transformants and is thus to be preferred over the *tef1* promoter under these conditions.

Discussion

T. reesei is a model system for the degradation of plant biomass to monomeric sugars applied in biofuel production. Its genome was recently sequenced (Martinez et al. 2008) and several sophisticated tools such as strains with improved gene targeting, strain crossing, HTP gene manipulation, and genome sequencing have been developed (reviewed by Seiboth et al. 2011). One step which asks for improvement is the testing of properties of its gene products involved in plant cell wall degradation. Since most of the cellulases and hemicellulases are glycosylated proteins, the use of commonly available hosts may produce misleading results since prokaryotic hosts are not able to perform the necessary posttranslational modifications at all and other eukaryotic hosts produce usually overglycosylated proteins with altered activities (Boer et al. 2000;

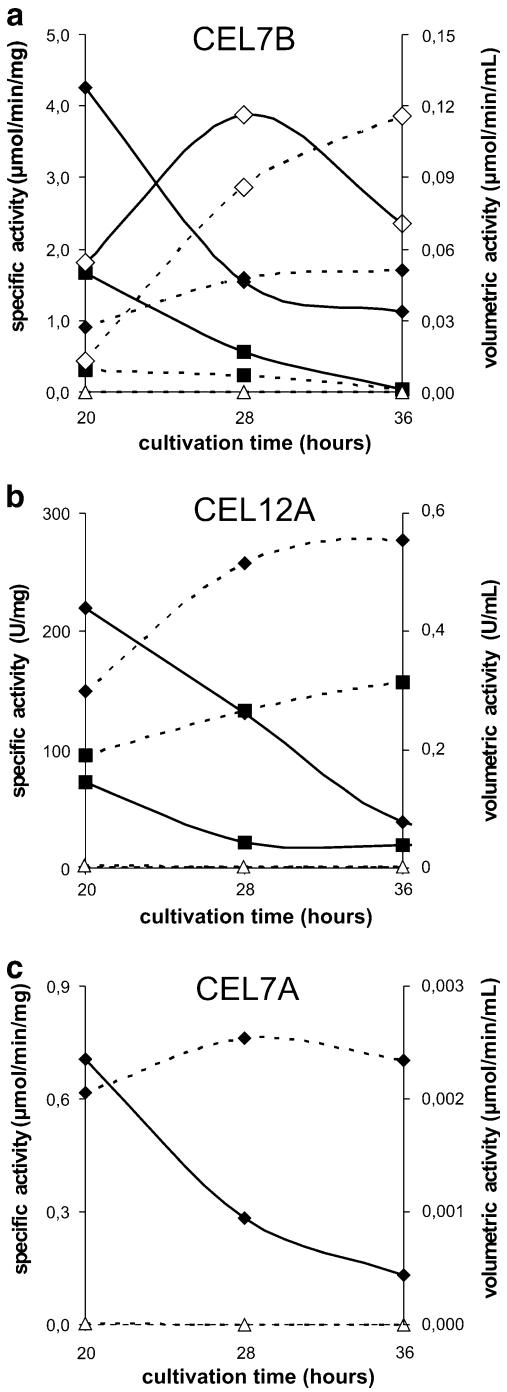

Fig. 3 Cellulase activities in the supernatants of the *cel7b*, *cel7a*, and *cel12a* transformants. Hydrolysis rates of MUC, MULAC, and CMC by culture supernatants of CEL7B (**a**), CEL12A (**b**), and CEL7A (**c**) producing strains respectively were assayed at each sampling point. Volumetric activities (*dashed lines*) are reflective of total active enzyme amount in the culture supernatant and specific activities (*solid lines*) are reflective of enzyme purity. Compared to the transformants the parental strain *T. reesei* Δ*xyr1* (empty triangle) has negligible activities towards MUC, MULAC, and CMC. The strains selected are identical to Fig. 2 and express the respective cellulase either under the *cDNA1* (*filled diamond*) or *tef1* promoter region (*filled square*). In the case of strain c-cel7b-6, extra D-glucose (10 g/L) was added after 20 h of incubation (*empty diamond*)

Godbole et al. 1999; Nakazawa et al. 2008; Jeoh et al. 2008). The aim of this study was therefore to test whether *T. reesei* could be used as the native host for overexpression of selected cellulases with high purity and free of other cellulase contaminants. Such cellulase contaminants are inevitable even if the overexpressed protein would be tagged for purification, because cellulases are known to tightly adsorb to each other, particularly in diluted solutions (Sprey and Lamberta 1984). The hypothesis that was tested was whether a strain in which the XYR1 transcriptional regulator of cellulase and hemicellulase gene transcription is nonfunctional would produce cellulases at a reasonable level and in high purity when fused to a strong expression signal. Using three model cellulase (CEL7A, CEL7B, and CEL12A), we show that strong expression signals such as those from the *tef1* and especially the *cdna1* expression signals result in a reasonable high production of these enzymes on D-glucose as carbon source. In contrast to earlier reports where the cloning of an expression vector based on the *cDNA1* promoter region failed (Nakari-Setälä and Penttilä 1995), we encountered no problem to construct such an expression vector and provide a readily usable expression vector based on the *cDNA1* promoter region. We should note that the present studies were performed in shake flasks, and the secreted protein concentrations reached under these conditions can easily be increased tenfold and more by using controlled fermentation conditions (unpublished data). Interestingly, the Δ*xyr1* strain was not completely devoid of extracellular protein production and secreted a low amount of extracellular proteins during cultivation on D-glucose. However, our data rule out that these proteins would affect cellulase activity assays under these conditions: all three enzyme activity assays specifically demonstrated activity only in the overexpressing strains and were absent from the Δ*xyr1* strain. Zymograms for cellulase activity confirmed that the major band detected in the SDS-PAGE nicely overlapped with the cellulase activity (our unpublished results). In addition, the culture filtrates did not reveal any signs of proteolytic degradation which is frequent during cultivation on inducing carbon sources (Haab et al. 1990; Hagspiel et al. 1989). The nature of the two proteins secreted by Δ*xyr1* has not been examined in this study. However, the lower Mr band exhibits a mass resembling that of the elicitor of plant response protein EPL1. The orthologue of this gene has been shown to be constitutively expressed in another *Trichoderma* sp., *Trichoderma atroviride*, during growth on D-glucose (Seidl et al. 2006b), and it is possible that the respective protein produced by Δ*xyr1* strain is thus *T. reesei* EPL1.

In this study, we used D-glucose as a low-cost carbon source. Due to the use of the Δ*xyr1* strain as host, carbon sources such as cellulose, xylan, lactose, or D-xylose cannot be applied as substrates since the host strain would fail to assimilate them (Stricker et al. 2006; Seiboth et al. 2007;

our unpublished data). However, glucose may not be always the best carbon source for protein production because it is known to nonenzymatically form Schiff's bases with amino group side chains in proteins and eventually lead to their activation (Sullivan 1996). Therefore alternative carbon sources such as glycerol or protein hydrolysates, whose utilization is not XYR1 dependent could be used with probably equal results. *Tef1* is known to be well expressed under all these conditions. The *cdna1* promoter is currently tested by us for its response on nonglucose carbon sources. The system configured here for shake flask experiments can further be refined since conditions for highly reproducible growth of *T. reesei* in microtiter plates have been established which allow also the testing for the formation of extracellular enzyme activities (Seidl et al. 2006a). Taken together, our expression system based on the *cDNA1*- and *tef1*-driven expression vectors and the cellulase negative host strain Δ*xyr1* is suitable for a high-throughput system for testing of cellulase muteins.

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