Enhancing saccharification of wheat straw by mixing enzymes from genetically-modified *Trichoderma reesei* and *Aspergillus niger*

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**Abstract**

**Objectives** To increase the efficiency of enzymatic hydrolysis for plant biomass conversion into renewable biofuel and chemicals.

**Results** By overexpressing the point mutation A824 V transcriptional activator Xyr1 in *Trichoderma reesei*, carboxymethyl cellulase, cellobiosidase and β-D-glucosidase activities of the best mutant were increased from 1.8 IU/ml, 0.1 IU/ml and 0.05 IU/ml to 4.8 IU/ml, 0.4 IU/ml and 0.3 IU/ml, respectively. The sugar yield of wheat straw saccharification by combining enzymes from this mutant and the *Aspergillus niger* genetically modified strain ΔcreA/xlnR/araR₆ was improved up to 7.5 mg/ml, a 229 % increase compared to the combination of wild type strains.

**Conclusions** Mixing enzymes from *T. reesei* and *A. niger* combined with the genetic modification of transcription factors is a promising strategy to increase saccharification efficiency.

**Keywords** *Aspergillus niger* · Genetically-modified strains · Saccharification · *Trichoderma reesei* · Wheat straw

**Introduction**

Depleting fossil energy sources and related environmental issues have stimulated great interest in alternative renewable biofuel sources (Nigam and Singh 2011).
Plant biomass is a promising source of raw material for biofuel production (Klemm et al. 2005). A key bottleneck for the bioconversion of plant biomass into biofuels is the high cost of enzymes and their insufficient efficiency in releasing sugars from lignocellulose (Falkoski et al. 2012). To achieve cost-effective bioconversions, maximizing the enzyme yield and optimizing enzyme mixtures is considered an attractive approach with great potential (Pal and Chakraborty 2013).

Trichoderma reesei and Aspergillus niger are established industrial producers of enzymes that degrade plant biomass (Dos Santos Castro et al. 2014). The main activators of cellulolytic and xylanolytic gene expression in T. reesei and A. niger are Xyr1 and XlnR, respectively (Stricker et al. 2006). Constitutive versions of A. niger the enzymes produced by this strain with those from an T. reesei (van Peij et al. 1998). Constitutive expression of Xyr1 leads to a significant elevation of the production of the main biomass degrading enzymes (Mach-Aigner et al. 2008). The mutation A824V of Xyr1 in T. reesei leads to a significant elevation of the production of the main biomass degrading enzymes (Mach-Aigner et al. 2008). The mutation A824V of Xyr1 improves xylanase and basal cellulase gene expression in T. reesei (Dernst et al. 2013). In A. niger, constitutive activation of XlnR also results in constant xylanase production even under repressing conditions (Hasper et al. 2004). A. niger contains a second hemicellulolytic transcriptional activator, AraR, which has sequence homology to XlnR but is only present in the Eurotiales (Battaglia et al. 2011). This regulator controls the production of extracellular L-arabinose releasing enzymes in A. niger.

Although T. reesei produces an efficient set of cellulolytic enzymes, β-D-glucosidase production is low, while the opposite was reported for A. niger (van den Brink and de Vries 2011). Therefore, plant biomass hydrolysis can be improved by using T. reesei cellulase complemented with enzymes from other organism (Tabka et al. 2006). In this study, improvement of wheat straw saccharification was achieved by overexpressing a constitutive version of xyr1 in T. reesei and combining the enzymes produced by this strain with those from an A. niger strain in which creA was deleted and constitutive versions of araR and xlnr were inserted.

Materials and methods

Microbial strains, plasmids and cultivation

Escherichia coli DH5α was used for cloning, Agrobacterium tumefaciens AGL-1 served as a T-DNA donor for T. reesei transformation. All fungal strains are listed in Supplementary Table 1. A uridine auxotrophic ura5- negative strain 2-3 was used for overexpression of native or mutated A824V xyr1. A T-DNA binary vector, Npxbthg, containing the selecting marker gene ura5 coding for orotate phosphoribosyl-transferase from Penicillium oxalicum under control of the Aspergillus nidulans gpdA promoter and trpC terminator, was used to construct the transformation vectors for T. reesei.

Transformants of T. reesei were selected as described previously (Gruber et al. 1990), with cefotaxime (300 μM) and 0.2 % Triton X-100 (v/v). Each positive transformant was used to generate genetically stable and correct mono-conidia, which were confirmed by PCR. All Aspergillus strains were cultivated in Aspergillus minimal medium (de Vries et al. 2004). Wheat straw, xylan from beechwood, Avicel, glucose and xylose were used as carbon sources. An A. niger strain with a creA deletion was used (van den Brink et al. 2014).

The expression of araR and xlnR was made constitutive by deleting the C-terminus from Pro646 and Leu668, respectively (Hasper et al. 2004). The A. niger ΔcreA background was used in both case.

Overexpression of native or constitutive T. reesei xyr1

The native and mutated A824V xyr1 PCR fragment were amplified from genomic DNA of QM9414 by using primers in Supplementary Table 2 and cloned between the pdc promoter and trpC terminator into the BamHI site of Npxbthg using the ClonExpress MultiS kit (Vazyme Biotech) resulting in vector Npxbthg-OEXyr1 and Npxbthg-OEmXyr1. These vectors were transformed into the recipient strain using A. tumefaciens-mediated fungal transformation.

Cellulase activity, SDS-PAGE and silver staining

Activity of carboxymethyl cellulase (CMCase), cellobiosidase (pNPCase) and β-D-glucosidase (pNPGase) were assayed as described previously (Ma et al. 2011). Significance analysis was performed by Student’s t-test using a two-tailed test. SDS-PAGE (12 %, w/v) with silver staining was performed to analyze extracellular protein profiles. Due to interference of wheat straw with the usual protein determination methods, the total
protein determination was estimated by visual inspection of the SDS-PAGE loaded with 20 μl of samples.

Saccharification of wheat straw

Saccharification was performed in 10 ml containing 2 ml enzyme mixtures, 8 ml saline/sodium acetate buffer (pH 5, 50 mM) and 0.3 g washed wheat straw, and incubated at 30 °C and 200 rpm. The ratio of the enzyme mixtures from T. reesei and A. niger was 1:1 taken from wheat straw liquid (3 %, w/v) after 4 days of cultivation and filtered through a 0.22 μm nylon filter (van den Brink et al. 2014). For control measurements, 2 ml saline/sodium acetate buffer (pH 5, 50 mM) substituted the enzyme mixtures. The reducing sugar yield was quantified daily using the DNS method (Ma et al. 2011). HPLC was used to determine individual sugar concentrations. Sugar analysis was performed on a Thermo Scientific 5000 + HPLC-PAD system. A multi-step gradient was used to analyze the sugars. A flow rate of 0.3 ml/min was used on a CarboPac PA1 column. Guard column: Dionex CarboPac PA1 BioLC 2 × 50 mm. Main column: Dionex CarboPac PA1 BioLC 2 × 250 mm. A pre flow of 18 mM NaOH was used before injection to equilibrate the column. During a total running time of 50 min, the following solutions were used: A, water; B, 100 mM NaOH; C, 100 mM NaOH with 1 M sodium acetate. 18 % B was used for the first 20 min followed by a 10 min linear gradient to 40 % C, 0 % B. 100 % C for 5 min. To rinse the acetate, 100 % B was used for 5 min. 10 min 18 % B was used for 10 min to rinse the column. Quantification was performed by external standard calibration. Reference sugars (Sigma-Aldrich) were used from 2.5 to 200 μM. The data obtained are the results of two independent biological replicates and for each replicate three technical replicates were assayed.

Results and discussion

Overexpression of native and constitutive xyl1 leads to significant increase of enzyme yields

The three T. reesei strains: wild type, overexpressed xyl1 and overexpressed constitutive xyl1, were grown
on minimal medium with 1% carbon source (xylan, xylose, glucose, wheat straw and Avicel). No significant growth improvement between the T. reesei wild type and Xyr1 mutant strains was observed on glucose, Avicel or wheat straw (Fig. 1). In contrast, on xylan and xylose, the colony size of the Xyr1 mutants was considerably increased compared to the wild type. This correlates with the previously demonstrated role of Xyr1 in xylan and xylose utilization (Stricker et al. 2006). Furthermore, overexpression of the constitutive xyr1 resulted in better growth than that of native xyr1, suggesting the point mutation (A824V) in the gene encoding Xyr1 enhances the utilization ability of xylan and xylose by T. reesei.

Cellulase activities of T. reesei wild type and the native or constitutive xyr1 transformants OExyr1-1, OExyr1-2, OEmxyr1 were measured in supernatants from Avicel and wheat straw cultures. All three strains produced a larger amount of secreted proteins than the wild type (Fig. 2). This result contrasts with the overexpression of XlnR in Aspergillus vadensis where additional copies of the transcriptional activator had no effect on protein production (Bouzid and de Vries 2014). OEmxyr1 produced the highest protein level and showed the earliest protein production, particularly in Avicel (Fig. 2a). The other two mutants showed an earlier secretion of proteins with wheat straw than with Avicel (Fig. 2b). This may be linked to a higher fungal biomass formation as suggested by their growth profiles (Fig. 1).

Consistent with extracellular protein production, cellulase activity of the mutant strains was considerably improved compared to the wild type, in agreement with earlier reports (Mach-Aigner et al. 2008; Derntl et al. 2013). Interestingly, the overexpressed constitutive mutant OEmxyr1, had an even greater effect. After 4 days of growth, both in Avicel and wheat straw, only OEmxyr1 produced high cellulase activity. Moreover, after 7 days’ growth on Avicel, OEmxyr1 showed increased CMCase, pNPCase and pNPGase activities from 1.8 IU/ml, 0.1 IU/ml and 0.05 IU/ml to 4.8 IU/ml, 0.4 IU/ml and 0.3 IU/ml,
respectively, (Fig. 3a–c). No significant differences were observed for the \( p \)NPGase in wheat straw medium, but OEmxyr1 produced more of this activity in Avicel medium (Fig. 3c), demonstrating the influence of the carbon source on the regulation of the \( \beta \)-D-glucosidase in the constitutive Xyr1 strain (Castro et al. 2014).

To evaluate the effect of the overexpressed constitutive Xyr1, saccharification experiments were performed on wheat straw using supernatants of wheat straw-grown cultures. \textit{T. reesei} OExyr1 and OEmxyr1 showed higher release of sugar compared to the reference strain (Fig. 4). The OEmxyr1 released by far the largest amount of sugar which is in line with the measured activities. Interestingly, this increased saccharification capacity is mainly due to xylose release and not due to release of glucose (Fig. 4b).

A triple mutant from \textit{Aspergillus niger} was further added to the experiment. This triple mutant is a double constitutive \textit{araR} and \textit{xlnR} made from a \textit{ΔcreA} background (\textit{ΔcreA/xlnR,araR}_{\text{CRE}}).

### Enhancing wheat straw saccharification

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A triple mutant from \textit{Aspergillus niger} was further added to the experiment. This triple mutant is a double constitutive \textit{araR} and \textit{xlnR} made from a \textit{ΔcreA} background (\textit{ΔcreA/xlnR,araR}_{\text{CRE}}).
Combining the enzyme mixtures of these strains with the enzyme mixtures of the *T. reesei* wild type significantly improved saccharification. Combining enzyme mixtures of OE\textit{xyr1} and OE\textit{emxR} with *A. niger \textit{ΔcreA/xlnR}\textit{araR}\textit{c* resulted in even better wheat straw saccharification and the best sugar yield (7.51 mg/ml) was achieved by combining enzymes from OE\textit{emxR} and mutant \textit{ΔcreA/xlnR}\textit{araR}\textit{c}. This is 229 \% of the combination of wild type strains (Fig. 4), which goes beyond results obtained previously (van den Brink et al. 2014).

**In conclusion**, we significantly increased the saccharification of wheat straw by combining the enzyme mixtures of two engineered strains: *T. reesei* OE\textit{emxR} \textit{and A. niger \textit{ΔcreA/xlnR}\textit{araR}\textit{c*}.

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**Supporting information** Supplementary Table 1—List of *T. reesei* OE\textit{emxR} and \textit{A. niger \textit{ΔcreA/xlnR}\textit{araR}\textit{c* strains.}

Supplementary Table 2—List of primers.

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