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Cellulose and hemicellulose-degrading enzymes in *Fusarium commune* transcriptome and functional characterization of three identified xylanases

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ABSTRACT

Specific enzymes from plant-pathogenic microbes demonstrate high effectiveness for natural lignocellulosic biomass degradation and utilization. The secreted lignocellulolytic enzymes of *Fusarium* species have not been investigated comprehensively, however. In this study we compared cellulose and hemicellulose-degrading enzymes of classical fungal enzyme producers with those of *Fusarium* species. The results indicated that *Fusarium* species are robust cellulose and hemicellulose degraders. Wheat bran, carboxymethylcellulose and xylan-based growth media induced a broad spectrum of lignocellulolytic enzymes in *Fusarium commune*. Prediction of the cellulose and hemicellulose-degrading enzymes in the *F. commune* transcriptome using peptide pattern recognition revealed 147 genes encoding glycoside hydrolases and six genes encoding lytic polysaccharide monooxygenases (AA9 and AA11), including all relevant cellulose decomposing enzymes (GH3, GH5, GH6, GH7, GH9, GH45 and AA9), and abundant hemicellulases. We further applied peptide pattern recognition to reveal nine and seven subfamilies of GH10 and GH11 family enzymes, respectively. The uncharacterized XYL10A, XYL10B and XYL11 enzymes of *F. commune* were classified, respectively, into GH10 subfamily 1, subfamily 3 and GH11 subfamily 1. These xylanases were successfully expressed in the PichiaPink™ system with the following properties: the purified recombinant XYL10A had interesting high specific activity; XYL10B was active at alkaline conditions with both endo-1,4-β-D-xylanase and β-xylosidase activities; and XYL11 was a true xylanase characterized by high substrate specificity. These results indicate that *F. commune* with genetic modification is a promising source of enzymes for the decomposition of lignocellulosic biomass.

1. Introduction

Fusarium species include important phytopathogenic and toxigenic fungi that cause numerous diseases in the world's most economically important crop species. Most research on *Fusarium* species has focused on pathogenicity [1,2] and on understanding the infection process and molecular basis of pathogenicity [3,4]. Interestingly, plant-pathogenic microbes can breach plant barriers to invasion and host colonization due to their ability to break down the lignocellulose present in host cell walls [5]. Plant pathogenic *Fusarium* species produce abundant and diverse lignocellulose-degrading enzymes [6], which are of potential interest for biotechnological applications. The biochemical and functional properties of several xylanases belonging to the GH10 and GH11 families from the wild type fungus *Fusarium oxysporum* have been reported [7–11], which indicates that this fungus is a strong xylanase producer. Furthermore, significantly higher production of cellulase and xylanase resulted when *F. oxysporum* was co-cultured with *Aspergillus niger* on forest waste [12]. *F. oxysporum* has attracted interest due to its cellulose and hemicellulose-degradation system and ability simultaneously to ferment hexoses and pentoses to ethanol through consolidated bioprocessing [5,13]. The ethanol productivity was increased by homologous overexpression of one xylanase [14]. *Fusarium commune*, which is closely related to but independent of its sister taxon *F. oxysporum*

Abbreviations: PPR, peptide pattern recognition; CAZy, Carbohydrate-Active enZYmes; GH, glycoside hydrolase; AA, auxiliary activities; LPMOs, lytic polysaccharide monooxygenases; AZCL assay, insoluble chromogenic AZurine Cross-Linked assay; CMC, carboxymethylcellulose; Hotpep, homology to peptide patterns; *p*NPX, *p*-nitrophenyl-β-D-xylopyranoside; *p*NP, *p*-nitrophenol; RIN, RNA integrity number; CDS, coding sequence.

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[15], has been reported only as a discoloration, damping off and root rot pathogen [1,2].

The isolate of *F. commune* used in this study was obtained from the CBS-KNAW Fungal Biodiversity Center (accession number: CBS 131819) and had been isolated from rotting sweet potato tubers in China. Decrease in viscosity of sweet potato mash during the putrefaction process is mainly induced by microorganisms. Viscosity reduction of sweet potato mash can be also achieved by treatment with cellulases and hemicellulases [16,17]. Therefore microorganisms isolated from rotting sweet potato may exhibit considerable potential for the production of cellulolytic and hemicellulolytic enzymes. *F. commune* is able to produce a broad spectrum of enzymes, which demonstrate hydrolytic activity toward cellulose, hemicelluloses, pectin, starch and even protein when the fungus is cultured in liquid medium with corncob agricultural residue as sole carbon source. The crude culture supernatant from *Penicillium ochrochloron*, which was also isolated from rotting sweet potato, was able to reduce the viscosity of a fermentable mash of roots and tubers and significantly increase bioethanol fermentation efficiency [17,18]. The enzymes produced by *F. commune* may therefore also be of potential use for degrading agricultural residues.

Xylanases from *F. oxysporum* have been thoroughly studied. However, although this fungus has the exceptional ability of bioconverting lignocellulose directly to ethanol, there is limited information on its other lignocellulose-degrading enzymes [19]. For example, no comparison has yet been made between the cellulose and hemicelluloses-degrading enzymes of *Fusarium* spp. and the enzymes of other biomass degrading fungi to demonstrate the potential of *Fusarium* spp. for biotechnological applications. Heterologous expression of interesting enzymes is a potent approach for obtaining high-level purified enzymes and avoiding toxins. Yet so far only one xylanase (GH11 family) with low activity from *F. oxysporum* has been expressed in *Pichia pastoris* [19].

The aim of this study was to discover efficient cellulose and hemicelluloses-degrading enzymes in *F. commune* through transcriptome mining. A new approach, peptide pattern recognition (PPR) [20], was used to simultaneously compare multiple cellulose and hemicellulose-degrading enzyme sequences among the classical biomass-degrading fungi and *Fusarium* spp. and to identify the characteristic features and relatedness of these enzymes. The PPR approach was also applied to uncover new protein subfamily groupings in the GH10 and GH11 families that can increase understanding of structure and function relationships for the different GH10 and GH11 enzymes in *F. commune*.

2. Materials and methods

2.1. Microbial growth

F. commune (accession number: CBS 131819) was ordered from the Centraal Bureau voor Schimmelcultures, Fungal Biodiversity Center, Royal Netherlands Academy of Arts and Sciences (CBS-KNAW). This strain was grown on potato glucose agar (Sigma) at 30 °C for seven days and maintained at 4 °C. Subculturing was performed every month. Carboxymethylcellulose (CMC), avicel, xylan, wheat bran, corncob and sweet potato peel powder were used as substrates for enzyme production.

Enzyme-inducing media were prepared by adding 20 g/l agricultural residue (wheat bran, corncob and sweet potato peel added individually), 15 g/l agar, adjusted to pH 5.5. As a reference medium, 3.0 g/l peptone and 0.5 g/l yeast extract were added.

Additional enzyme-inducing media were prepared as described [21] with certain modifications: 20 g/l xylan/CMC/avicel (substrates were added individually), 2.0 g/l KH_2PO_4, 1.5 g/l $(NH_4)_2SO_4$, 0.15 g/l $MgSO_4{\cdot}7H_2O$, 0.3 g/l $CaCl_2$, and 3.3 g/l Tween-80, agar 15 g/l, pH 5.5. After sterilization, 0.1% trace element solution (0.05 g/l $FeSO_4$, 0.014 g/l $ZnSO_4{\cdot}7H_2O$, 0.016 g/l $MnSO_4$, and 0.016 g/l $CoCl_2$) was added. As a reference medium, 3.0 g/l peptone and 0.5 g/l yeast extract were added.

F. commune was grown on enzyme-inducing medium at 30 °C for eight days.

To further optimize the enzyme production conditions, *F. commune* was grown on wheat bran medium as described above without yeast extract and peptone. Single factor analysis was performed as follows. First, the fungus was grown under different temperatures (30, 40 and 50 °C). Then, the fungus was grown using different concentrations of wheat bran (10, 20, 30, 40 g/l) at the temperature found to be most optimal for enzyme production. Finally, the fungus was grown at different pH (3.5, 4.5, 5.5, 6.5 and 7.5). After growth under the above culture conditions, 16 agar plug squares of $2 \times 2\,mm^2$ were cut from each treatment culture and incubated overnight in a 50 ml tube containing 5 ml 0.1% Tween-20 solution at 90 rpm at room temperature. The supernatant was used for enzyme assays.

2.2. Enzyme activity assay

2.2.1. AZCL-assay

The enzyme profile of the supernatant was investigated using insoluble chromogenic AZurine Cross-Linked (AZCL) polysaccharides dispersed in agarose plates. Assay plates were prepared according to the manufacturer (Megazyme, Ireland). After incubation at 30 °C for 22 h, the diameter of the blue haloes was measured and reported in millimeters.

2.2.2. AZO-xylan and AZO-cellulose assay

Xylanase and cellulase activity were measured using AZO-xylan and AZO-cellulose (Megazyme, Bray, Ireland) as substrates, respectively, in a modified assay as described [22].

2.2.3. p-*nitrophenyl-β-*D-*xylopyranoside (pNPX) assay*

The assays were performed using 5 mM *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) (Sigma) as the substrate in 2× McIlvaine Buffer (pH 6) [23]. 15 μl purified enzyme was mixed with 150 μl substrate and incubated at 50 °C for 30 min. 30 μl of the reaction sample was transferred to a microtiter plate and terminated by adding 50 μl 1 M Na_2CO_3. The absorbance was measured at 405 nm. One unit of β-xylosidase activity was defined as the amount of enzyme required to produce 1 μmol *p*-nitrophenol (*p*NP) per minute under the described assay conditions.

2.3. Total protein analysis

Total protein concentration in the crude culture supernatant was determined by the Bradford method using the BCA Protein Assay Kit (Thermo Scientific) and bovine serum albumin (BSA) as standard.

The protein concentration of the purified recombinant enzyme was calculated according to the molar extinction coefficient of the each protein sequence (http://encorbio.com/protocols/Prot-MW-Abs.htm). The absorbance of the protein solution at the ultraviolet wavelength of 280 nm was measured by Nanodrop1000 (Thermo Scientific).

2.4. RNA extraction

Mycelium was harvested from *F. commune* grown on wheat bran under optimal conditions (Section 2.1), then ground in liquid nitrogen, and total RNA extracted using TRI reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to the manufacturer. The concentration, purity and RNA integrity number (RIN) were