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Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants

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ABSTRACT

The efficient degradation of complex xylans needs collaboration of many xylan degrading enzymes. Assays for xylan degrading activities based on reducing sugars or PNP substrates are not indicative for the presence of enzymes able to degrade complex xylans: They do not provide insight into the possible presence of xylanase-accessory enzymes within enzyme mixtures. A new screening method is described, by which specific xylan modifying enzymes can be detected.

Fermentation supernatants of 78 different fungal soil isolates grown on wheat straw were analyzed by HPLC and MS. This strategy is powerful in recognizing xylanases, arabinoxylan hydrolases, acetyl xylan esterases and glucuronidases.

No fungus produced all enzymes necessary to totally degrade the substrates tested. Some fungi produce high levels of xylanase active against linear xylan, but are unable to degrade complex xylans. Other fungi producing relative low levels of xylanase secrete many useful accessory enzyme component(s).

1. Introduction

In order to convert lignocellulosic biomass to ethanol, a whole array of enzymes is needed to degrade such a complex structure to monomers (Carvalheiro et al., 2008). An important part of cereal and wood hemicellulose is the class of xylans, comprising a backbone of β-1,4-xylopyranosyl units containing various side groups like, amongst others, 4-*O*-methyl-α-D-glucopyranosyl uronic acid, ferulic acid, arabino-furanose and *O*-acetyl groups (Ebringerova and Heinze, 2000). The composition of the lignocellulose determines the enzymes required for complete degradation of the substrate to monomers.

Wheat arabinoxylan (WAX) and Eucalyptus xylan hydrolysate (EXH) have been used before to identify and characterize an array of xylan degrading enzymes (Christov et al., 2000; Pouvreau et al., 2011; Tenkanen and Siika-Aho, 2000), since these substrates contain the most abundant substituents present in cereal lignocellulosic xylans (Gírio et al., 2010), except for ferulic acid (Vidmantiene et al., 2006). WAX contains mono and/or double α-L-arabinosylated xylopyranosyl units through *O*-3 and/or *O*-2 (Ebringerova and Heinze, 2000). The degradation of WAX requires, next to the presence of endo-xylanases (EC 3.2.1.8), the activity of AXH-m (releasing mono substituted arabinose: EC 3.2.1.55), AXH-d3 (releasing double substituted arabinose from position *O*-3: EC 3.2.1.55) or β-xylosidase (EC 3.2.1.37) within the fermentation supernatants (Gírio et al., 2010; Van Laere et al., 1997). The soluble EXH consists of *O*-acetyl-(4-*O*-methylglucurono)xylooligosaccharides (Christov et al., 2000). The acetyl substituents are closely associated with the 4-*O*-methylglucuronic acid (Evtuguin et al., 2003). The 4-*O*-methylglucuronic acid substituent may be substituted at *O*-2 with α-D-galactose (Shatalov et al., 1999). The degradation of EXH is only successful in the presence of acetyl xylan esterases (EC 3.1.1.72), endo- and exo-xylanases and α-glucuronidases (EC 3.2.1.131) (Christov et al., 2000; Tenkanen and Siika-Aho, 2000). α-Glucuronidases are enzymes that are able to hydrolyze the α-1,2-linkage between 4-*O*-methylglucuronic/glucuronic acid and xylose.

In the search for novel enzymes, in which the enzyme activity is often monitored using dyed substrates or via the formation of reducing end groups, no distinction can be made between different enzymes (Biely and Puchart, 2006; Ghatora et al., 2006). Next to these assays, proteomic approaches making use of genomic libraries will often result in the annotation of known enzymes instead of identifying real novel or desired enzymes (e.g. Wang et al., 2010).

Filamentous fungi are a good source of xylan degrading enzymes and their levels of enzyme excretion in the fermentation media are high, which makes them interesting for screening (Gírio et al., 2010; Handelsman et al., 1998; Polizeli et al., 2005). However, more precise assays are necessary to include a wide range of enzymes in such screening (Biely and Puchart, 2006).

In this paper a screening method is presented in which a range of xylan degrading enzymes in fungal fermentation liquids are

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identified. Analysis of the xylan digests was done by using HPAEC, HPLC and Maldi-TOF MS.

2. Methods

2.1. Fungi

Shake flask fermentation supernatants (78) of mesophilic lignocellulolytic fungi were obtained from the Budapest University of Technology and Economics (BUTE), Hungary. These fungi have been isolated from soil samples and decaying plant materials collected worldwide. A few taxonomically identified fungi were also introduced into the study. They were obtained from known culture collections.

2.2. Enzymes

As reference enzyme endo-(1,4)-β-D-xylanase-I of *Aspergillus awamori* (GH10) was used. The purification and mode of action of this enzyme is described elsewhere (Kormelink et al., 1993b). In addition, an acetyl xylan esterase (CE5) and an α-glucuronidase (GH67) of *Chrysosporium lucknowense* (Hinz et al., 2009) were used as reference enzymes.

2.3. Chemicals and substrates

All chemicals used were, if not mentioned otherwise, of analytical grade.

The substrates used were WAX, medium viscosity (Megazyme, Wicklow, Ireland) and an EXH as produced by hydrothermal treatment (Garrote et al., 1999), kindly provided by Prof. Dr. Parajo of the University of Vigo-Ourense, Spain.

2.4. Sugar composition of substrates

In order to determine the sugar composition, the substrates were hydrolyzed using 72% (w/w) sulfuric acid at 30 °C for one hour followed by hydrolysis with 1 M sulfuric acid at 100 °C for three hours. The neutral monosaccharides were analysed as their alditol acetates, using inositol as internal standard. A Focus gas chromatograph (Thermo Scientific, Waltham MA, USA) equipped with a Supelco SP 2380 column was used.

A part of the hydrolysate was used for the determination of the uronic acid content by the colorimetric m-HDP assay according to Ahmed and Labavitch (1978) using an auto-analyser (Skalar Analytical, Breda, The Netherlands) and using a galacturonic acid (0–100 μg/mL) calibration curve.

2.5. Isolation of lignocellulolytic fungi

Two types of agar media were used to isolate the cellulase and hemicellulase producing fungi. The medium contained either 30 g/L cellulose powder (Sigmacell Type 20, SIGMA) or 30 g/L finely milled wheat straw (<0.3 mm) as carbon source. The other components were similar for both media (in g/L); $NaNO_3$, 3; $(NH_4)_2SO_4$, 1; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; Difco yeast extract, 0.3; Bacto agar, 20; and (in mg/L) $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.6; $CoCl_2.6H_2O$, 2; $ZnSO_4.7H_2O$, 3.45. The pH (before sterilization) was set to 6.5 using sulfuric acid. Media were autoclaved routinely (30 min, 121 °C). The isolation agar media were supplemented with 100 μg/mL doxycycline hyclate (SIGMA) to suppress bacterial growth. After incubation at 30 °C primary fungal colonies were inoculated in Petri plates by streaking twice on potato-dextrose-agar (PDA) supplemented with 1 g/L Triton X100 in order to obtain single colonies. The fungal isolates were freeze-dried for long term storage.

2.6. Enzyme production by shake flask fermentation

The lyophilized fungi were revitalized on PDA medium at 30 °C and the properly sporulated Petri plate cultures were used for inoculation. Two types of shake flask cultivation media (LC-3 and LC-4) were used. Medium LC-3 contained (in g/L): finely milled wheat straw (<0.3 mm), 20; KH_2PO_4, 1.5; $(NH_4)_2HPO_4$, 2; defatted soybean meal, 1; corn steep liquor, 50% DM (SIGMA), 1; NaCl, 0.5; $CaCO_3$, 1; urea, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; and (in mg/L) $FeSO_4.7H_2O$, 2.5; $MnSO_4$, 0.8; $CoCl_2.6H_2O$, 1; $ZnSO_4.7H_2O$, 1.7. Medium LC-4 contained (in g/L): finely milled wheat straw (<0.3 mm), 20; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 1; $(NH_4)_2SO_4$, 1; defatted soybean meal, 1; corn steep liquor, 50% DM (SIGMA), 1; NaCl, 0.5; $CaCO_3$, 0.5; and (in mg/L) $FeSO_4.7H_2O$, 2.5; $MnSO_4$, 0.8; $CoCl_2.6H_2O$, 1; $ZnSO_4.7H_2O$, 1.7. For both media the pH (before sterilization) was set to pH 5.0 using sulfuric acid.

Of both media 150 mL was sterilized in 750 mL cotton-plugged Erlenmeyer flasks. They were inoculated with 3 loopful of spores per flask. Fermentation was carried out on a rotary shaker at 30 °C and 220 rpm for 5 days. Supernatants were stored at −18 °C for further analysis.

2.7. Enzyme screening

Endo-xylanase activity was determined using the colorimetric dinitrosalicylicacid (DNS) assay at pH5.0 using birch glucuronoxylan as substrate (Bailey et al., 1992). Xylanase activity was expressed as IU/ml.

The 1,4-β-D-xylosidase activity was assayed as described by Herr et al. (1978). The liberated 4-nitrophenol was measured at 400 nm.

α-L-arabinofuranosidase activity was assayed according to Poutanen et al. (1987). 4-Nitrophenol was used for the standard curve.

For novel screening approaches WAX and EXH (both 5 mg/mL) were dissolved in 0.05 M sodium acetate buffer, pH 5.0. Next, shake flask fermentation supernatant was added to a final concentration of 1% (v/v). For determination of the degree of acetylation, the sodium acetate buffer was replaced by 0.05 M sodium citrate buffer (pH 5.0). Incubation took 24 h at 500 rpm and 35 °C. Enzymes were inactivated by boiling the digests for 10 min.

2.8. Quantification and characterization of monomers and oligomers using HPAEC

The digests were 100× diluted with Millipore water and analyzed by High Performance Anion Exchange Chromatography (HPAEC) using an ICS3000 HPLC system (Dionex, Sunnyvale, CA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ISC3000 ED PAD-detector (Dionex). A flowrate of 0.3 mL/min was used with the following gradient of 0.1 M sodium hydroxide (NaOH) and 1 M sodium acetate (NaOAc) in 0.1 M NaOH: 0–45 min, 0–500 mM NaOAc in 0.1 M NaOH; 45–48 min washing step with 1 M NaOAc in 0.1 M NaOH; 48–60 min, equilibration with 0.1 M NaOH. Twenty μl of sample was injected each time. Quantification is based on the response factor of the standard xylose solutions of xylose to xylotetraose (X_1 Sigma Aldrich, Steinheim, Germany, X_{2-4} Megazyme, Wicklow, Ireland), D-arabinose and D-glucuronic acid (Sigma Aldrich).