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Novel thermophilic hemicellulases for the conversion of lignocellulose for second generation biorefineries

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ABSTRACT

The biotransformation of lignocellulose biomasses into fermentable sugars is a very complex procedure including, as one of the most critical steps, the (hemi) cellulose hydrolysis by specific enzymatic cocktails. We explored here, the potential of stable glycoside hydrolases from thermophilic organisms, so far not used in commercial enzymatic preparations, for the conversion of glucuronoxylan, the major hemicellulose of several energy crops. Searches in the genomes of thermophilic bacteria led to the identification, efficient production, and detailed characterization of novel xylanase and α-glucuronidase from *Alicyclobacillus acidocaldarius* (GH10-XA and GH67-GA, respectively) and a α-glucuronidase from *Caldicellulosiruptor saccharolyticus* (GH67-GC). Remarkably, GH10-XA, if compared to other thermophilic xylanases from this family, coupled good specificity on beechwood xylan and the best stability at 65 °C (3.5 days). In addition, GH67-GC was the most stable α-glucuronidases from this family and the first able to hydrolyse both aldouronic acid and aryl-α-glucuronic acid substrates. These enzymes, led to the very efficient hydrolysis of beechwood xylan by using 7- to 9-fold less protein (concentrations <0.3 μM) and in much less reaction time (2 h *vs* 12 h) if compared to other known biotransformations catalyzed by thermophilic enzymes. In addition, remarkably, together with a thermophilic β-xylosidase, they catalyzed the production of xylose from the smart cooking pre-treated biomass of one of the most promising energy crops for second generation biorefineries. We demonstrated that search by the CAZy Data Bank of currently available genomes and detailed enzymatic characterization of recombinant enzymes allow the identification of glycoside hydrolases with novel and interesting properties and applications.

1. Introduction

Glycosidases extracted from thermophilic organisms show many advantages over biocatalysts working at temperatures <50 °C and demonstrated remarkable utility in the bioconversion of carbohydrates by enzymes highly resistant to temperature, chemicals, and pH [1–5]. In particular, sustainable chemo-enzymatic lignocellulose conversions for the production of biofuels and precursors of fine chemicals [6–10] can take great advantage by hyperstable glycoside hydrolases [9,11–15].

In fact, a still critical aspect of lignocellulosic-derived second generation plants, exploiting the so-called energy crops as an alternative to crops used for human and animal food production, is the composition of the enzymatic cocktails specifically tailored for the biomass of choice (for reviews see [7,8,10,16,17]. A specific example is the degradation of glucurono-xylan [18], the major hemicellulose of *Arundo donax* (giant reed) a fast growing and low input-high yielding energy crop [19]

The enzymatic degradation of this linear polymer of β-1,4-linked D-xylopyranosyl units frequently substituted with α-1,2 linked glucuronate residues, which are often methylated on the C4 (MeGlcA) [20] occurs by the concerted action of xylanases and α-glucuronidases; thus, we embarked in the search for novel enzymes of this kind in the genomes of (hyper) thermophilic microorganisms

Abbreviations: CAZy, carbohydrate active enzyme; 3D, three-dimensional; MGX, 4-*O*-methyl-glucurono-xylan; 2Np-Cel, 2-nitrophenyl-β-cellobioside; 4Np-Xyl, 4-nitrophenyl-β-xylopyranoside; 4Np-Glc, 4-nitrophenyl-β-glucopyranoside; CMC, carboxy-methyl-cellulose; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography; ORF, open reading frame; 4Np-GluA, 4-nitrophenyl-α-glucuronide; XOs, xylooligosaccharides; Xyl1, xylose; Xyl2, xylobiose; Xyl3, xylotriose; Xyl4, xylotetraose; Xyl5, xylopentaose; Xyl6, xyloesaose; HPAEC-PAD, high performance anionic exchange chromatography with pulsed amperometric detection; MeGlcA, 4-*O*-methyl glucuronic acid.

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which are known for containing unique (hemi) cellulolytic systems [21–26].

α-Glucuronidases (EC 3.2.1.131 and .139), belonging to families GH4, GH67 and GH115 of carbohydrate active enzyme (CAZy) classification (www.cazy.org), are still not extensively studied with about 2, 20 and 4 members characterized from these families, respectively, [27]). Family GH4 contains several enzymatic activities and do not hydrolyze 4-*O*-methyl-α-glucuronoxylan or its oligosaccharidic fragments [28]. Instead, GH67 and GH115 enzymes, including only α-glucuronidases, show the formers selectively removing the MeGlcA-1,2 bound to the non-reducing end xylose of short oligosaccharides of glucurono-xylans [29,30]. Instead, GH115 enzymes remove glucuronic acid from both the terminal and the internal regions of xylooligosaccharides and xylans [31–33]. A more detailed inspection of these families revealed that enzymes from thermophilic microorganisms are concentrated in GH67 with only the uncharacterized enzyme from Thermobispora bispora belonging to GH115. Therefore, we focused our search among the thermophilic GH67 enzymes acting on MeGlcA bound to the non-reducing end of xylooligosaccharides.

Xylanases (EC 3.2.1.8 and .32) are currently classified in families GH5, GH8, GH10, GH11, GH26, GH30, GH43 and GH51. Among these, GH10 and GH11 enzymes, which are endo-β-xylanases whose catalytic apparatus and three-dimensional structures are well known, cleave glucuronoxylan chains when MeGlcA is linked to xylose at the +1 and +2 subsites, respectively [27,34,35]. Therefore, GH10 products, showing MeGlcA at the non-reducing end, are direct substrates of α-glucuronidases, making the xylanases from this family the ideal candidates for hydrolysis of glucuronoxylan in cooperation with GH67 enzymes.

Here, we show the exploitation of glycoside hydrolases belonging to families GH10 and GH67 from the thermophilic bacteria *Alicyclobacillus acidocaldarius* and *Caldicellulosiruptor saccharolyticus*, which contain a large survey of carbohydrate active enzymes proved to be useful for glycan biotransformations [36–40]. We demonstrate that these enzymes, which showed remarkable stability to high temperatures and unique substrate specificities, efficiently hydrolyzed (methyl)-glucurono xylans and smart cooking pre-treated *A. donax* biomass.

2. Materials and methods

2.1. Materials

All commercially available substrates were purchased from Sigma–Aldrich, Carbosynth and Megazyme. The synthetic oligonucleotides were from PRIMM (Italy). The biomass of *A. donax* used in this study derived from a pre-treatment step (smart cooking) described in patent WO2010113129A2. This treatment comprised the soaking of the ligno-cellulosic biomass feedstock in vapor or liquid water or mixture thereof in the temperature range of 100–210 °C for 1 min to 24 h to create a soaked biomass containing a dry content and a first liquid. Steam exploding of the former created a steam exploded stream comprising solids and a second liquid, which was used for the experiments described. This treatment was able to break up the lignocellulosic matrix and avoid the formation of inhibitor compounds using only steam and water without the requirement of additional chemical products.

2.2. Cloning and purification

The Aaci_2328 gene, coding GH10-XA xylanase, was amplified by PCR from the genome of *A. acidocaldarius* ATCC27009 using the synthetic oligonucleotides 2328-5′ (5′-CACCATGACGGATCAAGCGCCGT-3′) and 2328-3′ (5′-GTTTTGGGCGAGGCGCACCAC-3′). The amplification reaction was performed with the PfuUltra II Fusion HS DNA Polymerase (Stratagene) by using the following program: hot start 5 min at 95 °C; 5 cycles 1 min at 95 °C, 1 min at 50 °C and 1.5 min at 72 °C; 30 cycles 1 min at 95 °C, 1 min at 60 °C, and 1.5 min at 72 °C; final extension 10 min at 72 °C. The DNA fragment obtained was cloned in the expression vector pET101/D-TOPO (Invitrogen), obtaining the recombinant plasmid pET101/D-TOPO-Aaci_2328. Here, the gene is under the control of an isopropyl-1-thio-β-D-galactopyranoside (IPTG) inducible T7 RNA polymerase promoter and the C-terminal of the protein was fused to V5 epitope and 6xHis tag. PCR-generated construct was verified by sequencing and the ORF was expressed in *Escherichia coli* cells, strain BL21 Star (DE3) (Invitrogen), according to the manufacturer. The cells transformed with pET101/D-TOPO-Aaci_2328 were grown at 37 °C in 2 L of Luria–Bertani (LB) broth supplemented with ampicillin (50 μg mL^{-1}). Gene expression was induced by the addition of 0.1 mM IPTG when the culture reached an A_{600} of 1.0. Growth was allowed to proceed for 16 h, and cells were harvested by centrifugation at 5000 × *g*. The resulting cell pellet was resuspended in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl and 1% TRITON-X100 with a ratio of 5 mL g^{-1} cells and then was incubated at 37 °C for 1 h with 20 mg of lysozyme (Fluka) and 25 U g^{-1} cell of Benzonase (Novagen). Cells were lysed by French cell pressure treatment and cell debris were removed by centrifugation at 12,000 × *g* for 30 min. The free cellular extract (FCE) was loaded on a His Trap FF crude column (GE-Healthcare) equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl (Buffer A). After an initial wash-step (20-column volumes) with buffer A, the protein was eluted with a two-step gradient of imidazole in Buffer A (250 mM imidazole, 20-column volumes followed by 500 mM imidazole, 20-column volumes) at a flow rate of 1 mL min^{-1}. The protein was eluted at 250 mM imidazole. The active fractions were pooled, dialyzed against 20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl (PBS buffer) and then heat-fractionated for 30 min at 50 °C and, after centrifugation, for additional 30 min at 60 °C. The resulting supernatant was stored at 4 °C. The protein concentration was determined with the Bradford assay [41]. After this procedure GH10-XA was more than 95% pure by SDS-PAGE.

The Aaci_0060 gene, coding GH67-GA α-glucuronidase, was amplified by PCR from the genome of *A. acidocaldarius* ATCC27009 using the synthetic oligonucleotides 0060-5′ (5′-CACCCTTGACGAACATCCCTGA-3′) and 0060-3′ (5′-CGGGTAGATGTGGAGCCC-3′). The amplification reaction was performed with the PfuUltra II Fusion HS DNA Polymerase (Stratagene) by using the following program: hot start 5 min at 95 °C; 5 cycles 1 min at 95 °C, 1 min at 48 °C and 3 min at 72 °C; 30 cycles 1 min at 95 °C, 1 min at 55 °C, and 3 min at 72 °C; final extension 10 min at 72 °C. The DNA fragment obtained was cloned in the expression vector pET101/D-TOPO (Invitrogen), as described above, obtaining the recombinant plasmid pET101/D-TOPO-Aaci_0060. The Csac_2689 gene, coding GH67-GC α-glucuronidase, was amplified by PCR from the genome of *C. saccharolyticus* DSM8903 using the synthetic oligonucleotides 2689-5′ (5′-CACCATGGAACACGTCAAACAAAAA-3′) and 2689-3′ (5′-TGGATATATAAGTCTTCCTTTTTCATC-3′). The amplification reaction was performed with the Platinum Taq High Fidelity (Invitrogen) by using the following program: hot start 5 min at 94 °C; 5 cycles 1.5 min at 94 °C, 1 min at 50 °C and 3 min at 68 °C; 30 cycles 1.5 min at 94 °C, 1 min at 55 °C, and 3 min at 68 °C; final extension 10 min at 68 °C. The DNA fragment obtained was cloned in the expression vector pET101/D-TOPO (Invitrogen), as described above, obtaining the recombinant plasmid pET101/D-TOPO-Csac_2689. The ORFs were expressed in *E. coli* cells as described above, and gene expression was induced by the addition of 0.5 mM IPTG. After harvesting, the resulting cell pellets were resuspended,