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The effect of *Pleurotus ostreatus* arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion

Loredana Marcolongo [a], Elena Ionata [a], Francesco La Cara [a], Antonella Amore [b], Simona Giacobbe [b], Olimpia Pepe [c], Vincenza Faraco [b,*]

[a] Institute of Biosciences and BioResources, National Research Council, Napoli, Italy
[b] Department of Chemical Sciences, University of Naples "Federico II", Napoli, Italy
[c] Department of Agriculture, University of Naples "Federico II", Portici (Napoli), Italy

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ABSTRACT

The fungal arabinofuranosidase from *Pleurotus ostreatus* PoAbf recombinantly expressed in *Pichia pastoris* rPoAbf and its evolved variant rPoAbf F435Y/Y446F were tested for their effectiveness to enhance the enzymatic saccharification of three lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grains (BSG), after chemical or chemical–physical pretreatment. All the raw materials were subjected to an alkaline pretreatment by soaking in aqueous ammonia solution whilst the biomass from *A. donax* was also pretreated by steam explosion. The capability of the wild-type and mutant rPoAbf to increase the fermentable sugars recovery was assessed by using these enzymes in combination with different (hemi)cellulolytic activities. These enzymatic mixtures were either entirely of commercial origin or contained the cellulase from *Streptomyces* sp. G12 CelStrep recombinantly expressed in *Escherichia coli* in substitution to the commercial counterparts. The addition of the arabinofuranosidases from *P. ostreatus* improved the hydrolytic efficiency of the commercial enzymatic cocktails on all the pretreated biomasses. The best results were obtained using the rPoAbf evolved variant and are represented by increases of the xylose recovery up to 56.4%. These data clearly highlight the important role of the accessory hemicellulolytic activities to optimize the xylan bioconversion yields.

1. Introduction

Lignocellulose represents a favoured feedstock for development of a 'no oil' bioeconomy since it is the most abundant biomass, representing near 70% of the total plant biomass, and its use does not provide competition with food industries. The efficient enzymatic hydrolysis of pretreated biomass to achieve both high glucose and xylose yields requires finely tuned mixtures of enzymes acting on the complex carbohydrate matrix. Cellulases, hemicellulases (xylanases) and accessory enzymes (e.g., arabinofuranosidases, pectinases, mannanases) are needed for conversion of pretreated lignocellulose into fermentable sugars. Hemicellulose represents about 20–35% of lignocellulosic biomass and it is a very complex polysaccharide composed of a wide variety of sugars including xylose, arabinose, mannose, and galactose (Saha, 2003). The importance of side-chain acting hemicellulases, such as α-L-arabinofuranosidase (EC 3.2.1.55), feruloyl esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72), in lignocellulosic biomass degradation has been clearly evidenced in literature demonstrating marked increases of the efficiency of lignocellulosic hydrolysis in presence of these auxiliary enzymes (Gao et al., 2011; Ravalason et al., 2012).

The fungi that obtain their nutrition from the breakdown of plant biomass are interesting producers of cell wall-degrading enzymes including side-chain acting hemicellulases (Van den Brink and de Vries, 2011). Several fungi such as *Aspergillus terreus* (Le Clinche et al., 1997), *Aspergillus awamori* (Kaneko et al., 1998), *Penicillium purpurogenum* (De Ioannes et al., 2000), *Aspergillus niger* (vd Veen et al., 1991) and *Aspergillus nidulans* (Ramón et al., 1993) have been reported to be able to produce α-L-arabinofuranosidases.

The white rot fungus *Pleurotus ostreatus* has been recently investigated for its hemicellulolytic capability (Iandolo et al., 2011) and an α-L-arabinofuranosidase, named PoAbf, was identified during its solid state fermentation on tomato pomace (Amore et al., 2012a). The enzyme PoAbf recombinantly expressed in *Pichia pastoris*, rPoAbf, was characterized and further

* Corresponding author. Address: Department of Chemical Sciences, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, via Cintia, 4 80126 Napoli, Italy. Fax: +39 081 674313.
E-mail address: vfaraco@unina.it (V. Faraco).

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experiments of its directed evolution allowed developing a variant, rPoAbf F435Y/Y446F, with improved catalytic properties (Giacobbe et al., 2014). As a matter of fact, this variant when assayed with p-nitrophenyl α-L-arabinofuranoside showed a k_{cat} about 3-fold higher and a K_M 30% lower than that of the wild type, and an improved ability to hydrolyze insoluble substrates such as larch arabinogalactan, arabinoxylan and arabinan, suggesting its improved potential for hemicellulose conversion.

Our work was aimed at assessing the ability of the α -L-arabinofuranosidase rPoAbf and its directed evolved variant rPoAbf F435Y/Y446F to improve the biodegradation of three different lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grain. Since the commercially available hemicellulases are typically dominated by backbone-acting xylanases and β-xylosidases with low side-chain acting hemicellulases, the effect of the addition of PoAbf and its directed evolved variant to commercial cellulases, xylanases, β-xylosidase and β-glucosidase was analyzed. These enzymes were also tested in a cocktail containing the cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *Escherichia coli* (Amore et al., 2012b) in substitution to the commercial cellulase.

2. Material and methods

2.1. Raw materials

Different agro-industrial raw materials including giant reed (*A. donax*), brewery's spent grains (BSG) and corn cobs were utilized for the saccharification experiments. Untreated *A. donax* biomass was kindly provided by Enea Trisaia Research Center – Italy and consisted of chips of approximately 3 × 2 × 0.5 cm of size obtained from whole plants, including leaves. The biomass was then dried at 50 °C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. BSG were provided by Maltovivo micro-brewery, Avellino, Italy and corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal). Both materials were dried up at 50 °C to a moisture content less than 10% (w/w), milled to particles smaller than 3 mm and stored in sealed vials at room temperature until use.

2.2. Pretreatments and determination of chemical composition

The different raw materials were subjected to a pretreatment with an aqueous ammonia solution on a lab-scale as described by Maurelli et al. (2013). The biomasses reduced to a fine powder, were soaked in 10% (v/v) aqueous ammonia solution at a solid loading of 5% at 70 °C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800 × *g*, and the residues were extensively washed with 50 mM sodium acetate buffer until obtaining the required pH for the subsequent enzymatic saccharification process (pH 5.0).

The biomass from *A. donax* pretreated by steam explosion (Di Pasqua et al., 2014) was also analyzed.

Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis (1998). This procedure involved an acid hydrolysis carried out in two step which fractionate the polysaccharides into their corresponding monomers. First, the samples were soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30 °C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and the secondary hydrolysis was performed for 1 h at 120 °C. After filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analyzed via HPLC as described below. The acid insoluble lignin (Klason lignin) was determined by weighting the dried residue after total removal of the sugars.

2.3. Enzymatic activities

The following commercial enzymatic preparations: Accellerase 1500 (cellulase enzyme complex), Accellerase XY (xylanase enzyme complex), and Accellerase BG (β-glucosidase) were kindly provided by Danisco US Inc., Genencor Division (Palo Alto, CA, USA). Cellulase from *Trichoderma reesei* ATCC26921, Cellobiase from *A. niger*, Xylanase from *Trichoderma viride* and thermostable β-xylosidase were purchased from Sigma (St. Louis, MO). The cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *E. coli* (Amore et al., 2012b), the α-L-arabinofuranosidase from *P. ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf) (Amore et al., 2012a) and a mutant of rPoAbf indicated as rPoAbf F435Y/Y446F (Giacobbe et al., 2014) were utilized in combination with the commercial enzymatic preparations.

The enzymatic activities were measured at 50 °C in 50 mM sodium acetate buffer (pH 5.0). Cellulase activity was determined spectrophotometrically utilizing the soluble chromogenic substrate carboxymethyl cellulose-Remazol Brilliant Blue R (AZO-CM-Cellulose) (Megazyme Co., Bray Ireland). The standard assay mixture, containing 150 μL of 0.2% (w/v) substrate in 50 mM sodium acetate buffer, and 150 μL of properly diluted enzyme solution, was incubated for 30 min. The reaction was stopped by adding 750 μL of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation at 9000 × *g* for 10 min. The absorbance of the supernatant was read at 590 nm, and the enzyme units were calculated from a standard curve constructed with known amounts of cellulase from *T. viride*. The xylanase activity was evaluated by the same assay method utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) (Megazyme Co., Bray Ireland), as the substrate (Biely et al., 1985). One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 A min^{-1} under the experimental conditions. β-xylosidase activity was determined, according to Biely et al. (2000), utilizing the soluble chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP) (Sigma, St. Louis, MO). The assay was started by adding 50 μl of appropriately diluted enzyme in 50 mM sodium acetate buffer pH 5.0 to 450 μL of 2 mM *p*NPXP, in the same buffer. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the assay conditions. α-Arabinofuranosidase and β-glucosidase activities were evaluated by the same assay method utilizing *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside and *p*NP-β-D-glucopyranoside (Sigma, St. Louis, MO) as the substrates, respectively. All the enzymatic measurements were performed in triplicates.

2.4. Enzymatic hydrolysis

The saccharification experiments were carried out at 50 °C for 72 h in a total volume of 5 mL (50 mM sodium acetate buffer pH 5.0 plus enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated lignocellulosic materials was carried out with enzyme cocktails prepared with the following commercial products at the indicated amounts expressed as units per grams of pretreated biomass: 5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG (mix 1) or 5.4 U g^{-1} Cellulase from *T. reesei* ATCC26921, 145 U g^{-1} of Cellobiase from *A. niger*, 80 U g^{-1} of Xylanase from *T. viride*, 8 U g^{-1} of thermostable β-xylosidase (mix 2). Because of its high glucose content, Accellerase BG was dialyzed against 50 mM sodium acetate before addition to the pretreated biomass.