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Co-immobilization and stabilization of xylanase, β-xylosidase and α-L-arabinofuranosidase from *Penicillium janczewskii* for arabinoxylan hydrolysis

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ABSTRACT

Differently activated agarose-based supports were evaluated for co-immobilization of a crude extract from *Penicillium janczewskii* containing xylanase, β-xylosidase and α-L-arabinofuranosidase activities. Adequately selecting support and immobilization conditions (8 h, using agarose with 10% crosslinking) increased enzyme levels substantially, mainly in relation to the xylanase (2-fold). A coating with dextran aldehyde MW 6000 Da, partially oxidized, covalently attached the enzymes to the support. Optimum activity was verified in the pH range 2–4, and at 50, 65 and 80 °C for the xylanase, α-L-arabinofuranosidase and β-xylosidase, respectively. The xylanase was highly thermostable retaining more than 70% of activity even after 24 h incubation at 60 and 70 °C; and at 80 °C its half-life was 1.7 h. The half-lives of the β-xylosidase and α-L-arabinofuranosidase at 50 °C were 2.3 and 3.8 h, respectively. The co-immobilization of the enzymes on a single support give raise to a functional multi-enzymatic biocatalyst acting in the complete hydrolysis of different and complex substrates such as oat spelt and wheat arabinoxylans, with xylose yield higher than 40%. The xylanase and the α-L-arabinofuranosidase presented high stability retaining 86.6 and 88.0% of activity after 10 reuse cycles.

1. Introduction

Xylan is the second most abundant biopolymer in plant cell walls and the main hemicellulosic polysaccharide. It is composed of a β-(1 → 4) D-xylopyranosyl backbone substituted at various degrees by side chain residues such as glucopyranosyl, 4-*O*-methyl-D-glucurono-pyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl, and/or *p*-coumaroyl [1]. The precise composition of the polymer is strongly dependent on plant species and tissue. For instance, hard wood xylans often have D-glucuronic acid attached to their backbone, whereas L-arabinose is the most common branch in cereal xylans [2].

Given the diversity of xylan structures, their complete and efficient hydrolysis involves the synergistic action of main chain degrading enzymes, including *endo*-β-1,4-xylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37), and side chain cleaving enzymes, including α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterase (EC 3.1.1.72), and feruloyl esterases (EC 3.1.1.73). Endo-β-1,4-xylanase and β-D-xylosidase are the main enzymes responsible for the degradation of the polymer: xylanases cleave the internal β-(1 → 4) bonds in the xylan backbone, liberating different chain-length-(substituted) xylooligosaccharides, and β-xylosidases are exoglycosidases that release xylose from the non-reducing ends of these xylooligosaccharides. β-xylosidases are critical for the systems since they carry the greatest work load in terms of number of glycosidic bonds cleaved, as well as in relieving product inhibition of xylanases [3]. Among other accessory enzymes, α-L-arabinofuranosidases are

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exo-type enzymes that catalyze the cleavage of the terminal α-L-arabinofuranosyl residues from arabinosylated substrates. Xylan hydrolysis is not aleatory, *i.e.*, the degree of substitution in xylan influence the products of hydrolysis for xylanases [4]. In this sense, accessory enzymes such as α-L-arabinofuranosidases are important since the removal of side-chain residues from xylan backbone may have a synergistic effect with the other xylanolytic enzymes [5] and also the difference in substrate specificity among different xylanases has important implications in the deconstruction of xylan [4].

Enzyme immobilization poses as a possibility to improve the characteristics of an enzyme in terms of stability and catalysis, as well as for process improvement allowing the reuse of the biocatalyst for many operational cycles [6]. Immobilization of more than one enzyme on the same support, however, is especially challenging, as it has to preserve the catalytic activity of all enzymes involved in the system and ideally improve their stability [7]. Many xylanolytic enzymes have been individually immobilized by different methods; and some studies have investigated the co-immobilization of two xylanolytic enzymes [8]. In this sense, the xylanase and β-xylosidase from *Talaromyces thermophilus* were co-immobilized on chitosan and employed for the hydrolysis of oat spelt xylan, demonstrating the synergistic action of both enzymes by increasing the saccharification of the substrate [9]. In another study, co-immobilization of recombinant xylanase and α-L-arabinofuranosidase onglyoxyl agarose was evaluated through different approaches in the hydrolysis of arabinoxylan [10]. The effect of xylanase, β-xylosidase and α-L-arabinofuranosidase from *Aspergillus oryzae* in the decomposition of arabinoxylan was verified using the soluble enzymes in the moromi mash during soy sauce fermentation [11], nevertheless, co-immobilization of three xylanolytic enzymes acting cooperatively in the complete hydrolysis of complex substrates has not been reported to date.

This way, the aims of this work were to establish a protocol for simultaneous co-immobilization of the xylanase, β-xylosidase and α-L-arabinofuranosidase from *Penicillium janczewskii* present in the crude extracellular extract, as well as improve the stabilization of the immobilized enzymes via post-immobilization techniques. After that, the immobilized enzymes were biochemically characterized and evaluated in the hydrolysis of arabinoxylans.

2. Materials and methods

2.1. Materials

Agarose with 4, 6 and 10% of cross-linking BCL were purchased from Agarose Bead Technologies (Madrid, Spain). *p*-nitrophenyl β-D-xylopyranoside (*p*NPX), glycidol, potassium tetraborate tetrahydrate, sodium borohydride, sodium periodate, ethylenediamine, glutaraldehyde, *Leuconostocc* spp. dextran (MW 6000–100,000), polyethylenimine (PEI, MW 1300), oat spelt and beechwood xylans were obtained from Sigma-Aldrich Co (St. Louis, MO). D-xylose Assay Kit, xylose, *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra) and low viscosity wheat arabinoxylan were from Megazyme (Wicklow, Ireland). All reagents were of analytical grade.

2.2. Methods

2.2.1. Microorganism, enzyme production and preparation of enzyme extract

P. janczewskii (CRM 1348) is deposited in The Central of Microbial Resources, CMR-UNESP, Brazil. The microorganism was maintained on Vogel solid medium [12] and liquid cultures were prepared in the same medium with brewer's spent grain as substrate, under optimized conditions for xylanolytic enzymes production [13]. After cultivation, the mycelium was removed by vacuum filtration and the culture filtrate was centrifuged (10,000g, 4 °C, 15 min). The supernatant was dialyzed *overnight* against distilled water, 0.025 M sodium acetate buffer pH 5.0 or 0.025 M sodium phosphate buffer pH 7.0 before immobilization. A sample of the supernatant was also treated with 0.01 M sodium periodate for 1.5 h in order to oxidize sugar moieties of the enzymes and then dialyzed against 0.025 M sodium phosphate buffer pH 7.0.

2.2.2. SDS-PAGE

A sample containing 50 μg of protein prepared from the extracellular extract obtained under optimized conditions for xylanase production (medium with oat spelt xylan, pH 6.5, 7 days of cultivation, 30 °C) was applied to SDS-PAGE performed in 8–18% (w/v) gradient gels, according to Laemmli [14]. The resolved protein bands were visualized after staining with 0.1% Coomassie brilliant blue R-250 dissolved in methanol, acetic acid, and distilled water (4:1:5 v/v/v). Standard proteins (Sigma) were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa).

2.2.3. Enzyme assays

Xylanase activity was determined according to Bailey et al. [15] with 1% (w/v) beechwood xylan prepared in 0.05 M sodium acetate buffer pH 5.0 (before determining optimum pH) or pH 4.0 (after determining optimum pH) and appropriately diluted enzyme solution. Reducing sugars were quantified with DNS acid reagent [16]. β-xylosidase and α-L-arabinofuranosidase activities were determined in a reaction mixture containing, respectively, 3 mM *p*NPX and *p*NPAra prepared in 0.05 M sodium acetate buffer pH 5.0 (before determining optimum pH) or pH 4.0 (after determining optimum pH) and appropriately diluted enzyme solution to 1 mL final volume. Reactions were stopped by adding 1 mL of a saturated potassium tetraborate solution and the absorbance was measured at 405 nm [17]. One unit of activity was defined as the amount of enzyme required to release 1 μmol of product equivalent per min in the assay conditions at 25 °C.

2.2.4. Preparation of support

Monoaminoethyl-*N*-aminoethyl (MANAE)-agarose, was prepared as described elsewhere [18] and the glutaraldehyde-agarose support was prepared from MANAE agarose [19,20]. Briefly described, we used 10 g of MANAE in 20 mL of 0.5 or 15% (v/v) glutaraldehyde solution prepared in 0.2 M phosphate buffer pH 7.0. The suspensions were kept under mild stirring at 25 °C for 1 and 15 h in the case of the supports activated with 0.5 or 15% (v/v) glutaraldehyde, respectively. This treatment permitted to fully modify the primary amino groups of the support with one or two glutaraldehyde molecules, respectively [19]. After that, the supports were filtered and washed exhaustively with 0.025 M sodium phosphate buffer and then with distilled water. Glyoxyl-agarose was prepared with the maximal activation degree, as previously described [21]. Polyethylenimine (PEI) and dextran sulfate [22] and heterofunctional amino-glyoxyl and amino-epoxide [23,24] supports were prepared as described elsewhere.

The supports were initially prepared using agarose with 4% crosslinking. MANAE and 0.5% (w/v) glutaraldehyde supports were further prepared using agarose with 6 and 10% crosslinking. Activated supports were stored at 4 °C and, before use, washed with incubation buffer according to immobilization condition.

2.2.5. Enzyme immobilization

Immobilizations were performed by suspending 1:10 (w/v) the activated supports in the dialyzed/diluted enzyme solution. Buffers