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Synergistic action of co-expressed xylanase/laccase mixtures against milled sugar cane bagasse

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ABSTRACT

The primary plant cell wall is composed of cellulose, hemicellulose, lignin and protein in a stable matrix. The concomitant depolymerization of lignin by laccase and of hemicelluloses by xylanase can improve lignocellulose degradation in the production of second generation biofuels. A thermophilic variant of xylanase A (XynAG3) and the thermostable laccase (CotA), both from *Bacillus subtilis*, were produced in co-transformed *Pichia pastoris* strain GS115. Mobility changes in SDS-PAGE after Endo H digestion indicated that both enzymes were glycosylated. The maximum catalytic activity of the $XynAG3_{Pp}$ and the $CotA_{Pp}$ was observed at 58 °C and 75 °C, respectively, and both enzymes presented high activity at pH 5.0. The half-life at 60 °C of $XynAG3_{Pp}$ and $CotA_{Pp}$ was 150 min and 540 min, respectively. The relative levels of $CotA_{Pp}$ and $XynAG3_{Pp}$ in culture broths were altered by the concentration of methanol used for induction, and $CotA_{Pp}$:$XynAG3_{Pp}$ ratios of 1:1.5 and 1:2 were evaluated against milled sugar-cane bagasse. The highest activity was observed at a 1:2 ratio of $CotA_{Pp}$:$XynAG3_{Pp}$, and was 44% higher as compared to the sum of the activities of the individual enzymes in the same assay conditions. These results demonstrate the synergistic action between an endoxylanase and a laccase against the natural lignocellulosic substrate.

1. Introduction

Vegetal lignocellulosic material is a principal component of terrestrial biomass and consists mainly of cellulose, hemicellulose and lignin, in variable amounts depending on the plant source. Cellulose is embedded in a complex matrix of hemicellulose and lignin [1], and therefore delignification is of importance for the exploitation of cellulose in industrial processes. Laccases (EC 1.10.3.2) decompose lignin by oxidation of a wide range of aromatic and polyphenolic compounds, which is coupled to the reduction of dioxygen to water [2], and xylanases (EC 3.2.1.8) depolymerize xylan, the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in plant cell walls [3]. Applications of mixtures of laccases and xylanases have been reported [4], and more recently these enzymes have attracted considerable interest due to their potential for application in ethanol production using lignocellulosic residues, since removal of lignin and hemicelluloses can significantly enhance the hydrolysis of cellulose to release reducing sugars for subsequent fermentation to ethanol [5].

Laccases coordinate the binding of four copper atoms in three types of sites that differ in their environment and spectroscopic properties [6]. Redox potentials of laccases are usually lower in comparison with those of other lignin-modifying enzymes such as lignin peroxidase or manganese peroxidase [7], and they act in the degradation of polymers, the ring cleavage of aromatic compounds and the cross-linking of monomers. The best-studied bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis*. The cotA gene encodes a 65-kDa protein that exhibits a high thermal stability with a half-life of 2 h at 80 °C and an optimum catalytic temperature at 75 °C [8].

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Endo-1,4-β-xylanases (EC 3.2.1.8) catalyze the hydrolysis of the β-1,4 glycosidic bonds in the xylan backbone to produce xylooligosaccharides of varying length [9]. Xylanases from various bacteria, fungi and yeasts have been characterized [10], and are found predominantly in glycosylhydrolase families GH5, GH8, GH10 and GH11 and also in other families including GH7, GH16, GH30, GH43 and GH62 [11]. The xylanase A from the mesophile *B. subtilis* (XynA) is a GH11 enzyme, and has been extensively studied [12,13].

Several yeast expression systems have been developed for commercial production of recombinant proteins, and one of the most commonly used systems is the methylotrophic yeast *Pichia pastoris*, where expression is driven by the strong promoter for the alcohol oxidase I (AOX1), which is induced by methanol and is repressed by other carbon sources such as glucose, glycerol, and ethanol [14]. Another important feature of this system is the capacity for growth to very high cell densities in well-defined medium, which contributes to the efficient production and secretion of heterologous proteins [15]. For many applications the cost of enzymes is one of the main factors determining the economic viability of a process. Cost reduction may be achieved by optimizing the reaction conditions so as to be compatible with temperature and pH requirements of the enzymes, increasing enzyme production to lower unit cost, and using enzyme combinations that promote synergy of catalytic function. In this study we have investigated the ability of the eukaryote *P. pastoris* to efficiently co-express two *B. subtilis* enzymes, a thermophilic XynA derived from directed evolution and the CotA. The pH and temperature optima of the heterologous enzymes were characterized, together with their thermal stability, kinetics and synergistic effects against an insoluble lignocellulosic biomass.

2. Methodology

2.1. Construction of P. pastoris strains

The coding sequence of a thermophilic variant of the xylanase A (*xynAG3*) created by directed evolution [16] and the laccase gene, *cotA*, from *Bacillus subtilis* (BGSC 1A1 code 168), were cloned into pET28a as previously described [4]. A PCR reaction was performed with the construct pET28_*cotA* as template and the primers LacPN (5′-GGCAGCCTCGAGAAAAGAGAGGCTGAAGCTAGCAGCCATCATCAT-3′), LacPC (5′-GGGCCTAGGTTATTTATGGGGATCAGTTAT-3′) which introduced restriction sites for *Xho*I and *Avr*II, respectively (underlined sequences). Using the pET28_*xynAG3* construct as a PCR template, the primers XylPN (5′-GGCAGCCTCGAGAAAAGAGAGGCTGAAGCTAGCACAGACTAC-3′) and XylPC (5′-GATCAGCGGCCGCCGTTAGCTACCC-3′) introduced restriction sites for *Xho*I and *Not*I, respectively, in the thermophilic variant XynAG3. The PCR products encoding the CotA and the XynAG3 were digested with the appropriate restriction enzymes and cloned into plasmids pPIC9K and pPICZαA, respectively. The pPIC9k_*cotA* construct included an N-terminal polyhistidine sequence, and the pPICZαA_*xynAG3* construct included a C-terminal polyhistidine sequence. The pPIC9k_*cotA* and pPICZαA_*xynAG3* constructs were linearized with the restriction enzymes Stu*I* and *Sac*I, respectively, and the linearized DNA was used for transformation of *P. pastoris* GS115 by electroporation with a Gene Pulser (BioRad, Hercules, CA, USA). The linearized construct pPIC9k_*cotA* was inserted into the HIS4 gene by homologous recombination and selected by histidine auxotrophism and the linearized pPICZαA_*xynAG3* was inserted within the 5′ AOX1 region and selected by zeocin resistance. To obtain a double transformant, the previously constructed *P. pastoris* GS115/pPICZαA_*xynAG3* was electroporated with the pPIC9k_*cotA* construct. The strain transformed with double construct (*P. pastoris* GS115/pPICZαA_*xynAG3*/pPIC9k_*cotA*) was selected by zeocin (500 μg mL^{-1}) resistance on MD agar plates (0.34% yeast nitrogen base, 1% ammonium sulfate, 4×10^{-5}% (w/v) biotin, 100 mM phosphate buffer, pH 6.0, 2% dextrose, 1.5% bacteriological agar).

2.2. Protein expression and purification

Both XynAG3 and CotA were expressed individually and in a coexpression mixture. For expression of the XynAG3, a single colony of *P. pastoris* GS115/pPICZαA_*xynAG3* was inoculated into 100 mL of BMG medium (1.34% yeast nitrogen base with amino acids, 4×10^{-5}% (w/v) biotin, 100 mM potassium phosphate, pH 6.0, and 1.0% (v/v) glycerol) and grown at 30 °C in an orbital shaker until the culture reached an $OD_{600\,nm}$ of 3. Cells were harvested by centrifugation at 3000 × *g* for 5 min, and the cell pellet was resuspended to an $OD_{600\,nm}$ of 1 in 100 mL BMM medium (BMG medium in which glycerol was substituted with either 0.5% or 1% methanol) and the culture was grown for 6–8 days at 30 °C. Every 24 h methanol was added, and at the end of the culture, the cells were centrifuged (3000 × *g*, 15 min) and the supernatant was stored at 4 °C. For expression of the CotA and XynAG3/CotA mixtures, *P. pastoris* GS115/pPIC9k_*cotA* and the *P. pastoris* GS115/pPICZαA_*xynAG3*/pPIC9k_*cotA*, the yeast nitrogen base with amino acids was replaced with 0.34% yeast nitrogen base without ammonium sulfate and without amino acids plus 1% ammonium sulfate and 0.25 mM $CuSO_4$.

The supernatant expression medium of individual enzymes expressed in *P. pastoris*, Xylanase AG3 ($XynAG3_{Pp}$) and Laccase ($CotA_{Pp}$), was cleared of cell debris by centrifugation at 5000 *g* for 7 min at 4 °C and filtration with a 0.2 μm filter. The filtrate was concentrated and the buffer exchanged with an Amicon Stirred Cell 10000 MWCO (Merck Millipore, Billerica, MA, USA). The concentrate was applied to a HiTrap™ 3 mL Q-HP column (Promega, Madison, WI, USA) previously equilibrated with 500 mM NaCl, 40 mM HEPES buffer pH 8.0. The column was washed with buffer containing 40 mM HEPES pH 8.0, 300 mM NaCl, and 40 mM imidazole. Proteins were eluted with 40 mM HEPES buffer pH 8.0, 500 mM NaCl and 300 mM imidazole at a flow rate of 1.5 mL min^{-1}. Fractions containing the enzymes were concentrated and desalted using an Amicon Ultra-15 10000 MWCO (Merck Millipore, Billerica, MA, USA). Protein purity was routinely checked using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Deglycosylation of recombinant proteins by endoglycosidase H

The purified recombinant protein samples (10–20 μg) were boiled for 5 min in 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS. After cooling, the samples were mixed with 250 U of endoglycosidase H (Endo-H) (New England Biolab, Ipswich, MA, USA) and incubated at 37 °C for 3 h. The samples were subsequently analyzed by SDS-PAGE and mass spectrometry.

2.4. Mass spectrometry analysis

After SDS-PAGE analysis, slices containing the protein bands of the Endo H digested samples were cut from the gel, destained with 50% methanol, treated with 10 mM DTT (dithiothreitol) for 1 h at 56 °C and alkylated with 55 mM chloroacetamide for 1 h at 25 °C. After washing in 50 mM ammonium bicarbonate, the gel slices were shrunk in 100% acetonitrile. Digestion with trypsin was performed in 50 mM ammonium bicarbonate for 8 h at 37 °C with agitation. The peptides were extracted with 90% acetonitrile/0.5 M

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