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Research Paper

Comparison of different microbial laccases as tools for industrial uses

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Laccases from different sources are employed in a number of biotechnological processes, each characterized by specific reaction constraints and thus requiring an enzyme with suitable properties. In order to avoid the bias generated by different assay methodologies, in this work we investigated the main properties of ten laccases from fungi and bacteria under identical conditions. As a general rule, the optimal activity was apparent at pH 3–4 and was lost at pH $\geq$ 7.0 (all laccases were stable at pH $\geq$ 7.0); enzymes active at neutral pH values were also identified. For all tested laccases, activity increased with temperature up to 80°C and stability was good at 25°C. Interestingly, laccases insensitive to high salt concentration were identified, this favoring their use in treating waste waters. Indeed, bacterial laccases retained a significant activity in the presence of DMSO (up to 40% final concentration) and of surfactants, suggesting that they can be applied in lignin degradation processes requiring solvents. The available laccases are versatile and satisfy requirements related to different processes. Notably, the recombinant laccase from *Bacillus licheniformis* favorably compares with the tested enzymes, indicating that it is well suited for different biotechnological applications.

Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2), sometimes also referred to as polyphenol oxidases, belong to the group of blue multi-copper oxidases along with ceruloplasmin, ascorbate oxidase, bilirubin oxidase, and various manganese oxidases. Laccase is attracting great scientific interest because of its very basic requirements – it only uses oxygen from the air and releases water as the sole by-product – and great catalytic capabilities, rendering it one of the 'greenest' enzymes [1–3]. Laccase couples the four-electron reduction of dioxygen to water with the oxidation of a broad range of substrates (including phenols, polyphenols, arylamines, anilines, hydroxyindols, and thiols). Its substrate promiscuity can be even further expanded by using redox mediators, that is, diffusible electron carriers from natural or synthetic sources [4–6].

Laccases are widely distributed among fungi, higher plants and bacteria. Fungal laccases contain four Cu ions, organized into two copper clusters: one situated in a mononuclear T1 site close to the protein surface and three others buried at a trinuclear site, consisting of a mononuclear T2 site and a binuclear T3 site [7,8]. In the resting form of the enzyme, the four copper ions are in the +2 oxidation state [7,9]. The catalytic cycle begins by sequestering one electron at a time from the reducing substrate and transferring it to the trinuclear T2/T3 copper cluster through a highly conserved His-Cys-His pathway, where four electrons reduce dioxygen to two molecules of water.

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Laccases are commonly classified into two main groups: 'low-medium' and 'high' redox potential laccases according to their redox potentials at the T1 site, ranging from +430 mV in bacterial and plant laccases to +790 mV in some fungal laccases: the latter are typically secreted by ligninolytic basidiomycetes and are the most important for biotechnological applications [1,10].

The potential uses of laccases are numerous due to their low catalytic requirements and oxidative versatility. In fact, these enzymes catalyze a wide spectrum of reactions, which include cross-linking of monomers, degradation of polymers, ring cleavage, and oxyfunctionalization of aromatic compounds [4,5,8]. Laccases are used in several industrial sectors, including the textile (for bleaching) and dye industry, for effluent treatment and bioremediation, in the food industry (for beverage processing and baking), and in forest-product industry, where lignin removal is the main goal (paper manufacture) or where lignin polymerization is the main objective (e.g., manufacture of fiberboards and synthesis of novel materials) [6]. Furthermore, fungal laccases have been recently employed to improve the conversion of plant biomass in integrated lignocellulose biorefineries, in organic synthesis (i.e., for the enzymatic conversion of chemical intermediates and the synthesis of pigments and antioxidants through dimerization of phenolic and nonphenolic acids), in oxidative transformation of environmental pollutants (e.g., herbicides), and in bioelectrocatalysis for detecting phenolic pollutants [1,11,12]. Moreover, in the presence of redox mediators, laccases can transform compounds with higher redox potentials as well as complex polymers such as lignin, avoiding limits due to steric hindrance [13,14].

In the past several years, a number of laccases from different sources [15] were isolated and characterized. These enzymes have been assayed on different compounds using different experimental conditions, however, making it difficult to compare their characteristics: this limit is further hampered by the fact that various isoenzymes, which differ in biochemical properties, are produced by the same strain. A major challenge of laccase use is that the catalytic activity and stability are highly dependent on experimental variables such as pH, temperature, ionic strength, and co-solvents. In this study we evaluated the main biochemical properties of a number of commercial, natural and recombinant laccases under identical experimental conditions, with the final goal to compare their features in order to identify the best biocatalyst for specific applications.

Materials and methods

Reagents

The laccases from *Rhus vernicifera*, *Trametes versicolor*, and *Pleurotus ostreatus* were purchased from Sigma–Aldrich (L2157, S38429 and S75117, respectively) (Sigma–Aldrich, Milano, Italy). LAC enzymes were supplied by ASA Spezialenzyme GmbH as lyophylized powder: LAC C from *T. versicolor* (2.500 U/g on ABTS, 400 U/g on catechol as substrate) and LAC 4 from *Trametes* sp. (50 U/g on ABTS as substrate) are extracellular enzymes isolated from the supernatant of fermentation broth; LAC 3 from *Myceliophthora thermophila* (Mt, 2.200 U/g on ABTS, 3.000 U/g on catechol as substrate) is expressed in *Aspergillus niger* as extracellular enzyme and is isolated from supernatant of the fermentation broth; LAC A from *Agaricus bisporus* (Ab or LAC A, 2.200 U/g on ABTS, 6.000 U/g on catechol as substrate) is extracted and purified from the stems of the fruit body; LAC 5 from *Thielavia* sp. (1.100 U/g on ABTS as substrate) is an extracellular enzyme isolated from the supernatant of the fermentation broth.

The total protein concentration was quantified using the biuret method; purity was judged by SDS-PAGE and native-PAGE analyses (Supplementary Table S1). All LAC enzymes gave a single band by zymogram analysis with the only exception of LAC 5 that produced a smear containing at least two different forms. All chemicals were of analytical grade (Sigma–Aldrich).

Design and cloning of cDNA encoding for BALL laccase

The synthetic cDNA encoding the laccase from *Bacillus licheniformis* (BALL) [16] was designed by *in silico* back translation of the amino acid sequence reported in the GenBank database (Accession no. GU972589.1). In order to facilitate subcloning into the pET24b(+) plasmid (Merck Millipore, Vimodrone, Italy), sequences corresponding to *Nde*I (CATATG) and *Xho*I (CTCGAG) restriction sites were added at the 5′- and 3′-ends of the cDNA, respectively. The codon usage of the synthetic gene was optimized for expression in *Escherichia coli* and produced by GeneArt (Life Technologies, Monza, Italy) (Accession no. KR348913). BALL cDNA was inserted in the pET24b(+) vector using the *Nde*I and *Xho*I sites, resulting in a 6.9-kb construct (pET24-BALL). Six codons (encoding for six additional histidines) were added to the 3′-end of the BALL gene during the subcloning process.

BALL expression and purification

The pET24-BALL plasmid was transferred to the host BL21(DE3) *E. coli* strain (Merck Millipore). Cells were grown at 37°C in Terrific broth (TB) medium. Protein expression was induced at an $OD_{600nm} \approx 1.6$ by adding 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 2 mM $CuSO_4$; the cells were grown further at 18°C for 18 hours. In order to enhance copper incorporation, eighteen hours after adding IPTG the cells were incubated at different temperatures for further 6 hours without shaking. Cells were harvested by centrifugation ($8000 \times g$ for 10 min at 4°C) and lysed by sonication (four cycles of 30 s each, with 30 s interval on ice). The crude extract was heated at 75°C for 15 min and centrifuged at $39{,}000 \times g$ for 30 min at 4°C.

The enzyme was purified using a HiTrap chelating affinity column (1 mL) previously loaded with metal ions (1 mL of 100 mM $NiCl_2$) and equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 1 M NaCl and 5% glycerol. The column was washed with this buffer until the absorbance value at 280 nm was that of the buffer. Then, the bound protein was eluted with 50 mM Tris–HCl buffer (pH 7.0) containing 500 mM imidazole and 10% glycerol [17]. The fractions containing laccase activity were dialyzed against 50 mM sodium acetate, pH 5.0. The amount of protein was estimated by absorbance at 280 nm using a molar extinction coefficient of 84,739 M^{-1} cm^{-1} [18].

Design and cloning of cDNA encoding for OB1 laccase

The synthetic cDNA encoding the mutated α-factor prepro-leader and the OB1 variant of laccase from basidiomycete PM1 [19] was designed by *in silico* back translation of the amino acid sequence reported in the GenBank database (Accession no. CAA78144.1)