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Research review paper

Laccase engineering: From rational design to directed evolution

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ABSTRACT

Laccases are multicopper oxidoreductases considered by many in the biotechonology field as the ultimate "green catalysts". This is mainly due to their broad substrate specificity and relative autonomy (they use molecular oxygen from air as an electron acceptor and they only produce water as by-product), making them suitable for a wide array of applications: biofuel production, bioremediation, organic synthesis, pulp biobleaching, textiles, the beverage and food industries, biosensor and biofuel cell development. Since the beginning of the 21st century, specific features of bacterial and fungal laccases have been exhaustively adapted in order to reach the industrial demands for high catalytic activity and stability in conjunction with reduced production cost. Among the goals established for laccase engineering, heterologous functional expression, improved activity and thermostability, tolerance to non-natural media (organic solvents, ionic liquids, physiological fluids) and resistance to different types of inhibitors are all challenges that have been met, while obtaining a more comprehensive understanding of laccase structure–function relationships. In this review we examine the most significant advances in this exciting research area in which rational, semi-rational and directed evolution approaches have been employed to ultimately convert laccases into high value-added biocatalysts.

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Introduction

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductases) catalyse the oxidation of a wide array of compounds coupled with the four-electron reduction of oxygen to water (Morozova et al., 2007a). They belong to the multicopper oxidase family, which also comprises ceruloplasmin, ascorbate oxidase, bilirubin oxidases and ferroxidases

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(Solomon et al., 1996). These enzymes contain four copper atoms: one paramagnetic type 1 copper (T1 Cu) that is responsible for their characteristic blue colour and where the oxidation of the reducing substrate occurs, one type 2 copper (T2 Cu)and two type 3 coppers (T3 Cu) that conform a trinuclear cluster in which molecular oxygen is reduced to two molecules of water (Davies and Ducros, 2006; Mot and Silaghi-Dumitrescu, 2012) (Fig. 1).

Since a laccase was first extracted from the exudate of the Japanese lacquer tree *Toxicodendron vernicifluum* in the late nineteenth century (Yoshida, 1883), these enzymes have been identified in more than 20 bacterial species (Santhanam et al., 2011), in several higher plant species (Mayer and Staples, 2002) and in lichens (Laufer et al., 2009). Moreover, polyphenol oxidases with laccase-like activity have also been described in insect cuticles (Lang et al., 2012), in oysters (Luna-Acosta et al., 2010) and in metagenomic libraries of bovine rumen (Beloqui et al., 2006). However, laccases are particularly abundant in fungi, having been found in almost all wood-rotting fungi analysed to date (Brijwani et al., 2010). While bacterial laccases are intracellular or periplasmic enzymes, fungal laccases are typically extracellular proteins that show different glycosylation degrees.

Laccases play diverse biological roles that are determined by their origin and the life stage of the organism that produces them. In bacteria they participate in morphogenesis, pigmentation, oxidation of toxic compounds, and protection against ultraviolet radiation and oxidizing agents (Singh et al., 2011). In addition, plant laccases are involved in wound responses and lignin polymerization (Mayer and Staples, 2002), while the polyphenol oxidases with laccase activity discovered in insect cuticles participate in the sclerotisation (Miessner et al., 1991). In fungi, diverse functions are fulfilled by laccases, including morphogenesis, stress defence, fungal plant-pathogen/host interactions and lignin degradation (Alcalde, 2007).

The substrate range of laccases is very broad as they can oxidize aromatic compounds (*ortho*- and *para*-diphenols, methoxysubstituted phenols, diamines, benzenethiols), metal ions (Mn^{2+}), organometallic compounds (*e.g.* $[W(CN)_8]^{4-}$-, $[Fe(EDTA)]^{2-}$), organic redox compounds (*e.g.* 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS; 1-hydroxybenzotriazole, HBT) and the iodide anion (Morozova et al., 2007b; Xu, 1996). Furthermore, in the presence of both natural and synthetic redox mediators, the catalytic activity of these enzymes may be expanded to non-phenolic substrates that are very recalcitrant and hardly oxidized by laccase alone (*e.g.* polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls, azo-dyes or organophosphate pesticides) (Cañas and Camarero, 2010).

From an electrochemical viewpoint and based on the analysis of their molecular structures, these enzymes are classified into three different groups according to the redox potential of the T1 site ($E^{0'}_{T1}$): low-, medium-, and high-redox potential laccases (Mot and Silaghi-Dumitrescu, 2012) (Table 1). The $E^{0'}_{T1}$ of laccases is not determined by a single structural feature but it is rather the result of the combination of various factors, such as copper–ligand interactions, the effects of desolvation around the T1 site, the intermolecular electrostatic interactions, and the restrictions in protein folding (Li et al., 2004). Bacterial and plant laccases constitute the group of low-redox potential laccases, with an $E^{0'}_{T1}$ below +460 mV *vs.* NHE (Normal Hydrogen Electrode) and a Met residue as the T1 Cu axial ligand. Fungal laccases fall into the medium- and high-redox potential classes. The group of medium-redox potential laccases includes enzymes from ascomycetes and basidiomycetes fungi, with an $E^{0'}_{T1}$ ranging from +460 to +710 mV *vs.* NHE, and typically with a Leu as the non-coordinating axial ligand. High-redox potential laccases (HRPL) are mainly produced by basidiomycete white-rot fungi, with an $E^{0'}_{T1}$ ranging from +730 to +790 mV *vs.* NHE, and with a Phe as the non-coordinating axial ligand. This latter group is

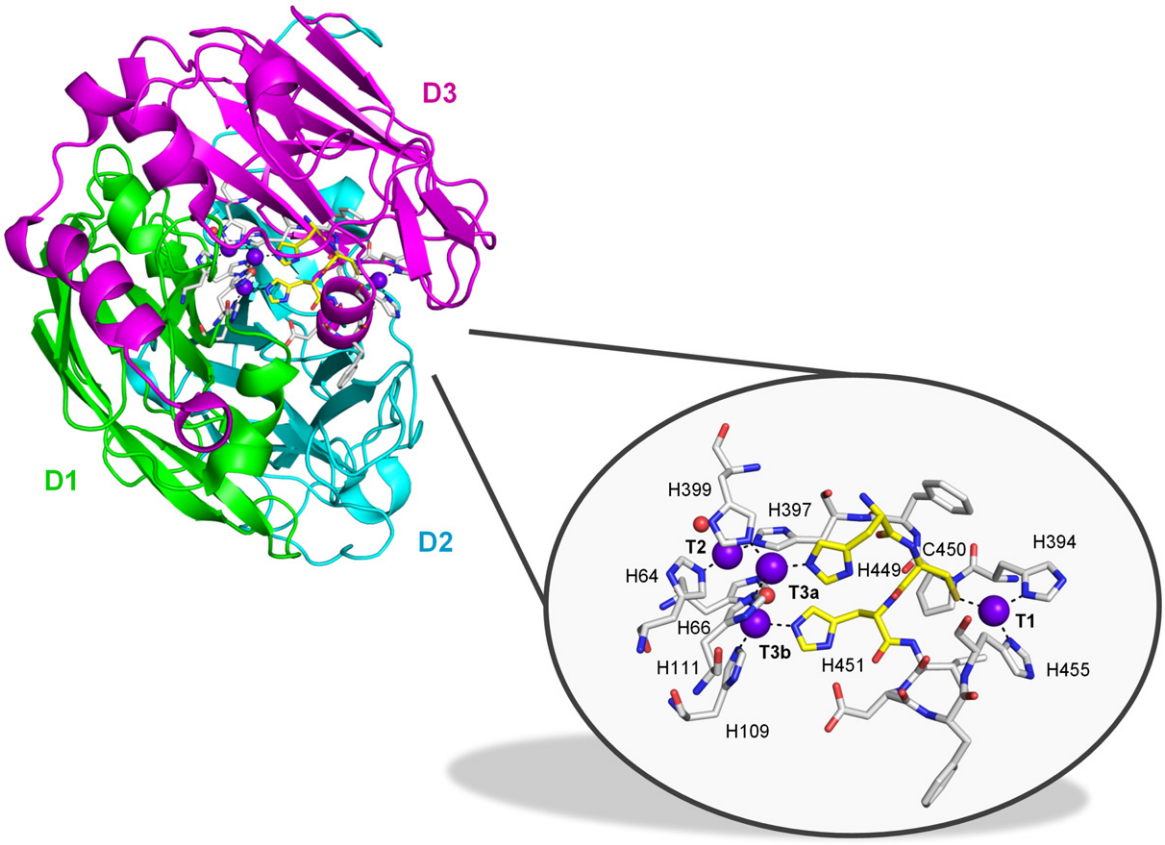

Fig. 1. General structure and details of the active site of laccase (*Trametes trogii* laccase, PDB ID: 2HRG). The three cupredoxin-like domains (D1, D2 and D3) are shown in green, cyan and magenta, respectively. Purple blue spheres represent copper ions and red spheres depict coordinating water molecules. The residues of the internal transfer pathway from T1 Cu to the T2/T3 trinuclear cluster are colored in yellow. Residues involved in the first coordination sphere of the catalytic coppers and their interactions (as black dashes) are also represented.