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Functional expression, production, and biochemical characterization of a laccase using yeast surface display technology

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ABSTRACT

A *Trametes versicolor* laccase was functionally expressed on the membrane surface of *Saccharomyces cerevisiae* EBY100. Laccase expression was increased 6.57-fold by medium optimization and surpassed production by the native strain. Maximal laccase and biomass production reached 19 735 ± 1719 Ug^{-1} and 6.22 ± 0.53 gL^{-1} respectively, after 2 d of culture. Optimum oxidization of all substrates by laccase was observed at pH 3. Laccase showed high affinity towards substrates used with Km (mM) and Vmax (μmol min^{-1}) values of 0.57 ± 0.0047 and 24.55 ± 0.64, 1.52 ± 0.52 and 9.25 ± 1.78, and 2.67 ± 0.12 and 11.26 ± 0.75, were reported for ABTS, 2, 6-DMP and GUA, respectively. EDTA and NaN_3 displayed none competitive inhibition towards laccase activity. The optimum temperature for activity was 50 °C; however, the enzyme was stable over a wide range of temperatures (25–70 °C). The biologically immobilized laccase showed high reusability towards phenolic substrates and low reusability with non-phenolic substrates. High affinity for a diversity phenolic compounds and great ethanol tolerance substantiates this laccase/yeast biocatalyst potential for application in the production of bioethanol.

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Abbreviations; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CA, Casamino acids; Gal, Galactose; GUA, Guaiacol; pETcon/6c, Expression plasmid pETcon + laccase 6c; SD-W, Synthetic drop-out medium with dextrose and without tryptophan; SGal-W, Synthetic drop-out medium with galactose and without tryptophan; YNB, Yeast nitrogen base without amino acids; YSD, Yeast surface display; YPD, Yeast extract peptone dextrose medium; YSD-6c, Laccase yeast surface display system (S. cerevisiae transformed with pETcon/6c); YSD-pETcon, S. cerevisiae transformed with pETcon (–); 2,6-DMP, 2,6-dimethoxyphenol

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Introduction

Laccase (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) is a blue multi-copper oxidase that catalyses the oxidation of phenols, aromatic amines, and other aromatic compounds concomitantly with the reduction of molecular oxygen to water (Trejo-Hernández *et al.* 2001). The wide variety of substrates oxidized by laccases and the use of oxygen as an electron acceptor continues to attract interest in these enzymes for industrial applications, including textile dye bleaching, pulp bleaching and soil bioremediation. For such reasons, many efforts have focused on the molecular and biochemical characterization, of native and heterologous expression of fungal laccases (Autore *et al.* 2009; Bertrand *et al.* 2014).

Laccase production by lignolytic fungi has been comprehensively investigated due to the ability of these microorganisms to grow on economic substrates, secretion of enzymes and their capacity to oxidize xenobiotic compounds (Shah & Nerud 2002; Ikehata *et al.* 2004). However, production in this area suffers from yet still, major drawbacks (low enzyme production, low catalytic activity, and extended production periods), that hinders the ideal demand/supply balance, for desired industrial application. As result, academic groups and companies have focused their energies in the use genetic engineering. This approach involves the cloning and expression of genes of interest using compatible hosts. Thus, the search of efficient heterologous expression systems is of utmost importance (Bertrand *et al.* 2014).

Cell-surface display is one of the most recent heterologous expression systems to be developed. This system involves the expression of peptides and proteins on the surface of living cells by fusing them to functional components of cells which are exposed to the environment of cells. This strategy can be carried out using different surface proteins of cells as anchoring motifs and different proteins from different sources as a passenger protein (Tafakori *et al.* 2012). There are some display systems using microorganisms, such as phages, bacteria, and yeasts. In the case of the yeast *Saccharomyces cerevisiae*, a-agglutinin, α-agglutinin, or flocculin has been used as an anchor protein. These proteins exist on the yeast cell surface and have glycosylphosphatidylinositol (GPI) anchors that play important roles in the surface localization of proteins. The GPI-anchored proteins translocate to the cell surface through the secretory pathway of *S. cerevisiae* (Kato-Murai & Ueda 2008).

Yeast surface display (YSD) is a potentially very powerful biotechnological tool. The potential of this technology includes the expression of membrane proteins, peptides and enzymes. In the future it will be fundamental in applications such as cell adhesion, molecular recognition, signal transduction, protein folding, cell physiology, protein engineering, immobilization of biocatalysts, change in cell function, biosensors and in bioremediation (Kato-Murai & Ueda 2008; Tafakori *et al.* 2012). Enzymes displayed on yeast as whole-cell biocatalysts have many advantages such as easy proliferation, simple genetic manipulation and easy production procedure (by cultivation and centrifugation only); thus, they are more favourable than immobilized enzymes (Kato-Murai & Ueda 2008; Chen *et al.* 2011). YSD has been used successfully for the production, immobilization, and application of enzymes such as lipases (Shiraga *et al.* 2005; Chen *et al.* 2011; Lui *et al.* 2014). However, up to date, only a few attempts have been made to heterologously express laccases in this system, with little or no success (Lu *et al.* 2012; Nakanishi *et al.* 2012; Bleve *et al.* 2014).

The aim of this study was to functionally express a laccase from *Trametes versicolor* using YSD, and characterize the system as a whole cell biocatalyst.

Materials and methods

Strains and media

Escherichia coli DH5 α F−Φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK−, mK+) *pho*A *sup*E44 λ−*thi*-1 *gyr*A96 *rel*A1 was used as a host for DNA manipulation. *Saccharomyces cerevisiae* strain EBY100 (ATCC® MYA-4941™) MATaAGA1::-*GAL*1-AGA1::*URA*3 *ura*3-52 *trp*1 *leu*2-Δ 200 *his*3-Δ 200 *pep*4::*HIS*3 *prb*11.6R *can*1 *GAL* was used as the host for protein expression. *E. coli* cells transformed by electroporation were recovered in 2X YT (16 gL^{-1} tryptone, 10 gL^{-1} yeast extract, 5 gL^{-1} sodium chloride) medium, and then grown in LB medium (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 10 gL^{-1} sodium chloride) containing 15 mg mL^{-1} ampicillin. YPD medium (10 gL^{-1} yeast extract, 20 gL^{-1} peptone, 20 gL^{-1} dextrose) was used to cultivate yeast host cells. Yeast transformants were cultivated in SD-W medium containing 20 gL^{-1} dextrose, 7 gL^{-1} yeast nitrogen base without amino acids (Amresco, Ohio) and 20 gL^{-1} casamino acids.

Laccase cloning and expression

Isolation and molecular characterization of laccase KR492187 (lac 6c) was previously described (Bertrand *et al.* 2015). The nucleotide sequence of lac 6c was amplified without its native *Trametes versicolor* signal peptide since the expression plasmid pETcon (−) already presented a signal peptide native to *Saccharomyces cerevisiae* (Fig 1A). The plasmid pETcon (a gift from Andrew Scharenberg) is a shuttle plasmid for expression in yeast presenting a GAL 1 promoter, and different tag/fusion proteins (HA, 3× (G4S) Linker, Stuffer, G3S Linker, Myc Tag). This plasmid also presents ampicillin and TRP1 as the selectable markers for *Escherichia coli* and yeast, respectively (https://www.addgene.org/41522/). The restriction sites for *NdeI* and *BamHI* were introduced via PCR at the 5′ and 3′ with oligonucleotides PC*NdeI* 5′-gctagccatatggccatcgggccggtg-3′ and PC*BamHI* 5′-gatgagaaggatcctcactggttagcctcgct-3′, respectively. Lac 6c PCR product and yeast surface display expression plasmid pETcon (−) were digested with endonucleases *BamHI* and *NdeI* and purified using DNA Gel Recovery Kit (Zymoresearch). The digested PCR product and the vector were ligated and purified. 1 μL of the purified of the ligation reaction was used for electroporation of *E. coli* DH5α in a Bio-Rad gene pulser®/microplate pulser™ with 0.1 cm electrode gap electroporation cuvettes (1800 V in 5.2 ms). Positive clones were analysed by digestion and PCR and subsequently purified. The resulting plasmid, pEtcon/6c (Fig 1B), is in the process of being deposited in the Addgene plasmid repository.

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