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Short Communication

Expression system of CotA-laccase for directed evolution and high-throughput screenings for the oxidation of high-redox potential dyes

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Laccases are useful biocatalysts for many diverse biotechnological applications. In this study we have established efficient and reliable expression systems and high-throughput screenings for the recombinant CotA-laccase from *Bacillus subtilis*. The expression levels of *cotA-laccase* were compared in five different *Escherichia coli* host strains growing in 96-well microtiter plates under different culture conditions. Lower coefficients of variance (around 15%) were achieved using crude cell lysates of BL21 and KRX host strains growing under microaerobic conditions. Reproducible high-throughput screenings for the decolorization of high redox potential azo and anthraquinonic dyes were developed and optimized for identification of variants with increased redox potential. The enzymatic assays developed were tested for the screening of one mutant library from CotA-laccase created by error-prone PCR.

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Laccases belong to the multicopper oxidase (MCO) family of enzymes [1]. Most MCOs contain ~500 amino acid residues and are typically composed of three Greek key β-barrel cupredoxin domains (domains 1, 2 and 3) that come together to form three spectroscopically distinct types of Cu sites, *i.e.*, type 1 (T1), type 2 (T2), and type 3 (T3) [2, 3]. T1 copper ("blue copper") is a mononuclear catalytic center involved in substrate oxidation, whereas T2 and T3 form a trinuclear center involved in dioxygen reduction to water. These enzymes are able to oxidize a range of aromatic substrates such as polyphenols, methoxy-substituted phenols, diamines and even some inorganic compounds reducing dioxygen to water [4]. Owing to their relatively high nonspecific oxidation capacity, laccases have been found to be useful biocatalysts for diverse biotechnological applications including the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, and, as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil [4–6]. Furthermore, laccases can be used in organic synthesis, as they can perform exquisite transformations ranging from the oxidation of steroid hormones or heteromolecular coupling for the production of new antibiotics derivatives to the enzymatic polymerization required for the synthesis of phenolic-based resins or water-soluble polymers [5, 6].

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Abbreviations: ABTS, 2,2 azino bis (3-ethylbenzthiazoline-6-sulfonic acid); **DMP**, 2,6-dimethoxyphenol; **HTS**, high-throughput screening; **MCO**, multicopper oxidase, **MTP**, microtiter plate

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Redox potential is a key property of laccases as substrate oxidation in these enzymes involves the Marcus "outer-sphere" mechanism in which the redox potential difference between the substrate and the T1 Cu site in laccases (together with the reorganization energy and the transmission coefficient) determines the electron transfer and thus, the oxidation rate [3, 7–11]. The redox potentials exhibited by the T1 copper sites of laccases span over a broad range of values, from 400 mV for plant laccases to 790 mV for some fungal laccases [1, 3]. The reasons for the wide potential range are not yet fully understood, but it is known that it is influenced by a variety of factors, including the solvent accessibility of the metal center and the electrostatic interactions between this center and the protein [3]. Most of the site-directed mutagenesis at the T1 Cu site of laccases have highlighted the limitation of rational approaches towards the modulation of the redox potential [12–17] and thus, encourage, as an alternative, the application of directed evolution techniques for this purpose. Directed evolution has been very successful in engineering proteins with altered substrate specificity, improved activity, affinity, thermostability, solvent stability or protein folding [18–25], and at the same time represents a complementary approach to structure-based protein design strategies [26].

CotA-laccase from *Bacillus subtilis*, our model system, is a suitable platform for a molecular evolution protocol, since it has the predictable robustness for the destabilizing conformational changes needed to accommodate beneficial functional mutations through evolution [28–30]. It is produced in high yields in *E. coli* [27, 28], the preferred host organism due to the ease of genetic manipulation, availability of efficient genetic tools, high transformation efficiency and stability of plasmid DNA. Furthermore, the evolution studies on laccases are still scarce and based only on two fungal systems [20–24] and there are reasonable expectations for the occurrence of a high frequency of beneficial random mutations towards a higher redox potential considering its relatively low value (525 mV *vs*. SHE [27]) in CotA-laccase. To address the evolution of CotA-laccase for a higher redox potential, a strategy of mutagenesis and screening was delineated. Low-error-rate random-mutagenesis PCR, which explores sequence space efficiently, was chosen as a method for generating molecular diversity. Taking into consideration that the redox potentials of variant enzymes from directed evolution libraries cannot be screened directly in crude cell lysates, several high-throughput screenings (HTSs) were developed for a group of high redox potential, structurally diverse, synthetic dyes. It is expected that the combination of HTS for these dyes would allow for the indirect identification of enzyme variants with increased redox potential. In this study, the protocol for the production of bacterial CotA-laccase by *E. coli* in 96-well microtiter plates (MTPs) was optimized and the development of effective HTS assays for standard laccase substrates and for selected high redox potential dyes was pursued. Furthermore, we assessed an error-prone PCR mutant library with the methodologies developed.

The growth of the recombinant *E. coli* Tuner strain expressing the *cotA-laccase* gene (strain AH3517 [28]) was tested in 96-well MTPs in different aeration conditions. A pre-culture started from isolated colonies was grown in 96-well MTPs containing 200 μL Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin, at 30°C and 600 rpm. For cultivation, 20 μL of 24-h pre-cultures (shorter periods affected negatively the homogeneity of final cultures ODs) were dispensed into new 96-well MTPs containing 180 μL culture medium with antibiotic and cultivated until the $OD_{600\,nm}$ reached values of ~0.6. At this time, the temperature was reduced to 25°C, the medium was supplemented with 250 μM $CuCl_2$ and *cotA-laccase* expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). Different aeration conditions were tested: (i) aerobic (shaking at 600 rpm), (ii) microaerobic (switching off the shaker 4 h after induction [27]) and (iii) anaerobic (static). After 24 h of cultivation the 96-well MTPs were centrifuged for 30 min at 3500 × *g*, 4°C. The cell sediments were suspended in 40 μL B-PER lysis solution (Bacterial Protein Extraction Reagent, Pierce), adjusted to 100 μL with 20 mM Tris-HCl buffer, pH 7.6, and centrifuged for 30 min at 4000 × *g*, 4°C. The resulting crude cell lysates were used for protein determination by the Bradford assay [31] or enzymatic activity measurements performed on a Synergy2 (BioTek) microplate reader at 37°C using the classical phenolic and non-phenolic laccase substrates 2,2'-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 2,6-dimethoxyphenol (2,6-DMP), respectively [16]. The assay mixtures contained undiluted lysates (1:10 dilution) in Britton and Robinson buffer and crude cell lysates (1:10 dilution) and reactions were initiated by adding the substrate at a final concentration of 1 mM. Reactions with ABTS were performed at pH 4 and followed at 420 nm (ε = 36 000 $M^{-1}cm^{-1}$), while reactions with 2,6-DMP were performed at pH 7 and followed at 468 nm (ε = 49 600 $M^{-1}cm^{-1}$). We found that the final $OD_{600\,nm}$ of cultures were similar after growth in aerobic (1.04 ± 0.22), microaerobic (0.85 ± 0.15) or anaerobic conditions

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(0.75 ± 0.17). However, aeration has a significant impact on the yield of recombinant active enzyme expressed per cell as it is shown in Fig. 1. In cells grown under aerobic conditions no activity could be detected in crude extracts in contrast to anaerobic or microaerobic cultures. This is presumably related with the copper content of the produced recombinant enzyme, which was shown to be strictly dependent on culture aeration conditions as previously described [27]. The reproducibility of *cotA-laccase* expression system was tested in four 96-well MTPs using a liquid handling robot (Microlab Starlet, Hamilton) by measuring the total protein content and the enzymatic activities for ABTS and 2,6-DMP in crude extracts of AH3517 cells grown under microaerobic conditions. In these conditions, a very high (25%) coefficient of variance (CV) was found with either 2,6-DMP and ABTS (Table 1). To correctly interpret the upcoming screening data and reduce the probability of missing positive variants, the variability of *cotA-laccase* expression was tested in additional recombinant *E. coli* strains: the plasmid pLOM10 coding for the *cotA-laccase* gene was transformed into strain BL21(DE3)pLysS (Novagen) producing strain LOM446, KRX (Promega) producing strain LOM447, strain Rosetta(DE3)pLysS (Novagen) producing strain LOM504 and strain Origami (DE3)pLysS (Novagen) producing strain LOM505. The expression of *cotA-laccase* is under the control of the strong T7*lac* promoter, induced upon addition of IPTG, in all strains with the exception of LOM447 strain, where recombinant gene expression is under the control of promoter *rha*P_{BAD}, induced by rhamnose. The lower variances for HTS were obtained with crude cell lysates of LOM446 and LOM447 strains (Table 1). The CV of 14–15% achieved are expected to allow variants to be identified with at least twofold improved activity [32]. The decision to continue with LOM447 strain was based on its higher transformation efficiency when compared with strain LOM446, besides its features of cloning and expression strain, which avoided an additional step of plasmid transference among strains in the evolution process.

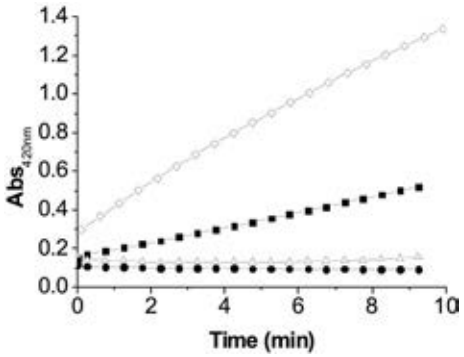

Figure 1. Time course for the oxidation of ABTS using supernatants of control cells (not expressing the *cotA*-laccase gene) (●), strain AH3517 grown at aerobic (△), microaerobic (◇) and anaerobic (■) conditions.

HTS for dye decolorization were optimized using crude cell lysates of strain LOM447 and a group of structurally diverse dyes that are substrates of the enzyme (Fig. 2, [33]). We selected three groups of dyes: (i) Sudan Orange G, Reactive Black 5 and Acid Blue 62 that are bleached with a high efficiency (85–98%) by the purified enzyme; (ii) Direct Black 38, Reactive Red R and Reactive Red 4 that present a intermediary susceptibility (38–60%) to degradation, and (iii) Disperse Yellow 3 that is hardly decolorized (8%). Table 2 shows that all dyes present a high redox potential as estimated from oxidation peaks in cyclic voltammograms. The decolorization was followed in four 96-well MTPs, in the absence and presence of 5 μM ABTS, by measuring changes in the absorbance at the maximum wavelength of each dye for 24 h (Fig. 3). The aim of adding ABTS to the reaction mixtures was to increase the rate of decolorization [33]. The rate and the percentage of decolorization were calculated and a low CV of 4–7% was achieved.

We have used a library of variants of the whole *cotA-laccase* gene created by epPCR to validate the HTS developed. *Mutazyme*II polymerase (Gen-

Table 1. Protein concentration and ABTS and 2,6-DMP activity in crude cell lysates of five different recombinant *E. coli* strains overproducing WT CotA-laccase. The validation of the HTS enzymatic assays were performed using data from four 96-well MTPs[a)]

E. coli strains	Protein		ABTS activity		2,6-DMP activity	
	(mg/mL)	CV (%)	(U/mL)	CV (%)	(U/mL)	CV (%)
AH3517	0.097 ± 0.015	15	456 ± 113	25	42 ± 5	21
LOM446	0.159 ± 0.019	12	464 ± 62	14	42 ± 5	13
LOM447	0.125 ± 0.019	15	436 ± 15	15	37 ± 5	14
LOM504	0.178 ± 0.033	19	950 ± 126	21	n.d	n.d.
LOM505	0.114 ± 0.026	23	179 ± 51	29	n.d	n.d.

a) n.d., not determined

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Table 2. Anodic peak potential of azo and anthraquinonic dyes used in the HTS protocols[a)]

Dye (dye content)	Functional group	Anodic peak potential (mV *vs.* SHE)
Sudan Orange G (98%)	Azo	700
Acid Blue 62 (98%)	Anthraquinone	987
Reactive Black 5 (55%)	Dis-azo	922
Direct Red R (91%)	Dis-azo	866
Direct Black 38 (50%)	Tris-azo	1220
Reactive Red 4 (50%)	Azo	1174
Disperse Yellow 3 (30%)	Azo	826

a) The cyclic voltammetry experiments were performed using a Voltalab 30 Potentiostat (Radiometer Analytical, France), controlled by the Voltamaster 4 electrochemical software. The working, counter and reference electrodes were respectively: glassy carbon electrode (0.07 cm^2), coiled platinum wire (23 cm) and an Ag/AgCl electrode filled with saturated KCl (BAS, Bioanalytical Systems, West Lafayette, IN, USA). Cyclic voltammograms were registered at a scan rate of 10 mV/s. All experiments were done in 0.1 M phosphate buffer, pH 7.4.

emorph kit II, Stratagene) was selected to catalyze the mutagenic PCR, since libraries created using this enzyme produce a low-biased mutation spectrum, with equivalent mutation rates at AT base pairs *versus* GC base pairs. The manufacture protocol for a low mutation rate (0-4.5 mutations/kb) was followed with minor modifications. The primers used for amplification were: Fwd-CotA (5′-ATACATATGGCTAGCTTGGAAAATTTAG-3′) and Rev (5′-CCAAGGGGTTATGCTAGTTATTGCTCAG-3′). Thirty cycles for amplification of 2 min at 95°C, 0.5 min at 55°C, 1 min at 72°C were performed. The pool of PCR-mutated *cotA* (1539 bp) was digested with *Dpn*I from Fermentas (St. Leon-Rot, Germany), purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and cloned into vector pLOM10 using the restriction sites *Nhe*I and *Xho*I. To avoid the re-cloning of the wild type (WT) caused by incomplete restriction and re-ligation of pLOM10, the 1539 bp DNA fragment coding for amino acids residues 1–513 of CotA-laccase were previously removed from pLOM10 by digestion with *Nhe*I and *Xho*I followed by insertion of a ΩSm/Sp cassette from plasmid pHRP316 [34] into the same sites. The ΩSm/Sp cassette was removed by enzymatic cleavage and the vector was recovered from agarose gel using the QIAquick Gel Extraction Kit from Qiagen and used to clone the pool of mutated DNA. The resulting plasmid was transformed into *E. coli* KRX strain. The DNA sequence analysis of 12 variants chosen randomly showed a mutation rate between 0-5, very close to the expected 0-4.5. Figure 4 shows the result of the mutant library screening (around 1800 clones) using the ABTS activity assay, plotted in order of decreasing activity. Around 4% of all clones screened (60 clones) present 1.5-2.5-fold higher activities than the parent strain and 53% of variants exhibit less than 10% of the parent enzyme activity. A re-screening with freshly transformed cells was performed to get a correct comparison of clones and discard false-positives to identify a few preliminary "winners" (Table 3). Two mutations (K461M and K351N) were found at the surface of the enzyme far from the Cu catalytic sites, while mutations I369T, K373N and L386F are closer to the T1 Cu center; *e.g.*, K373 is 14 Å and L386 5 Å from T1 Cu [35]. Since we are introducing 0–4 mutations in

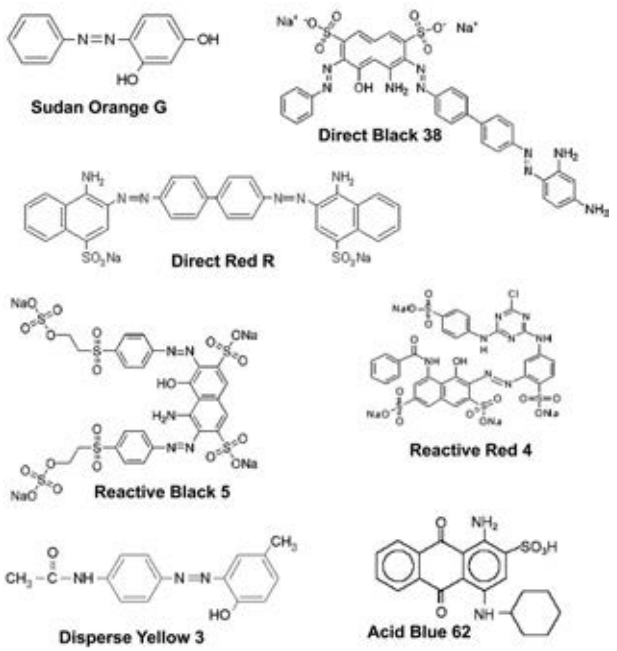

Figure 2. Structures of azo and anthraquinonic dyes used in the HTS protocols.

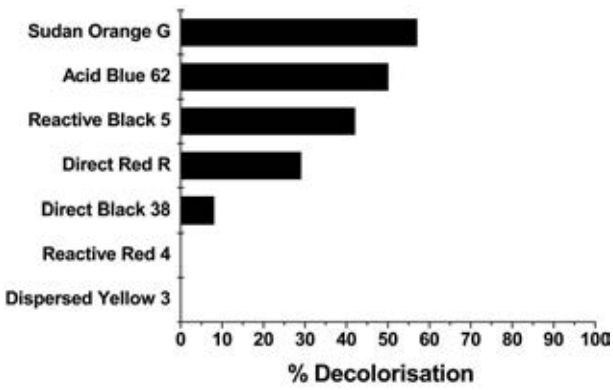

Figure 3. Dye decolorization (50 μM) in 0.1 M Britton and Robinson buffer, pH 8 using crude cell lysates of the strain LOM447 in the presence of 5 μM ABTS. The plates were sealed and incubated at 37°C with shaking (600 rpm) for 24 h.

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Table 3. "Winners" from the screening of the *cotA-laccase* epPCR library. Enzymatic activities in crude cell lysates of mutants as compared to the WT

Mutants	Enzymatic activities as compared to the WT			
	ABTS	Sudan Orange G	Acid Blue 62[a)]	Reactive Black 5[a)]
9A4 (K461M/2 silence)	1.6	1.2	1.6	1.2
9C8 (I369T/K461M)	1.7	1.3	1.9	1.3
9H8 (L386F/K461M)	1.7	1.1	1.4	1.2
10H2 (K461M)	1.9	1.5	1.7	1.2
16H5 (K351N)	2.5	1.5	1.7	1.3
19A12 (K373N)	1.5	1.1	1.1	0.9

a) With 5 μM of ABTS used as redox mediator [33].

the whole gene of CotA-laccase (~500 amino acids) we will continue to screen this library to cover a higher diversity of mutants created and to select the most suitable mutant for parenting the next generation of random mutagenesis. We will additionally focus the random mutagenesis in a shorter selected region of CotA-laccase, domain 3 that contains the catalytic T1 Cu site, contributes to the properties of the T2/T3 Cu center and includes the putative substrate binding site [35]; this would increase the chances of an efficient library screening. Interestingly, all the mutations found in the HTS are localized at the end of domain 2 or in domain 3 of the enzyme.

The key to designing directed enzyme evolution strategies, a purpose of current work in our laboratory, is the establishment of efficient expression and screening systems that accommodates the predicted diversity generated by the mutagenesis or combinatorial techniques, and thus it is expected that the future outcome of this study will lead to the generation of CotA-laccase variants with improved biotechnological effectiveness and versatility and/or to a better insight over the structure-function relationships in the MCO family of enzymes.

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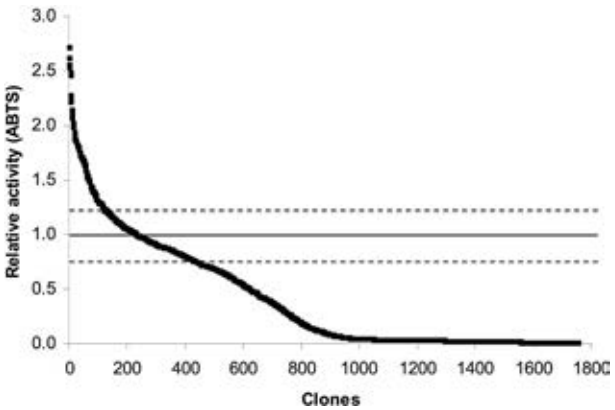

Figure 4. Activity profile for ABTS oxidation for whole gene *cotA-laccase* mutant library prepared by error-prone PCR. Activity is plotted in descending order. The solid horizontal line shows the activity of the parent WT CotA-laccase in the assay and dashed lines indicate the CV of the assay (15%).

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