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Short Communication

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Engineered *Bacillus pumilus* laccase-like multi-copper oxidase for enhanced oxidation of the lignin model compound guaiacol

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Abstract

Laccases and laccase-like multi-copper oxidases (LMCOs) are versatile and robust biocatalysts applied in a variety of oxidative processes, and various studies have attempted to improve their catalytic activity. Here we report the engineering of a bacterial LMCO for enhanced oxidation of the lignin-related compound guaiacol by a combination of structure-guided mutagenesis and DNA shuffling. Mutant L9 showed a 1.39 mM K_m for guaiacol and a 2.5-fold increase in turnover rate ($k_{cat}/K_m = 2.85{\cdot}10^4\ M^{-1}s^{-1}$).

Key words: DNA shuffling, engineered bacterial LMCO, guaiacol oxidation, laccase engineering, lignin, site-directed mutagenesis

Introduction

Laccase-like multi-copper oxidases (LMCOs) and the subgroup laccases (E.C. 1.10.3.2, *p*-benzenediol:oxygen oxidoreductase) are enzymes of high relevance for biotechnological processes such as the synthesis of pharmaceuticals, food processing (Giardina *et al.*, 2010), fragrance production, bioremediation (Miele *et al.*, 2010) and functionalization of materials (Božič *et al.*, 2012; Ihssen *et al.*, 2014; Fernandez-Fernandez *et al.*, 2015). The oxidation of substrates occurs at the T1 copper site located on the surface of the protein. The extracted electrons are transferred to an inner T2-T3 copper cluster where oxygen is reduced to water (Giardina *et al.*, 2010). In nature, fungal laccases are thought to play important roles in both the synthesis and in the degradation of lignin, while the function of bacterial LMCOs is still uncertain (Munk *et al.*, 2015; Pollegioni *et al.*, 2015; Wang *et al.*, 2015).

Constituting 15–40% of the dry weight of plants, lignin is the most available aromatic polymer on Earth (Ragauskas *et al.*, 2014). Lignin has a complex structure carrying p-hydroxyphenyl, guaiacyl and syringyl phenylpropanoid units in different ratios (Boerjan *et al.*, 2003; Mohammadali *et al.*, 2015). Currently burnt for heat or energy production, lignin could be used to obtain higher added-value products by introducing desirable functional groups or by modifying its physicochemical features (Boerjan *et al.*, 2003; Heap *et al.*, 2014; Laurichesse and Avérous, 2014; Ragauskas *et al.*, 2014). Most laccases cannot directly oxidise lignin and the presence of redox mediators (Martinez *et al.*, 2009) or other secreted enzymes (Munk *et al.*, 2015) is required. Evidence of the direct interaction between a laccase and lignin has however been provided for the small bacterial laccase from *Streptomyces coelicolor* A3(2) that crystallised in a state directly binding a non-phenolic lignin model compound (Majumdar *et al.*, 2014).

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The substrate specificity of laccases and LCMOs is broad and includes lignin-like molecules such as mono- and di-phenolic compounds, together with amino phenols and methoxy phenols (Madhavi and Lele, 2009). The *Bacillus pumilus* CotA-type LMCO used in this study offers good thermostability and it can be produced in high yields in *Escherichia coli* (Reiss *et al.*, 2011; Ihssen *et al.*, 2015). *Bacillus pumilus* LMCO oxidises the synthetic substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-suphonic acid) (ABTS) with a high catalytic efficiency of $3.6{\cdot}10^6\ M^{-1}\ s^{-1}$, however, the k_{cat}/K_m ration is 200-fold lower for phenolic lignin-related compounds such as 2,6-dimethoxyphenol (2,6-DMP) (Reiss *et al.*, 2011). In the present study, the LMCO from *B. pumilus* was genetically engineered to promote its direct activity with lignin-like compounds by using 2-methoxyphenol (guaiacol) as substrate.

Protein engineering by structure-guided, iterative randomisation of active site residues and DNA shuffling is a common strategy to tailor the performance of enzymes (Stemmer, 1994b; Reetz *et al.*, 2010). Properties of Basidiomycete laccases such as activity under blood-like conditions or resistance to organic cosolvents and ionic liquids have been improved by multiple rounds of *in vitro* evolution (Zumarraga *et al.*, 2007; Liu *et al.*, 2013; Mate *et al.*, 2013). Protein engineering studies have also focused on bacterial LMCOs (Mate and Alcalde, 2015), with one aiming at improving the thermostability of an enzyme from *Bacillus* sp. HR03 using site-directed point mutations (Mollania *et al.*, 2011), and another one enhancing expression and catalytic activity with ferulic acid of *B. licheniformis* LMCO by random and site-directed mutagenesis (Koschorreck *et al.*, 2009). In a third example substrate specificity of *B. subtilis* CotA was changed by simultaneous randomisation of residues in the active site (Gupta *et al.*, 2010). In our study, we used site-specific mutagenesis in combination with DNA shuffling to produce variants of *B. pumilus* LMCO with higher catalytic efficiency for the substrate guaiacol, a model lignin compound (Boerjan *et al.*, 2003) (Fig. 1).

Results and discussion

Starting point for the selection of mutagenesis targets was the construction of a homology model of *B. pumilus* LMCO. The positions of bound ABTS and the four copper atoms of the active sites were inferred from the crystal structure of *B. subtilis* CotA (PDB ID: 1UVW) (Enguita *et al.*, 2004), with whom *B. pumilus* LMCO shares a 65% sequence identity. Residues in close distances of 2.89–4.95 Å to the ABTS molecule in the *B. pumilus* LMCO model were selected for pairwise randomisation, i.e. P226 and F227, T260 and I494, L386 and G417, L415 and R416. Screening in 96-well plates yielded numerous variants with similar or slightly improved guaiacol oxidation rates (up to 2-fold) compared to the wild-type (wt) *B. pumilus* LMCO. Six double or single mutants, P226L-F227D, T260G-I494L, L386H-G417I, L386Q-G417R, L386Q-G417F and T415L were selected based on relative activity, frequency of occurrence and diversity for the next engineering step based on DNA shuffling.

We developed a novel agar plate screening protocol for LMCOs expressed in *E. coli*. By including an anoxic incubation step, which was previously shown to increase copper incorporation and activity yields of CotA in *E. coli* in liquid culture (Durao *et al.*, 2008; Ihssen *et al.*, 2015), we were able to achieve reproducible and homogenous coloration of LMCO-expressing colonies on solid medium after exposure to air. When the library with shuffled double and single mutants of *B. pumilus* LMCO library was screened on plates containing guaiacol, variants with different degrees of activity could be differentiated (Fig. 2).

Candidate clones with visually increased colorization were selected by manual picking of the colonies. Increased efficiency of guaiacol oxidation was first confirmed on agar plates (Fig. 2, right panel) and then for crude cell lysates from mini-cultures in 96-deep-well plates. The two best-performing clones L2 and L9 exhibited 9- and 14-fold higher guaiacol oxidation rates in the lysates, respectively, compared to the strain expressing the wt *B. pumilus* LMCO (data not shown). Sequencing of the isolated plasmids from these clones revealed that both were mutated at positions L386 and G417 (Fig. 2, right panel). The same residues were also mutated in variant L386W-G417L of the related enzyme *B. subtilis* CotA that had been shown to have an increased catalytic efficiency with ABTS (Gupta *et al.*, 2010). In the structural model of *B. pumilus* LMCO (Fig. 3), the tip of the side chain of L386 was in 3.2 Å distance to an ethyl group of ABTS, and residue G417 was also in close proximity (3.7 Å) to another ethyl group located on the opposite side of the substrate molecule. One can assume that the amino acid substitutions L386Q and G417R/I of variants L2 and L9 lead to a narrower binding pocket and offer additional possibilities for electrostatic interactions. Both changes may lead to enhanced binding of the relatively small guaiacol molecule.

Due to the high number of PCR cycles during reassembly and amplification, DNA shuffling often introduces additional mutations

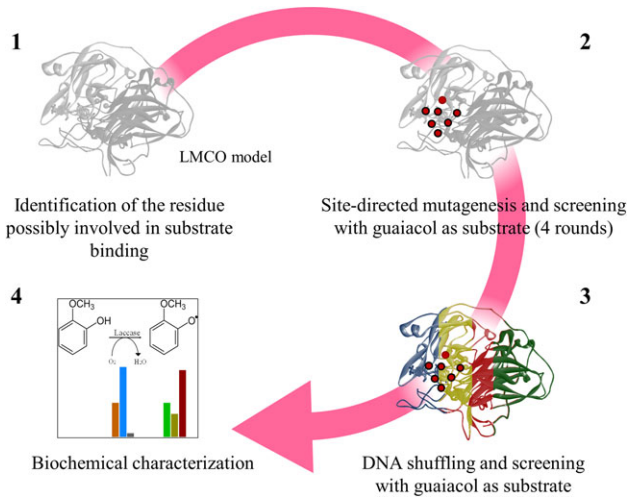

Fig. 1 Four-step approach used for the selection of *B. pumilus* LMCO mutants with high activity with guaiacol.

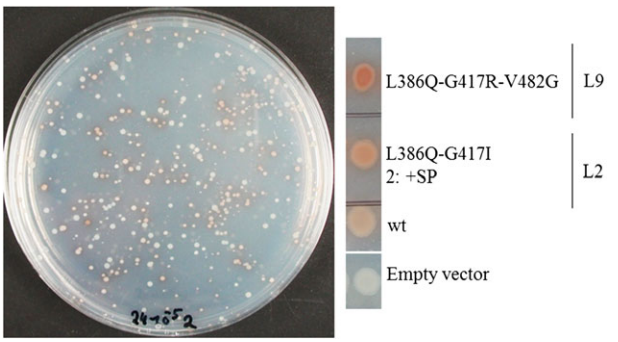

Fig. 2 Solid phase screening of a shuffled library of *B. pumilus* LMCO variants expressed in *E. coli* for guaiacol oxidation. Left: representative screening plate; right: confirmation of selected improved variants L2 and L9 after transfer to new plate. wt: wild-type plasmid pBuL, empty vector: pET22b(+) without insert.

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at random positions (Stemmer, 1994a). Variant L2 was found to harbour the insertion of two amino acids (Ser and Pro) after the N-terminal methionine, in addition to the L386Q-G417I substitutions. The N-terminal end is not part of the active site of *B. pumilus* LMCO (Fig. 3); nevertheless, the insertion may induce structural changes with indirect positive effects on activity or may compensate for potential destabilising effects of L386Q-G417I. Variant L9 contained a third amino acid substitution V482G that was located at the channel allowing access of oxygen and water to the trinuclear copper centre (Fig. 3). This mutation could enhance guaiacol oxidation indirectly via accelerated oxygen diffusion and/or may compensate for the destabilising effects of L386Q-G417R substitutions.

Increased activity of LMCO variants in culture could be due to either an enhanced expression/solubility of the protein (Koschorreck *et al.*, 2009) or to an improved catalytic efficiency. *B. pumilus* LMCO variants L2 and L9 were thus purified in parallel with the wt enzyme from shake flask cultures (Fig. S1). The catalytic efficiency of variants L2 and L9 for guaiacol was increased 2.8- and 3.0-fold, respectively (Table I). Interestingly, catalytic efficiency of variant L9 was also 4.5-fold increased with the related phenolic substrate 2,6-DMP, while the same compound was less efficiently oxidised by variant L2 when compared to the wt enzyme (Table I).

In order to investigate the individual contribution of the three amino acid changes of L9 to the overall improvement, the mutant plasmid was back-mutated step-wise to obtain variants with single amino acid changes. Substitution L386Q facilitated improvements mainly at higher guaiacol concentrations, indicating accelerated substrate turnover (Fig. 4). Substitution G417R exclusively increased activity at 0.2 and 0.5 mM guaiacol, while oxidation rates at high substrate concentration were slightly decreased. This suggests that substrate binding and thus affinity were enhanced. Clearly, the beneficial effects of L386Q and G417R were cumulative as the double mutant with both amino acid substitutions outperformed the single mutants at all tested guaiacol concentrations (Fig. 4). Substitution V482G alone had an unexpectedly strong positive effect on guaiacol oxidation rates both at low and high substrate concentrations (Fig. 4), indicating a general improvement of either activity or expression yield of functional enzyme. The combination of L386Q, G417R and V482G was required for maximal activity at high and in particular at low guaiacol concentrations (Fig. 4), suggesting a synergistic effect of all three mutations.

The catalytic efficiency of both variants with ABTS and in particular substrate affinity, reflected in a higher K_m, was decreased (Table I, Fig. S2), indicating a trade-off between the rather large substrate ABTS and the much smaller guaiacol during *in vitro* evolution. The opposite effect was observed for *B. subtilis* CotA where catalytic efficiency of ABTS oxidation (screening target) was improved 7-fold for variant L386W-G417L, while the same parameter was decreased 18-fold in case of the substrate syringaldazine (Gupta *et al.*, 2010).

The catalytic efficiency of *B. pumilus* LMCO towards 2,6-DMP decreased slightly for the L2 variant (1.6-fold) but increased 4.5-fold for L9 variant approaching values reported for fungal laccases (Baldrian, 2006; Maté *et al.*, 2010). Additionally, the oxidation rate of guaiacol increased considerably for the L9 variant eventually leading to 3.7-fold higher efficiency while retaining affinity constants similar to fungal laccases (Kiiskinen *et al.*, 2004; Chairin *et al.*, 2014).

Structural changes in enzymes to improve the catalytic efficiency may have unintended negative effects on other properties such as heat stability or pH-activity range. We analysed temperature stability of

Table I. Kinetic constants of purified wt *Bacillus pumilus* LMCO and variants L2 and L9 for one non-phenolic and two phenolic substrates

Substrate	Variant	K_m (mM)	V_{max} (nmol·min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
ABTS	wt	0.11 ± 0.01	0.71 ± 0.01	42.2	$3.83 \cdot 10^5$
	L2	0.41 ± 0.05	0.57 ± 0.02	23.2	$5.70 \cdot 10^4$
	L9	1.05 ± 0.17	0.59 ± 0.04	11.9	$1.13 \cdot 10^4$
2,6-DMP	wt	1.17 ± 0.08	1.17 ± 0.03	69.7	$5.96 \cdot 10^4$
	L2	2.16 ± 0.01	2.96 ± 0.04	80.4	$3.72 \cdot 10^4$
	L9	0.24 ± 0.03	3.22 ± 0.09	64.9	$2.71 \cdot 10^5$
Guaiacol	wt	2.06 ± 0.42	0.26 ± 0.03	15.7	$7.61 \cdot 10^3$
	L2	1.83 ± 0.18	0.95 ± 0.04	38.8	$2.12 \cdot 10^4$
	L9	1.39 ± 0.12	1.96 ± 0.07	39.5	$2.85 \cdot 10^4$

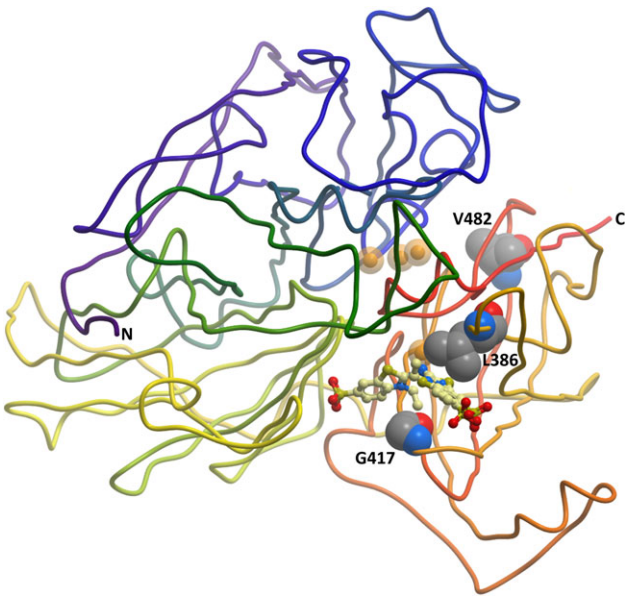

Fig. 3 Structural model of wt *B. pumilus* LMCO. Residues mutated in variants L2 and L9 are shown as atom coloured spheres, copper atoms of the active site are shown as semitransparent spheres, bound ABTS (position derived from *B. subtilis* crystal structure PDB ID: 1UVW) is shown with a ball stick representation.

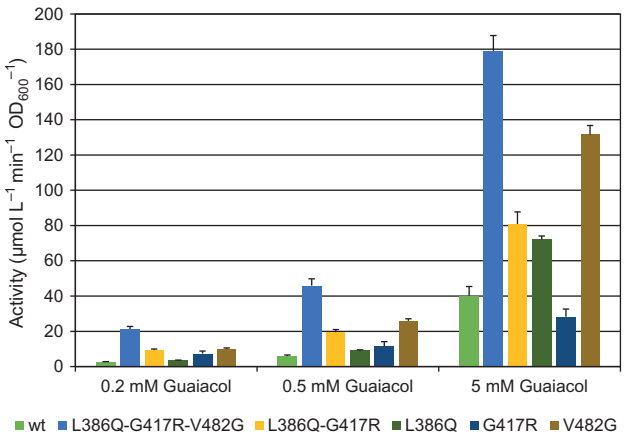

Fig. 4 Effect of individual amino acid changes of LMCO variant L9 on the guaiacol oxidation activity as biomass-normalised activity. Average values and standard deviations of $n = 3$ independent cultures in shake flask per variant.

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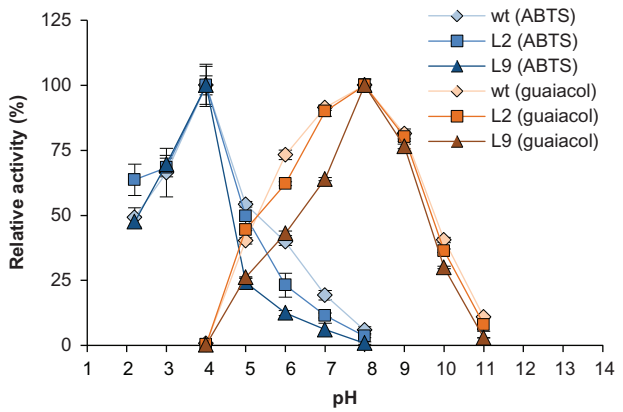

Fig. 5 pH-activity profiles of wt *B. pumilus* LMCO and variants L2 and L9 with 5 mM ABTS or guaiacol as substrates. The values are relative to the maximum measured activity.

variants L2 and L9 and found no difference compared to the wt *B. pumilus* LMCO, i.e. all enzymes retained more than 50% activity on both ABTS and guaiacol upon incubation at 70 or 80°C for 2 h (Figs. S3 and S4). Accordingly, both variant enzymes required extended incubation at 95°C in denaturing buffer for complete denaturation for SDS PAGE analyses. Apparently, CotA-type enzymes are rather tolerant to amino acid substitutions, as heat stability was also unaffected in *B. subtilis* CotA variants with changed substrate specificity (Gupta *et al.*, 2010). However, the decline in relative activity from the maximum at pH 4 towards more alkaline pH values was sharper for L9 compared to the wt with ABTS (Fig. 5). *Vice versa*, the relative activity with guaiacol as substrate (maximum at pH 8) decreased more rapidly towards acid pH with variant L9 than with wt *B. pumilus* LMCO (Fig. 5).

In conclusion, we report a multi-step mutagenesis approach to give an engineered *B. pumilus* LMCO variant L9 that has an increased activity towards the lignin-like model compound guaiacol while retaining the very good thermostability and activity at neutral-to-basic pH of the wt enzyme.

Materials and methods

Protein modelling and selection of mutagenesis targets

A homology model of *B. pumilus* LMCO (GeneBank: EDW21710.1, previously ZP_03054403.1) was generated using the 3D-JIGSAW software (http://bmm.crick.ac.uk/~3djigsaw). The positions of copper atoms and bound substrate ABTS were inferred from the crystal structure of *Bacillus subtilis* CotA (PDB ID: 1UVW) using PyMOL (https://www.pymol.org/). Visualisation of 3D structures was carried out with the software molsoft ICM browser software (http://www.molsoft.com/icm_browser).

Mutagenesis methods

DNA libraries with pairwise randomisation of residues of the ABTS binding site were generated (QuikChange, Agilent) using primers with NDT degeneracy at the desired positions (Reetz *et al.*, 2010) and plasmid pBuL (Ihssen *et al.*, 2015) as template. A primer with NDK degeneracy was used to include codon TGG (tryptophan) for L386. Six variant genes containing slightly beneficial and neutral double mutations were shuffled as previously described (Stemmer, 1994a). Briefly, PCR products of mutated *B. pumilus cotA* were digested for 2.5–3.5 min with DNAseI at room temperature. Fragments of 50–200 bp were separated and reassembled by self-primed PCR (30 cycles, Phusion HF polymerase). Full-length assemblies were amplified by PCR using forward and reverse primers binding to the 5′ and 3′ ends of *B. pumilus cotA* (20 cycles, Phusion HF polymerase, New England Biolabs). PCR product with shuffled mutations was cloned into the vector pET22(+) by isothermal assembly (Gibson *et al.*, 2009). Libraries were constructed, multiplied and maintained in *E. coli* XL-10 Gold (Stratagene, chemical transformation). Quality of libraries was verified by sequencing of randomly picked clones. For screening, plasmid libraries were transformed into *E. coli* Rosetta DE3 by electroporation. Mutations of improved variants were identified by sequencing (Microsynth, Switzerland).

Screening of mutant libraries in liquid phase

Libraries with pairwise randomised residues were screened in 96-deep well plates (DWP) containing 0.25 mL/well of selective LB medium with ampicillin (100 mg L^{-1}) and chloramphenicol (34 mg L^{-1}) per well. Expression plates were inoculated 1:50 from fresh overnight DWP cultures and incubated at 30°C and 500 rpm in a shaker until OD_{600} = 0.2–0.3 was reached. Then, 0.25 mM $CuCl_2$ and 1 mM isopropyl beta-D-thio-galactopyranoside (IPTG) were added and plates were shifted to static incubation at 25°C for 16–20 h. Cells were lysed in 96-well plates by addition of 10× CellLytic B (Sigma).

Screening of mutant libraries on guaiacol-containing plates

The shuffled library was screened on agar plates containing 0.8 mM guaiacol. The agar medium contained 13.3 g L^{-1} Na_2HPO_4•2 H_2O, 3 g L^{-1} KH_2PO_4, 1.8 g L^{-1} $(NH_4)_2SO_4$, 1.0 g L^{-1} NZ amine A, 15 g L^{-1} agar, 10 mL L^{-1} of 100 x trace element solution (Ren *et al.*, 2013), 2 mM $MgSO_4$• 7 H_2O, 1.5 mg L^{-1} thiamine•HCl, 100 mg L^{-1} ampicillin, 34 mg L^{-1} chloramphenicol 34 mg/mL in ethanol (final conc. 34 mg L^{-1}), 0.15 mM $CuCl_2$ 4 g L^{-1} glucose, 10 µM IPTG. Screening plates inoculated with *E. coli* library clones were incubated for 16–20 h at 30°C, followed by incubation at room temperature for 20–24 h under nitrogen atmosphere. Colour formation was initiated by removing plates from the anoxic atmosphere by opening the cover lid in a sterile hood. Active clones were visible after 10–30 min as brownish colonies that were hand-picked upon visual selection.

Analysis of the contribution of individual mutations

The step-wise back-mutation to wt codons was performed with the Quickchange mutagenesis kit (Agilent). Performance of L9 variants with single and dual amino acid substitutions were analysed in triplicate shake flask experiments, using growth conditions which yielded the highest volumetric activities of *B. pumilus* LMCO (Ihssen *et al.*, 2015). After overnight induction under oxygen-limited conditions, cells were harvested by centrifugation and resuspended to a final OD_{600} = 10 in 1× Cellytic B lytic buffer and incubated for 20 min at room temperature. Appropriately diluted cell lysates were assayed for guaiacol oxidation as described below.

Biochemical methods

LMCO variants were purified by (i) precipitation of heat-labile proteins at 70°C, (ii) anion exchange chromatography and (iii) size exclusion chromatography (Reiss *et al.*, 2011). Enzymatic assays for determination of ABTS and 2,6-DMP oxidation rates were performed at room temperature (21–23°C) as described previously (Reiss *et al.*, 2011). The thermal stability was assayed after

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incubating aliquots of the purified enzyme at 70 or 80°C for a maximum of 24 h and then measuring the residual activity. Activity with the substrate guaiacol was determined in Teorell–Stenhagen or McIlvaine buffer pH 7.0 (Ihssen *et al.*, 2015) at room temperature in microplate wells (V_{tot} = 0.2 mL, sample volume 10 µL). Guaiacol oxidation rates were calculated using an extinction coefficient of 12 000 M^{-1} cm^{-1} at 470 nm (Wong *et al.*, 2013). Values for Michaelis-Menten parameters (k_{cat}, K_m) were calculated using the Enzyme Kinetics module of SIGMA-PLOT (Systat Software, San Jose, CA). One unit was defined as amount of McIlvaine buffer series withzyme that oxidised 1 µmol of substrate per minute. pH-activity profiles were determined in ABTS or Teorell–Stenhagen buffer series with guaiacol at a substrate concentration of 5 mM.

Supplementary data

Supplementary data are available at *Protein Engineering, Design & Selection* online.

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