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SITE-DIRECTED MUTATION OF A LACCASE FROM *THERMUS THERMOPHILUS*: EFFECT ON THE ACTIVITY PROFILE

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Abstract - A site-directed mutant R453T of a laccase from *Thermus thermophilus* HB27 (*Tth*-laccase) was constructed in order to investigate the effect on laccase catalytic properties. The mutated gene was cloned and overexpressed in *Escherichia coli*. Nickel-affinity purification was achieved and followed by copper ion incorporation. The mature mutated enzyme was quantitatively equal to the wild type. A photometric assay based on the oxidation of the substrate 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was employed in comparison with the wild-type *Tth*-laccase on catalytic properties. The R453T mutant exhibited improvement in substrate affinity and specific activity at room temperature, whereas those parameters were not significantly influenced when the temperature increased up to 65°C or higher. The mutant had better catalytic activity than that of the wild type at acidic pH. Investigated by circular dichroism spectroscopy, the mutant *Tth*-laccase displayed similar profiles at low and high temperatures.

Key words: Thermus thermophilus, laccase, site-directed mutagenesis, enzyme

INTRODUCTION

Laccases are blue multicopper oxygenases that can oxidize a wide variety of organic (mostly phenolic) as well as inorganic substrates with a concomitant reduction of O_2 to H_2O (Solomon et al., 1996; Thurston et al., 1994). The enzymatic activity of laccases is dependent on the presence of four Cu (II) ions, which are arranged in two main sites. Type-1 copper (T1 copper) is responsible for pulling electrons from a substrate, which are subsequently delivered to a metal cluster formed by the three other copper ions (one T2 copper and two T3 coppers). This trinuclear cluster is capable of binding oxygen, which is the final electron acceptor (Ducros et al., 1998; Garavaglia et al., 2004; Li et al., 2007). Based on a wide range of comparative studies, including sequence homology and crystal structure analysis, the copper site coordination is very similar among multicopper oxidases. The main difference is in the coordination sphere of the T1 copper site. The typical T1 site, such as that found in an *Escherichia coli* laccase (CueO) and a *Bacillus subtilis* laccase (CotA), contains two histidines, a cysteine that forms a short S-Cu bond, and a methionine that forms a long S-Cu bond which is common in bacteria laccases. These four ligands bind T1 copper in a distorted tetrahedral coordination of geometry. Except the CHM ligand group, second shell residues contributed from the coils around the ligands in the crystal structures are believed to influence a lot to the functional properties of laccases (Karlin et al., 1997). As Fig. 1 shows, the copper-ligand cysteine is at the C-cap of a β-strand, followed by a loop, and a short helix containing the second histidine. A glycine is putatively structurally important to form a tight turn at the end of the helix, and the copper ligand methionine is right after the glycine. Many of the engineered mutations around the T1 site of laccases have been reported to affect various properties of laccases. For example, the D500G mu-

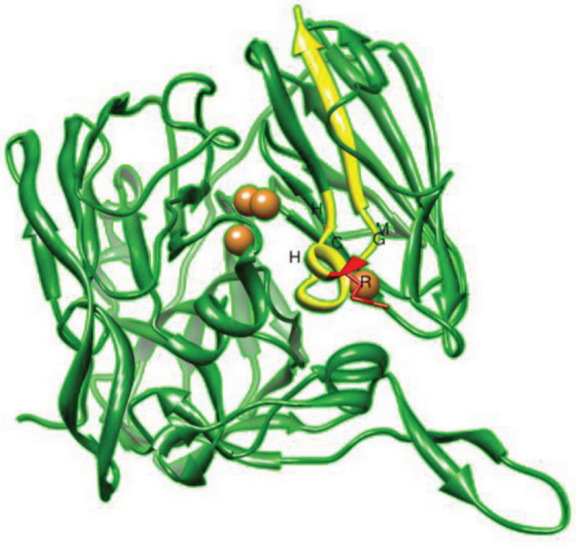

Fig. 1. Representation of the three-dimensional structure of *Tth*-laccase (PDB-ID 2XU9), with its C-terminal including T1 copper site colored (Arg453 in red, other amino acid residues in yellow, the copper ions cofactors in brown).

tation in a *Bacillus licheniformis* laccase had a major influence on the expression of the laccase (Koschorreck et al., 2009). The replacement of the axial ligand methionine by leucine or phenylalanine in *B. subtilis* CotA led to an increase in the redox potential of the corresponding mutants and had a negative effect on the catalytic constants (Mate et al., 2010).

Biotechnological applications of laccases are important in many industrial and environmental applications (Thurston et al., 1994), and thus, studies geared toward understanding their mechanism and providing a scientific basis for the employment of these enzymes in biotechnological processes have been a focus of increased interest (Quaratino et al., 2007; Papinutti et al., 2008; Mishra and Kumar, 2009). Among the more interesting studies are those dealing with the identification of enzymes that are stable under harsh temperature conditions (Chemykh et al., 2008; Uthandi et al., 2010). In that regard, one such laccase has been isolated from the extremely thermophilic bacterium, *Thermus thermophilus* (locus tag TTC1370) (Henne et al., 2004). Studies have revealed that the optimal reaction temperature of this thermostable laccase is 92°C, with a half-life of thermal inactivation at 80°C of over 14 h, making it an extraordinary thermophilic laccase (Miyazaki, 2005). Though valuable and interesting, *Tth*-laccase has low activity at moderate temperatures (Liu et al., 2011), which is also an important consideration in commercial and industrial applications of hyperthermophilic enzymes. To optimize the properties of *Tth*-laccase, a site-directed mutation was designed based on comparison of the amino acid sequences and the three-dimensional structures of the T1 domains of laccases, and the catalytic characteristics of the mutated *Tth*-laccase at different temperatures will be evaluated.

MATERIALS AND METHODS

The QuikChange site-directed mutagenesis kit was purchased from Stratagene (Santa Clara, USA). Molecular weight markers were from New England BioLabs (Ipswich, USA). Primers were synthesized by Invitroen Biotechnology Co., Ltd (Shanghai, China). Plasmid pQE-70 containing the cDNA of *Tth*-laccase was a gift from Dr. Bachas (University of Kentucky, USA). *E. coli* strain Top10 was used for cloning, and *E. coli* strain BL-21 (DE3) was used for protein expression. The Ni-NTA Superflow resin was acquired from Qiagen China Co., Ltd. (Shanghai, China). All other chemicals were of reagent grade or better and commercially available. Deionized water was used in the preparation of all solutions.

Site-directed mutagenesis and expression

The mutant R453T was constructed by PCR using a QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol. PQE-70 plasmid containing the cDNA of laccase was used as the template for introduction of the site-directed mutation. Primers were designed as follows: 5'-ATC GTG GAG CAC GAG GAC **ACC** GGG ATG ATG GGA GTC CTC GA, sense; and 5'-TC GAG GAC TCC CAT CAT CCC **GGT** GTC CTC GTG CTC CAC GAT, antisense. The PCR temperature program was initiated at 95°C for 5 min, followed by 18 cycles of 95°C for 60 s, 55°C for 45 s, 68°C for 90

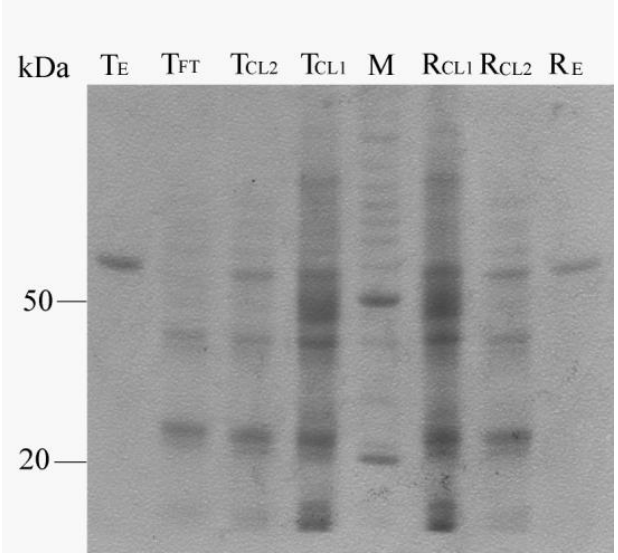

Fig. 2. SDS-PAGE analysis of R453T mutant and wild-type *Tth*-laccase (53 kDa).
Lane M: molecular weight marker, the two most distinct bands correspond to 20 and 50 kDa, respectively. Lane R_{CL1}: Cell lysate of the wild-type *Tth*-laccase after IPTG induction. Lane R_{CL2}: Supernatant portion of the cell lysate after heating at 65°C for 20 min. Lane R_E: Purified 6His-*Tth*-laccase. Lane T_{CL1}: Cell lysate of Lane M: molecular weight marker, the two most distinct bands correspond to 20 and 50 kDa, respectively. Lane R_{CL1}: Cell lysate of the wild-type *Tth*-laccase after IPTG induction. Lane R_{CL2}: Supernatant portion of the cell lysate after heating at 65°C for 20 min. Lane R_E: Purified 6His-*Tth*-laccase. Lane T_{CL1}: Cell lysate containing mutated *Tth*-laccase. Lane T_{CL2}: Supernatant portion of the cell lysate after heating. Lane T_{FT}: Portion flowing through the column. Lane T_E: Purified R453T mutant.

s, and a final extension at 68°C for 5 min. The mutant was verified by DNA sequencing.

A one-liter culture of *E. coli* BL-21 (DE3) harboring pQE-70-laccase was grown in LB medium containing ampicillin with shaking until the optical density at 600 nm reached 0.7. Expression of the laccase was then induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside into the medium with additional incubating at 30°C overnight. The cells were collected by centrifugation, resuspended in 40 mL of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and stored at -20°C for subsequent purification.

Purification and copper binding of Tth-laccase apo-enzyme

The cells were thawed and disrupted by sonication on ice. Cell debris was removed by centrifugation at 18,000×g, 4°C, for 10 min. The protein was partially purified by heating the lysate at 65°C for 20 min and then chilling it over ice for 30 min to precipitate thermally unstable proteins (Li et al., 2002; Iyer et al., 2002). After centrifugation at 18,000×g, 4°C, for 20 min, the precipitated thermolabile proteins were removed. The supernatant, which contains recombinant laccase, was loaded onto a Ni-NTA column equilibrated with lysis buffer. The column was capped and shaken in an end-over-end mixer for 1 h. Then, the column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 60 mM imidazole, pH 8.0). Proteins were eluted under gradient elution by increasing the concentration of imidazole up to 500 mM in the wash buffer. At every step, the protein samples were collected and analyzed by 10% SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by Coomassie brilliant blue R250 staining.

After purification, the eluted protein was dialyzed twice against 1 l of 20 mM acetate buffer containing 0.1 mM $CuSO_4$ (pH 6.0) at 4°C for 48 h. Then, the protein solution was dialyzed two times against 1 l of 20 mM acetate buffer (pH 4.5) without copper ion at 4°C for another 48 h. The precipitate was removed by centrifugation at 18,000×g, 4°C, for 10 min. The supernatant was collected, and the protein concentration was determined using the Bradford method (Bradford, 1976).

Laccase activity assay

The oxidase activity of laccase was measured spectrophotometrically in air-saturated 20 mM sodium acetate buffer with 100 mM $CuSO_4$, pH 4.5 (Li et al., 2007). The method was based on the oxidation of ABTS, which results in a colored product that absorbs at 420 nm with an absorbance coefficient value ε=36,000 M^{-1} cm^{-1}. The reaction was carried out for 5 min at pH 4.5, the referenced optimum pH of re-

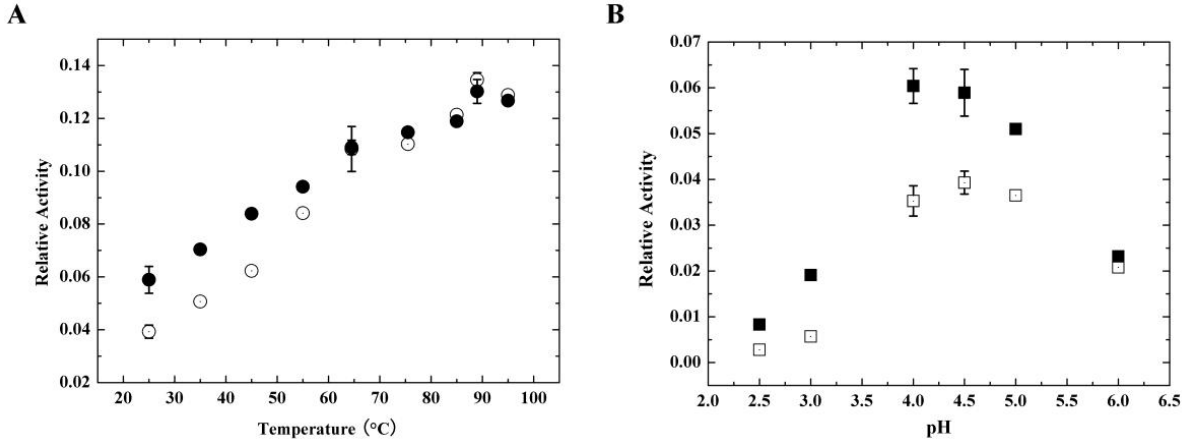

Fig. 3. Catalytic properties of recombinant wild-type *Tth*-laccase and R453T mutant. A. Temperature dependence of activity (open circles wild-type *Tth*-laccase, closed circle R453T mutant). B. pH dependence of activity (open square wild-type *Tth*-laccase, closed square R453T mutant).

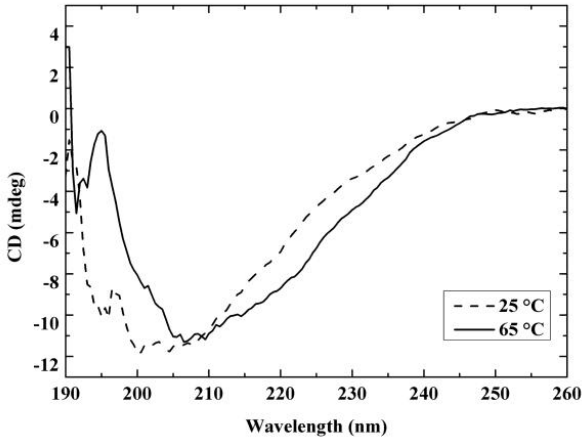

Fig. 4. CD spectra in the UV region of a 0.2 mg/mL laccase solution of the R453T mutant in 20 mM acetate buffer, pH 4.5, after 1 min incubation at 25°C or 90°C (solid line 25°C, broken line 90°C).

combinant *Tth*-laccase. The specific activity was expressed as micromole of ABTS oxidized per minute per milligram of protein. The concentration of the purified protein in the assay solution was 10 μg/ml. Buffer without protein was used as control. By using the enzyme activity assay, the temperature dependence and the pH dependence of enzymes were investigated. Reactions were performed in triplicate at different temperatures using a water-jacketed cuvette. The exact temperatures of the reactions were measured with a temperature sensor.

Circular dichroism (CD) spectroscopic characterization of laccase

CD spectra of the laccase (0.2 mg/mL in 20 mM sodium acetate buffer with 100 mM $CuSO_4$, pH 4.5) were recorded at 21°C or 90°C under a N_2 atmosphere on a Jasco J-810 spectropolarimeter, employing a 0.1 cm pathlength quartz cell.

RESULTS AND DISCUSSION

Construction of R453T Tth-laccase

The steps of our work started with the R453T site-directed mutagenesis of *Tth*-laccase, which is a result of the comparison on the amino acid sequences of T1 area between *Tth*-laccase and other laccases. Position 453 in *Tth*-laccase is not a conserved site among laccases; most laccases do not exhibit an amino acid residue with electrical charged side chain at the corresponding position, whereas *Tth*-laccase has Arginine at this position (Table 1). However, as mentioned earlier, the three-dimensional structures of T1 domains share high similarity. The corresponding positions of arginine 453 in laccases are commonly

Table 1. C-terminal amino acid sequence alignments of fungal and bacterial laccase genes (targeted mutant site is framed; corresponding sites of the mutant in this study are represented against a gray background; the numbers across the top refer to the sequence positions in protein; boldface lettering indicates amino acids that act as copper ligands in laccases).

Microorganism	Sequence alignment of laccases																										
M. albomyces	530	H	C	H	I	A	W	H	V	S	G	G	L	S	V	D	F	L	E	R	P	A	D	L	R	Q	R	I	S
P. cinnabarinus	451	H	C	H	I	D	F	H	L	D	A	G	F	A	V	V	M	A	E	D	T	P	D	T	K	A	A	N	P
P. ostreatus	450	H	C	H	I	D	W	H	L	E	I	G	L	A	V	V	F	A	E	D	V	T	S	I	T	A	P	-	-
A. bisporus	451	H	C	H	I	D	W	H	L	E	A	G	L	A	I	V	F	A	E	A	P	E	D	N	V	S	G	P	Q
S. lavendulae	570	H	C	H	L	L	E	H	E	D	M	G	M	M	R	P	F	V	V	M	P	A	E	A	M	K	F	-	-
B. licheniformis	490	H	C	H	I	L	E	H	E	D	Y	D	M	M	R	P	L	E	V	T	D	V	R	-	-	-	-	-	-
B. subtilis	491	H	C	H	I	L	E	H	E	D	Y	D	M	M	R	P	M	D	I	T	D	P	-	-	-	-	-	-	-
E. coli	499	H	C	H	L	L	E	H	E	D	T	G	M	M	L	G	F	T	V	-	-	-	-	-	-	-	-	-	-
T. thermophilus	444	H	C	H	I	V	E	H	E	D	R	G	M	M	G	V	L	E	V	G	-	-	-	-	-	-	-	-	-

Table 2. Kinetic properties of recombinant *Thermus thermophilus* HB27 and the R453T mutant in solution (data are averages ± standard deviation, n=3).

Tth-laccase types	Temperatures (°C)	K_M (mM)	Specific activity (μmol min^{-1} mg^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
Wild-type	25	1.03±0.03	0.219±0.018	2.12±0.17
	61.5	0.30±0.02	0.396±0.002	69.80±5.1
R453T	25	0.75±0.06	0.279±0.022	19.50±1.86
	61.5	0.31±0.03	0.410±0.039	69.33±6.2

at the end of the helix close to the T1 site (Fig. 1), adjacent to the glycine turn and the copper ligand methionine 455. According to the result of calculating the related structures for *Tth*-laccase, CueO from *E.coli* is on the top of this list of all proteins. The amino acid in CueO instead of 453R of *Tth*-laccase is 508T, a polar-neutral residue. Based on the above considerations, the A453T mutant of *Tth*-laccase was constructed and the sequence was proved.

The expression of the recombinant mutant *Tth*-laccase in *E. coli* was performed and followed by the purification of the enzyme. The wild-type recombinant *Tth*-laccase was harvested in the same way. The cell lysate was heated to precipitate the native *E. coli* proteins (Li et al., 2002; Iyer et al., 2002). After that, the supernatant was subjected to nickel ion affinity chromatography. The eluent was dialyzed against a copper-containing buffer (pH 6.0) to allow the metal ion to become incorporated into the active sites of the laccase. The excess unbound Cu^{2+} was removed by a series of dialysis steps in a copper-free buffer (pH 4.5). SDS-PAGE analysis showed that the overall expression of the R453T mutant was quantitatively similar to that of wild-type *Tth*-laccase (Fig. 2). In addition, the purification of the mutant was verified by this protocol, which could equally yield approximately 2.4 mg of the 53 kDa blue-colored mature wild-type protein per liter of culture or of the A453T mutant. As the result showed, the substitution of an arginine by a threonine did not bring on denatured structural change at T1 site. The C-terminal His-tag could still bind with a nickel column, and the T1 site could still capture the copper to complete the mature blue protein (Messerschmidt, 1990).

Catalytic properties of R453T mutant

In order to investigate the kinetic behavior of the *Tth*-laccase mutant R453T, the effect of the substrate con-

centration on the rate of ABTS oxidation was studied under both low and high temperatures. Lineweaver-Burk analysis was used to obtain the specific activity and K_M values (Table 2) (Lineweaver, 1934). At room temperature, the K_M value of the R453T mutant was obviously lower than that of wild-type *Tth*-laccase. The specific activity of the mutant was 27% higher. It can be assumed that the increase in activity was related to a decrease in Km, which indicates an increase in the affinity for the substrate.

Previous studies reported that the wild-type *Tth*-laccase has an increase of activity from 25°C to 92°C (Miyazaki, 2005; Liu et al., 2011). This behavior was also observed with its mutant R453T (Fig. 3A). The relative activity of the R453T mutant was 50% higher than the wild type at room temperature. Along with the temperature increase, the activity ascendance of the mutant appeared to fall gradually. After it reached 60°C, the temperature-activity profile of the mutant was very similar to that observed for the recombinant wild-type *Tth*-laccase. In addition, the mutant did not exhibit obviously different K_M. This result indicates that this mutant does not influence the temperature-dependent structure change of the enzyme. Additionally, when a high temperature markedly raised the function, the mutant did not benefit more from the substrate affinity or the special activity of the enzyme.

There is a commonly held belief that functional change usually happens with exchange of amino acid in ligand group and second shell of the enzymatic activity center (Karlin et al., 1997). For laccases, the hydrophobic part of the second shell generally involves multiple methionine residues and one or more large aromatic residues. The copper center and its direct ligands are almost always totally buried. Position 453 is between histidine 450 and methionine 455 in the amino acid sequence of *Tth*-laccase, both of which are super conservative in blue-copper enzyme families. This notable position has not had any mutation reported in laccases because its responding positions in most laccases are not conservative. However, arginine is less hydrophobic compared to many other amino acids at this position of laccases. Although it was hard to fully prove, the neutral polar side chain of threonine 453 might benefit the catalytic activity of laccases. Besides, because of its position between the T1 center and the protein surface, the shorter side chain of threonine may contribute to the higher substrate affinity by providing an easier pathway for substrate interaction. As the R453T mutation did not obviously affect the function at high temperature, it can be assumed that the special thermal property of *Tth*-laccase is owed to other structure specifities.

As Fig. 3B shows, the activity of the wild-type and mutated laccase decreased in slightly different manners when varying the pH from 2.5 to 6.0 in sodium acetate buffer. R453T mutant activity was systematically higher than the wild type until pH 6.0. At pH below 4.0, the activity loss of the wild-type laccase was more rapid than for the mutant. In addition, the mutant enzyme exhibited a slight shift of the maximum activity towards pH 4.0, whereas the wild type has the highest activity at pH 4.5.

At first, it was reported that threonine is a common amino acid constituent in the helical, beta-sheet of acid proteins (Kumar et al., 2000; Zhang et al., 2009). Because of the hydrogen bond on the side chain, threonine can interact with water and help the proteins to remain stable at low pH, whereas the employment of arginine on the surface makes the protein take more positive charge at acidic status, which does not favor protein stability at low pH. Secondly, a more hydrophobic shell putatively inhibits solvent ions and/or water from reaching the active site (Karlin, 1997). Therefore, it is not surprising that the R453T mutant exhibited stronger endurance in an acidic environment.

CD Spectroscopic characterization of R453T mutant

The far-UV CD spectra of R453T were carried out at 25 and 90°C to understand its temperature dependence on secondary structure (Fig. 4). The mutant enzyme exhibited a similar spectral shape at both temperatures. The far-UV CD regions had minima at around 210 nm at room temperature and at around 200-210 nm at 90°C, which is indicative of dominant

β-sheet structures (Kelly et al., 2005), and consistent with the structure of laccases in reference (Bonomo et al., 2001; Liu et al., 2011). The negative trough during 195-200 nm decreased as the temperature was raised to 90°C, which reveals the random coil content reduction. A similar profile was also observed in the referenced CD spectra of the wild-type *Tth*-laccase (Liu et al., 2011). Usually, for most methophilic laccases, the higher temperature usually causes them to have less helical and more random coil conformation (Bonomo et al., 2001). It is deduced that the structure change of random coils might be of benefit to the raised enzyme affinity ability and the catalytic function at high temperature. In spite of some structural change, the major conformation of the enzyme remained stable whether at 25°C or 90°C. Therefore, the R453T mutation has little influence on *Tth*-laccase thermostability.

In conclusion, a site-mutation in the T1 domain of *Tth*-laccase was constructed and investigated. This R453T mutation enhanced the specific activity at room temperature and exhibited better stability in an acidic environment, which supports that the composition of the amino acid residues around the T1 center could affect the properties of laccases.

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