

# **Investigating the role of conformational effects on laccase stability and hyperactivation under stress conditions.**

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### **Notes**

The authors declare no competing financial interest.

**ABSTRACT:** Fungal laccase from *Steccherinum ochraceum* 1833 displays a remarkable stability under different harsh conditions, namely organic/buffer mixtures, thermal treatment and microwave radiations. The behavior appears even more significant in the light of the sharp inactivation observed for two different fungal laccases. Laccase from *Steccherinum ochraceum* 1833 also displays a hyperactivation under mild thermal treatment (60 °C). Molecular dynamics simulations at 80 °C explained how this laccase retains the geometry of the Electron Transfer Pathway that assures the transfer of electrons through the copper ions thus maintaining its catalytic activity at high temperature. Spectroscopic studies revealed that the thermal activation corresponds to specific conformational changes in the protein. The results indicate that this laccase is potentially applicable under denaturing conditions that might be beneficial for the biotransformation of recalcitrant substrates.

**KEYWORDS:** Laccase stability, Electron Transfer Pathway, molecular dynamics, circular dichroism, fluorescence, *Steccherinum ochraceum*

## **Introduction**

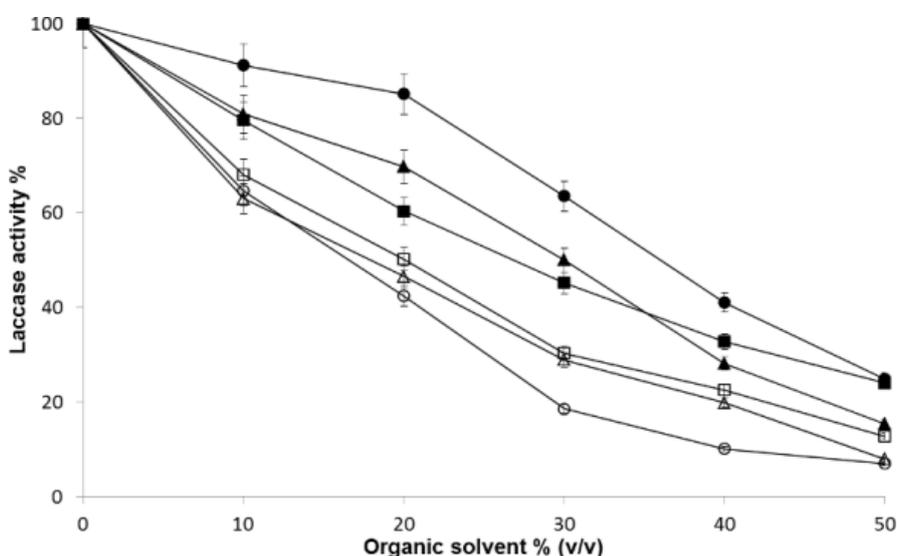
Laccases (EC 1.10.3.2) are metalloenzymes representing the largest subgroup of blue multicopper oxidases (MCO) found in plants, fungi, bacteria and insects. The majority of fungal laccases are extracellular monomeric globular glycosylated proteins of approximately 60-70 kDa, with glycans accounting for about 10-30% of their molecular weight.<sup>[1,2,3]</sup> They catalyze the reduction of an oxygen molecule to water accompanied by the oxidation of four substrate molecules according to a mechanism that involves four copper ions.<sup>[4]</sup> Laccases are some of the few oxidoreductases commercialized as industrial catalysts, with applications in biopulping and biobleaching, dye decolorization, xenobiotic detoxication and many other industrial, environmental, diagnostic, and synthetic uses.<sup>[5]</sup> These enzymes exhibit broad substrate specificity towards monophenols, diphenols, aminophenols, polyphenols, aryl amines, which can be further enhanced by addition of redox mediators.<sup>[6,7]</sup> The capability of laccases to catalyze the formation of reactive radicals of aromatic

substrates has been exploited for the depolymerization of lignin and for targeted modifications of wood fibers in order to improve their chemical or physical properties.<sup>[8,9,10]</sup> On the other hand, laccases are able to catalyze the formation of radicals that undergo homo- and hetero-polymerization, representing route for the synthesis of new hybrid molecules and biomaterials for pharmaceutical, food and cosmetic needs.<sup>[11,12]</sup> The potential usability of enzymes in industrial processes under non-physiological conditions is strictly connected to the robustness of the enzymes towards denaturant agents, as co-solvents and temperature. In many cases substrates of industrial interest are poorly soluble in water, so that laccases stability in aqueous/solvent mixtures is of interest for enlarging their industrial exploitation.<sup>[13,14]</sup> Furthermore, it has been demonstrated that higher temperatures and microwave radiations can improve kinetics of enzymatic reactions.<sup>[15,16]</sup> The present study reports on the properties of three promising fungal laccases from *Lentinus (Panus) tigrinus* 8/18 (LtL),<sup>[17,18]</sup> *Steccherinum ochraceum* 1833 (SoL)<sup>[19]</sup> and *Lentinus strigosus* 1566 (LsL).<sup>[20]</sup> More specifically, solvent and temperature stability were experimentally investigated for evaluating their potential application under industrially relevant conditions. Moreover, the effect of microwave radiations was assessed for exploring new routes for increasing reaction kinetics. Experimental data revealed the remarkable stability of SoL and Molecular Dynamic (MD) simulations tried to explain at molecular level the structural basis of this behavior, by disclosing how the catalytic activity at high temperature is maintained by retaining a correct geometry of the Electron Transfer Pathway responsible for the transfer of electrons through the copper ions. Circular dichroism (CD) and fluorescence spectroscopy also revealed conformational modifications that are compatible with the observed thermal activation of SoL, which is accompanied by protracted stability.

## **Results and Discussion**

## Activity of laccases in buffer/organic solvent mixtures

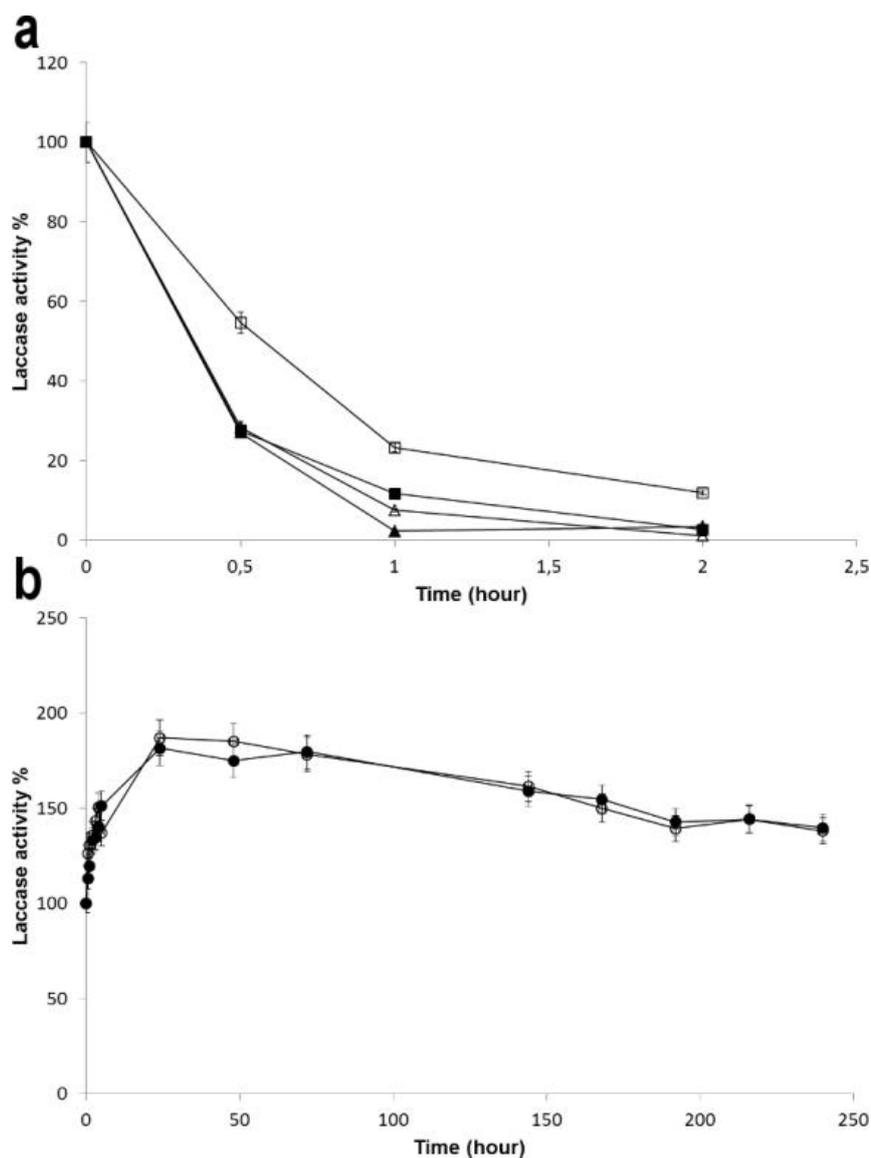
The detrimental effects of water miscible solvents on the activity and stability of enzymes are generally concentration-dependent but also related to the physical-chemical properties of the solvents.<sup>[21]</sup> In this study the effects of dioxane (LogP = -1.1) and ethanol (LogP = -0.18) were evaluated by measuring the activity of laccases at 25 °C in buffer/solvent mixtures at increasing co-solvent concentrations. Results in Figure 1 show a distinct concentration-dependent decrease of activity, taking pure buffer as a reference system. Dioxane exerts a stronger detrimental effect and 20 % of co-solvent causes already a 50 % loss of activity, whereas in 50 % mixture less than 20 % of enzymatic activity is preserved. The effect of ethanol is less pronounced, with SoL maintaining 90 % of activity in 20 % buffer/ethanol mixtures, but still all laccases display less than 40 % of their original activity when the percentage of solvent reach 50 % (Figure 1). It is important to note that the activities were measured as initial rates monitored within a time range of less than 1 minute. Accordingly, the solvent effects reported in Figure 1 account for enzyme inactivation only at a very limited extent.



**Figure 1.** Activity retained by laccases in the presence of increasing concentrations of ethanol (solid symbols) and dioxane (empty symbols): LtL (▲), LsL (■) and Sol (●). The activity displayed in pure aqueous buffer is taken as a reference (100%). Each data point is the average of at least four measurements.

Therefore, the time-dependence of the effect of co-solvents on the stability of laccases was analyzed by incubating the three enzymes in 50/50 mixtures for different times and taking the  $t_0$  activity as a

reference. A distinctive behavior of SoL was observed (Figure 2b) since its activity increases progressively during the first 24 hours of incubation resulting almost doubled. Then activity decreases and after 200 h it stabilizes at a value around 150% of initial activity, which is maintained until the end of incubation (240 hours totally). This behavior might be partially related to some non-specific solvent effect, such as an initial improvement in the dispersion of the enzyme particles in the mixture but the formation of an activated conformation of SoL cannot be excluded. Interestingly, both co-solvents exert identical effects. Globally, SoL demonstrates a remarkable stability, even upon prolonged incubation in 50/50 buffer/solvent mixtures. In conclusion, although the initial activity expressed by SoL in the presence of co-solvents is considerably lower as compared to pure aqueous buffer, during the incubation the enzyme increases its activity and finally maintains a constant performance for protracted time, which is of considerable importance for practical applications and for the recyclability of the biocatalyst. This finding results even more notable if considered that both LtL and LsL lose most of their activity in one hour with both solvents (Figure 2a).

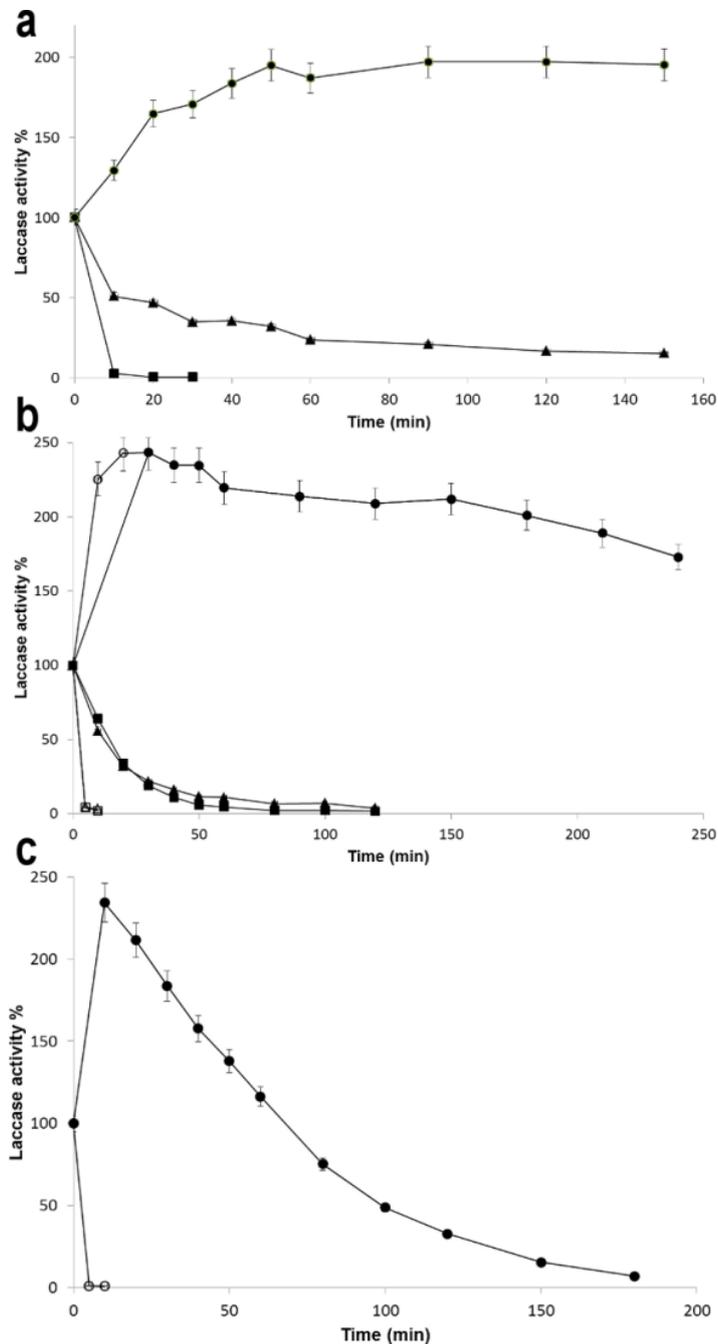


**Figure 2.** Stability (expressed as % of retained activity) of laccases exposed for different times to 50% co-solvent. Dioxane (empty symbols); ethanol (solid symbols; LtL (▲); LsL (■); SoL (●). Each data point is the average of four measurements.

### Thermal stability and effects of microwave radiations

Microwave irradiation was recognized since decades as an efficient heating source for chemical synthesis: reactions requiring several hours under conventional conditions can often be completed in few minutes.<sup>[22,23]</sup> Nowadays microwave radiation is a very reliable and common heating technique, which has shown its applicability also in biocatalysis for increasing reaction kinetics, also with solid-phase biotransformations.<sup>[24,25]</sup> Therefore, the effect of microwave heating on the three laccases was

evaluated by irradiating aqueous solutions of the enzymes and then measuring the residual activity on aliquots withdrawn at defined times. The residual activity was calculated by taking as a reference the initial rate measured under standard assay conditions at 25 °C.



**Figure 3.** Stability of LtL (▲), LsL (■) and SoL (●) upon exposure to conventional heating or microwave irradiation.

a) Residual activity after microwave irradiation with 50 W at 50 °C; b) comparison between effect of conventional heating at 60 °C (solid symbols) and effect of microwave radiation (60 °C, 10W; empty symbols); c) comparison of stability of SoL upon conventional heating at 80 °C (solid symbols) and after microwave irradiation (80 °C, 10 W; empty symbols).

Each data point is the average of at least three measurements.

Figure 3 confirms the remarkable stability of SoL even upon microwave irradiation. As observed already in the study of co-solvent effect (Figure 2b), there is an initial hyper-activation, which occurs within the first 60 minutes of exposure to 50 W of irradiation at 50 °C (Figure 3a). The enzyme displays about 180 % of its original activity (measured at 25 °C, no irradiation) without any apparent inactivation effect for at least 150 minutes.

It is interesting to note that the increase of activity of SoL occurs gradually within the first hour, so that it suggests some time-dependent conformational rearrangement at protein level. This behavior appears even more surprising if considered that the other two laccases undergo a fast and severe inactivation when incubated under the same conditions. Indeed, the detrimental effect of microwave is very harsh even at 10W irradiation at 60 °C but data in Figure 3b allow to discriminate between the effect of temperature and the effect of microwave radiation on the inactivation of LtL and LsL.

On the contrary, the activity profiles of SoL incubated at 60 °C with or without 10 W of microwave radiation are very similar, with an increase of 200 % of activity within the first 30 min, and this observation indicates that the hyper-activation depends mainly on the temperature rather than on microwave radiations. Figure 3b shows also how LtL and LsL undergo a progressive thermal inactivation within the first hour of incubation at 60 °C with a conventional heating system. A modest microwave radiation (10 W) at 60 °C accelerates the inactivation, which occurs within the first five minutes. Such *non-thermal microwaves effect* has been already reported in the literature for other classes of enzymes,<sup>[26,27,28]</sup> along with proofs that the electromagnetic field can cause protein unfolding within few minutes.<sup>[29,30]</sup> Fluorescence spectroscopy and circular dichroism studies confirmed that microwaves induce structural rearrangements not associated to the temperature.<sup>[31]</sup> In order to observe some inactivation phenomena on SoL, it was necessary to incubate the laccase at 80 °C (Figure 3c). Conventional heating causes an initial sharp increase of activity but after the first 10 min of thermal treatment it is possible to observe the occurrence of inactivation, although after one hour the enzyme displays the same activity measured at  $t_0$ . Irradiations with 10 W at 80 °C cause a severe non-thermal microwaves effect leading to immediate inactivation of SoL. In conclusion, data

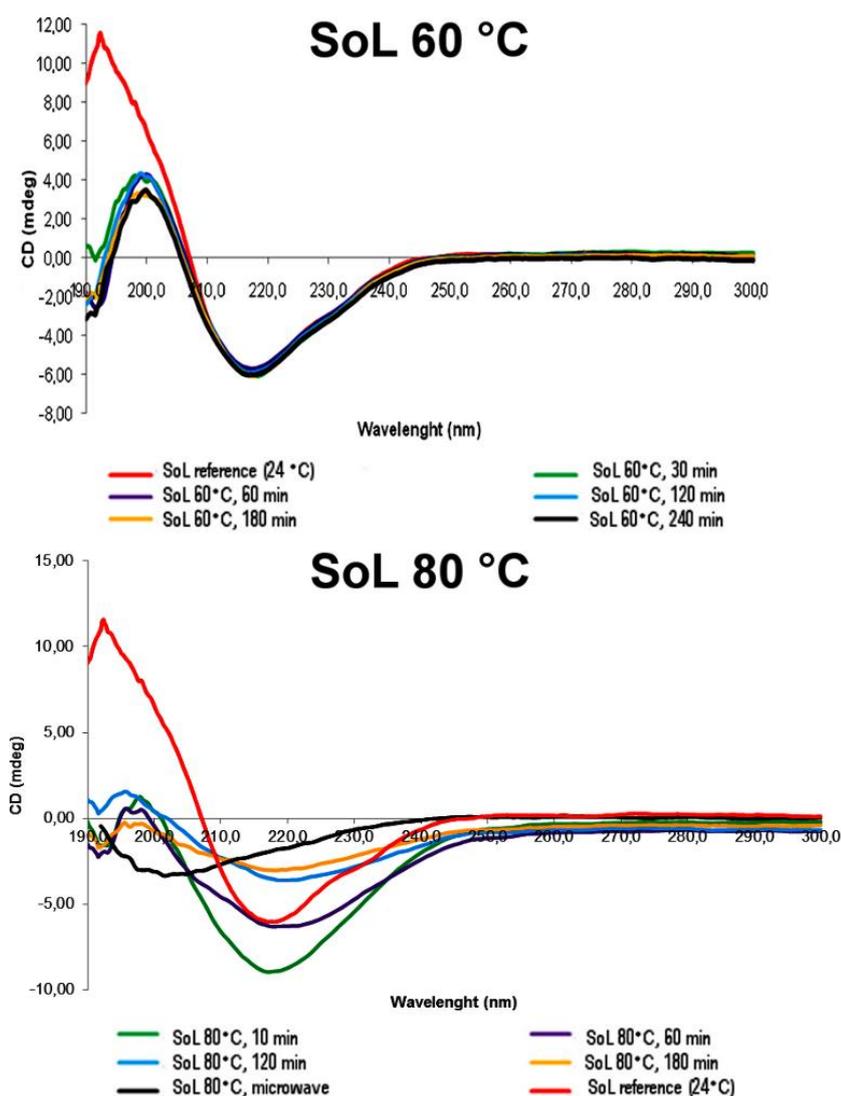
suggest that the hyperactivation of SoL is microwave independent whereas 10 W of irradiation at 80 °C exerts disruptive effects, most probably on some intermediate conformations that originate from the thermal treatment.

### **Spectroscopic analysis of *S. ochraceum* 1833**

It is known that secondary structure can be determined by Circular Dichroism (CD) spectroscopy in the "far-UV" spectral region (190-250 nm) exploiting typical absorptions.<sup>[27]</sup>  $\alpha$ -helices have the most distinctive and strongest CD spectrum with two negative bands of comparable magnitude at 218 and 208 nm and a stronger positive band near 190 nm. A negative band near 217 nm and a positive band in the 195 to 200 nm region are characteristic of beta-sheets. All models for unordered random coil polypeptides have a strong negative band near 200 nm. In the present study, we tried to recognize possible conformational changes that might explain the observed hyper-activation of SoL when incubated at 60 °C and also the time-dependent inactivation observed at 80 °C. Crystal structure shows that SoL consists of 11.4% of  $\alpha$ -helices, 35.3% of  $\beta$ -sheets, 12.3% of turns and 41.0% of random coils. The comparison of CD spectra of SoL recorded at 60 °C and at 24 °C (Figure 4) reveals remarkable differences in the range 190-205 nm, which are consistent with conformational changes of secondary structures. According to experimental data of Figure 3, such conformation changes are not detrimental to SoL activity, but rather there are evidences of a hyper-activation of SoL at 60 °C within the first hour of incubation and an extended stability even after protracted incubations (Figure 3b). These data are also in agreement with the CD spectra recorded at 60 °C, which appears perfectly superimposable at incubation times longer than 1 hour, whereas the spectrum recorded after 30 min indicates the existence of an intermediate conformational condition.

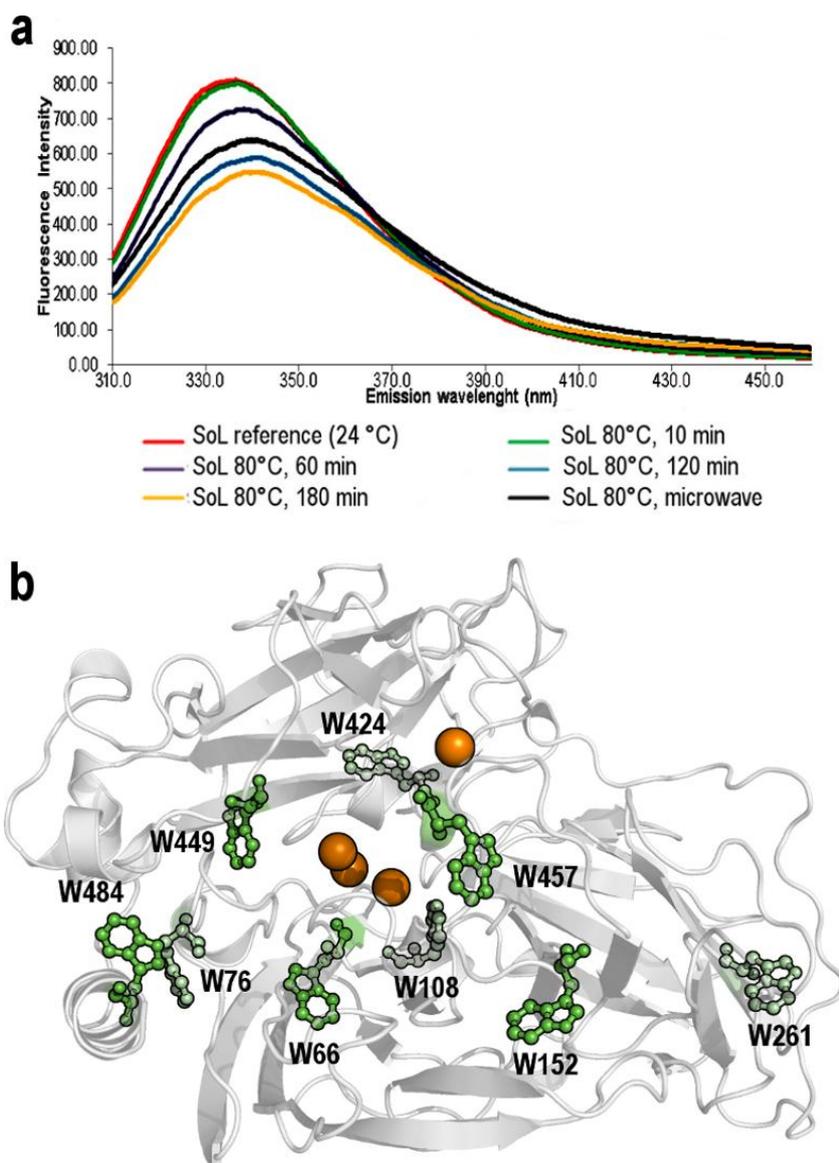
As expected, severe time-dependent variations of SoL conformations are documented by the CD spectra at 80°C. The band at 218 nm is maintained for the first 10 minutes, in agreement with experimental data of Figure 3c that indicate how the enzymatic activity is still preserved and even increased under such conditions. The same band undergoes a progressive decrease after 120 and 180

minutes, in agreement with the thermal inactivation that was experimentally evident after the first hour of incubation (Figure 3c). The band at 195 nm, decreases significantly already after 10 minutes and at 180 minutes becomes negative, with a shift to 215nm. It must be noted that previous CD analysis<sup>[27]</sup> on laccases from *Rigidoporus lignosus* and from *Pleurotus ostreatus* reported the disappearance of the positive band at 195 nm upon thermal treatment, accompanied by a shift of the negative band from 215 nm to lower wavelengths. These changes were ascribed to perturbations of  $\beta$ -sheets domains and a concomitant formation of random coils structures. Indeed, CD spectra obtained after treating SoL with microwave radiations for 10 minutes at 80 °C (black line) indicate that the band at 195 nm disappears completely and the band at 218 nm shifts to 201 nm, in agreement with the formation of highly disordered structures.



**Figure 4.** CD spectra of SoL recorded after different incubation times at 60 and 80 °C. The CD spectrum of SoL at 80 °C was also recorded after exposure to microwave radiation. The CD spectrum SoL at 24 °C is always indicated as a reference (red profile).

In the attempt to gain further details on these conformational phenomena, SoL was also investigated by exploiting the fluorescence emission spectra of its nine tryptophan residues (Figure 5).<sup>[32]</sup> More specifically, it has been reported that an increase of the polarity of the microenvironment surrounding the Trp residues translates into a red-shift of the spectra. That is caused by a Stoke-shift of the  $\lambda_{\max}$  of emission, which also leads to a strong quenching and reduction of the intensity of the signal.<sup>[33]</sup> The structural analysis of SoL (Figure 5b) shows that 5 Trp residues are more accessible to the solvent: W66, W152 and W449, which are located on  $\beta$ -sheets, and W457 and W484 that are inserted on  $\alpha$ -helices. Three Trp residues appear more shielded from the solvent: W76, which is positioned on an  $\alpha$ -helix, W261 and W424 located on  $\beta$ -sheets. Finally, W108 is situated in the proximity of T2-T3 centers and it is the most buried inside the protein core.



**Figure 5.** a) Fluorescence emission spectra of SoL recorded at 80 °C after different incubation times and after microwave radiation. The spectrum of SoL at 24 °C is reported as reference. b) SoL structure represented in cartoon mode. Copper ions are indicated as orange spheres and Trp residues are highlighted in stick mode. The Trp residues more exposed to solvent (66, 152, 449, 457, 484) are in bright green whereas buried Trp residues (76, 108, 261, 424) are in light green.

Fluorescence emission spectra recorded at 24 °C and 60 °C are perfectly superimposable, even after prolonged incubation (see Supporting Information Figure S1). Similarly, the spectrum recorded after 10 minutes of incubation at 80 °C (green line of Figure 5a) is also superimposable to SoL spectrum at 24 °C. Therefore, conformational variations occurring under these conditions do not determine a

change in the exposure of Trp residues to the solvent or substantial variations of the secondary structures.<sup>[33]</sup> Spectra recorded at 80 °C show a decrease of emission intensity directly proportional to the incubation time, and this is accompanied by a red shift of  $\lambda_{\text{max}}$  (see details in Supporting Information, Table S1). This means that after prolonged exposure to 80 °C some buried Trp residue become oriented towards more hydrophilic environments, most probably due to an increased exposure to the solvent. In conclusion, CD and fluorescence analysis of SoL indicates that both hyper-activation and inactivation phenomena documented by experimental data are the consequence of specific conformational changes. The conformational modifications occurring at 60 °C appears more superficial and do not modify significantly the exposure of Trp residues to the solvent. Such form of SoL is not only compatible with enzyme activity but rather it can be associated to the hyper-activation of SoL that was experimentally observed. All these observations set the basis for further investigations aiming at describing in detail the hyper-activation of SoL at 60 °C. On the other hand, at 80 °C SoL undergoes a time-dependent unfolding and denaturation. Microwaves apparently do not exert any detrimental effect on the activated conformation observed at 60 °C, since the core regions surrounding the copper ions, which are rich in  $\alpha$ -helices, are still shielded from the solvent. Microwaves, instead, exert a disruptive denaturant effect at 80 °C. This is evident since the first instant of incubation (Figure 3c) because the high temperature most probably causes a conformational change with a widening of the surface accessible to the solvent as also suggested by fluorescence spectra. It must be underlined that fluorescence spectra of LtL recorded after 10 min of incubation at 60 °C (Figure S2 in Supporting Information) show no important difference. However, relevant modifications of the spectra are evident upon prolonged incubations (180 min). Data suggest that no disruptive conformational change occurs within the first 10 min, although in the same range of time LtL loses 40% of its activity.

In order to elucidate these factors and gain more insights on SoL conformational behavior at 80°C, MD analysis was performed.

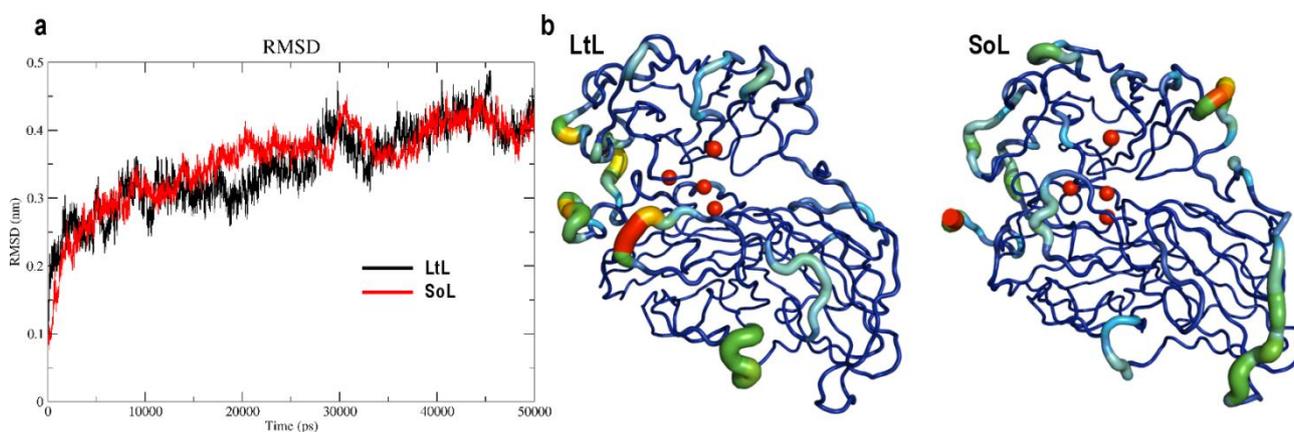
## **Molecular Dynamic simulations of thermal effects**

The structure of most fungal laccases is organized in 3 domain (A, B and C-terminal), displaying a similar Greek key  $\beta$ -barrel architecture. Generally, their structures contain one or two disulfide bridges connecting domains A and B.<sup>[34]</sup> The reduction of the oxygen molecule to water and the concomitant oxidation of four molecules of substrate involve four copper ions, which are classified according their spectroscopic features. The oxidation of the substrate occurs at the mononuclear Type 1 copper center (T1), located in the C domain. The oxygen is then reduced at the tri-nuclear site, composed by a Type 2 (T2) copper and a pair of Type 3 (T3) coppers.<sup>[35]</sup> More in detail, T1 is coupled to the tri-nuclear site through a T1-Cys-His-T3 Electron Transfer Pathway (ETP).<sup>[36,37]</sup>

Molecular modeling and more specifically molecular dynamics (MD) were used to simulate the behavior of laccases upon thermal treatment with the intent of explaining the different enzyme behavior. LsL was excluded from this investigation since neither its structure nor its sequence are available for simulating a reliable model of this enzyme. Crystal structures of SoL and LtL were retrieved from the Protein Data Bank (PDB),<sup>[38]</sup> pre-processed (see Experimental Section and Supporting Information) and protonation was set at pH 5.0. Structural models were explicitly solvated with a water environment at 20 mM ion strength, corresponding to the experimental conditions used for the activity assay. Particular attention was paid to the simulation of the copper ions, since they are involved in the ETP, and distortions of ETP were hypothesized to be involved in the inactivation and biological turnover of the enzyme.<sup>[36,37]</sup> In previous MD simulations the ion coordination had been treated by defining fix bonds for constraining their relative positions,<sup>[39]</sup> which makes, ultimately, MD simulations of scarce utility for describing phenomena affecting ETP. In order to avoid such limitations, coppers ions were modeled by defining each coordination sphere as pair interactions (see Experimental Section for details). Each copper was considered in its fully oxidized state as  $\text{Cu}^{2+}$  and the coordination spheres were defined by considering the initial situation taken from their crystal structures (see Supporting Information Figure S3 and S4 for LtL and SoL respectively).

MD simulations were run for the two laccases at 80 °C for 50 ns. It must be underlined that evidences coming from MD analysis cannot be directly correlated to spectroscopic/experimental data since they simply refer to phenomena occurring at different temporal scale. MD simulations provide a “snapshot” of some phenomena occurring within a very limited time frame (e.g. 50 ns), whereas experiments and spectroscopic analysis monitor changes occurring within minutes or hours. By simulating the two laccases at 80 °C we intended to analyze the early instants of some phenomena that are initially beneficial only to SoL activity but that anticipate disruptive changes for both proteins. Indeed, experimental data show how after 10 min incubation at 80°C SoL undergo a hyperactivation. However, after 20 min incubation SoL already starts losing its activity. On the other side, LtL loses immediately its activity under the same conditions.

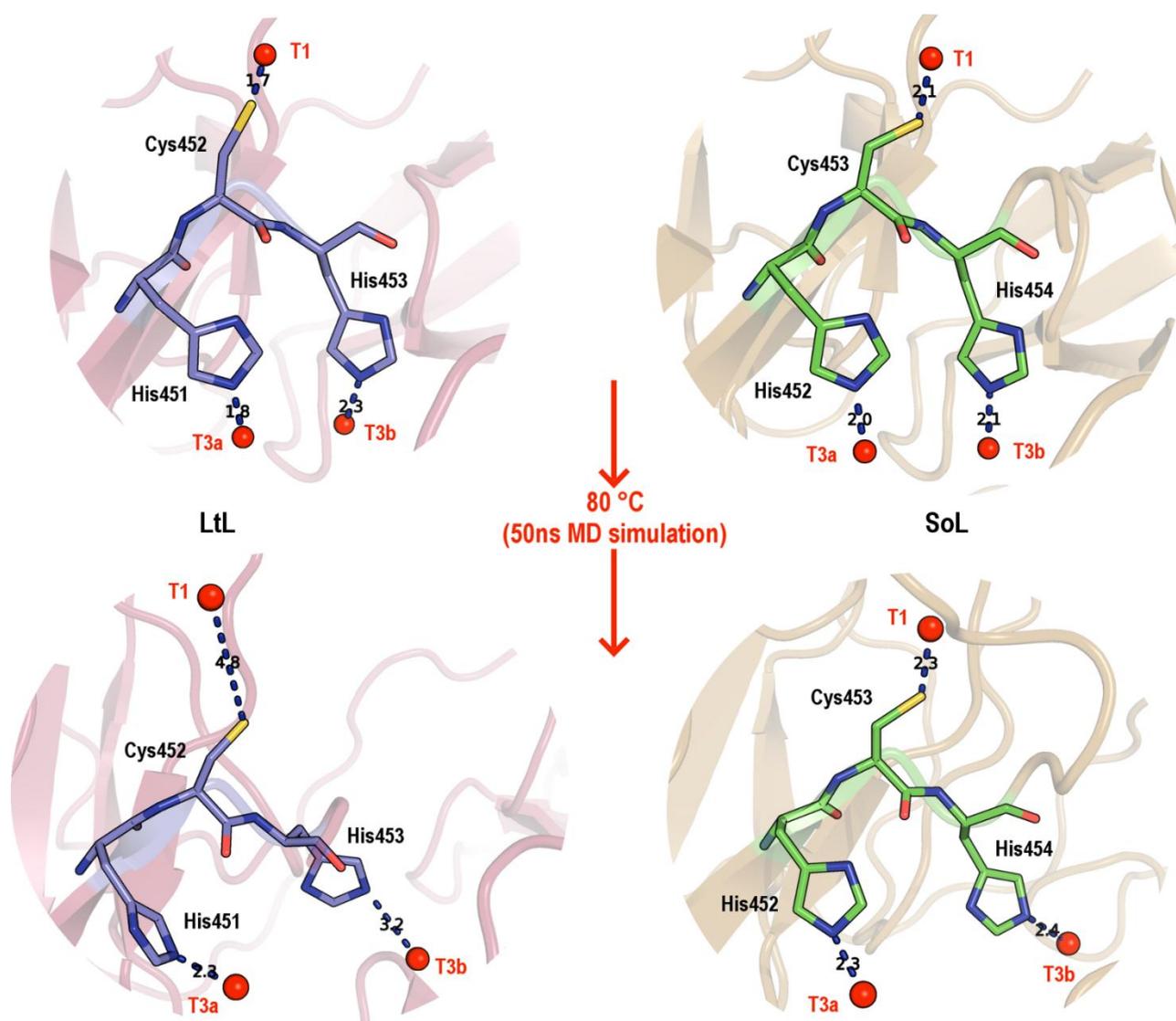
Each laccase was equilibrated for 5 ns at 300 K and MD simulations of 50 ns were performed at 353 K (80 °C). The results obtained for SoL and LtL were compared by calculating Root Mean Square Deviations (RMSD) illustrated in Figure 4a, which consider only the C $\alpha$  of the protein backbone and represent an average picture of the whole apo-enzyme structural plasticity. Figure 4b reports a 3D image of the mobility of each single residue (calculated by means of Root Mean Square Fluctuation, RMSF) and it highlights the domains endowed with higher mobility.



**Figure 6.** a) RMSD calculated for all C $\alpha$  during 50 ns MD simulation at 80 °C. b) Representation of RMSF on the three-dimensional structures of LtL (on the left) and SoL (on the right). The thickness and color *temperature* (from blue to red) are correlated with the fluctuation entity: thicker and red regions correspond to the highest RMSF values. Copper ions are highlighted as red spheres.

Both LtL and SoL present highly mobile residues at the edges of domain A and C and in the proximity of the copper ions (Figure 4b, red spheres) but fluctuations are more pronounced in LtL (thicker and red backbone) than in SoL. We calculated that the fluctuations of this flexible region of SoL in the proximity of T2 and T3 centers (Figure 4b) determine, at 80 °C, an increase of surface accessible to the solvent from 117 to 125 nm<sup>2</sup> (see Supporting Information, Figure S5). However, further investigations would be necessary to verify whether these conformational changes can be correlated to the observed hyper-activation of SoL.

The conformational variations appear of limited extent for both SoL and LtL proteins. However, since in the present study the enzyme stability was experimentally expressed in terms of residual activity, the observed differences are not necessarily related to drastic changes in protein conformation. Rather, minor conformational changes can affect the enzyme efficiency as long as they prevent the accomplishment of the catalytic mechanism, for instance by interfering with the transfer of electrons from the T1 copper ion to the tri-nuclear site.<sup>[40]</sup> Starting from these concepts, RMSD were calculated and analyzed for each copper ion of both enzymes and results are summarized in Supporting Information Figure S6. Overall, the mobility of T1 center at 80 °C in LtL represents the major difference between the two laccases and we investigated whether such small dynamic variations could impede a correct ETP. As illustrated in Figure 5, a Cys is coordinated to the T1 center and two adjacent His residues coordinate the two T3 copper ions.<sup>[36,37]</sup>



**Figure 7.** The evolution of the Electron Transfer Pathway for LtL (on the left) and SoL (on the right) upon 50 ns MD simulations at 80 °C. The starting conformations are reported in the upper part of the figure whereas the final situations are illustrated underneath.

The analysis of distances between copper ions and these crucial residues during the MD simulations of LtL reveals that the distance between T1 and Cys452 increases from 1.7 Å to 4.8 Å, and this is compatible with a distortion and perturbation of the electron transfer. More importantly, SoL retains the original geometry and distances throughout the 50 ns simulation, with a very limited mobility of this region. In conclusion, MD simulations suggest that LtL loses its activity upon thermal treatment not necessarily because of fast and complete unfolding and denaturation but most probably because of the immediate distortion of the ETP. On the other side, SoL displays a restricted mobility of the

region involved in the ETP and this factor is in agreement with its extraordinary thermal stability. Therefore, SoL combines a remarkable resilience to unfolding with specific structural properties of the core of the protein. Consequently, the enzyme exploits beneficial thermal effect and superficial conformational modifications leading to hyperactivation, whereas LtL is promptly inactivated by heating. The observed hyperactivation of SoL in aqueous-organic solvent mixtures suggest that the phenomenon is not ascribable to simple thermal effect but that specific conformational changes are involved, as also supported by CD spectra.

Concerning the structural basis of SoL stability to unfolding, it is widely recognized that glycosylation patterns alters the behavior of the native protein, by increasing thermal stability. <sup>[41]</sup>

However, state of the art of computational methodologies does not allow simulating accurately the conformational effect of glycans on the whole protein due to the wide variability of glycosylation patterns. Therefore, the present study has focused the attention on factors referring only to the apoprotein and that can be of interest also for engineering strategies. Intrinsic protein rigidity is most often correlated to a more general resilience towards denaturing agents and this fact makes SoL an optimal candidate for laccase mediated transformations of recalcitrant substrates under harsh conditions. <sup>[42]</sup>.

## **Conclusions**

Laccase from *Steccherinum ochraceum* 1833 (SoL) displays remarkable stability when incubated in the presence of co-solvents or exposed to 60 °C and microwave irradiation. More importantly, these harsh conditions cause a hyper-activation of this fungal laccase, whereas induce rapid inactivation of other fungal laccases, such as those from *Lentinus tigrinus* 8/18 (LtL) and *Lentinus strigosus* 1566 (LsL). The thermal stability of SoL is so pronounced that inactivation becomes evident only after one

hour of incubation at 80 °C. Non-thermal inactivating effect of microwaves was documented only at 80 °C, whereas at 50 °C and 60 °C microwaves can be safely used as heating method. The structural basis of the higher stability of SoL was disclosed by Molecular Dynamic simulations that illustrated how at high temperature (80 °C) SoL retains after 50 ns the original and productive geometry of the Electronic Transfer Pathway responsible for the transfer of electrons from the T1 copper ion to the tri-nuclear site, whereas the pathway is distorted in LtL. Therefore, the simulations suggest that the loss of activity of LtL is not necessarily related to immediate severe protein unfolding but rather minor distortion of the Electronic Transfer Pathway can prevent the accomplishment of the oxidative mechanism.

The present work brings also to the attention the hyperactivation phenomena of SoL that appear related to limited superficial conformational changes occurring under harsh conditions but without inducing severe unfolding and disruption of ETP, as supported by spectroscopic and MD analysis. These features make SoL a very promising biocatalyst under conditions that might be beneficial for the biotransformation of recalcitrant substrates.

In conclusion, the new structural/conformational factors here documented should be taken into account in future studies addressing the conservation (or loss) of activity of laccases. Moreover, structural features observed so far only in SoL can have a more general impact on future studies aiming at engineering laccases with improved stability and activity.

## **Experimental Section**

### **Cultivation**

The basidial fungi *Steccherinum ochraceum* 1833 and *Lentinus strigosus* 1566 were obtained from basidiomycete collection of V. L. Komarov Botanical Institute of the Russian Academy of Sciences (BIN RUS, Russia). White rot basidiomycete *Lentinus (Panus) tigrinus* 8/18 was isolated from rotting wood in Dushanbe (Tadzhikistan) and stored in collection of Laboratory of Enzymatic Degradation of Organic Compounds (IBPM RAS). All cultures were supplied on slant wort agar. For laccase

purification the fungus *S. ochraceum* 1833 was cultivated as previously described.<sup>[18]</sup> Submerged cultivation of *L. tigrinus* 8/18<sup>[41]</sup> and *L. strigosus* 1566<sup>[42]</sup> were performed at 29 °C with shaking (200 rpm) and full details are provided in Supporting Information.

### **Purification**

The predominant laccase isoform II from *S. ochraceum* 1833 was purified as described earlier.<sup>[18]</sup> Predominant laccase from *L. tigrinus* 8/18 and predominant laccase isoform from *L. strigosus* 1566 were purified using 3 subsequent steps involving anion-exchange chromatography and one step of gel filtration. Complete details are available in Supporting Information. All final electrophoresis grade laccase solutions were desalted, concentrated, and used in the following experiments.

### **Activity assay in aqueous buffer**

All laccases were stored in 20 mM Na-acetate buffer pH 5.0. Laccase activity was determined at 25 °C in 20 mM Na-acetate buffer (pH 5.0) by adding defined volumes of laccase stock solutions (SoL solution with the concentration of 0.037 mg/mL; LtL solution with the concentration of 0.022 mg/mL; LsL solution with the concentration of 0.022 mg/mL) and finally 10 µL catechol solution (1 M) for a total volume of 1 mL. Laccase activity was determined at 25 °C. The progress of the enzymatic oxidation was monitored at 400 nm every second for 1 min, then extrapolating initial rates for each enzyme. One enzymatic Unit corresponds to the amount of laccase able to catalyze the oxidation of 1 µmol of substrate in 1 minute.

### **Activity in buffer/organic solvent mixtures**

The activity of each laccase was evaluated by considering the oxidation of catechol in different buffer/organic solvent mixtures. Two different solvents were considered: dioxane (LogP = -1.1) and ethanol (LogP = -0.18). Each solvent (dioxane or ethanol) was mixed in different proportions with 20 mM Na-acetate buffer (pH 5.0) for a total volume of 1 mL. Aliquots of laccase stock solutions described above (10 µL of SoL; 50 µL of LtL and LsL) and 10 µL catechol solution (1 M) in 20 mM Na-acetate buffer (pH 5.0) were then added. The activity of each laccase at  $t_0$  was considered as 100 % activity (193, 55 and 49 U/mg for SoL, LtL and LsL respectively).

### **Time-dependent stability in buffer/organic solvent mixtures**

The stability of each laccase was evaluated by considering the oxidation of catechol in 10 mL mixtures of 20 mM Na-acetate buffer at pH 5.0 buffer and organic solvent (dioxane and ethanol) at 50 % v/v. Aliquots of laccase stock solutions described above (100  $\mu$ L SoL; 500  $\mu$ L of LtL or LsL) were added and mixed with each buffer/solvent mixture at 25 °C. At different times, 1 mL of these solutions was withdrawn and employed for the spectrophotometric assay by adding 10  $\mu$ L catechol solution (1 M). The progress of the enzymatic oxidation was monitored at 400 nm every second for 1 min, then extrapolating initial rates for each enzyme. The activity of each laccase at  $t_0$  was considered as 100 % activity.

### **Thermal and microwave stability**

100  $\mu$ L of laccase stock solutions (see above) were diluted to 10 mL using 20 mM Na-acetate buffer (pH 5.0). Solutions were incubated at constant temperature (50, 60 or 80 °C) with or without microwave radiation (50 or 10 W). At different times, aliquots of 1 mL were employed for the spectrophotometric assay by adding 10  $\mu$ L catechol solution (1 M). The progress of the enzymatic oxidation was monitored at 400 nm for 1 min. The activity of each laccase at  $t_0$  was considered as 100 % activity. Microwave irradiation was performed using a Discover<sup>®</sup> CEM instrument.

### **Computational investigations**

Crystal structures of laccases from *L. tigrinus* 8/18 and laccase from *S. ochraceum* 1833 were retrieved from the Protein Data Bank (PDB)<sup>[38]</sup> (PDB ID: 2QT6<sup>[28]</sup> and 3T6V<sup>[45]</sup> for *L. tigrinus* 8/18 and *S. ochraceum* 1833 respectively). Protein structures were visualized inspected and pre-processed using the PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC). Only the apo-protein with their copper ions and crystal waters were retained (i.e. by deleting inhibitors, glycosylation residues, etc.). The protonation state of each structure was calculated at pH 5.0 using the PDB2PQR server<sup>[46]</sup> based on the software PROPKA.<sup>[47]</sup>

Each structure was simulated using software GROMACS version 4<sup>[48]</sup> and defined into OPLS-AA force-field.<sup>[49]</sup> The four copper ions were considered in fully oxidized state as Cu<sup>2+</sup>. Information

concerning the coordination of each copper ion was taken from the pdb file of each laccase (see Supporting Information Figure S3 and S4 for *L. tigrinus* 8/18 laccase and *S. ochraceum* 1833 laccase respectively) and specified into the pairs section of the GROMACS topology file. Full details of protocols applied for the computational studies are available in Supporting Information.

### **Circular dichroism analysis**

CD spectra were recorded on a Spectrometer Jasco-710 on 0.22  $\mu$ M solutions of SoL in 20 mM Na-acetate buffer (pH 5.0). Full details of the set up and recording parameters are available in Supporting Information.

### **Fluorescence spectroscopy**

Spectra were recorded on a Fluorescence Spectrophotometer Hitachi F-4500 in 20 mM Na-acetate buffer (pH 5.0) at different temperatures. Full details of the set up and recording parameters are available in Supporting Information.

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