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Acceleration of Oxidations Promoted by Laccase with Red Light

Valentina Giraldi,†[a] Marianna Marchini,†[a] Matteo Di Giosia,[a,b] Andrea Gualandi, [a,b] Martina Cirillo,[a] Matteo Calvaresi, [a,b] Paola Ceroni, [a,b] Daria Giacomini,* [a,b] and Pier Giorgio Cozzi* [a,b]

Irradiation with red light is able to improve yields and shorten the reaction time in enzymatic reactions. A commercially available Laccase from Trametes versicolor, used in conjunction with catalytic amount of TEMPO (20 mol%), was irradiated under red light (630 nm) for the clean oxidation of benzylic alcohols and amines in a faster reaction compared to the one conducted in the dark, affording the desired product in excellent yields (up to 99%) using air as terminal oxidant. The observed acceleration is due to the strongest oxidation ability of Laccase, in its excited state, towards TEMPO

Introduction

The search for new, efficient, and environmentally benign “green” catalysts for oxidation processes is an important matter for the textile, pulp, paper, and chemical industries.[1] One big challenge in this search is to find an effective catalytic system able to perform the oxidation reaction using air as terminal oxidant and producing water as by-product.

On this perspective, a significant group of oxidoreductases, the Laccases, received an increased interest in the organic chemistry community[2] when enzymes became commercially available.[3] The exploitation of these enzymes in catalytic oxidation was also favoured by the deep knowledge gained in the last decade about their biochemistry and enzymatic mechanism.[4]

Laccases (*p*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) are glycoproteins produced by plants and fungi, but also by some bacteria and insects, capable of catalysing polymerization or depolymerization of lignin.[5] Polymerization occurs via monoelectronic oxidation of suitable substrates, principally phenols and amines, to reactive radicals that initialize the polymerization process. The process is a redox-mediated reaction aided by a cluster of four copper atoms that constitutes the catalytic core of the enzyme. Inferred from the 3D-crystal structure of the fungal Trametes versicolor Laccase, (Figure 1A),[6] one of the four catalytic Cu ions is located at the T1 site, and the other three atoms are placed at a tri-nuclear cluster, the T2 and T3 sites (Figure 1B). The T1 site is located at the enzyme's surface, and it holds the blue copper-binding site appointed to the substrate oxidation and responsible for the intense blue colour of the oxidized enzyme.

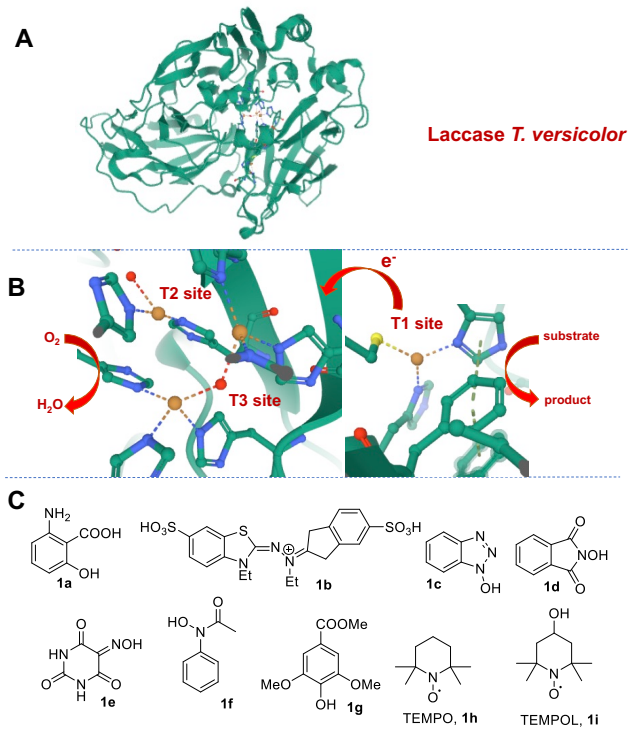


Fig. 1A) Crystal structure of Laccase from *Trametes versicolor* (Protein Databank code 1GYC); B) Copper clusters at the active site of Laccase Tv; C) Useful mediators for the laccase-mediator-system.

This site exhibits a Cys(S)→Cu(II) charge-transfer (CT) band in Laccase absorption spectrum (ε ≈ 5000−6000 M^{-1} cm^{-1} at about 610 nm).[7] The coordination geometry and ligands of the T1-Cu might determine the high redox potential (0.8 V vs NHE) observed in Laccase from Trametes.[8] The 4-electron reduction of O_2 to water occurs at T2/T3 sites that are buried within the protein. The T2 copper shows no significant absorption, whereas the T3 site bridging a hydroxo ligand between the coppers gave intense absorbance at 330 nm regulating the internal electron transfer from the T1 to the T2/ T3 cluster.[7]

Laccases are used for the distinctive redox ability of copper ions to catalyze the oxidation of a wide range of phenolic substrates, in parallel with the reduction of molecular oxygen to water. Regarding the application in organic synthesis, non-phenolic compounds cannot be directly oxidized, due to high oxidation potentials (above ~ 1V vs NHE)[8] or because too bulky to enter into the active site.[2a] In this case, small molecules (mediators) that are readily oxidized by Laccases could transfer electrons between the enzyme and the target substrate in the so-called Laccase-Mediator System (LMS).[9] Suitable mediators (Figure 1C) such as 3-hydroxyanthranilic acid **1a**,[10a] 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid **1b**,[10b] *N*-hydroxybenzotriazole **1c**,[10c] *N*-hydroxyphtalimide **1d**,[10d] violuric acid (VLA) **1e**, *N*-hydroxyacetanilide **1f**, methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid) **1g**[10e] and (2,2,6,6-tetramethylpiperidine-1-yl)oxy (TEMPO) **1h**[10f,e] are currently used. As an example, the oxidation of TEMPO (**1h**) by Laccase gives oxoammonium cation, that in turns oxidizes the non-phenolic substrates via an Anelli-type oxidation.[11]

Photocatalysis has emerged as a powerful tool to generate reactive intermediates under mild reaction conditions.[12] The combination of photocatalysis and biocatalysis, has recently become an object of study.[13] Combination of these two branches of catalysis brings the advantages of the reactivity of photocatalysts with the selectivity of enzymes,[14] and studies in photobiocatalysis are in progress, included the regeneration of cofactors, generation of H_2O_2 in situ, cascades reactions involving a photobiocatalytic transformation, and the photoinduced enzymatic reactions inspired by photoactive cofactor within the enzyme.[14] More specifically, Hyster has published a remarkable example of the use of co-factor dependent enzymes in photoredox applications, by irradiation with visible light.[15] Nicotinamide-dependent double bond reductases in combination with $Ru(bpy)_3Cl_2$ under visible light irradiation have been used in the reduction of ketones by a radical mechanism.[16] Similarly, $Ru(bpy)_3Cl_2$ and Flavin-dependent ene'-reductases, that are capable of reducing enones, enoates, and nitroalkenes using the native hydride transfer mechanism,

can be “reprogrammed” by photoredox catalysis to reduce vinyl pyridines.[17] Brunstad and Nicewicz[18] have recently described the bio-conjugation of 9-mesityl-10-phenyl acridinium photocatalyst with thermostable proteins to generate a new class of artificial enzymes, able to promote the photoredox oxidation of aryl sulphides by visible light irradiation. Finally, electron transfer from a biotinylated electron donor to photochemically generated Ru(III) complexes, covalently anchored to streptavidin, was recently demonstrated.[19] [Ru(II)L_3 (L = bipyridine, phenanthroline) complexes were bioconjugated to the exposed cysteines of streptavidins to prepare an artificial metalloenzyme able to perform photoredox catalysis transformations.

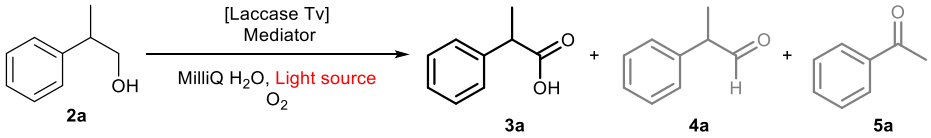
Concerning Laccases, application of photoredox methodologies was reported. Laccase enzymes have been coupled with some photosensitizers as zinc-tetramethylpyridinium porphyrin[20a] or ruthenium-polypyridine-type complex in the presence of EDTA as a sacrificial reductant[20b] to reduce O_2 to H_2O.[21] In these interesting applications, the photosensitizers were able to reduce the copper ions of the Laccase upon light irradiation, by electron transfer. Some years ago, we applied commercially available Laccase enzymes in green oxidation of benzylic alcohols towards the corresponding acids, in order to describe convenient access to Profen drugs.[22,23] Although the methodology was successful, producing different biologically active Profens without racemisation, it suffers from quite longer reaction time (more than 6 days to achieve 65% yield in certain cases, see further discussion and examples). With the purpose of using metalloenzymes in photoredox processes, focusing on the absorption properties of enzymes and their photophysical (electron or energy transfer) properties, we have started to investigate the oxidation of organic substrates by a photo-Laccase system under visible light irradiation. As Laccase is a metalloenzyme with interesting absorption properties, we want to explore the possibility to improve the oxidation of organic substrates by a photo-Laccase system under visible light irradiation. Due to the presence of the mentioned charge transfer (CT) band, a photoexcited state of the Laccase could be populated by absorption of visible light and its enhanced oxidation ability could be beneficial in the redox reaction with organic mediators, improving the catalytic performance. In this paper, we report spotless and light-accelerated oxidation reactions of benzylic alcohols and amines mediated by Laccase, in the presence of a catalytic amount of TEMPO (**1h**). By irradiation with visible light (red LED centred at 630 nm, see Supporting Information or emission profile) the excited state of Laccase is involved in improving the oxidative capability of Laccase in our reactions.

Results and discussion

Based on the previous experience in the oxidative properties of Laccase,[2] we started our investigation using 2-phenyl-1-propanol **2a** as the model substrate, due to its importance as a starting material for drug synthesis. The alcohol **2a** was diluted in MilliQ water, mediator, and Laccase from *Trametes versicolor* (Laccase Tv) were added and O_2 bubbled for 30 seconds, then the reaction mixture was subjected to red LED irradiation at 630 nm (23 W).

The alcohol **2a** was completely consumed in three days (72 h) and the only product obtained was the 2-phenylpropanoic acid (**3a**) in quantitative yields after acid-base extraction (Table 1, entry 2). Under the same reaction conditions but without irradiation with red light the reaction was slower (6 days were needed for complete consumption of **2a**), with lower selectivity and yield (Table 1, entry 1). When the reaction was not irradiated, acetophenone **5a** was observed as by-product derived from a slow degradative oxidation of the aldehyde **4a** that occurs in long-standing reactions.[23] In the absence of TEMPO, the reaction did not proceed (Table 1, entry 4), thus confirming the need for a mediator. When examining the influence of the amount of TEMPO on the reaction outcome, we found that lower its amount from 20 to 10 and 5 mol% (Table 1 entries 5 and 6), almost quantitative conversions were obtained, but the yields of **3a** notably decreased and, at the same time, increased the amount of aldehyde **4a** and acetophenone **5a**.

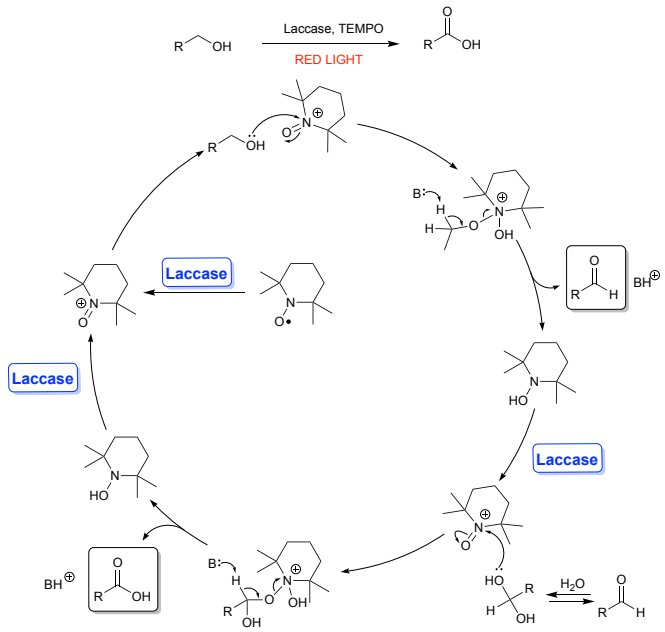
Table 1. Screening of the reaction conditions for the Laccase oxidation of **2a**.



Entry[a]	Light source	Mediator (mol%)	Temperature (°C)	Time (h)	Conversion (%)[b]	**2a**/**3a**/**4a**/**5a**[b]	Yield (%)[c] **3a**
1[d]	No light	**1h** (20)	30-35	**144**	99	0/96/tr[e]/4	**65**
2[d]	Red LED	**1h** (20)	30-32	**72**	99	0/99/0/tr[e]	**99**
3	Red LED	**1h** (20)	30-32	**24**	99	0/99/tr/tr[e]	**77**
4	Red LED	--	30-32	120	0	100/0/0/0	–
5	Red LED	**1h** (10)	30-32	72	99	0/87/tr/13	76
6	Red LED	**1h** (5)	30-32	72	90	tr[e]/47/44/9[f]	27
7	Red LED	**1g** (20)	30-32	96	0	100/0/0/0	–
8	Red LED	**1c** (20)	30-32	72	0	100/0/0/0	–
9	Red LED	**1i** (20)	30-32	72	99	0/36/57/7[f]	27
10	CFL	**1h** (20)	25-30	24	99	0/77/19/4[f]	51
11	CFL	**1h** (10)	25-30	24	80	16/33/51/tr[e,f]	27
12[f]	Red LED	**1h** (20)	30-32	72	99	0/90/tr[e]/9[f]	56

[a]The alcohol **2a** was diluted in MilliQ H_2O, mediator and Laccase Tv were added and O_2 bubbled for 30 seconds, then the reaction mixture was subjected to LED or CFL irradiation. See SI for reaction set up. Reaction conditions: **2a** (0.25 mmol), MilliQ H_2O (3 mL), mediator (mol% in table), and Laccase Tv (2.5 mg, 6U). [b] Evaluated by ^{1}H NMR analysis of the crude reaction mixture. [c] Isolated yields after acid-base extraction. [d] Reaction conditions: **2a** (0.5 mmol), MilliQ H_2O (6 mL), mediator (0.1 mmol), and Laccase Tv (5 mg, 12 U). [e] tr = traces (less 1%). [f] The presence of other not identified by-products was observed. [g] Solvent medium: AcOH/AcONa buffer 0.5 M, pH = 4.5.

The oxidation of primary alcohols to the corresponding carboxylic acids is considered a two-step oxidation: 1) the oxidation of alcohol to give the aldehyde; 2) the oxidation of aldehyde to the carboxylic acid through the formation, in aqueous conditions, of the intermediate aldehyde hydrate (Scheme 1). The yield of **3a** depends on TEMPO concentration, probably due to the slower oxidation of the *gem*-diol with respect to the first oxidation step. As was reported in our precedent studies,[22] oxidation of benzylic alcohols is a slow reaction. In entries 1 and 2, we have compared the yields and reaction rate of two reactions with and without red light irradiation. With the commercially available Laccase used in our studies, the reaction in the presence of red light resulted accelerated (see also ESI for full details). The ^{1}H-NMR of the crude reaction mixture (Table 1, entry 3) shown no traces of by-products other than aldehyde, acid, or acetophenone. A poor recovery of the acid **3a** in this experiment could be due to a lower scale of the reaction respect entry 2 and to a possible scanty extraction of **3a** in the acid/base separation.

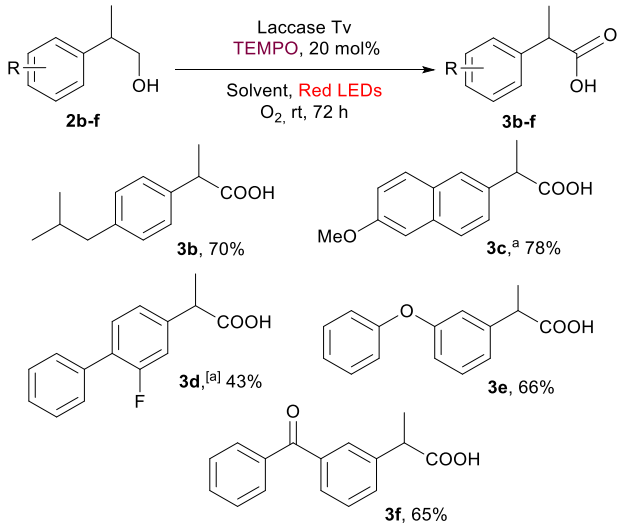


Scheme 1. Two-step oxidation of primary alcohols to carboxylic acids.

Some other mediators were tested (Table 1, entries 7-9), but only TEMPO or TEMPOL were effective in the reaction. Irradiation with fluorescence lamp (CFL, see supplementary information for emission profile) gave quantitative conversions in 24 h when 20 mol% of TEMPO was employed, but a lower production of acid **3a** (Table 1, entry 10) with the presence of aldehyde **4a** and acetophenone **5a**. Bio-oxidations by Laccases are usually conducted in acetate buffer at pH = 4.5 – 4.8. However, at pH = 4.5 the acid is isolated with only 56% yields (Table 1, entry 12). This could be probable due to a partial decomposition of the mediator. It is known, in fact, that the stability of nitroxyl radical TEMPO in acidic medium, such as in acetate buffer, is low, and for longer reaction times it decomposed to a greater extent.[24] Moreover, on measuring the activity of the Laccase, we observed higher and constant values over the time in MilliQ water, but a progressive loss of activity in acetate buffer (see supplementary information).

To get information on the effect of irradiation on the two-step oxidation of primary alcohols, we followed the time course of the reaction with Laccase Tv, and TEMPO (20 mol%) in MilliQ water under irradiation and in the dark. The relative amounts of the starting alcohol (**2a**), the aldehyde (**4a**) as intermediate product, and the final carboxylic acid (**3a**) were monitored at fixed times by ^{1}H NMR analysis of the crude reaction mixture (Figure S4). On a direct comparison of the two conditions, irradiation or in the dark, it is clear how the light increased the rate of both steps: the consumption of starting alcohol was faster upon irradiation (complete at 3.75 h) than in the dark (not complete at 3.75 h), and the next oxidation to the carboxylic acid was accelerated as well.

The optimized conditions established with 2-phenyl-1-propanol **2a** were further tested on a series of 2-arylpropan-1-ols, important precursors of the Profen class of anti-inflammatory drugs (Scheme 2).

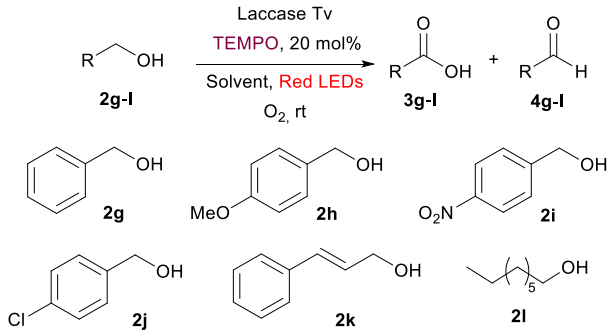


Scheme 2. Oxidation of some 3-aryl-propanols for the synthesis of selected aryl-propionic acids (Profens); [a] 10% in volume of acetone as co-solvent was used.

Racemic ibuprofenol **2b** was successfully oxidized to Ibuprofene **3b** with satisfactory yields after 72 h. It is noteworthy that the reaction time was considerably reduced with respect to the previously reported conditions (79%, reaction time 6 days).[21b] As ascertained in the previous study,[21b] to obtain Naproxen (**3c**) and Flurbiprofen (**3d**) the addition of a 10% acetone as co-solvent improved the yields due to a better solubility of the starting alcohols **2c** and **2d**, respectively. Some variability in the isolated yields of Profens occurred due to the presence of certain amounts of the corresponding acetophenones as by-products. Starting from optically active alcohols it is possible to have access to optically active Profens without racemization of the stereogenic center during bio-oxidation as demonstrated from our previous paper.[21b]

Oxidation of benzyl alcohols (**2g-l**) proceeded with quantitative conversions within a one-day reaction time, and predominantly gave the corresponding aldehydes (Table 2). The amounts of carboxylic acids increased with time. *p*-Chloro-benzyl alcohol (**2j**) was chosen as substrate to point out the effect of irradiation of the reaction outcome. From the results obtained with and without irradiation (Table 2, entries 5-6) it is possible to observe that a higher content in the carboxylic acid **3j** was obtained with red-light irradiation, thus confirming the rate improvement of irradiation versus the carboxylic acid formation. 1-Octanol (**2l**) selected as model of aliphatic primary alcohols gave a quantitative conversion after 48 hours and a mixture of octanal (**4l**) and octanoic acid (**3l**) was obtained.

Table 2. Oxidation of benzyl alcohols and octanol.[a]

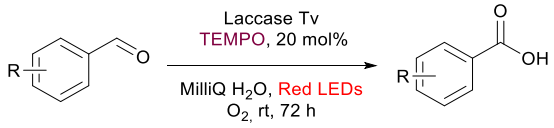


Entry	Substrate	Solvent	Time (h)	Conversion (%)	**3**/**4**[b]
1	**2g**	MilliQ H_2O	24	99	27/73
2	**2g**	MilliQ H_2O	48	99	36/64

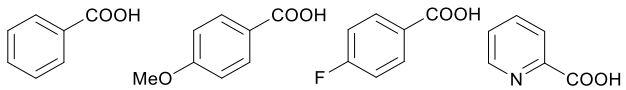
3	**2h**	MilliQ H_2O	16	99	12/88
4	**2i**	MilliQ H_2O/ acetone 10%	24	99	35/65
5	**2j**	MilliQ H_2O/ acetone 10%	24	99	34/66
6[c]	**2j**	MilliQ H_2O/ acetone 10%	24	99	14/86
7	**2k**	MilliQ H_2O	24	99	7/93
8	**2l**	MilliQ H_2O	48	99	41/59

[a] Reaction conditions: alcohol (0.25 mmol), TEMPO (0.05 mmol), and Laccase Tv (2.5 mg, 6 U), red light irradiation, temperature: 28-30°C. [b] Ratio determined by ^{1}H NMR analysis of the crude. [c] Reaction conducted in the dark.

Table 3. Oxidation of aldehydes to carboxylic acids.[a]



4g,**h**,**m**,**n** **3g**,**h**,**m**,**n**



3g **3h** **3m** **3n**

Entry	Substrate	Time (h)	Conversion (%)	3/4[b]
1	4g	72	40	40/60
2	4h	72	12	12/88
3	4m	72	35	35/65
4	4n	4.5	99	99/tr

[a]Reaction conditions: aldehyde (0.25 mmol), TEMPO (0.05 mmol), and Laccase Tv (2.5 mg, 6 U), red light irradiation, temperature: 28-30°C.[b] Ratio determined by ^{1}H NMR analysis of the crude.

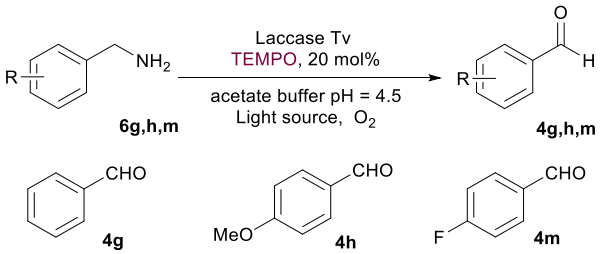
Oxidation of aldehydes (**4g**, **h**, **m**, **n**) to the corresponding acids (**3g**, **h**, **m**, **n**) was also studied (Table 3). Excellent results were obtained with 2-pyridine carboxaldehyde **4n** in terms of photo-acceleration of the process as it was completed in only 4.5 hours, whereas other aldehydes were not completely converted in 72 hours. Two factors could play a main role in this oxidation step: low water solubility of the aldehyde and the hydration equilibrium that should favor both solubility and the oxidation.

The photo-Laccase-mediator system was further explored in the oxidation of benzyl amines. The bio-oxidation of amines was initially performed starting from the optimized reaction conditions previously reported.[21a] The use of acetate buffer at pH = 4.5 was necessary because of the inactivation of Laccase Tv at the basic pH generated by amine dissolution in H_2O.[25] Benzylamines **6g**,**h**,**m** smoothly underwent oxidation with excellent conversions, and the corresponding aldehydes **4g**,**h**,**m** were isolated in good to excellent yields (Table 4). The irradiation of the reaction mixture with CFL lamp in the oxidation *p*-methoxy-benzylamine **6h** was most effective than that obtained by irradiation with LEDs 630 nm (Table 4, entry 2) with a quantitative conversion of **6h** and affording the aldehyde **4h** in high yield after 6.5 h of irradiation (Table 4, entry 3). By lowering the amount of TEMPO, the reaction slowed down and in 2 days 88% conversion and 82% yields of aldehyde **4h** were obtained (Table 4, entry 4). A slight substituent effect was observed since *p*-fluoro-benzaldehyde (**4m**) was isolated in quantitative yields after 5 h of irradiation only (Table 4, entry 5).

Photochemical studies

The activity of Laccase Tv was examined under LED-irradiation at 630 nm in comparison to a normal day-light exposure to understand whether the enzyme would be deactivated or not by the LED-light. Four conditions were tested over 6 days, see SI for details. Data in Table S2 and the plot of Figure S5 clearly shows that the Laccase Tv was not deactivated upon LED irradiation.To get insights into the photochemical mechanism of the reaction, we investigated the photophysical properties of the Laccase and their change in the presence of the redox mediator TEMPO. The absorption spectrum of the commercially available Laccase (Figure S6) does not show the typical absorption band at 610 nm, reported in the literature and responsible for the blue colour. Only after concentration of the enzyme (see supporting information), we saw the typical absorption band in the red spectral region (see Figure S6). Upon excitation at 340 nm, a weak fluorescence band is observed at 447 nm (Figure S7): this band has been attributed to the T3 site in agreement with literature.[26] Upon addition of TEMPO, the lifetime of this fluorescent excited state decreases: a quenching constant of 9.5 x 10^9 $M^{-1}s^{-1}$ is estimated by the Stern-Volmer plot (Figure S8). However, this quenching is not responsible for the observed acceleration of the oxidation of benzylic alcohols and amines under red LED irradiation as the T3 site is not absorbing red light.

Table 4. Oxidation of benzyl amines.[a]



Entry	Light source	Product	TEMPO (mol%)	Time (h)	Yield (%)[b]	**4**
1	Red LEDs	**4g**	20	5	70	
2	Red LEDs	**4h**	20	20	90	
3	CFL 23W	**4h**	20	6.5	95	
4	Red LEDs	**4h**	5	48	82	
5	Red LEDs	**4m**	20	5	99	

[a] Reaction conditions: amine (0.25 mmol), TEMPO (0.05 mmol), and Laccase Tv (2.5 mg, 6 U), light irradiation, temperature 25-30 °C. [b] Isolated yields after acid-base extraction.

We investigated the effect of red light on a model reaction, i.e. catechol oxidation.

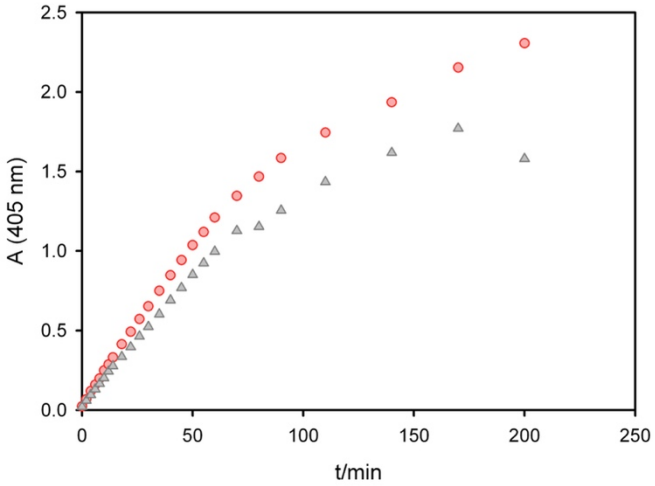


Figure 2. Comparison between absorbance variation at 405 nm of a solution containing catechol 18 mM and Laccase Tv (6 mg in 5mL) in acetate buffer 0.5 M pH = 4.5, kept 280 minutes in the dark at 6°C (grey triangles) and irradiated 200 minutes with red led at 6°C (red dots).

This well-known reaction, used to estimate the activity of Laccase enzymes,[27] yields an oxidation product, *o*-quinone, that absorbs in the visible region of the spectrum (λ_{max} 405 nm), so that its formation can be easily detected. The conversion of catechol to *o*-quinone was studied at 6°C to slow down the reaction rate. The kinetics of catechol oxidation in the dark is slower than that performed with red LED irradiation, as evidenced by the amount of *o*-quinone formed as a function of time (Figure 2).

We can thus conclude that red-light excitation accelerates the oxidation of catechol promoted by Laccase and this is likely due to the higher oxidation ability of the excited state of the T1 site. If the reaction was more exoergonic we expect a higher electron transfer rate constant in the normal region of the Marcus theory.[28] A similar behaviour is expected for the redox reaction of Laccase with TEMPO, which is a reversible redox mediator and cannot be accumulated under steady-state irradiation.

Conclusions

In summary, we have reported a novel concept in photoredox arena, using the excitation of metal's copper cluster inserted in Laccase, a commercially available enzymes, for enhancing the oxidation properties of the enzyme towards organic substrates. The concept could be extended to other metal enzymes, expanding the arsenal of synthetic transformations in Biocatalysis. The reduced reaction time compared to "dark" reaction, the use of visible light and air as terminal oxidant make this process environmentally friendly. Further application of oxidation promoted by Laccase under irradiation and studies about metal containing enzymes in photoredox conditions are planned in our laboratories.

Author Contributions

P.G. C., A. G., and D. G. conceived the studies and experiments; V.G. and M.C. performed the reactions and measured activity of enzymes, supervised by D.G.; M. M. and P. C. performed and analyzed photophysical measures on samples of concentrated enzymes in model reactions; Concentration of commercial sample of enzymes was carried out by M. D.G. and M.C. The manuscript was written and prepared with contributions from all authors.

Conflicts of interest

There are no conflicts to declare.

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