Direct heterogeneous electron transfer reactions of *Trametes hirsuta* laccase at bare and thiol-modified gold electrodes

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Abstract

Direct electron transfer reactions of high redox potential *Trametes hirsuta* laccase on bare and 15 different thiol-modified gold electrodes were studied using cyclic voltammetry and potentiometry. Well-pronounced Faradaic processes were obtained for the enzyme adsorbed on bare gold, whereas reproducible and stable electrochemistry was obtained only when 4-aminothiophenol was used for gold modification. Moreover, the laccase-4-aminothiophenol-modified gold electrode showed the highest value of the steady-state potential under aerobic conditions equal to 660 mV vs. NHE compared with the other 15 different thiol modified electrodes and also the bare electrodes with immobilized enzyme. However, this value is still too far away from the equilibrium potential of the oxygen electrode and *Trametes hirsuta* laccase-modified graphite electrode, for which a well-pronounced high potential process of oxygen bioelectroreduction is shown at 800 mV vs. NHE. Possible mechanisms of the enzyme function on bare and thiol-modified gold electrodes are discussed in correlation with the structure and orientation of the enzyme on the surface of the various electrodes.

Keywords: Laccase; T1, T2, and T3 sites; Redox potential; Gold electrode

Abbreviation used: DET, Direct electron transfer; ET, Electron transfer; Em, Midpoint potential; CVs, Cyclic voltammograms

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1. Introduction
Only a limited number of enzymes, e.g., “blue” multicopper oxidases, are able to reduce molecular oxygen directly to water without formation of highly toxic oxygen intermediates. Laccase is one of the simple “blue” multicopper oxidases, which contain four copper ions classified as a T1, T2, and T3 sites in accordance with their spectroscopic characteristics [1]. Laccase is able to oxidize a large number of organic and inorganic substrates with concomitant reduction of molecular oxygen [2]. One electron from the substrate is transferred to the T1 site, the primary electron acceptor of the enzyme, which is then transferred through an intramolecular electron transfer (ET) mechanism via a His-Cys-His bridge to the T2/T3 cluster, where oxygen is reduced to water [1].
As early as in 1978, the unique property of some redox enzymes, bioelectrocatalysis based on direct electron transfer (DET) reactions, was demonstrated for a fungal laccase adsorbed on carbon [3]. Laccase is widely used for construction of biosensors [4-6] and biofuel cells [7-10]. DET can be exploited for the creation of simple, cheap, and non-toxic laccase-based biosensors and biocathodes for biofuel cells, where no mediator is utilized. This possibility stimulated many electrochemical studies of laccases from different sources on metal and carbon electrodes in the last few years [11-17].
One of the important thermodynamic parameters of a redox protein or a redox enzyme is the formal redox potential of the cofactor in the active site. Due to the presence of four differently coordinated copper ions in the structure of laccase one can expect the presence of different redox potentials of the enzyme corresponding to its different intermediate redox states. However, only the potential of the T1 site has been determined and its value is now known for numerous plant and fungal laccases. The potentials of the T1 site were found to be in the range from 430 to 790 mV vs. NHE for laccases from different sources [16, 18, 19]. The other three coppers, which form the T2/T3 cluster of the enzyme, are much more difficult to titrate due to several factors. The absorbance of many of the mediators used for redox titration of the enzyme, coincides with the absorbance changes of the T2/T3 cluster during its redox transformation (approximately 330 nm). Additionally, several redox potentials of the cluster should be suggested corresponding to the different intermediate redox states of the cluster, such as peroxy and native intermediates, and possibly some others [20, 21]. However, one cannot produce all these intermediates under anaerobic conditions during the redox titrations because these forms are formed during the catalytic turnover of the enzyme in the presence of oxygen. The values of the redox potentials of the T2 and T3 sites of two enzymes, which have been determined so far (Trametes fungal laccase and Rhus plant laccase [17, 18, 22]), are, most likely, too far away from the real values of the redox potentials of the T2/T3 cluster during the enzymatic reduction of oxygen. Obviously, the values of the redox potentials of the intermediate forms of laccase are not yet known and call for further investigations. As for the T1 site, its formal redox potential seems to be independent on the presence or absence of oxygen. This is why the value obtained during the anaerobic redox titration most likely is the same for the native enzyme during its catalytic turnover and can be used to explain the function of the enzyme.
Along with redox potentiometry, wildly used for determination of the redox potential of biological objects for many years [23, 24], other methods can be exploited for the estimation of the midpoint potential \((E_m)\) of redox proteins and enzymes, e.g., different types of voltammetry [25]. One huge advantage of these methods is the possibility \((i)\) to study electrochemical transformations of such oxidases in the presence and in the absence of oxygen and \((ii)\) to follow the formation of several intermediates of the enzyme by the appearance or disappearance of Faradaic processes in the voltammograms. However, relatively often an enzyme adsorbed on the electrode changes its native conformation and the values of the \(E_m\) obtained during the voltammetric measurements cannot be directly attributed to the value of the redox potential of the active site of the free nonadsorbed enzyme. Thus, the
best scientific way to obtain solid thermodynamic information about the redox transformation of oxidoreductases is to combine both potentiometric and voltammetric data. One possible application of high redox potential laccases having values of the redox potential of the T1 site close to 800 mV [15] is in the construction of biofuel cells [9, 26]. However, this would require basic knowledge about the electrochemical transformation of the enzyme on different electrode materials. Moreover, as mentioned above, this knowledge can also be very useful for understanding the mechanism of oxygen reduction to water by blue multicopper oxidases, which is not entirely known in all its details today. As the objective for our studies we have chosen the high redox potential fungal laccase form the basidiomycete *Trametes hirsuta*. The redox potential of the T1 site of this enzyme determined both by the mediated and direct redox titrations is equal to 780 mV vs. NHE [15, 17, 19]. The biochemical, spectral, and kinetic properties of this and other laccases have been previously intensively studied [19]. It is important to mention that different potentiometric studies of this enzyme, based on direct and mediated spectroelectrochemical redox titrations, were previously performed [17]. Moreover, the electrochemical behavior of this laccase on different carbon electrodes was also studied [15]. Thus, the goal of this work was to extend our electrochemical investigations of DET reactions of *Trametes hirsuta* laccase to bare and thiol-modified gold electrodes.

2. Experimental
2.1. Reagents and chemicals
H₂SO₄, Na₂HPO₄, KH₂PO₄, NaOH, KCl, and NaF were obtained from Merck (Darmstadt, Germany). Catechol, cysteamine, L-cysteine, and 4-aminophenol were from Sigma Chem. Comp. (St. Louis, MO, USA). Absolute ethanol (99.7%) was from Solveco Chemicals AB (Täby, Sweden). All chemicals were of analytical grade. Buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, Milford, CT, USA).

2.2. Enzyme
The basidiomycete *Trametes hirsuta* (Wulf. ex Fr.) Quél. (*Coriolus* (*Polyporus*) *hirsutus*), strain T. hirsuta 56 producing extracellular laccases, was obtained from the laboratory collection of the Moscow State University of Engineering Ecology (Moscow, Russia). Accounts of the production of laccase on a preparative scale and detailed biochemical characterization of the enzyme have been previously published [19]. Homogeneous preparations of the laccase were stored in 0.1 M phosphate buffer, pH 6.5, at −18 °C. The enzyme activity was measured spectrophotometrically using a spectrophotometer Uvikon 930 (Kontron Instrument, Everett, MA, USA) as described in [27].

2.3. Electrochemical measurements
2.3.1. Determination of the steady-state potentials of the laccase-modified electrodes
For the steady-state potential measurements an in-house made two electrode voltammeter (Lund University, Sweden) was used. The reference electrode was an Hg/Hg₂Cl₂/KCl_sat electrode (SCE, 242 mV vs. NHE), and the laccase-modified electrodes were used as the indicator electrodes. In all these experiments a one-compartment electrochemical cell (volume 10 ml) was used. The equilibrium potential values were registered under aerobic conditions.

2.3.2. Cyclic voltammetry
Electrochemical measurements were performed using a three-electrode potentiostat BAS CV-100W Electrochemical Analyzer with BAS CV-100W software v. 2.1. (Bioanalytical Systems, West Lafayette, IN, USA). The reference electrode was an Hg/Hg₂Cl₂/KCl_sat electrode (SCE, 242 mV vs. NHE), and the counter electrode was a bare gold electrode. The supporting electrolyte was a 100 mM phosphate buffer at pH 5.0. The cyclic voltammograms (CVs) were recorded in a broad potential range and with different scan rates. The cleaning
procedure of the working gold electrode included the following steps. The gold electrodes were first immersed in freshly prepared Piranha solution (3:1 H$_2$SO$_4$ 98%: H$_2$O$_2$ 25%) for 5 min. Next, the electrode surface was polished with DP-Suspension and with alumina FF slurry (0.25 µm and 0.1 µm, Stuers, Copenhagen, Denmark), rinsed with Millipore water, and sonicated between and after polishing for 10 min. Then the electrode was electrochemically treated in 0.5 M NaOH solution (100 mV/s scan rate; between -60 and -1360 mV vs. NHE), in 0.5 M H$_2$SO$_4$ (100 mV/sec; between 60 mV and 1840 mV vs. NHE), and finally in 100 mM EDTA in 0.1 M phosphate buffer at pH 5.0 for 30 min at a constant applied potential of +790 mV vs. NHE. The electrode was then kept in 0.5 M H$_2$SO$_4$ until use, when it was rinsed thoroughly with water. The gold counter electrode was cleaned by dipping it for 5 min into Piranha solution and then rinsed thoroughly with water.

2.3.3. Preparation of the electrodes

For physical adsorption of laccase on either a bare or a thiol modified gold electrode surface, the electrode was mounted with its surface facing up and 10 µL of the laccase solutions of different concentrations were placed on the gold surface. The electrode was covered to avoid evaporation, and the solution was let to react for 0.5 - 3 h. The thiol-modification was provided by submerging of the clean electrode into a thiol solution (10 mM of thiol in water or ethanol) for 24 h, followed by rinsing with Millipore water.

Covalent binding of laccase onto the electrode was accomplished through formation of a self-assembled monolayer of 4-aminophenol. First, gold electrodes were immersed into a 10 mM 4-aminophenol solution in absolute ethanol [12]. The self-assembled monolayer was let to assemble for at least 4 h. In parallel, 10 µL of the laccase solution (same concentrations as taken for physical adsorption) were transferred into 0.2 ml of a 10 mg/ml NaIO$_4$ solution in H$_2$O at pH 5.0 and allowed to react for 30 min (pH is given by the periodate salt), in order to oxidize the sugar residues on the enzyme surface. After that, the pH was raised to 7 by adding 100 mM Na$_2$HPO$_4$. The oxidized sugar residues were allowed to react with the primary amine functionality present in the self-assembled monolayer, forming a Schiff base, when immersing the water-rinsed thiol-modified electrode into the modified enzyme solution for 1 h. Afterwards, the electrodes were ready to be used.

3. Results and Discussion

The standard redox equilibrium potential of the O$_2$/H$_2$O couple, which is known to be +1229 mV vs. NHE, can be established only in a specially pure solution at specially pre-treated platinum [28]. Laccase, which is able to reduce molecular oxygen under mild conditions, when adsorbed (immobilized) on an electrode, can shift the electrode potential, if a bioelectrocatalytic reaction occurs [3], i.e., if a DET communication is established between the adsorbed laccase and the electrode. The shift in the value of the steady state potential depends on many factors, such as electrode material, source of the enzyme, solution pH, etc. [3, 15, 29]. The highest reported potential, i.e., 912 mV at pH 5.0, which is only 22 mV lower than the formal redox potential of the O$_2$/H$_2$O couple at pH 5.0, was obtained at a carbon-black electrode, which had been exposed for one day to a *Trametes versicolor* laccase containing solution. It should be emphasized that in the absence of oxygen or where no DET reaction is established between the enzyme and electrode, the steady-state potential of the electrode is far away from the equilibrium potential of the oxygen electrode or the redox potential of the adsorbed enzyme. Thus, one of the simplest methods for screening the possibility of bioelectrocatalytic reduction of oxygen by laccase is the measurement of the steady-state potential of laccase-modified electrodes in the presence of oxygen.

The steady state potentials of numerous bare and thiol-modified electrodes with adsorbed *Trametes hirsuta* laccase were measured in citrate-phosphate buffer, pH 3.0 under airsaturated conditions (Table 1). For all electrodes a pronounced difference between their steady-state potentials and the equilibrium potential of the oxygen electrode was obtained.
However, in the case of *Trametes hirsuta* laccase-modified spectrographic graphite electrode under the same conditions, for which well-pronounced bioelectroreduction of oxygen was previously shown, a very high steady-state potential could be obtained reaching values up to 910 mV [15]. The values of the steady-state potentials in Table 1 show that DET reaction between the enzyme and bare and thiol modified gold electrodes is not so efficient or the mechanism of this reaction is more complicated compared to the mechanism of DET between *Trametes hirsuta* laccase and spectrographic graphite electrodes. As can be seen from Table 1 only a few number of thiol-modified electrodes with adsorbed laccase exhibited higher potentials compared with that registered for bare gold electrode with adsorbed enzyme. Electrodes modified with the combination, laccase-4-aminothiophenol, yielded the highest value of the steady-state potential (Table 1), and was thus further investigated and the results were compared with the electrochemical behavior of *Trametes hirsuta* laccase adsorbed on bare gold electrodes.

Cyclic voltammograms of adsorbed *Trametes hirsuta* laccase on a bare gold electrode resulted in two well-pronounced Faradaic processes with a low and a high $E_m$, approximately at 500 mV and 900 mV vs. NHE. It should be emphasized that the electrochemical behavior of the enzyme strongly depends on many different factors, such as enzyme concentration, adsorption time, state of the electrode surface, and possibly many others, which are really difficult to control. For instance, at a high concentration of pure enzyme (18 mg/ml) and after a long adsorption time (approximately 1 h) at room temperature the CV exhibited only a well-pronounced low redox potential process ($E_m = 530$ mV) (Fig. 1A) with a peak separation ($\Delta E_p$) of 140 mV. Moreover, under certain conditions (9 mg/ml laccase; an adsorption time of approximately 3 h; 8°C) along with the well-pronounced low redox potential process ($E_m = 530$ mV; $\Delta E_p$ equal to 100 mV), an additional anodic peak at 840 mV could be found (Fig. 1B). In this case a low potential bioelectrocatalytic oxygen reduction process could be realized simultaneously with the disappearance of the high potential anodic peak. The results for another situation (18 mg/ml laccase; an adsorption time of approximately 3 h; room temperature) are observed in Fig. 2. Initially, both the low and the high potential processes are clearly visible and almost reversible ($\Delta E_p$ is less than 50 mV). However, the cathodic peaks were much less pronounced compared with their anodic counterparts. Upon addition of $F^-$, known to be a very strong inhibitor of laccase activity both in its dissolved and adsorbed states [2, 30], the electrochemical behavior of laccase (Fig. 2) in the absence of oxygen was strongly affected, whereas in another case, equivalent to what is presented in Fig. 1A, such an effect of $F^-$ was negligible (data not shown). Finally, $F^-$ did not inhibit the catalytic process of oxygen reduction, presented in Fig. 2B, whereas a relatively strong inhibition was found for the case presented in Fig. 1B (data not shown).

One of the simplest proposed mechanisms of laccase in homogeneous solution is presented in Fig. 5A. The substrates of the enzyme (e-donors) are oxidized close to the T1 site and then the electron is transferred via the amino-acid bridge (His-Cys-His) to the T2/T3 cluster, where molecular oxygen is reduced to water. However, possibly another different situation can be observed in the case when the enzyme is adsorbed on a bare gold electrode (Fig. 5B). Taking into account that a random orientation of laccase on bare gold is expected, one can suggest the presence of some enzyme molecules, which are not in direct electronic contact with the electrode (Fig. 5B, molecule in the middle of the electrode). Some molecules might be oriented by their T1 site to the electrode, and the natural electron transfer process, i.e., from the T1 site to the T2/T3, cluster might be obtained (Fig. 5B, molecule on the right side of the electrode). However, due to a partial denaturation of laccase on bare gold, the normal high potential process, which is clearly observed when the enzyme is adsorbed on graphite, could not be seen. Such a behavior of the enzyme possibly corresponds to the situation, presented in Fig. 1. A portion of the molecules can be oriented by their T2/T3 cluster and in this case both a low and a high potential processes, corresponding to the redox transformation of the T2 and T3 sites are observed (Fig. 5B, molecule on the left side of the electrode). Moreover, $F^-$,
which strongly bind to the T2/T3 cluster of the enzyme, drastically changes the electrochemistry of the adsorbed laccase, as shown in Fig. 2A. In the presence of the e⁻ acceptor of the enzyme, molecular oxygen, only the low redox potential bioelectrocatalytic process is observed, possibly due to the abnormal electron transfer pathway. In this case what occurs is the production of hydrogen peroxide instead of water, as was recently confirmed [31]. As mentioned above F⁻ is a well known and strong inhibitor of laccase, blocking the internal electron transfer pathway, presented in Fig. 5A. However, F⁻ did not affect the bioelectrocatalytic reduction of oxygen to hydrogen peroxide caused by laccase adsorbed on bare gold due to the different mechanism of this reaction compared with the natural function of the enzyme, when oxygen is reduced directly to water.

A completely different situation was observed for thiol-modified gold electrodes with adsorbed or covalently bound Trametes hirsuta laccase. For many thiols no electrochemical activity of the enzyme was found under anaerobic or under aerobic conditions when the enzyme was either adsorbed or covalently attached to the thiol layers. Reproducible and stable Faradaic processes were obtained only when 4-aminothiophenol was used for gold modification. As can be seen in Fig. 3A, two well-pronounced redox couples were obtained under anaerobic conditions. However, both processes are significantly shifted compared to the electrochemistry of the enzyme on bare gold. The two redox processes have Eₘ-values of about 380 mV and 800 mV, respectively, with ΔEₚ values of less than 30 mV. These two values are in good agreement with previously proposed values of the T1 and T2 sites of Trametes hirsuta laccase, which were determined to be 400 mV and 780 mV, respectively [15, 17, 19]. Moreover, addition of F⁻ did almost not affect the redox process with the Eₘ of 780 mV, only decreased the anodic peak current to some extent (Fig. 3A). Thus, one can suggest that this redox process corresponds to the electrochemical transformation of the T1 site of the enzyme, which is in DET contact with the gold surface, as presented in Fig. 4C (molecule on the right side of the electrode). In this case a high potential process of oxygen electroreduction should be obtained under aerobic conditions, as well as a clear inhibition effect of F⁻ on the oxygen bioelectroreduction reaction. Indeed, addition of F⁻ drastically decreased the current of bioelectroreduction of oxygen on the laccase-4-aminothiophenol-modified gold electrode (Fig. 3B, curve 2). In contrast, the low potential process of oxygen reduction, similar to that for bare gold with adsorbed laccase, could be still seen even in the presence of 8 mM F⁻ (Fig. 3B, curve 3). Moreover, addition of F⁻ affected the low potential electrochemical process (Fig. 3A) in the same manner as for the T2 site of the enzyme in homogeneous solution, e.g., the value of the redox potential increased approximately with 25 mV [17, 18]. Furthermore, an additional cathodic process at 230 mV appeared after binding of F⁻ to the enzyme (Fig. 3A). Thus, most likely the low potential process (Fig. 3A) corresponds to the redox transformation of the T2/T3 cluster, which is in this case in DET contact with the 4-aminothiophenol-modified gold electrode, as shown in Fig. 4C (molecule on the left side of the electrode). It should be noticed, however, that even if both schemes presented for the enzyme immobilized on bare and thiol-modified electrode, where the T2/T3 cluster is in DET contact with the electrodes (Fig. 4B and C, molecule on the left side of the electrodes), are identical, undoubtedly, significant differences in the electrochemical behavior of the enzyme were found in these two cases, which can be attributed to conformational changes of the enzyme adsorbed on bare gold electrodes compared with those at the 4-aminothiophenol modified electrodes. Moreover, two additional slopes on the curve of oxygen bioelectroreduction by Trametes hirsuta laccase immobilized on the 4-aminothiophenol electrode can be seen (Fig. 3B, arrows), possibly suggesting some other fractions of the enzyme on the self-assembled monolayer-gold, corresponding to a different orientation of the enzyme molecules and/or conformational changes during enzyme binding to the thiol-layer. However, it would be too speculative to present such cases in Fig. 4C. In order to clarify these additional studies of laccases from different sources would be needed, which are in the scope of further investigations in our laboratories. As was shown previously Trametes hirsuta
laccase contains 12% carbohydrates of the total mass of the protein [19], distributed around the enzyme globule, as was already shown for another high redox potential basidiomycete laccase; *Trametes versicolor* [32]. Thus, even on 4-aminothiophenol-modified gold electrode with the enzyme covalently attached, obviously different orientations of *Trametes hirsuta* laccase can be obtained. Moreover, when high potentials are applied, thiol layers are usually not stable and partial desorption of the enzyme might occur, which affects both the stability of the laccase-thiol-modified gold electrodes and, possibly, also the electrochemical behavior of the enzyme.

4. Conclusions
The direct electrochemistry of a high redox potential fungal laccase on bare and thiol-modified gold electrodes was found to be very complex. In both cases the steady-state potentials of *Trametes hirsuta* laccase modified electrodes were too far away from the redox potential of the equilibrium potential of the oxygen electrode. Two electrochemical processes, one with a low and one with a high potential, of the adsorbed enzyme were exhibited on CVs of bare and 4-aminothiophenol-modified gold electrodes. It is concluded that in the case of both types of electrodes only a small fraction of the bound laccase is oriented to the electrode through the T1 site of the enzyme, and some fraction of the enzyme is partly denatured on the bare gold surface. Another fraction of the enzyme molecules is oriented through the T2/T3 copper cluster, and bioelectroreduction of oxygen to hydrogen peroxide could be realized in this case. As for the thiol-modified electrode, denaturation of the enzyme is much less pronounced and bioelectroreduction of oxygen to both water and hydrogen peroxide was obtained. However, the pronounced problem for oxygen reduction at high potentials by laccases immobilized on self-assembled monolayers can be related to the instability of the thiol layers at potentials as high as that of the T1 site of the enzyme, and from which follows possibly an improper orientation of the laccase during covalent binding via the carbohydrate moieties of the enzyme.

5. Acknowledgement
The authors thank Dr. E. S. Gorshina for the cultivation of *Trametes hirsuta*. The work was supported by the Swedish Research Council, the Swedish Institute (SI), the Consejería de Educación de la Comunidad de Madrid, and the European Social Funding (F.S.E.). The SI is acknowledged for the support of a postdoctoral fellowship for S.S.
6. References

Table 1. Steady-state potentials of *Trametes hirsuta* laccase-modified gold electrodes (0.1 M citrate-phosphate buffer, pH 3.0)

<table>
<thead>
<tr>
<th>Number</th>
<th>Electrode modification</th>
<th>Potential, mV (vs. NHE)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>4-aminothiophenol</td>
<td>660</td>
</tr>
<tr>
<td>2</td>
<td>11-Mercapto-1-undecanoic acid</td>
<td>635</td>
</tr>
<tr>
<td>3</td>
<td>Cystamine</td>
<td>620</td>
</tr>
<tr>
<td>4</td>
<td>L-cisteine</td>
<td>610</td>
</tr>
<tr>
<td>5</td>
<td>Aldrithiol</td>
<td>580</td>
</tr>
<tr>
<td>6</td>
<td>Bare gold</td>
<td>575</td>
</tr>
<tr>
<td>7</td>
<td>11-Mercapto-1-undecanol</td>
<td>560</td>
</tr>
<tr>
<td>8</td>
<td>3,3'-Dithiodipropionic acid</td>
<td>555</td>
</tr>
<tr>
<td>9</td>
<td>3-Carboxypropil disulfide</td>
<td>550</td>
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<tr>
<td>10</td>
<td>3,3'-thiodipropionic acid</td>
<td>520</td>
</tr>
<tr>
<td>11</td>
<td>Mercaptopropionic acid</td>
<td>490</td>
</tr>
<tr>
<td>12</td>
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<tr>
<td>13</td>
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<tr>
<td>16</td>
<td>2-Mercaptoethanol</td>
<td>200</td>
</tr>
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</table>

Note: The standard deviations have been calculated from results of three independent experiments and were found to not exceed 10%.
Legends to figures

Figure 1. Cyclic voltammograms of *Trametes hirsuta* laccase immobilized on bare gold electrode (0.2 M citrate-phosphate buffer, pH 4.0).
(A) Scan rate - 100 mV/sec; start potential - +100 mV; second scan.
(B) Scan rate - 10 mV/sec; start potential - +1040 mV; second scan. 1 – anaerobic conditions; 2 – air saturated solution.

Figure 2. Cyclic voltammograms of *Trametes hirsuta* laccase immobilized on bare gold electrode (0.1 M phosphate buffer, pH 5.0).
(A) CVs under anaerobic conditions in presence or absence of sodium fluoride (scan rate - 250 mV/s, concentration of NaF - 8 mM, second scans). (B) CVs of the enzyme from *Trametes hirsuta* under aerobic and anaerobic conditions with and without NaF (start potential - +140 mV, scan rate - 10 mV/s; second scan).

Figure 3. Cyclic voltammograms of *Trametes hirsuta* laccase covalently attached to a 4-aminothiophenol-modified gold electrode (0.1 M phosphate buffer, pH 5.0).
(A) CVs of the thiol-modified electrode, after the enzyme attachment and in presence of NaF 8 mM (start potential - +1040 mV, scan rate - 10 mV/s; second scans). (B) CVs of the enzyme from *Trametes hirsuta* under aerobic or anaerobic conditions, and further NaF 8 mM addition, starting at +1040 mV potential (start potential - +1040 mV, scan rate - 10 mV/s; second scans).

Figure 4. Simple mechanisms of the homogeneous (catalysis in the solution) and heterogeneous (electrochemical reduction based on DET reactions) reduction of oxygen by fungal laccase.
Figure 1

A

Potential / mV vs. NHE

B

Potential / mV vs. NHE

Current / nA

Current / nA

1

2

A

B
Figure 2

A

Current / μA

Potential / mV vs. NHE

Anaerobic

NaF Anaerobic

B

Current / μA

Potential / mV vs. NHE

aerobic (1)

anaerobic (2)

NaF (3)

NaF anae (4)
Figure 3

A

![Graph A](image)

B

![Graph B](image)
Figure 4

- **A**
  - $S_{\text{red}}$ and $S_{\text{ox}}$
  - Copers
  - IET

- **B**
  - Laccase in solution
  - T1 copper
  - T2/T3 coppers

- **C**
  - Bare gold electrodes
  - 4-aminothiol-modified gold electrodes

- **T1**
- **T2/T3**

- **H**$_2$O
- **O**$_2$