1 Immobilization of *Rhus vernicifera* laccase on sepiolite; effect of chitosan and copper
2 modification on laccase adsorption and activity

3 Yaniv Olshansky^{a,b+}, Segula Masaphy^{c,d}, Robert A Root^b, Giora Rytwo^{a,e,*}

4 ^a Environmental Physical Chemistry Laboratory, MIGAL – Galilee Research Institute

5 ^b Department of Soil, Water and Environmental Science, University of Arizona, Tucson, USA

6 ^c Applied Microbiology and Mycology Department, MIGAL- Galilee Research Institute

7 ^d Dept. of Food Sciences, Tel Hai College, Upper Galilee

8 ^e Dept. of Environmental Sciences, Tel Hai College, Upper Galilee

9 +Current address

10 *Corresponding author: Giora Rytwo, Environmental Physical Chemistry Laboratory, MIGAL, POB 831 Kiryat
11 Shmona, +972-4-6953553, giorarytwo@gmail.com

12 **Abstract:** The enzyme laccase, a multi-copper oxidase found in many plants and fungi, can be
13 used in water treatment processes for the removal of pollutants. Commercially available laccase,
14 derived from *Rhus vernicifera*, was adsorbed on sepiolite, sepiolite modified with chitosan,
15 sepiolite plus Cu(II), and sepiolite modified with both chitosan and Cu(II) to investigate
16 enzymatic activity. Adsorption of laccase on unmodified sepiolite increased its activity by 250
17 ±40% compared to the non-adsorbed enzyme, whereas for sepiolite-Cu–chitosan and sepiolite–
18 chitosan the activity was enhanced by up to 700% and 500%, respectively. The stronger
19 enhancement for the Cu-containing adsorbent suggests that exchangeable Cu has an effect on the
20 adsorbed laccase. Desorption of the adsorbed laccase was less than 10%, and the non-desorbed
21 enzyme retained high activity, indicating robust adsorption. This study suggests that chitosan-
22 sepiolite based composites might be used as efficient support for laccase scaffolding and
23 immobilization, thus providing in an effective adsorbent surface for catalytic oxidation of
24 organic pollutants sensitive to laccase activity.

1. Introduction

Laccases are oxidase proteins with a globular structure and three Cu domains. In the T1 domain, Cu(II) is coordinated to two histidines and a cysteine (Durante et al., 2004; Giardina et al., 2010), and it oxidizes the reduced substrate via an outer-sphere mechanism. The redox potential for this complex ranges between 440 and 760 mV (Xu et al., 1996). The non-specific activity of laccases is not dependent on the presence of additional co-factors such as Mn(II) or H₂O₂, but the presence of Cu(II) is known to increase this activity (Rodríguez-Couto, 2015). Laccases are active on several organic compounds, and can be used in a broad range of industrial water-treatment processes, such as degradation of dyes or polyaromatic hydrocarbons (Chang et al., 2015; Lu et al., 2007). Immobilization of laccase on solid adsorbent is considered an essential step for its efficient use in water treatment (Fernández-Fernández et al., 2013). Such immobilization can be performed by: (1) entrapment or retention in a porous medium (Fernández-Fernández et al., 2013); (2) encapsulation in a semi-permeable matrix (Lu et al., 2007); (3) adsorption to a solid matrix (Wang et al., 2013). The latter approach was found to decrease laccase activity after adsorption on TiO₂ or surfactant-modified montmorillonite surfaces (Chang et al., 2015; Wang et al., 2013). In contrast, covalent binding of laccase to chitosan, using carbodiimide as a cross-linking agent, resulted in increased laccase activity (up to 260%) compared to free laccase (Cabana et al., 2011). A higher stability for thermal and protease degradation was observed for laccase immobilized on mineral surfaces, and the protein remained active for longer periods than free laccase (Fernández-Fernández et al., 2013). The present study evaluates the activity of laccase from *Rhus vernicifera* adsorbed on pristine or chitosan-modified sepiolite, with and without Cu(II) as the exchangeable cation.

2. Materials and Methods

2.1 Chemicals and reagents

Laccase (EC. 1.10.3.2) from *R. vernicifera* (#L2157, CAS Number 80498-15-3), medium molecular weight chitosan (#448877 CAS Number 9012-76-4, , 190-310 kDa, 75 to 85% deacetylated) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, #10102946001, CAS Number 30931-67-0) were obtained from Sigma Aldrich (Rehovot, Israel). Sepiolite from Vallecas-Vicálvaro clay deposits (Madrid, Spain) was kindly provided by TOLSA S.A. as a commercial product named Pangel® S9. The unit cell formula of the mineral is reported as $\text{Si}_{12}\text{O}_{30}\text{Mg}_8(\text{OH},\text{F})_4(\text{H}_2\text{O})_4 \cdot 8\text{H}_2\text{O}$ (Santaren et al., 1990), a cation exchange capacity (CEC) close to $0.15 \text{ mmole}_c \text{ g}^{-1}$ (Darder et al., 2006), a specific surface area of app, $340 \text{ m}^2 \text{ g}^{-1}$, a large micropore volume (around $0.44 \text{ cm}^3 \text{ g}^{-1}$) (Rytwo et al., 1998), and it contains >95% of pure sepiolite (Gómez-Avilés et al., 2013).. All other chemical were reagent grade or higher. All materials were used without additional purification.

2.2 Preparation of modified sepiolite

Cu saturated sepiolite (Cu-S9) prepared by sequential reacting S9 with 1N CuCl_2 . Excessive salts were removed by sequential washing with ultrapure water (Barnstead) until the supernatant was free of chlorides and the electrical conductivity (EC) was reduced to $<0.005 \text{ dS m}^{-1}$. Chitosan-S9 and Cu-S9 chitosan were prepared following Darder et al. (Darder et al., 2006), briefly dispersions of 1.5% (w/w) S9 and Cu-S9 were prepared in ultrapure water. Solution of chitosan (1.5% w/w) and acetic acid (1% w/w) added dropwise to continually stirred clay dispersion. The final S9 to chitosan ratio was 1:1 (w/w) and the dispersion stirred for additional 24 hours at room temperature. Excessive chitosan was removed by six sequential washing of the clay-polymer composites with ultrapure water. All modified S9 samples were freeze-dry before

further use. Total organic carbon (TOC) and total nitrogen (TN) were measured with N/C analyzer 2100S-AG (Analytik Jena, Germany). X-ray fluorescence (XRF) spectra of the dried powders was measured in an He atmosphere at 10, 20, 40 & 50 keV using a S2 Ranger Energy Dispersive XRF (Bruker GmBh, Karlsruhe, Germany). (. Semiquantitative evaluation of the XRF spectrum was performed using an adapted EQUA-ALL application in Spectra EDX v.2.4.2 software program (Bruker GmBh).

2.3 Laccase preparation and activity measurement

Laccase solution was prepared by dispersing 12 mg of dry laccase powder in 8 mL of phosphate buffer (10 mM, pH 7) solution, which was then homogenized and filtered through a 0.45- μ m membrane. Activity level (U mL^{-1}) was measured by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (Johannes and Majcherczyk, 2000), a method proven effective for the measurement of laccase activity (Solis-Oba et al., 2005). Briefly, 37.6 μ L of sample was added to 200 μ L of phosphate buffer in a Greiner flat-bottomed 96-well plate. Then a 12.5 μ L aliquot of 5 mM ABTS solution was added to all wells. Optical density (OD) was measured at 405 nm every min for 30 min using a plate reader, Infinite M200 Pro (TECAN, Switzerland). Samples were held at 30 $^{\circ}\text{C}$ and agitated for 5 second prior to each OD reading. The slope (OD min^{-1}) was calculated from the linear fit of light absorbance vs time, with 1 U laccase oxidizing 1 μ mol ABTS per min. Therefore, laccase activity was calculated using eq.1:

$$U = \frac{\Delta E \times V_t}{\varepsilon \times l \times V_s} \quad (1)$$

Where, ΔE is the change in OD with time (OD min^{-1}), V_t is the total volume (mL) of the solution in the well, ε is the extinction coefficient ($36.8 \text{ mL } \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$ for oxidized ABTS), and l and V_s are the light path length (cm) through the sample and its volume (mL), respectively.

2.4 Laccase adsorption

Adsorption of laccase to all S9 adsorbents was performed in duplicates batch experiments: aliquots of laccase solution was added to 1.5-mL tubes (Eppendorf) containing dispersion of a single adsorbent (e.g. Cu-S9). The adsorbent concentration was 1 g L^{-1} , and the final laccase concentration ranged from 1 to 45 U mL^{-1} . The tubes were agitated in the dark at 25°C for 24 h. Then aliquot of the dispersion removed for activity measurement. The remaining dispersion was centrifuged for 15 min at $7,000g$; aliquot of the adsorbent free supernatant removed for laccase activity measurement. Adsorbed enzyme activity determined using equation 2 and 3.

$$U_{\text{adsorbed-1}} = \frac{(U_i - U_f) \times V}{m} \quad (2)$$

Where $U_{\text{adsorbed-1}}$ is the adsorbed activity U mg^{-1} adsorbent, U_i is the activity of unreacted laccase solution (U mL^{-1} solution), U_f is the activity of reacted laccase solution (U mL^{-1} solution), V is the solution volume and m is the mass of added adsorbent.

$$U_{\text{adsorbed-2}} = \frac{(U_d - U_f) \times V}{m} \quad (3)$$

Where $U_{\text{adsorbed-2}}$ is the adsorbed activity U mg^{-1} adsorbent, U_d is the activity of laccase in the reacted dispersion (U mL^{-1} solution), U_f is the activity of reacted laccase solution (U mL^{-1} solution), V is the solution volume and m is the mass of added adsorbent.

Since adsorption of laccase may affect its activity, enhanced activity of laccase was calculated by the ratio between equation 3 and 2. A scheme with graphical details of the

evaluation process is available as *Additional Material*. Following the adsorption step, desorption of laccase from the adsorbent assessed by re-dispersing the precipitate in fresh buffer solution. Dispersion were further agitated and analyzed under the same conditions. One to three sequential desorption steps were performed.

3. Results and discussion

The abundance of each element in the XRF spectrum (Figure 1) was evaluated against a Si reference, where Si was assumed to remain constant upon cation exchange. The pure S9 had Mg/Si = 49.3%, and emission bands consistent with Fe (6.404 and 7.058 keV), but no Cu (8.048 and 8.905 keV). The spectrum of S9–chitosan (not shown) was almost identical to that of S9. For Cu–S9, the Cu emission band can be observed (Cu/Si = 1.8%), accompanied with a slight decrease in Mg amount (Mg/Si = 41.2%) which could be ascribed to the Cu–Mg exchange process. Considering S9 unit-cell formula and CEC (section 2.1), the Cu/Si ratio (1.8%) fits well to the expected value for homoionic Cu(II) S9 (1.74%). In the Cu–S9–chitosan composite similar levels of Mg were measured (Mg/Si = 40.8%), however. the amount of Cu decreased considerably (Cu/Si = 1.0%), effect that could be attributed to cationic chitosan exchanging Cu. TOC/TN analysis indicated chitosan load of 0.57 ± 0.03 g biopolymer g⁻¹ sepiolite for both S9–chitosan and Cu–S9–chitosan. Therefore, the amount of Mg exchanged with Cu on sepiolite surfaces did not affect the amount of chitosan adsorption.

Adsorption of laccase on all adsorbents (Figure 2) exhibited a linear partition-mode (C-type isotherm), suggesting that the applied concentrations were below saturation for all adsorbents. Chitosan modified adsorbent-exhibited higher adsorption than chitosan-free adsorbents. The mean laccase adsorption for chitosan-modified adsorbents ($65 \pm 7\%$ and $62 \pm 5\%$ for S9–chitosan and Cu–S9–chitosan, respectively) were significantly higher ($p < 0.05$ by

Kruskal-Wallis test (Mangiafico, 2016)) than the raw S9 and Cu-S9 ($52 \pm 2\%$ and $55 \pm 4\%$, respectively). The presence of a Cu surface did not affect the amount of laccase adsorption on either chitosan-modified or raw S9.

Laccase adsorption to all tested adsorbents resulted in enhanced activity (calculated as the ratio between equation 3 and 2) (Figure 3). This enhancement was stronger for S9-chitosan at lower laccase loadings, where activity enhanced by up to 700% and 500% for Cu-S9-chitosan and S9-chitosan, respectively. At higher laccase loadings, activity enhancement reduced to 350% and 200% for Cu-S9-chitosan and S9-chitosan, respectively. The stronger enhancement for the Cu-containing adsorbent suggests that exchangeable Cu has an effect on the adsorbed laccase. In chitosan free adsorbent, the activity of the adsorbed laccase increased by $250 \pm 40\%$ and $240 \pm 45\%$ for S9 and Cu-S9, respectively, and did not change with enzyme loading. Desorption of laccase was insignificant: in all adsorbents less than 10% of the adsorbed laccase desorbed even after three desorption steps (Figure 4), indicating robust stability of the laccase-adsorbent complexes in this experimental setup. The laccase that remained complexed with the adsorbent following desorption step showed higher activity than the adsorbent-free laccase solution at the same concentration. The enhanced activity was up to 580% for the S9-chitosan modified adsorbent and $130 \pm 50\%$ and $190 \pm 23\%$ for laccase complexed S9 and Cu-S9, respectively (Figure 5). Laccase-Cu-S9 complex retained enhanced activity of $140 \pm 30\%$ even after three desorption steps (Figure 5). Enhanced activity measured in the current study were comparable or exceeded previously reported values for laccase complexed to chitosan (Cabana et al., 2011).

Most laccase-complexes have an isoelectric point of $\text{pH} \sim 4$ (Baldrian, 2006; Viswanath et al., 2014), and indeed, some studies specify a negative surface charge (Li et al., 2015).

However, laccase from *R. vernicifera* is known to have an isoelectric point of pH 7.9 - 8.9 (Lu and Miyakoshi, 2012) and therefore at pH 7 would be positively charged (McCaig et al., 2005; Yang et al., 2006). Chitosan bound to S9 has been shown to form highly positively charged nanocomposites (Rytwo et al., 2013). Polymer chain cross-linkers have been used to covalently bond laccase and chitosan (Cabana et al., 2011). Although a cross-linker was not used in the present study, efficient *Rhus* laccase interactions were achieved with chitosan-S9, as reflected by the activity and the stability of the complex, suggesting that electrostatic interactions only might not be enough to explain the results, and probably van der Waals interactions might be also important. Thus, detailed characterization of laccase interactions with chitosan-sepiolite requires further research.

4. Conclusions

Minor desorption and enhanced activity resulting from the laccase-adsorbents suggest that a laccase- and chitosan-modified S9 complex can be stable for several cycles of reaction without losing substantial content of the enzyme to the reaction solution. The higher activity of adsorbed vs. free laccase has been attributed to conformational changes in the protein structure (Cabana et al., 2011). In the current study, higher activity of laccase adsorbed to chitosan-modified clays compared to bare clays may have resulted from higher flexibility of the S9-chitosan matrix and increased accessibility of the substrate. In addition, laccase adsorbed to Cu-containing clays exhibited higher activity than clays without Cu, indicating the involvement of Cu ions in the electron transfer between laccase and the substrate. Alternatively, it may imply different modes of laccase adsorption to the adsorbent that might result in conformational changes in the enzyme.

Previous studies have presented several applications for sepiolite–chitosan nanocomposites (Darder et al., 2006; Rytwo, 2012). This study shows that a sepiolite–chitosan composite can serve as an efficient support for laccase immobilization, and exchanging the surface cations with Cu can further increase the activity of the adsorbed enzyme. Additional research is needed to evaluate the stability of these complexes under various and specific water treatment scenarios including the details of the specific interactions. Embedding of laccase complexes within a porous polymeric matrix (Wicklein et al., 2013) or semi-permeable membrane, which would prevent mass losses of the complex from the system, or use in a sequential batch device (Rytwo et al., 2007) or recirculating filtering device (as used for photocatalysis in (Rytwo et al., 2015)) could result in an effective active adsorbent for the removal of lacase oxidative-activity sensitive chemicals.

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Figure Legends:

Figure 1. XRF spectra of sepiolite S9 (full line), Cu-S9 (dashed line) and Cu-S9-chitosan (dotted line) irradiated with 20 KeV Ag lamp.

Figure 2. Isotherms of laccase adsorbed on Cu-S9-chitosan (open squares), S9- chitosan (filled squares), Cu-S9 (open circles) and S9 (filled circles).

Figure 3. Activity enhancement of laccase adsorbed on Cu-S9-chitosan (open squares), S9- chitosan (filled squares), and Cu-S9 (open circles) and S9 (filled circles).

Figure 3. Activity enhancement of laccase adsorbed on Cu-S9-chitosan (open squares), S9- chitosan (filled squares), and Cu-S9 (open circles) and S9 (filled circles).

Figure 4. Adsorption (filled squares) and desorption (open circles, filled triangles and open diamonds for first second and third desorption steps respectively) isotherms of laccase.

Figure 5. Activity enhancement of laccase retained after first desorption step on Cu-S9-chitosan (open blue squares), S9-chitosan (filled squares), Cu-S9 (open circles), S9 (filled circles) and Cu-S9 after three desorption steps (filled rhombus).

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