Biotransformation of Natural Compounds by Free and Immobilized Fungal Laccase using Mediator Combinations in the LMS Reaction Model.

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Abstract:
The ternary systems constructed on the basis of isotropic properties of various compositions of a hydrocarbon and a short chain alcohol in a low water content were used to carry out the oxidation of Mastic oil, d-limonene and α-Pinene at 30 °C using fungal laccase from Trametes versicolor in the presence of different concentrations and combinations of three mediators, 2,2'-azino-bis 3-
ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole (HBT) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) in Laccase Mediator System (LMS) model. The biotransformation was conducted with the use of either free or immobilized laccase on organogels prepared by introducing appropriate amounts of Dioctyl Sulphosuccinate (AOT) microemulsions containing laccase to a second solution of polymer Hydroxy Propyl Methyl Cellulose (HPMC). The main oxidation product of \( \alpha \)-pinene was verbenone and verbenol, but low amounts of \( \alpha \)-pinene oxide was also detected. Similarly, \( d \)-limonene was oxidized to limonene epoxide, while mastic oil was oxidized to \( \alpha \)-pinene oxide, limonene epoxide, verbenol, verbenone. In all cases, some of the reaction products remained unidentified. The amounts of unidentified products were rather high especially in the case of \( d \)-limonene and crude mastic oil. Mediator Combinations with HBT in different ratio were used for further transformation optimization of the three natural compounds in the ternary systems. Ascorbic and Gallic acids in 40mM concentration also were used in combination with HBT to transform the natural compounds. All combinations revealed different level of oxidation and only one combination of Gallic acid and HBT was unable to oxidize any of three compounds. The natural compounds oxidation rate correlated with the mediator concentration in the reaction mixture, and clear synergetic effect were found when two mediators were present, 10mM ABTS and 80mM HBT, while the best triple combination was 10mM ABTS: 40mM HBT: 40mM TEMPO.

Introduction:

Laccases (EC 1.10.3.2), p-diphenol: dioxygen oxidoreductases) are multicopper proteins that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism. Fungal laccases have many, biological functions such as lignin degradation, removal of potentially toxic phenols, morphogenesis, pigment synthesis, sporulation, phytopathogenesis and fungal virulence. The enzyme uses molecular oxygen instead of hydrogen peroxide as electron accepter, and is a potentially attractive biocatalyst, while the other peroxidases require hydrogen peroxide. The poor solubility in water of most potential laccase substrates has raised interest in the behaviour of these oxidases in non-conventional reaction media with restricted water content. The addition of organic solvents affects various physical and chemical properties of the medium of enzymatic reaction, such as its hydrophobicity, dielectric constant, pH, varying the chemical potentials of all reactants present in the solutions, and the free energy of substrate binding by the enzyme, even when the observed maximum reaction rates remain practically not affected.[1, 2]

Enzyme catalysis in low water containing organic solvents is finding an increasing number of applications in diverse areas. Different strategies for obtaining higher activity and stability in organic media. Control of water activity...
and medium engineering are two crucial approaches in optimization of catalytic behavior in non aqueous enzymology. The greater understanding of enzyme behavior in non aqueous media is expected to lead to larger and even more diverse kinds of applications [3].

The laccase-mediator system (LMS) has yet to be applied on the process scale due to the cost of mediators and the lack of studies that guarantee the absence of toxic effects of these compounds or their derivatives. This model have undertaken a systematic investigation of several mediators towards non-phenolic lignin compounds. The mediators ABTS, HBT, and TEMPO (Figure 1), for various reasons, exhibited the most promising features as catalysts in this oxidation. Experimental determination of the electrochemical properties of mediators, and of their bond dissociation energies via semiempirical calculations, enabled us to rationalize the origin of their different mechanistic behavior. The mechanistic details of these processes are still a matter of conjecture but they are generally believed to involve one-electron oxidation of the mediator by the oxidized (cupric) form of the laccase, followed by reaction of the oxidized mediator with the substrate, either via electron transfer (ET), e.g., with ABTS, or via hydrogen atom transfer (HAT), e.g., with N-hydroxy compounds which form nitroxy radicals [4].

TEMPO and its derivatives form a unique case and are assumed to involve the formation of the corresponding oxoammonium cation via electron transfer to the copper(II) of laccase, i.e., via the Semmelhack mechanism proposed for CuCl/TEMPO. It is generally accepted that the substrate, in this case the mediator, undergoes one electron oxidation at the T1 copper site while reduction of oxygen to water occurs at the trinuclear T2/T3 site, with electrons being shuttled between the two sites. With N-hydroxy mediators, such as HBT, it is generally believed that laccase-catalyzed oxidations involve one electron oxidation of the mediator, followed by loss of a proton from the intermediate radical cation, to afford the corresponding nitroxy radical. This is followed by hydrogen abstraction from the alcohol substrate by the nitroxy radical [4, 5, 6].

![Figure 1. The role of HBT on laccase activity for the oxidation of α-pinene](image)

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The aim of this study is to investigate the biocatalytic biotransformation of natural compounds, such as $\alpha$-pinene, Mastic oil and $d$-limonene into biologically active oxidizing derivatives such as Verbenol, Verbenone, Pinene oxide and limonene epoxide using fungal laccase from *Trametes versicolor*, in the presence of small molecules capable to act as electron transfer mediators, like; ABTS, HBT and TEMPO. The three mediators were used in different concentrations and Combinations ratio in the micro aqueous surfactant less System with free laccase.

**Materials and Methods**

**Materials**

Gallic acid, 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole (HBT), laccase (EC 1.10.3.2) from the fungus *Trametes versicolor* (3.9 mg protein ml$^{-1}$) were purchased from Fluka Chemicals (Switzerland). 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) was obtained from Aldrich. Mastic oil from *Pistacia lentiscus* var. *chia* was a generous gift from Chios mastiha growers association. $\alpha$-pinene, $d$-limonene as well as other chemicals and organic solvents were of the highest purity commercially available.

**Methods**

**Ternary phase diagrams**

The ternary phase diagrams were constructed on the basis of isotropic properties of various compositions of a hydrocarbon ( $\alpha$-pinene, $d$-limonene or Mastic oil), a short chain alcohol (tert-butanol) and water at 30 °C as described elsewhere [7].

**Preparation of microemulsions and Organogels**

AOT-microemulsions were prepared by adding appropriate amounts of laccase from *Trametes versicolor* in acetate buffer (100 mM, pH 4.6) in a 100mM AOT in isooctane solution. The amount of the aqueous phase was adjusted so as to maintain the desired water content (Wo: 40).

Laccase-containing organogels were prepared by introducing appropriate amounts of AOT microemulsions containing laccase from *Trametes versicolor* to a second solution of polymer (HPMC) in water in a similar manner as described elsewhere [8]. In a typical experiment, 1 ml of the microemulsion (AOT-based) containing 6.24 mg of laccase from *Trametes versicolor* (2.5 mg protein or 140units enzyme) was mixed with 1.0 g HPMC and 2 ml of water at room temperature. The mixture was vigorously shaken and stirred until homogeneous (about 5–10 min). The gels were stored in a freezer (at −20 °C) until used.

**Biotransformation of natural compounds by laccases in Ternary Systems**
The ability of *Trametes versicolor* laccase to catalyze oxidations of hydrophobic substrates in surfactantless microemulsions as a ternary system, 16% natural compound 65.1% t-Butanol and 18.9% water) were investigated. In a typical reaction, the substrate (1000 mM) was added to vial containing the appropriate amounts of organic solvent and the aqueous phase (100 mM acetate buffer, pH 4.6) containing 10U ml⁻¹ as free enzyme. The reaction was initiated with the addition of a mediator (ABTS, HBT, and TEMPO) in different concentrations and combinations ratio and the reaction mixture was incubated at 25°C for 120 hours with 180 rpm shaking. Two Controls were performed in the absence of the enzyme or mediator. Duplicate Samples, each include 50 µL, were withdrawn periodically and analyzed by Gas Chromatography (GC).

**Gas Chromatography:**

Gas Chromatography (GC) was performed using a Beta 120 (Supelco, USA) fused silica capillary column 30m*0.25mm*0.25µm film thickness. The initial oven temperature was 100°C and the temperature was increased by 15°C/min to 180°C. Detection was made by Flame Identified Detector (FID).

**Results and Discussion:**

*T. versicolor* laccases in free or immobilized forms were tested in order to discover whether they could express their biocatalytic activity against natural compounds from the gum *Pistacia lentiscus* Var. *chia*. More specific α-pinene, d-limonene and mastic oil were oxidized, in the presence of small molecules capable to act as electron transfer mediators, into biologically active oxidizing derivatives. The oxidations were the effect of a two-step process, in which the enzyme first catalyzed the oxidation of primary substrate, the mediator, and then the oxidized mediator oxidized the secondary substrate, the alkene.

Laccase catalyzed oxidation of the terpenes only in the presence of the mediator. Neither laccase nor HBT alone was able to cause oxidation. The degree of conversion depended on the terpene used. The main oxidation products of α-pinene was verbenone, verbenol and low amounts of α-pinene oxide. Similarly, d-limonene was oxidized to limonene epoxide, while mastic oil was oxidized to α-pinene oxide, limonene epoxide, verbenol, verbenone. In all cases, some of the reaction products remained unidentified. The amounts of unidentified products were rather high especially in the case of d-limonene and crude mastic oil.

In the present work the influence of the amount of HBT on the oxidation of α-pinene by free laccase from *T. versicolor* in α-pinene based ternary system was investigated. The study was conducted within the range of 40 to 250 mM HBT, figure 2. In all cases the conversion of α-pinene to α-pinene oxide, verbenone and verbenol was succeed. We found that high concentrations of HBT had a positive effect on the enzymatic reaction. However, 40mM of HBT was enough to carry the reaction to completion and depended in most of experiments.
The mediators appeared to differ from each other in specificity towards a given alkene. More specific, ascorbic acid, Gallic acid, ABTS, TEMPO were as tested alone or in combination with HBT for the oxidation of α-pinene. All the mediators proved to be substrates for laccase (data not presented), but only a few combinations were able to oxidize α-pinene. Bourbonnais et al [9] found that the oxidation of HBT by laccase is very slow, more than 85 times slower than oxidation of ABTS (as measured by O2 uptake), even though, HBT mediation activity is as good as or even better than ABTS mediation activity. ABTS or TEMPO alone didn’t lead to any oxidative products comparing to HBT alone.

In stead of HBT, the effect of ascorbic acid, as a mediator and radical scavenger, on the reaction mechanism was examined. α-pinene oxide concentration as a product was increased in the presence of ascorbic acid, while low product concentration of verbenol and verbenone was observed comparing to HBT alone. On other hand, 40 mM Gallic acid alone or in combination with 10mM of ABTS or with 10mM of ABTS and 40mM of HBT to transform α-pinene in ternary system was tested. No products were observed within 48 hours of reaction.

The effect of the concentrations and combinations ratio of ABTS and HBT were investigated. Up to 40 mM ABTS and 80 mM HBT, oxidation of α-pinene was increased in parallel with the increasing of mediator concentrations. More than this concentration ratio, the concentration of products were decreased (Figure 3). Similar results were observed for the oxidation of athracene [10].
Figure 3. Effect of the concentration of ABTS and HBT on verbenol production

A positive effect on α-pinenes oxidation was observed when three mediators were studied. The combination of 10 mM ABTS, 40 mM HBT and 40mM TEMPO proved to be the best among other combinations. Generally, the oxidation of α-pinene correlated with the mediator concentration in the reaction mixture, and a clear synergetic effect was found when two or three mediators were present, HBT, TEMPO and ABTS. The mechanism of this synergistic interaction has yet to be elucidated. Similar results were observed with immobilized laccase on HPMC organogels. The enzyme was tested for its capability to oxidize α-pinene into its oxidizing derivatives with the use of single, double or triple mediators, figure 4.

Figure 4. Effect of the combination of HBT alone or in combination with ABTS and TEMPO on verbenone production by using laccase immobilized on HPMC.
Oxidation of Limonene by free or immobilized laccase on HPMC in the presence of different mediator combinations were tested. Combinations of the three mediators in different concentration were able to catalyze the oxidation of limonene (data not presented). We found that the best combination in this reaction mixture was also the triple combination of 10 mM ABTS, 40 mM HBT and 40mM TEMPO in both free and immobilizes laccase, figure 5.

![Graph showing concentration vs hours for free and immobilized laccase](image_url)

**Figure 5.** Production of limonene epoxide using free or immobilized laccase on HPMC, with 10 mM ABTS, 40 mM HBT and 40mM Tempo as mediators.

The effect of combinations ratio as well as the effect of mediator concentrations were investigated to transform mastic oil into different oxidative derivatives using free or immobilized laccase. The results revealed that, as in the case of α-pinene and limonene oxidations, higher yields of products were observed when two or three mediators (HBT, TEMPO, ABTS) presented in the reaction mixture. Triple combinations of 10 mM ABTS, 40 mM HBT and 40mM TEMPO proved to be the best in both free and immobilizes laccase in a ternary system consisted of mastic oil (16.0%), tert-butanol (65.9 %) and buffer (18.9%) , figure 6.
Figure 6. Oxidation of mastic oil into $\alpha$-pinene oxide, verbenol and verbenone using immobilized laccase on HPMC, with 10 mM ABTS, 40 mM HBT and 40mM Tempoas mediators.

**Acknowledgements**

I would like to give a unique thanks to Prof. Haralambos Stamatis from the university of Ioannina , Biological Applications and Technologies Department, for imparting to me a portion of his exceptional efforts to provide me a platform along my research. Thanks must go to my friends Katerina, Costa, Manolis and Aggliki for their enhancement and encouragement. Thank you so much I must send to the Ministry of National Education and Religious Affairs in Greece, especially Deli and Christina for seeing fit to award me and funding this research.

**References:**


Recived  .......................................................................................... (9/6/2010)
Accepted ................................................................. (24/8/2010)