

Laccase of *Lentinus edodes* Catalyzed Oxidation of Amines and Phenolic Compounds: A Semiempirical Quantum Chemical Consideration

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Based on the study by Leatham and Stahmann concerned with the rates (v) of amines and phenolic compounds oxidation catalyzed by laccase of basidiomycete *Lentinus edodes* (Berk.) Sing., as well as on the results of semiempirical quantum chemical computations using the PM3 method, the linear correlations of v and $\ln v$ values with first vertical ionization potentials of the substrates molecules and radicals derived from them, spin densities on N and O atoms of the above radicals, and with the radicals reorganization energies have been found.

Keywords: Amine, Laccase catalyzed oxidation rate, *Lentinus edodes*, Phenol, Quantum chemical prediction.

Introduction

It is important to identify the pathways by which the reaction rates of various substrates oxidation, catalyzed by enzymes, can be theoretically estimated. In searching for the quantitative correlations appropriate for the enzymatic activity predictions, the semiempirical quantum chemical methods seem promising.

To realize a comparison with results of quantum chemical computations, the reactive agents series (in which the experimental determinations were performed under the same conditions) must be selected. This requirement restricts to a great extent the choice of the systems enzyme-substrate under study.

In the present communication we considered 12 compounds of the amino and phenolic nature in air oxygen oxidation at pH 4.5, catalyzed by laccase from mature fruiting bodies of a commercially cultivated mushroom *Lentinus edodes* (according to the data presented in the fundamental work [Leatham and Stahmann, 1981]).

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is produced abundantly by certain fungi. It catalyzes the oxidation of *ortho* and *para* diphenols and diamino aromatic compounds by one-electron abstraction giving radicals. Amino and phenolic substances are oxidized *via* the aminyl and phenoxy radicals formation (Bourbonnais and Paice, 1990; Marzullo *et al.*, 1995).

Commercial cultivation of *L. edodes*, the shitake mushroom, is a major industry in many countries and is currently the only significant bioconversion process utilizing wood. Efficient colonization of growth substrates depends upon the capacity of the fungus to produce the extracellular enzymes required to degrade the major components of lignocellulose (Buswell *et al.*, 1995). Two classes of oxidative enzymes, i. e. laccases and ligninases, have received the greatest attention (Giardina *et al.*, 1996).

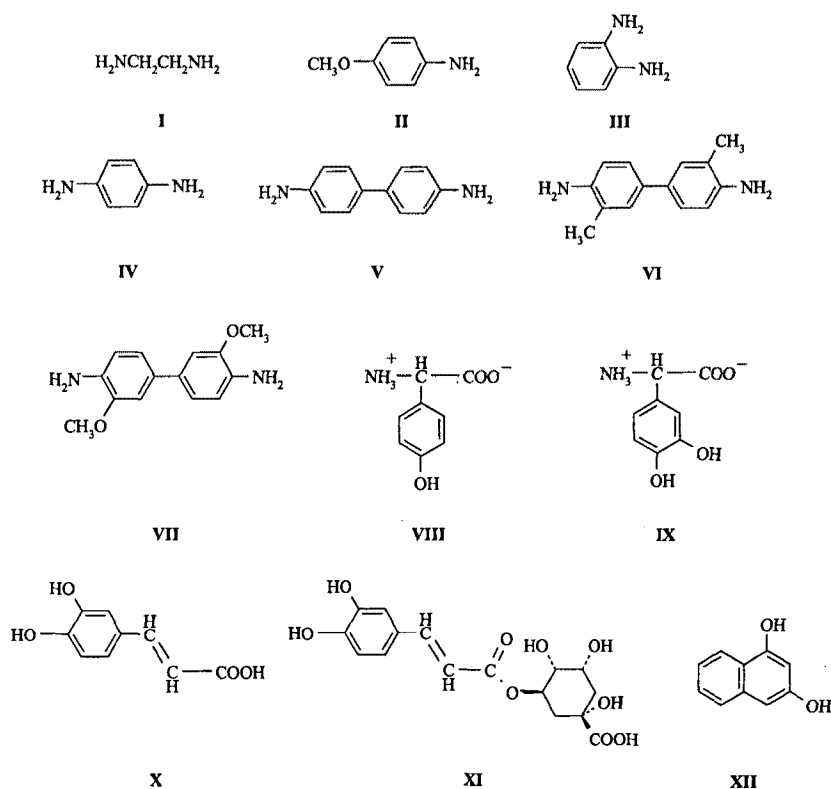
Laccase oxidative reactions on monomeric and polymeric phenolic compounds are involved in the pattern of medium degradation, in growth, and development. A better knowledge of the biochemistry of substrate degradation by *L. edodes* is needed in order to develop a better growth media and optimize cultivation techniques (Leatham, 1985). When *L. edodes* grows on lignocellulosic substrates, laccase has been shown to be the most abundant of phenol oxidases secreted. The relationship between biomass production and phenol oxidase activity in the shitake mushroom is being proven (Okeke *et al.*, 1994). The role of laccase has recently been reevaluated, since more information about its biodegradative mechanism has been investigated in several fungal species (Giardina *et al.*, 1996).

Extracellular phenoloxidase activity of basidiomycetes during mycelial cultures takes distinguishing courses according to species (Tanabe *et al.*, 1989). Thus, understanding the fungal corresponding degradative abilities and their theoretical backgrounds is important. Our investigation is aimed at establishing correlations of the substrates oxidation rates with the quantum chemical reactivity indices.

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Methods

The computations were performed using the semiempirical quantum chemical PM3 method (Stewart, 1989a; 1989b) and the software from the *MOPAC* package (Stewart 1983; Clark, 1985) with the complete geometry optimization.

For molecules with closed electronic shells, the computations were carried out within the restricted Hartree-Fock (RHF) formalism (Clark, 1985; Minkin *et al.*, 1997), i. e. the PM3-RHF approach was applied. For radicals, the unrestricted Hartree-Fock (UHF) method was used (PM3-UHF computations were performed). In calculating the rotational contributions to thermodynamic functions the symmetry number was taken to be 1.

Results and Discussion

The survey compounds (researched also in the work [Leatham and Stahmann, 1981]) were ethylenediamine (**I**), *p*-anisidine (**II**), *o*-phenylenediamine (**III**), *p*-phenylenediamine (**IV**), benzidine (**V**), 3,3'-dimethylbenzidine (**VI**), 3,3'-dimethoxybenzidine (**VII**), tyrosine (**VIII**), L-DOPA [3-(3,4-dihydroxyphenyl)-L-alanine] (**IX**), caffeic [*trans*-3,4-dihydroxycinnamic, *trans*-3-(3,4-dihydroxyphenyl)-2-propenoic] acid (**X**), chlorogenic {*trans*-3-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy] 1,4,5-trihydroxycyclohexanecarboxylic} acid (**XI**), 1,3-dihydroxynaphthalene (**XII**). Criterion of choosing the subjects under study has been substantiated in the *Introduction*. The said species comprise the series of compounds of similar structure, within which oxidation rate

measurements were carried out under adequate conditions. The substances **VIII** and **IX** were regarded in the zwitterionic form, since α -amino acids in aqueous solutions exist as internal salts (Gauptmann *et al.*, 1976).

The molecules under study are not too large, thus being appropriate for *ab initio* treatment. However, the results of the *ab initio* calculations performed at a low level of sophistication depend significantly, and not always monotonically, on the selected basis and consideration of correlation effects (Wong and Wiberg, 1992; Minkin *et al.*, 1997). Rigorous *ab initio* computations with wide basis sets are too expensive for moderate size molecules. For rather large systems perfect consideration, including complete geometry optimization, cannot be realized.

On the other hand, we established a correctness of the most important thermodynamic and molecular characteristics reproduction by the semiempirical MNDO, AM1 and PM3 methods (Pankratov and Shchavlev, 1997; Pankratov, 1998), as well as of electronegativity, inductive and mesomeric parameters of atomic groups (Pankratov and Shchavlev, 1998), in the series of organic compounds belonging to different classes and with various functional groups. Moreover, a semiempirical approach is preferential from the viewpoint of the oxidation rate evaluation simplicity.

In the present work, the electronic structures of the **I-XII** molecules, as well as aminyl and phenoxy radicals derived from them, were computed. In the case of the **IX-XI** systems, the radical centre is the oxygen atom in the *para* position of the aromatic ring, and for the substrate **XII** the oxygen atom is

in α -position of the naphthalene ring.

In principal, radical centers may occur both on the *para* and *meta* oxygen atoms of molecules IX-XI. However, it is known that the enzymatic oxidation of the phenolic species yields products of quinoid structure (Bourbonnais and Paice, 1990; Marzullo *et al.*, 1995). The latter can form only from the intermediates in which the *para* oxygen atom represents the radical centre. Such a case is also confirmed by a similar chemical behavior of the compounds IX-XI on one hand, and the substance VIII (the molecule of which contains no *meta* OH group) on the other hand. That is why we considered radicals formed by the *para* OH substituent.

We regarded the above mentioned radicals to be electroneutral ones, since just the latter form in the reactive system (Bourbonnais and Paice, 1990; Marzullo *et al.*, 1995). Cation radicals hypothetically appearing at the initial step of the oxidation reaction should be strong acids or even superacids (Holton and Murphy, 1979; Morkovnik and Okhlobystin, 1980; Todres, 1986; Albini *et al.*, 1994). A drastic shift of deprotonation equilibrium to the right is observed, in particular, for cation radicals containing mobile proton (Todres, 1986). This is also true for the cation radicals of amines and phenols. For example, in an aqueous solution at 22.5°C the cation radical of 2,4,6-triphenylphenol is featured by the pK_a value of -5 (Holton and Murphy, 1979). Thus, the cation radicals of the compounds I-XII, even occurring initially, should lose proton readily under the experimental conditions (pH 4.5).

For establishing correlations of reactivity with electronic structure, we calculated the following indices: first vertical ionization potentials (I), electron affinities (E), dipole moments of molecules I-XII and radicals derived from them, various characteristics of radicals, as spin densities on atoms (Pedersen *et al.*, 1973; Abronin and Zhidomirov, 1976; Boyd *et al.*, 1990; Pankratov *et al.*, 1993; Pankratov, 1994;

Pankratov and Stepanov, 1997), absolute electronegativities (Parr and Pearson, 1983; Pearson, 1989):

$$\chi = 0.5 (I + E),$$

absolute hardness (Parr and Pearson, 1983; Pearson, 1989):

$$\eta = 0.5 (I - E),$$

stabilization energies (differences of both heats and free energies of radicals and corresponding molecules formation), reorganization energies (E_r , differences between heats of formation for radicals with equilibrium geometry and geometry of initial molecules) (Khudyakov *et al.*, 1980; Leroy *et al.*, 1986; Denisov, 1987; Leroy and Sana, 1988; Luzhkov, 1989; Luzhkov and Yakushchenko, 1991; Lefort *et al.*, 1992) (Table 1).

Gibbs energies of formation (ΔG_f) were calculated from heats of formation (ΔH_f) and entropies (S):

$$\Delta G_f = \Delta H_f - T\Delta S_f,$$

where the standard entropies of formation ΔS_f were calculated by the formula:

$$\Delta S_f = S - \sum S_i,$$

in which S_i are the entropies of the elements constituting a molecule in their standard states (Stull *et al.*, 1969) in view of hydrogen, nitrogen, oxygen being the two-atomic molecules; $T = 298.15$ K.

The following has to serve as certain theoretical foundations of the linear dependences discussed below.

According to Arrhenius equation (Denisov, 1988) a rate constant of chemical reaction is presented as

Table 1. Computed dipole moments (μ , D), first vertical ionization potentials (I, eV), electron affinities (A, eV), absolute electronegativities (χ , eV), absolute hardness values (η , eV), stabilization energies ($\Delta\Delta H_f$ and $\Delta\Delta G_f$, kcal/mol), reorganization energies (E_r , kcal/mol), spin densities on radical centre N or O (ρ), and experimental oxidation rates [1] (v , $\Delta A \cdot \text{min}^{-1}$, where A is absorbance)*

Compound	μ_M	I_M	μ_R	I_R	A_R	χ_R	η_R	$\Delta\Delta H_f$	$\Delta\Delta G_f$	$-E_r$	ρ	V	Inv
I	2.16	9.39	1.37	9.52	-0.377	4.57	4.95	30.82	26.71	1.23	1.035	0.640	-0.446
II	1.59	8.32	1.76	8.75	-0.299	4.23	4.53	25.12	20.83	5.24	0.779	0.026	-3.65
III	2.54	8.23	1.39	8.32	-0.455	3.93	4.39	22.00	17.77	6.83	0.762	0.500	-0.693
IV	0.009	8.06	2.82	8.41	-0.430	3.99	4.42	24.83	20.59	5.39	0.771	0.706	-0.348
V	0.012	8.02	2.28	8.36	0.060	4.21	4.15	24.04	19.76	5.52	0.758	0.943	-0.0587
VI	2.34	7.97	2.87	8.30	-0.007	4.15	4.15	23.60	19.27	5.50	0.746	1.640	0.495
VII	2.25	7.95	2.88	8.22	0.055	4.14	4.08	23.83	19.30	5.96	0.724	2.836	1.04
VIII	10.2	9.38	7.66	9.63	0.479	5.05	4.57	25.78	20.94	12.45	0.424	0.027	-3.61
IX	9.13	9.13	8.56	9.56	0.429	4.99	4.57	16.77	13.81	17.97	0.406	0.075	-2.59
X	3.50	8.90	1.01	9.35	0.674	5.01	4.34	18.03	13.57	17.51	0.376	0.064	-2.75
XI	4.55	9.11	2.72	9.39	0.757	5.07	4.32	28.92	15.79	14.83	0.356	0.054	-2.92
XII	0.725	8.49	4.08	9.30	0.132	4.72	4.59	17.61	12.93	15.59	0.334	0.036	-3.32

*Subscript M is attributed to the initial substrates molecules, subscript R-to the radicals derived from them

$$k = B \exp(-E_a/RT),$$

where B is exponential multiplier, E_a is activation energy. On the other hand, provided that the principle of linear free energy relationships (LFER) (Shorter, 1983) is fulfilled, the activation energy must be proportional to the reaction free energy. The component of the latter is just the reorganization energy of the radical.

The LFER principle validity is a condition of the possible linearity of the dependences $\ln v$ vs I_M and $\ln v$ vs I_R , because the ionization potential has thermodynamic quantity. In general, the occurrence of linear correlations of this type could be assumed even out of the framework of the LFER principle, since the ionization potential could be discussed as an analog of the activation barrier on oxidation (in the process of which an electron moves away from the highest occupied molecular orbital in common with ionization). Also, a linear dependence of rate logarithm upon spin density should be expected as it takes place in a number of cases for charges on atoms and residual valency indices (Minkin *et al.*, 1997). Thus, possible linear relationships between $\ln v$ and quantum chemical reactivity indices can be explained in view of both the fundamental Arrhenius equation and the LFER principle. This implies linear interrelation of $\ln v$ with x . Within moderately small intervals of variables changes (as is the case in our work), the logarithmic function could be approximated by the linear one. Thus the dependences v vs x , like that of $\ln v$ vs x ($x = I_M, I_R, \rho_N, \rho_O, E_r$), would also be valid.

We have shown that oxidation rates (v), and $\ln v$ values for both aromatic amines and compounds of phenolic series under the uniform reproducible conditions (Leatham and Stahmann, 1981), correlate linearly to the spin densities on radical centre -nitrogen or oxygen atom, respectively. Under the same conditions the following dependences involving v and $\ln v$ are observed: for aromatic amines upon first ionization potentials of initial molecules and radicals, for phenolic substances upon the radicals reorganization energies (Table 2). The regression analysis was performed with the confidence level of 0.95. In all of the cases presented in Table 2, the correlation coefficient r is surprisingly high for biological experiments, even in two series for which the $|r|$ values difference from zero is statistically unnoticeable. The above quantities did not reach the critical values equal to 0.95 and 0.81, respectively, for the cases mentioned in Table 2.

For the dependences $v = a + b\rho_N$ and $\ln v = a + bI_R$ (aromatic amines), even the confidence limits about the intercept and the slope are rather narrow: $a = 38.84 \pm 11.65$, $b = -49.87 \pm 15.39$ in the first case, and in the second one $a = 74.26 \pm 9.01$, $b = -8.90 \pm 1.07$. Electronic structure-reactivity relationships could be expected for the series of compounds analogous in structure. The interrelations $v = a + bx$ and $\ln v = a + bx$, where $x = I_M, \rho_N$, are valid for all of the amino compounds studied, excluding **I**. For $x = I_R$, linear equations were obtained in the example of substances containing, in molecular aromatic fragment, one primary amino group or two NH_2 substituents

Table 2. Linear dependences of oxidation rates [1] (v , $\Delta A \cdot \text{min}^{-1}$) on first vertical ionization potentials of initial substrates molecules (I_M) and radicals from them (I_R), spin densities on nitrogen (ρ_N) and oxygen (ρ_O) atoms, reorganization energies (E_r) (r -correlation coefficient)

$v = a + bx$				
Compounds	x	a	b	r
II-VII	I_M	45.71	-5.51	-0.826
II, V-VII	I_R			
II-VII	ρ_N			
IX-XII	ρ_O			
VIII-XI	E_r			
$\ln v = a' + b'x$				
Compounds	x	a'	b'	r
II-VII	I_M	79.71	-9.92	-0.903
II, V-VII	I_R	74.26	-8.90	-0.999
II-VII	ρ_N	50.66	-67.66	-0.805*
IX-XII	ρ_O	-6.52	9.84	0.954
VIII-XI	E_r	-5.59	-0.167	-0.954

*The r value difference from zero is statistically unnoticeable

separated by a large number of chemical bonds.

We should note that the oxidation rate data for ethylenediamine (**I**), differing dramatically in its structure from the other substrates under consideration, are not included in any correlation equations obtained. To search for the quantitative regularities related to **I**, experimental and semiempirical studies on a series of substituted **I** compounds are required. When $x = \rho_O$, linearity occurs for the compounds with two hydroxy groups in aromatic ring. As for $x = E_r$, linear equations were obtained in respect to the molecules containing 1,2-dihydroxyphenyl fragment.

The restrictions mentioned on the substrates structure determine a set of compounds, concerning which the correlations we found could be expected to manifest their predictive power. As Table 2 shows, correlations, both with high and mediocre r -values, are observed for the same compounds. Thus, the r -value depends more on the variable x than on the chemical structure. Considering the relationships $\ln v$ vs x as the most substantiated ones, a poorer correlation coefficient r would be observed for the dependence $\ln v$ on ρ_N . This may be due to the fact that spin densities contain admixed components of higher multiplicities. At the same time, the validity of spin densities analysis as reactivity indices is well known (Pedersen *et al.*, 1973; Abronin and Zhidomirov, 1976; Boyd *et al.*, 1990; Pankratov *et al.*, 1993; Pankratov, 1994; Pankratov and Stepanov, 1997). In particular, homolytic (oxidative and reductive) coupling regioselectivity for 52 organic compounds and their derivatives correlates with spin densities on atoms in the corresponding intermediates-radicals, cation radicals or anion radicals (Pankratov *et al.*, 1993; Pankratov, 1994; Pankratov and Stepanov, 1997).

Let us consider the reasons for the appearance of the relationships obtained. The antipate quantitative relation of the oxidation rate with I_M and I_R is simple to explain: the easier the electron is withdrawn from the molecule or aminyl radical, the more readily they are oxidized. The oxidation rate of the phenolic substrate is higher for the more negative reorganization energy of radical. This could be explained by the essential contribution of reorganization energy to radical stabilization free energy. The greater the gain is in energy through reorganization, the lesser the Gibbs energy is in the reaction of phenoxyl radicals oxidative formation. This could be favourable to the redox process acceleration for two reasons. 1. A decrease in activation energy of radical formation from molecule following the LFER principle. 2. An increase in reverse reaction activation barrier. The tendencies for the oxidation rate change depending on the spin density on heteroatom (nitrogen or oxygen) are opposite for radicals derived from amines and hydroxy compounds. In the case of the aminyl radicals, the aromatic amines oxidation rate is higher for the lower spin density on nitrogen atom. The decrease in spin density on heteroatom means its delocalization over aromatic rings, that is a factor of radicals stabilization. On this point the character of dependences v vs ρ_N and $\ln v$ vs ρ_N for amines appears explicable analogously to the correlations v vs E_r and $\ln v$ vs E_r taking place for the phenolic species. For the phenoxyl radicals, the oxidation rate increases with the gain in spin density on the oxygen atom. Since the radicals mentioned are intermediates, spin density presents an important factor in determining the rates of their further transformations, and therefore the oxidation reaction rates as a whole.

Conclusion

The correlation equations $v = a + bx$ and $\ln v = a + bx$ found, where $x = I_M, I_R, \rho_N, \rho_O, E_r$, could be used for express facilitated (at semiempirical level) predicting v values, i. e. for the directive *a priori* selection of substrates (within the series of compounds similar in structure), for molecular design of substrates with the given behaviour on enzymatic oxidation (maximal or optimal oxidation rate, exclusive ability to be oxidized mediated by certain enzyme, etc.), along with the expert decisions working out in respect to promising substances synthesis. Thus sensitivity and selectivity of oxidation reactions would be ruled by the choice of substrates with the necessary properties.

The results may be of interest for mechanistic studies. Namely, directive choice of substrates lets one form a series of compounds for systematic investigations on enzyme catalysed oxidation mechanisms. Moreover, the existence of the linear correlations obtained confirms the formation of the aforementioned aminyl and phenoxyl radicals as reaction intermediates. The latter is an important peculiarity of the laccase catalysed oxidation mechanism. This interpretation of the interrelations obtained allows one to assume fine details in

the mechanisms of amines and phenolic species enzymatic oxidation.

The increase in the amines oxidation rate, with a decrease in the spin density on the nitrogen atom, attests (in view of the LFER principle) that the radical intermediates formation is included in the limiting steps of the reaction. On the other hand, the prominent dependence of the amines oxidation rate upon both I_M and I_R shows that the steps of intermediate transformations are also among the steps affecting the amines oxidation rate.

For phenolic compounds oxidation, symbate character of the behaviour of v and ρ_O indicates that the steps of further transformations of radical intermediates are involved in limiting the steps of the reaction. However, the character of the dependences v vs E_r and $\ln v$ vs E_r shows that the phenoxyl radicals formation also influences the oxidation rate.

Thus, the enzymatic oxidations of both amines and phenolics are featured by complicated mechanisms; the steps of radical intermediates formation and their further transformations affect the oxidation rate of the whole process.

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