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Transformation of Different Dyes by Extracellular Ligninases from *Phanerochaete chrysosporium*

Vpliv ekstracelularnih ligninaz glive *Phanerochaete chrysosporium* na barvila

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Summary

More than 50 structurally different dyes were tested for decolorization by extracellular ligninases from *Phanerochaete chrysosporium*. They can be divided into four main groups according to the type of decolorization. The influence of dyes structure on decolorization was not observed. The time course of spectral changes was followed spectrophotometrically and kinetic curves at specific wavelength were taken after that. The rate of decolorization for majority of dyes could be calculated. This value indicated which dyes could be used for ligninolytic activity measurements and how fast they could be biodegraded by ligninases.

Povzetek

Članek opisuje poskus razbarvanja vrste strukturno različnih barvil s pomočjo ekstracelularnih ligninaz glive *Phanerochaete chrysosporium*. Glede na način njihovega razbarvanja z ligninazami barvila lahko razvrstimo v štiri skupine. Pri tem nismo zaznali vpliva strukture barvil na način razbarvanja. Na osnovi zasledovanja časovnega spreminjanja absorpcijskih spektrov barvil se izbere ustrezna valovna dolžina za snemanje kinetičnih krivulj razbarvanja. Za precejšnje število barvil je bilo mogoče izračunati hitrost razbarvanja. Ta vrednost lahko služi pri izbiri ustreznega barvila za zasledovanje ligninolitične aktivnosti oz. za določitev hitrosti biorazgradnje barvil z ligninazami.

Introduction

The basidiomycetous fungus *Phanerochaete chrysosporium* catalyzes the oxidation of lignin. Lignin is a complex biopolymer composed of phenylpropanoid subunits linked with different bonds. The ability of the fungus to degrade these bonds gave an idea that it might be able to degrade other complex compounds as well. Among them are different dyes, which are released into the environment in effluents mainly from textile, paper printing and dyestuff industries. The majority of research was done on degradation of azo dyes, the largest of the groups used in industry with the greatest variety of colours (1). Under aerobic conditions, azo dyes are essentially nondegradable by bacteria (2). Under anaerobic conditions, however, the azo linkage can be reduced to form aromatic amines, which are colourless, but toxic and some of them carcinogenic (3). In mammals they can be oxidized to the corresponding N-hydroxy derivatives, capable of forming covalent linkages with DNA (3). Azo

dyes are so recalcitrant to oxidative biodegradation due to their azo group, which is not naturally occurring (4).

The ability of *Phanerochaete chrysosporium* for oxidative degradation is greater than of other known microorganisms. It was also shown that azo dyes are not only decolorized but also mineralized to CO₂ with this fungus (4).

The dyes decolorization could be observed during submersed cultivation of the fungus in a substrate with added dye or with extracellular fluid of ligninolytic culture. The experiments with three different azo dyes clearly showed decolorization dependence on fungal secondary metabolism (5). It was established that lignin degrading system of *Phanerochaete chrysosporium* is involved in dye degradation but some results suggest the involvement of enzymes other than lignin peroxidases (LiP) in the mineralization of some dyes (5, 6). Veratryl alcohol, a secondary metabolite of *Phanerochaete chryso-*

sporium is very important in degradation too. Its presence probably helps to complete the catalytic cycle of enzyme (7).

The aim of the present work was to investigate the action of LiP on dyes of most structural classes containing different functional groups to obtain some insight into dependence of LiP action on dyes structure. Some of these dyes may be useful for ligninolytic activity determination.

Experimental

Phanerochaete chrysosporium MZKIBK 223 (ATCC 24725) was grown under nitrogen limitation in 500 mL Erlenmeyer flasks on rotatory shaker (125 rpm). LiP activity (LA) was determined spectrophotometrically according to Tien and Kirk (8). One unit of ligninolytic activity (U) was defined as 1 μ mol of veratraldehyde formed during 1 min. Between days 5 to 8 of cultivation, LiP activity reached appropriate value for dye degradation experiments. Reaction mixture consisted of 2 mM veratryl alcohol (VA), 50 mM sodium tartarate (pH=3) and 1 mL of extracellular fluid. Dyes were added in such a concentration that initial absorbance was approximately 1.5. If dyes solubility was not sufficient, experiments were done with saturated solution in Na-tartarate buffer. Reaction was initiated by the addition of 1.08 mM H₂O₂. Decolorization was monitored spectrophotometrically. All dyes were divided into four main groups according to degradation. Wherever it was possible the rate of degradation was determined from kinetics data. The first derivative of kinetic measurement (dA/dt) gave us the initial rate of degradation. In order to compare the rate of dye decolorization at different ligninolytic activities the initial rate of decolorization was divided with ligninolytic activity of extracellular fluid ((dA/dt)/(LA)). This value served for determination of relative decolorization rate. A wide variety of commercially used dyes as well as indicators was tested on decolorization with ligninases.

Results

First group: Table 1 presents dyes, where absorbance decreases from the initial value to zero throughout the visible spectrum. The rate of degradation was measured

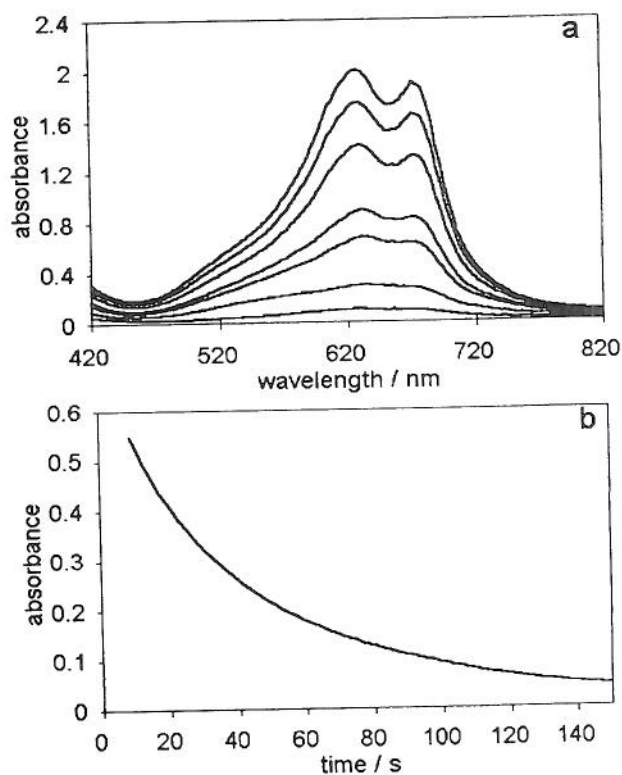


Fig. 1. a) Absorption spectra of the dye Ariabel blue (C.I. 74180). b) Kinetic curve of the dye decolorization at 620 nm; 3.3 mg/L of dye, LA = 240 U/L
Slika 1. a) Absorpcijski spektri barvila Ariabel blue (C.I. 74180). b) Kinetika razbarvanja barvila z ligninazami pri 620 nm; 3,3 mg/L barvila, LA = 240 U/L

Table 1. The group of dyes, where the rate of decolorization with ligninases was measured in absorption maximum
Tabela 1. Skupina barvil, kjer se hitrost oksidacije z ligninazami meri v absorpcijskem maksimumu

Dye name or colour index (C.I.) Ime barvila ali oznaka	Initial concentration Začetna koncentracija mg/L	Maximum wavelength Val. dolžina maksimuma nm	LiP activity Ligninolitična aktivnost U/L	Decolorization rate Hitrost razbarvanja s ⁻¹	Relative decolorization rate Relativna hitrost razbarvanja L/(U · s)
10316	16.6	428	520	0.022	4.23 · 10 ⁻⁶
11055	20.0	537	370	0.018	4.86 · 10 ⁻⁵
12719	33.0	382	600	0.09	1.50 · 10 ⁻⁴
16290	40.0	518	520	0.035	6.73 · 10 ⁻⁵
28440	30.0	574	670	0.032	4.80 · 10 ⁻⁵
29025	53.3	403	600	0.028	4.67 · 10 ⁻⁵
42053	8.3	624	370	0.034	9.20 · 10 ⁻⁵
45100	16.7	563	370	0.04	1.08 · 10 ⁻⁴
45380	3.0	520	410	0.0022	5.36 · 10 ⁻⁶
47005	23.0	412	1130	0.006	5.30 · 10 ⁻⁶
50240	100.0	518	370	0.031	8.40 · 10 ⁻⁴
61570	53.3	680	410	0.0058	1.41 · 10 ⁻⁵
61710	53.3	610	410	0.0052	1.27 · 10 ⁻⁵
62095	53.3	657	410	0.0048	1.17 · 10 ⁻⁵
74180	3.3	630	240	0.015	6.25 · 10 ⁻⁵
Basic red 14	16.7	513	520	0.01875	3.60 · 10 ⁻⁵

in the absorption maximum. Here belongs a subgroup of dyes where degradation proceeded too fast to take kinetic measurements (Table 2). Fig. 1 shows spectra of the dye Ariabel blue (C.I. 74180) (9) and kinetics of its decolorization.

Second group: Table 3 introduces dyes where a new transient peak was observed. The kinetic measurements of decolorization took place at the wavelength of the new peak maximum as well as at the wavelength of original peak maximum. Fig. 2b compares the kinetic of the dye Ariabel blue (C.I. 74220) original peak decolorization with the development of new peak formation and decolorization.

Third group: Some dyes underwent the hyperchromic effect during their decolorization with ligninases (9). The absorption peak wavelength has become (usually) lower. The kinetic measurements were recorded some nanometers higher than the original absorption maximum (Table 4) to diminish the influence of the hyperchromic effect. Fig. 3 presents the spectra of the dye Methylene blue · ZnCl₂ (C.I. 52015) and the kinetics of its decolorization.

Fourth group: The last group of dyes was very slowly or not at all decolorized, so the kinetics of decolorization could not be measured. Table 5 presents these dyes and

Fig. 4 presents the spectra of the dye Disperse blue 1 (C.I. 64500).

Discussion

Extracellular ligninases from *Phanerochaete chrysosporium* are very successful oxidants of different dyes. Their ability to decolorize different dyes has two main applications. First in several cases dyes are very useful indicators of lignin degrading ability. According to the

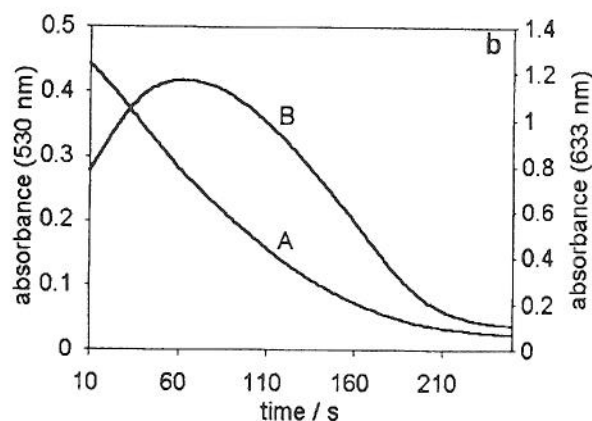
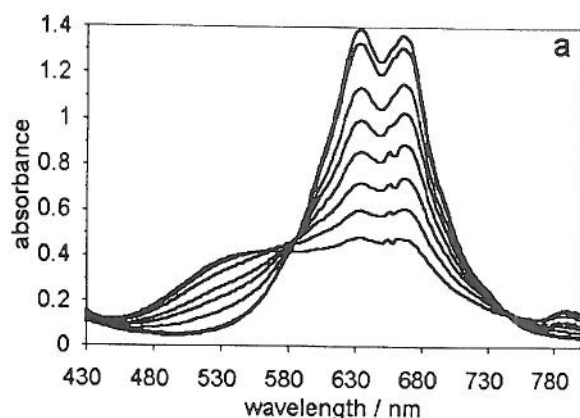


Fig. 2. a) Absorption spectra of the dye Ariabel blue (C.I. 74220). b) Kinetic curves of initial peak decolorization (A) at 633 nm and new peak formation (B) at 530 nm; 63.3 mg/L of dye, LA = 140 U/L

Slika 2. a) Absorpcijski spektri barvila Ariabel blue (C.I. 74220). b) Kinetika razbarvanja osnovnega vrha (A) pri 633 nm in kinetika tvorbe novega vrha (B) pri 530 nm; 63,3 mg/L barvila, LA = 140 U/L

Table 2. The group of dyes with immediate decolorization
Tabela 2. Skupina barvil, ki so se v hipu razbarvala

Dye colour index (C.I.) Oznaka barvila	Initial concentration Začetna koncentracija mg/L	Maximum wavelength Val. dolžina maksimuma nm	LiP activity Ligninolitična aktivnost U/L
11020	saturated	508	115
11210	33.3	520	590
12245	saturated	498	115
12251	saturated	452	150
14010	53.3	310	650
14700	53.3	620	360
16105	saturated	490	660
27755	167	585	670
27905	53.3	552	600
28240	53.3	522	300
34270	saturated	682	600
34280	53.3	623	600
44090	10	636	520
56059	1	619	150
62125	saturated	678	410
63000	saturated	660	520
73015	33.3	613	560

Table 3. The group of dyes with the new peak formation
Tabela 3. Skupina barvil, kjer je prišlo do tvorbe novega absorpcijskega vrha

Dye name or colour index (C.I.) Ime barvila ali oznaka	Initial concentration Začetna koncentracija mg/L	Maximum wavelength Val. dolžina maksimuma nm	Transient peak wavelength Val. dolžina prehodnega vrha nm	LiP activity Ligninolitična aktivnost U/L	Transient peak decolorization rate Hitrost razbarvanja prehodnega vrha s ⁻¹	Relative decolorization rate Relativna hitrost razbarvanja L/(U · s)
12250	133.0	489	388	240	0.0048	2.00 · 10 ⁻⁵
59040	1.0	403/370	445	240	0.0035	1.46 · 10 ⁻⁵
63010	100.0	600	564	420	0.01	2.38 · 10 ⁻⁵
74220	63.3	633	530	140	0.0025	1.78 · 10 ⁻⁴
Ethyl orange	saturated	495	463	300	0.01283	4.27 · 10 ⁻⁵

relative rate of decolorization $((dA/dt)/(LA))$ appropriate dyes for measurement of very low (high relative decolorization rates; for instance: C.I. 12719) as well as of very high ligninolytic activity (low relative reaction rates; for instance: C.I. 10316) can be found. It is known that the method with veratryl alcohol oxidation has a lot of deficiencies: absorbance at 310 nm interferes with absorbance of different aromatic compounds; at lower wavelengths the sensitivity to optical dispersion and turbidity is higher (10). That could be overcome with the monitoring of dye decolorization. In order to diminish the influence of dispersion and turbidity the use of dyes with

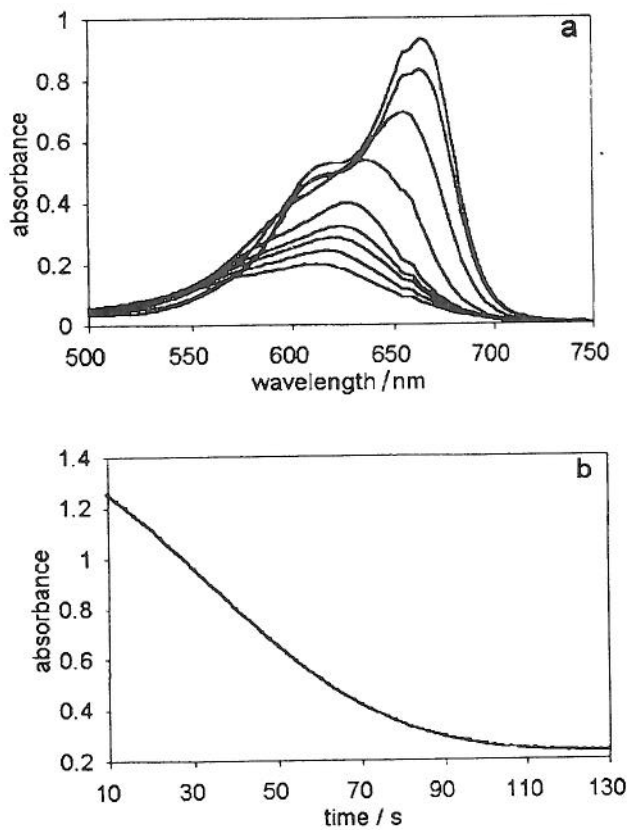


Fig. 3. a) Absorbance spectra of the dye Methylene blue (C.I. 52015). b) Kinetic curve of the dye decolorization at 679 nm; 4.5 mg/L of dye, LA = 190 U/L
Slika 3. a) Absorpcijski spektri barvila metilensko modro (C.I. 52015). b) Kinetična meritev razbarvanja barvila pri 679 nm; 4,5 mg/L barvila, LA = 190 U/L

Table 4. The group of dyes which undergo the hyperchromic effect
Tabela 4. Skupina barvil pri katerih prihaja do hiperkromnega efekta

Colour index (C.I.) Oznaka barvila	Initial concentration Začetna koncentracija mg/L	Maximum wavelength Val. dolžina maksimuma nm	Kinetic measurement wavelength Val. dolžina kinetične meritve nm	LiP activity Ligninolična aktivnost U/L	Decolorization rate Hitrost razbarvanja s^{-1}	Relative decolorization rate Relativna hitrost razbarvanja $L/(U \cdot s)$
11025	1.0	490	530	80	0.007	$8.75 \cdot 10^{-5}$
52010	4.0	647	655	264	0.0027	$1.02 \cdot 10^{-5}$
52015	4.5	664	679	190	0.0227	$1.19 \cdot 10^{-4}$
52030	4.5	632/594	650	264	0.0027	$1.02 \cdot 10^{-5}$

absorbance peaks at higher wavelengths, especially dyes of blue (C.I. 52015, C.I. 74220) or red (C.I. 11055, C.I. 45100) colour, is recommended.

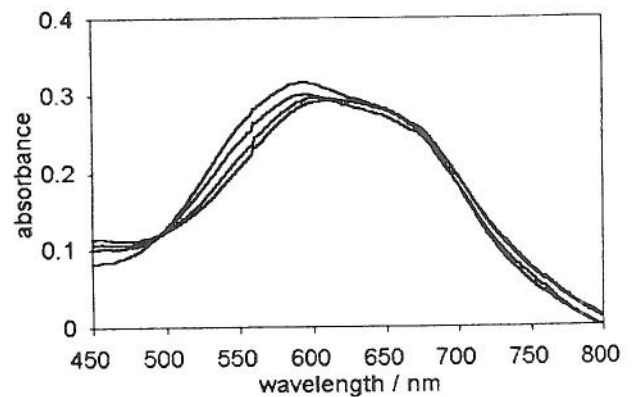


Fig. 4. Absorption spectra of the dye Disperse blue (C.I. 64500); 53.3 mg/L of dye, LA = 360 U/L
Slika 4. Absorpcijski spektri barvila Disperse blue (C.I. 64500); 53,3 mg/L barvila, LA = 360 U/L

Table 5. The group of dyes, where decolorization was very slow or negligible
Tabela 5. Skupina barvil, kjer je bilo razbarvanje zelo počasno ali zanemarljivo majhno

Dye name or colour index (C.I.) Ime barvila ali oznaka	Initial concentration Začetna koncentracija mg/L	Maximum wavelength Val. dolžina maksimuma nm	LiP activity Ligninolična aktivnost U/L
13058	saturated	570	650
13065	34.0	432	340
13080	1.0	432	150
17070	53.3	499	650
25135	53.3	370	600
26370	saturated	591	600
34140	saturated	580	600
45175	8.3	558	360
61105	53.3	583	590
61505	53.3	729	590
61512	saturated	565	166
62550	53.3	664	410
62500	53.3	604	590
64500	53.3	590	360
Reactive blue 160	63.0	620	520
Reactive red 120	33.0	537	520

Second, dyes are easily noticed in the environment and can be hazardous. Their biodegradation is thus of ecological importance. From the results in Tables 1 through 5 and Fig. 1 through 4 it can be concluded that a lot of dyes can be decolorized by ligninases and majority of them can be decolorized very fast at higher ligninolytic activities. It is very promising that the majority of tested dyes belonging to most structural classes could be effectively decolorized with *Phanerochaete chrysosporium* extracellular fluid.

Some of the tested dyes (Table 1) decolorize uniformly throughout the visible spectrum like Ariabel blue (C.I. 74180) shown in Fig. 1. Another group of dyes (Table 2) decolorizes almost immediately after coming in contact with LiP and H₂O₂. A limited number of dyes (Table 3) decolorize by forming a new and transient absorption band as e.g. Ariabel blue (C.I. 74220) shown in Fig. 2; a feature, to our knowledge, first observed by us. Further study of these dyes may allow new insight into decolorization mechanism of the dyes.

The hyperchromic effect of the azine dyes cited in Table 4 has been already observed (10). According to our observation an azo dye also undergoes this effect. Several dyes (Table 5) are decolorized by *Phanerochaete chrysosporium* culture filtrate very slowly or nearly not at all. Present data indicate no clear-cut relationship between the dyes structures and their ability to be degraded by LiP. On the one hand, in each structural class could be found dyes easily degradable by LiP as well as those degradable slowly, or nearly not at all. On the other hand, no dependence on dyes charge could be noticed, anionic as well as cationic dyes be found in Tables 1 to 5. The reasons are not clear at present and deserve further study.

Conclusions

Dyes of different structural classes can be completely decolorized with *Phanerochaete chrysosporium* extracellular fluid. The decolorization can proceed in any of the following three ways: absorbance may decrease uniformly throughout the visible spectrum, a new transient absorption peak may be formed, the hyperchromic effect may be observed. Dyes susceptibility to decolorization can be presented as relative initial decolorization rate. According to the value of the relative decolorization rate suitable dyes for very high as well as for very low ligninolytic activity measurements can be selected.

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