CHEMIJA. 2020. Vol. 31. No. 3. P. 178–190

Biosynthesis, purification, characterization and immobilization of laccase from *Lithothelium* sp.

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Novel fungal laccase isoenzymes (namely L95-1 and L95-2) produced by the Ascomycete *Lithothelium* sp. isolated from the forest soil were purified. However, only one of them was characterized, because the other isoenzyme lost its activity during purification. Extracellular L95-1 laccase was purified 30-fold using ion-exchange and hydrophobic interaction chromatography, with an overall yield of 88%. The molecular mass of purified L95-1 was estimated to be 85 kDa by SDS-PAGE analysis. L95-1 laccase was stable at temperature 4–22°C and pH 6.0–6.5. The substrate specificity of L95-1 laccase was examined with various compounds. Determined affinity constants (K_M) varied in a wide range of 3.7–2020.0 μ*M*, whereas catalytic efficiency constants (k_{cat}/K_M) covered a range of 0.008–1.9 $\mu M^{-1}\ s^{-1}$. The optimum pH for most substrates varied in a range from pH 5.0 to 6.0. Sodium azide and fluoride strongly inhibited L95-1 activity, whereas sulphate salts inhibited weakly.

The laccase was immobilized on the Fe_3O_4 nanoparticles and characterized. Residual activity remained at 20% after ten cycles of ABTS oxidation reaction. The immobilized laccase showed higher tolerance to various metal salts. The properties of L95-1 laccase make it potentially useful in the biotechnological applications.

Keywords: laccase, biosynthesis, *Lithothelium* sp., immobilization, magnetic nanoparticles

INTRODUCTION

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a family of copper-containing oxidases with important applications in bioremediation and other various industrial and biotechnological areas, such as textile, food, biofuel, organic synthesis, bioremediation, paper and pulp, pharmaceutical and cosmetic. Laccase-based biocatalysts fit well with development of industries that are efficient, sustainable and environment-friendly. Laccases were found in bacteria, fungi, insects and plants. Laccases display a wide substrate specificity, and oxidation is coupled with the reduction of an oxygen molecule to water. Although laccases act mainly towards phenolic compounds, such as mono-, di-, poly- and methoxy- phenols, aromatic and aliphatic amines, inorganic/organic metal compound, etc., their action spectrum is wide and the range of oxidizable substrates depends on the specific laccase used [1, 2]. Activity of laccase can be inhibited by several agents such as CN^-, F^- and fatty acids, which are able to bind to Cu^{2+} ions thus not proceeding the internal electron transfer, and Hg^{2+} ions that can induce protein conformations changes [1]. Therefore, newer and novel laccases attract a considerable

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attention due to its promising and valuable multiple applications in the biotechnological industry.

Although laccases are highly active catalysts, they are not always useful for biotechnological applications because of their low stability. This can be increased through the immobilization of the enzyme on solid supports [1, 3]. Enzyme-nanoparticles conjugates designed as industrial biocatalysts are poised to revolutionize many technological processes through ground-breaking new properties and applications. Iron is one of the most abundant metallic elements in living organisms and compared with many other nanoparticles, iron oxide nanoparticles are biologically tolerated. The most common biocompatible magnetic nanomaterials are pure iron oxides, such as maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) [4]. Especially in liquid-phase catalytic reactions, such small and magnetically separable particles may be useful as quasi-homogeneous systems that combine the advantages of high dispersion, high reactivity and easy separation [5, 6].

Laccases produced by the Ascomycetes, Basidiomycetes, and Deuteromycetes are playing a very important role in many biotechnological applications [7]. *Lithothelium* is a genus of fungi in the family Pyrenulaceae (lichenized Ascomycete). The Pyrenulaceae family is a widespread distribution [8]. However, information on purified from *Lithothelium* sp. laccase failed to find. For this reason, we presented a newly isolated laccase from a fungus *Lithothelium* sp. Laccase was purified, characterized and immobilized on the Fe_3O_4 nanoparticles. Laccase characteristic changes after immobilization on the Fe_3O_4 nanoparticles were investigated.

EXPERIMENTAL

Materials

Materials required for the synthesis of Fe_3O_4 nanoparticles: iron (III) chloride, ammonia (CarlRoth GmbH) and potassium borohydride (Fluka).

Substrate: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), promazine hydrochloride (PZ), syringaldazine (SYR) and vanillin (VAN) (Sigma-Aldrich, Switzerland). Methyl syringate (MS) as a product of Lancaster Synthesis was additionally recrystallized from ethanol. 2,6-Dimethoxyphenol (2,6-DMP) was obtained from Alfa Aesar, Germany. *N,N'*–Dimethylamine-4-(4-morpholine)benzene (AMB) was synthesized according to [9]. 3-(10*H*-Phenoxazin-10-yl)propanoic acid (PPA) was synthesized as described in [10]. Catechol (CAT), hydroquinone (HQ) and potassium hexacyanoferrate (11) (FERO) were obtained from Reachim, Russia. Catechin, quercetin, fisetin, gallic, syringic, synapic and chlorogenic acids were obtained from Sigma-Aldrich (Switzerland), p-coumaric, o-coumaric, m-coumaric, caffeic and ferulic acids were obtained from Fluka (Switzerland).

Metal salts: AlF_3, KBr, $AlCl_3$, $CaCl_2$, $CoCl_2$, $CuCl_2$, KCl, $MgCl_2$, $MnCl_2$, $NiCl_2$, NaOH, NaCl, KI, $AgNO_3$, KNO_3, $Co(NO_3)_2$, $CoSO_4$, $CuSO_4$, $Cu(CH_3COO)_2$ and CH_3COONa were obtained from Reachim, Russia; NaF were obtained from Merck, Germany; Li_2SO_4, $MgSO_4$, $MnSO_4$, Na_2SO_4, $ZnSO_4$, $Hg(CH_3COO)_2$, $Zn(CH_3COO)_2$ and NaN_3 were obtained from Sigma-Aldrich, Switzerland.

The solutions of the investigated substrates were prepared by weight and dissolved in triple distilled water (ABTS, FERO, HQ, CAT, vanillin) or ethanol (AMB, MS, CAA, PPA, PZ, DMP, SYR, catechin hydrate, quercetin, fisetin and ferulic, synapic, gallic, syringic, chlorogenic, p-coumaric, o-coumaric and m-coumaric acids). The final ethanol concentration in the reaction mixture did not exceed 1% (v/v).

Fungal strain. The mycelia of fungi taken from the forest soil (Lithuania) were diluted in sterile distilled water, the aliquots were spread on agar medium with ABTS (1 mM), and the plates were incubated at 20°C for five days. The culture plate was observed for colour change in the media. ABTS was used for screening of *Lithothelium* sp. for laccase production, and the front and the back morphology of the ABTS screening-green zone were formed. The laccases were screened and purified at the Department of Biochemistry and Molecular Biology of Life Sciences Center of Vilnius University, Lithuania.

Medium and culture conditions

The strain was kept on agar plates with 2% malt extract and 0.4% yeast extract.

The liquid pre-cultures were prepared by adding 1 cm^2 of fungal culture from the agar plates to 50 ml of the medium consisting of (g/l): malt extract – 3.5, yeast extract – 2.5, $MgSO_4$ – 0.5, glucose – 8.0, KH_2PO_4 – 2.0, pH 5.5–6.0. Pre-cultures were grown for 3 days at 30°C with agitation on a rotary shaker.

The production of extracellular laccase was carried out in the media, consisting of malt extract – 3.5 g/l, yeast extract – 2.5 g/l, KH_2PO_4 – 2.0–10.0 g/l, $MgSO_4$ – 0.5 g/l, $CuSO_4$ – 0.2–2.5 mM, pH 3.5–6.5.

Erlenmeyer flasks with 200 ml of the medium were inoculated with 8 ml of a liquid fungal suspension and cultivated for 4–5 days on the rotary shaker at 180 rpm and 22–30°C.

Enzyme assays

Purification of laccase isoenzymes. Culture flasks were harvested just after the activity of laccase had reached its production peak. Fungal mycelium was removed by centrifugation at 1500 × g or filtration. The laccase was purified using various chromatography methods.

CM sepharose 650 M (10 ml/l, TojoSoda, Japan) was added to the culture liquid with L95-1 laccase isoenzymes and was stirred for 4–5 h (or overnight). The sorbent was precipitated, washed with 5 mM potassium phosphate buffer, pH 6.0, poured to the column, and the adsorbed enzyme was eluted with the 0–0.5 M $(NH_4)_2SO_4$ gradient. Fractions with laccase activity were collected, supplemented with $(NH_4)_2SO_4$ up to 2.0 M and applied to the PHE FF (GE Healthcare) column equilibrated with the 5 mM potassium phosphate buffer, pH 6.0, with 2.0 M $(NH_4)_2SO_4$. Laccase was eluted with the 2.0–0 M $(NH_4)_2SO_4$ gradient. Fractions with laccase activity were collected, concentrated and dialyzed against the 2.5 mM potassium phosphate buffer, pH 6.0. The enzyme with the lowest hydrophobicity (L95-1) was eluted first. L95-2 was eluted at higher $(NH_4)_2SO_4$ concentration. The L95 isoenzymes were purified separately using ion-exchange chromatography. The Source Q15 (GE Healthcare) sorbent was precipitated, washed with the 5 mM potassium phosphate buffer, pH 6.0, poured to the column, and the adsorbed L95-1 isoenzyme was eluted with the 0–0.5 M $(NH_4)_2SO_4$ gradient. The isoenzyme L95-2 was purified analogously. Isoenzyme L95-1 was eluted when the ionic strength was 32 mM and L95-2 isoenzyme 114 mM. Both isoenzymes were concentrated and dialyzed against the 5.0 mM potassium phosphate buffer, pH 6.0, and were stored at –20°C.

Laccase activity assay. Activity of laccase during isolation and purification was determined spectrophotometrically using ABTS as the substrate. 1 ml of the reaction mixture consisted of 0.975 ml 100 mM sodium citrate buffer, pH 3.0, 20 µl 10 mM ABTS and 5 µl of laccase. ABTS oxidation was monitored by the increase of absorbance at 420 nm (ε = 36 000 M^{-1} cm^{-1}) at 30°C. One unit of enzyme was defined as an amount of enzyme that oxidized 1 µmol of ABTS per min. Protein concentration was determined by the method of Lowry using bovine serum albumin as standard [11].

Determination of molecular mass. The molecular mass of the purified laccase was estimated by SDS-PAGE analysis. Electrophoresis was performed in 14% polyacrylamide gels by using molecular mass standards (170, 130, 100, 70, 55, 40, 35, 25, 15, 10 kDa). Protein bands after SDS-PAGE were stained with Coomassie blue R-250 and the molecular masses were determined from the calibration graph.

Kinetic measurements and calculations

Oxidation of the selected substrates was monitored spectrophotometrically by using a computer controlled Nicolet evolution 300 spectrophotometer (Thermo Electron Corporation, USA) in the 50 mM acetate buffer solution, pH 5.5, at 25°C. The kinetic curves were recorded at the wavelength corresponding to the maximum of absorbance. The concentration of the oxidized substrates was calculated using the molar absorption coefficients: ε_{514} = 8.9 mM^{-1} cm^{-1} for the radical cations of phenothiazine (PZ) and ε_{530} = 16 mM^{-1} cm^{-1} for phenoxazine derivatives (PPA) [12], ε_{414} = 36 mM^{-1} cm^{-1} for ABTS [13], ε_{420} = 1.02 mM^{-1} cm^{-1} for hexacyanoferrate (II) (FERO) [14], ε_{530} = 65 mM^{-1} cm^{-1} for SYR [15], ε_{604} = 13.1 mM^{-1} cm^{-1} for AMB [16], ε_{410} = 0.74 mM^{-1} cm^{-1} for CAT [17], ε_{248} = 17.5 mM^{-1} cm^{-1} for HQ [18] and ε_{468} = 49.6 mM^{-1} cm^{-1} for DMP [19]. The value of the molar absorption coefficient of the oxidation product of caffeic acid (ε_{310} = 9.5 mM^{-1} cm^{-1}), chlorogenic acid (ε_{325} = 14.1 mM^{-1} cm^{-1}), p-coumaric acid (ε_{285} = 13.7 mM^{-1} cm^{-1}), ferulic acid (ε_{310} = 13.2 mM^{-1} cm^{-1}), gallic acid (ε_{310} = 2.7 mM^{-1} cm^{-1}), syringic acid (ε_{290} = 5.6 mM^{-1} cm^{-1}), MS (ε_{280} = 9.8 mM^{-1} cm^{-1}), vanillin (ε_{350} = 1.1 mM^{-1} cm^{-1}), quercetin (ε_{370} = 15.2 mM^{-1} cm^{-1}), fisetin 360 nm (ε_{360} = 15.6 mM^{-1} cm^{-1}) and catechin hydrate (ε_{440} = 5.6 mM^{-1} cm^{-1}) was determined experimentally.

The initial rate (V) of substrate oxidation was calculated by curve fitting. In the case of exponential

function $V = k \cdot C_0$, k is the first order reaction constant and C_0 is the initial concentration of a substrate. For the linear dependence the initial rate was calculated as a slope. To analyse the dependence of V on the substrate concentration and determine the apparent kinetic parameters V_{max} and K_M of the reactions, the Michaelis–Menten equation was used. Catalytic constant (k_{cat}) was calculated as ratio V_{max} and the total enzyme concentration ([E]): $k_{cat} = V_{max}/[E]$, catalytic efficiency constant was calculated as a ratio of k_{cat} and K_M:

Catalytic efficiency = k_{cat}/K_M.

Effect of pH, temperature and salts

The pH and temperature stability

For determination of pH storage stability, samples of enzymes were 10-fold diluted with 200 mM potassium phosphate buffers with various pH and mixed in tubes. The tubes were closed and stored for 24 h at temperature of 4, 22 and 30°C. After 24 h, the residual activity was determined as described above with 100 μM ABTS. The activity measured immediately after mixing equates to 100%.

For determination of the temperature storage stability of the diluted enzyme solution (samples of enzymes were 500-fold diluted with water), the samples of diluted enzymes were mixed in the tubes. The tubes were closed and stored for 1 month at different temperatures. The residual activity was determined as described above with 100 μM ABTS. The activity measured immediately after mixing equates to 100%.

The optimum pH of laccases with different substrates was measured with a spectrophotometer in a 1 cm quartz cuvette at 25°C using the universal 60 mM Britton-Robinson buffer [20] in a pH range from 3.0 to 9.0 and with different substrates. The highest activity was equated to 100%.

The effect of metal salts on activity of laccases was investigated spectrophotometrically using 100 μM of ABTS as substrate, 50 mM sodium acetate buffer solution, pH 5.5, and 0.1–100 mM different salts, at 25°C. The activity of enzyme without added salt was equated to 100%.

Synthesis of Fe_3O_4 magnetic nanoparticles

The magnetic Fe_3O_4 nanoparticles were synthesized according to the method described previously [21]. Specifically, 5 g of $FeCl_3 \cdot 6H_2O$ was dissolved in 400 ml of deionized water under vigorous stirring at room temperature. Subsequently, 1.43 g of KBH_4 was dissolved in 50 ml of the ammonia (3.5%) solution and quickly added to the aqueous Fe(III) solution. Soon after the occurrence of reduction reaction (the solution turning black), the temperature of the solution was increased to 100°C and was refluxed for 2 h under vigorous stirring conditions. Then the solution was cooled to room temperature and aged in water for 24 h. The resulting reaction product was separated by applying an external magnetic field and subsequently washed 5 times by deionized water and dried.

Immobilization of L95-1 on the Fe_3O_4 nanoparticles

The immobilization was carried out by physical adsorption. For enzyme adsorption, 10 mg of Fe_3O_4 nanoparticles were sonicated and mixed with the enzyme solution (in volume ratio 1:1), and then the mixture was suspended and kept for 10 min at room temperature. The immobilized L951-1 was separated by a magnetic field. The immobilization yield (IY) was calculated according to the following formula [22]

$$IY(\%) = \frac{P}{P_o} \cdot 100\%,$$

wherein P was the amount of enzyme immobilized and P_0 was the amount of initial enzyme.

The effectiveness factor (EF) was calculated according to the following formula [23]:

$$\text{Effectiveness factor} = \frac{V_{\max(\text{immobilized enzyme})}}{V_{\max(\text{free enzyme})}}.$$

Reusability of laccase from the magnetic nanoparticles was investigated by separating the Fe_3O_4 nanoparticles with adsorbed laccase and resuspending them in double-distilled water (DD) water or buffer solution several times and activity of the enzyme was determined as described above. The procedure was repeated for 10 consecutive cycles.

RESULTS AND DISCUSSION

Laccase biosynthesis

Lithothelium sp. fungal produces two laccase isoenzymes under the same conditions. Both laccase isoenzymes were purified, but it was observed that one of the isoenzymes was very unstable and rapidly

loses enzymatic activity. Therefore, one of the isoenzymes was investigated in the study. The laccase biosynthesis strongly depended on the cultivation media content. Laccase activity growth was observed for 4 days, and the 5th-day activity was reduced.

The maximum activity of laccase was observed after 4 days when the Cu^{2+} (inducer) concentration was 2–2.5 mM (Table 1). It was observed that the pH in the culture medium shifts to the alkaline pH region during cultivation in the presence of an insufficient concentration of potassium phosphate. Therefore, the effect of potassium phosphate in the enzyme biosynthesis has been studied (Table 2). By cultivation of the laccase, the maximum enzymatic activity was obtained when the inducer concentration was 2 mM, and, at the same time, the concentration of potassium salts was 28 mM in the cultivation media at pH 6.0, 22°C.

Laccase purification

The purification of laccase isoenzymes was carried out in three stages: ion-exchange (SuperQ 650M sorbent), hydrophobic interaction (Phenyl FF sorbent) and ion-exchange (Source Q15 sorbent) chromatography (results in Table 3).

After the first purification phase using ion-exchange chromatography, the specific enzyme activity increased from 1.5 to 3.9 U/mg, the protein content decreased to 253 mg, the purification fold was 2.6 and the yield was 100%. After hydrophobic interaction chromatography, the specific activity of L95-1 isoenzyme was 16 U/mg and that of

Table 1. **The dependence of biosynthesis efficiency on the Cu^{2+} concentration at different pH of cultivation media. Conditions: KH_2PO_4 – 14.0 mM, 30.0°C**

pH / Cu^{2+}	5.5				6.0				6.5			
	4 days		5 days		4 days		5 days		4 days		5 days	
	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*
0.75 mM	0.21	6.9	0.10	7.0	0.34	7.1	0.14	7.2	0.08	7.3	0.03	7.4
1.0 mM	0.04	6.8	0.12	7.0	0.08	6.9	0.08	7.1	0.01	7.1	0.04	7.2
1.5 mM	0.20	6.7	0.11	6.9	0.06	6.9	0.07	6.7	0.08	7.3	0.05	7.2
2.0 mM	0.13	6.7	0.18	6.8	0.21	6.8	0.12	6.9	0.18	7.1	0.07	7.2
2.5 mM	0.14	6.5	0.17	6.8	0.20	6.8	0.12	7.0	0.31	7.0	0.13	7.1

* pH is pH of cultivation media during biosynthesis.

Table 2. **The dependence of biosynthesis efficiency on Cu^{2+} and KH_2PO_4 concentrations. Conditions: pH of cultivation media 6.0, 24.0°C**

Cu^{2+} / KH_2PO_4	0.5 mM		1.0 mM		2.0 mM		2.5 mM	
	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*	A, U/ml	pH*
14.0 mM	0.09	7.2	0.19	6.7	0.19	7.1	0.14	6.8
35.0 mM	0.07	6.7	0.10	6.7	0.17	6.5	0.16	6.5
70.0 mM	0.03	6.5	0.05	6.4	0.07	6.4	0.08	6.3

* pH is pH of cultivation media during biosynthesis.

Table 3. **The purification profile of laccase isoenzymes produced by *Lithothelium* sp.**

Purification steps	Isoenzyme	Volume, ml	Total enzyme activity, U	Total protein content, mg	Specific activity, U/mg	Purification fold	Yield, %
Culture filtrate (crude extract)	L95	2550	998	688	1.5	1.0	100
Ion-exchange chromatography		36	995	253	3.9	2.6	100
Hydrophobic interaction chromatography	L95-1	8	888	55	16	11	89
	L95-2	3	108	53	2	1.3	11
Ion-exchange chromatography	L95-1	5	880	20	44	30	88
	L95-2	3	16	13	1.2	0.8	1.6

isoenzyme L95-2 was 2 U/mg. The isoenzyme L95-1 purification fold was 11 and the yield of laccase 89%, while the purification fold of isoenzyme L95-2 was 1.3 and the yield was only 11%. After the last purification phase, the yield of L95-1 was 88% and that of L95-2 was 1.6%.

The molecular mass of laccase L95-1 determined by the SDS-PAGE method is 85 kDa.

pH and temperature storage stability of laccase

The storage conditions of laccase (i.e. pH and temperature) are very important factors effecting the stability of the enzyme activity as they affect the active site of the laccase, thus effecting the binding to amino acids [24]. Therefore, the effect of pH on enzyme storage stability was also investigated (Fig. 1). In this study, L95-1 laccase was stable in a pH range from 6.0 to 6.5 when the temperature ranged from 4 to 22°C. The activity of laccase was almost undetectable at 30°C when the pH was 7.5.

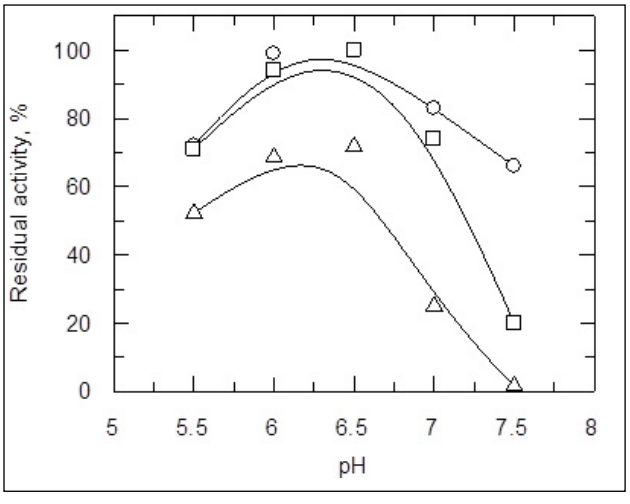

Fig. 1. Analysis of the optimal storage pH of L95-1 at different temperatures: 4°C (○), 22°C (□), 30°C (△). The activity measured immediately after mixing equated to 100%

Also, testing of the storage temperature of pure and diluted until working concentrations L95-1 at different temperatures showed that enzyme remained stable after 12 days at 4°C temperature (residual activity up to 80%) and the activity of enzyme decreased to 30% on day 28 (Fig. 2).

The laccase remained stable at –20°C temperature when the enzyme solution was rewarmed and frozen (the residual activity of approximately 90%), but the activity decreased to 40% after 28 days.

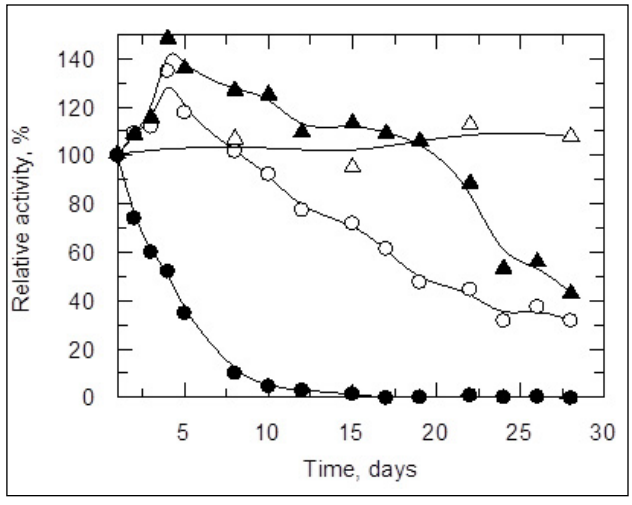

Fig. 2. Diluted L95-1 laccase stability at different temperatures: 4°C (○); room temperature (●); –20°C, rewarmed just before measurement (△); –20°C, rewarmed just before measurement, after again refrozen (▲). Conditions: 50 mM sodium acetate buffer solution (pH 5.5), 100 μM ABTS, 4.4 nM L95-1, 25°C

The isoenzyme remained stable (100% of activity) for 28 days when stored at –20°C and thawed before measurement. Laccase was unstable at room temperature, and its activity was rapidly dropped. After 4 days, the enzyme activity decreased by 50% and the activity was not detected since the 17th day.

Kinetic properties and substrate specificity

The activity of the purified L95-1 was investigated with various phenolic and non-phenolic compounds as substrates, as presented in Table 4. The results obtained by oxidizing typical laccase substrates showed that the L95-1 isoenzyme was the most affined for SYR, and the minimum value of K_M (3.7 μM) was obtained. Analysis of the literature data shows that syringaldazine is the preferred substrate for the laccase from *Rigidoporus lignosus*, *Lentinus edodes*, *Polyporus pinsitus*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Trametes villosa* and *Trametes pubescens*, no matter which fungus is producing the enzyme [25]. Generally, catalytic efficiency (k_{cat}/K_M) is considered an indicator of enzymatic effectivity [26]. The maximum L95-1 catalytic efficiency was determined by oxidizing syringaldazine, and the k_{cat}/K_M constant value was equal to 1.9 $\mu M^{-1} s^{-1}$. The catalytic efficiency of laccase may be indicated as follows: SYR>ABTS>AMB>FERO>CAT>DMP>PPA>HQ>vaniline>PZ. Similar results have been observed for recombinant laccase Lac1326, the catalytic efficiency decreased as follows: ABTS>SYR>CAT>DMP [26].

Table 4. **Kinetic parameters of purified laccase L95-1 for various substrates**

Substrate	K_M, μM	k_{cat}, s^{-1}	k_{cat}/K_M, $\mu M^{-1} s^{-1}$
Typical laccase substrates			
ABTS	14.2 ± 1.2	13.0 ± 0.5	0.9 ± 0.1
PZ	1050.0 ± 150.0	2.6 ± 0.3	$(2.5 \pm 0.1) \cdot 10^{-3}$
PPA	148.0 ± 10.8	17.9 ± 2.6	$(1.2 \pm 1.8) \cdot 10^{-1}$
FERO	645.0 ± 10.0	19.0 ± 1.0	$(3.0 \pm 0.1) \cdot 10^{-2}$
SYR	3.7 ± 0.3	6.7 ± 1.0	1.9 ± 0.3
AMB	1200.0 ± 180.0	44.0 ± 2.0	$(4.0 \pm 1.0) \cdot 10^{-2}$
CAT	2020.0 ± 200.0	30.0 ± 6.0	$(1.5 \pm 0.1) \cdot 10^{-2}$
HQ	995.0 ± 105.0	7.6 ± 1.7	$(8.0 \pm 2.0) \cdot 10^{-3}$
DMP	70.0 ± 15.0	4.4 ± 0.5	$(6.4 \pm 1.0) \cdot 10^{-2}$
MS	–	–	$(6.4 \pm 2.0) \cdot 10^{-3}$
Vanilline	–	–	$(2.5 \pm 0.2) \cdot 10^{-3}$
Hydroxycinnamic acids			
Caffeic acid	55.1 ± 6.2	5.9 ± 0.2	$(11.0 \pm 0.2) \cdot 10^{-2}$
Chlorogenic acid	130.0 ± 25	11.0 ± 2.0	$(9.0 \pm 2.0) \cdot 10^{-2}$
p-Coumaric acid	1330.0 ± 300.0	0.9 ± 0.2	$(1.0 \pm 0.1) \cdot 10^{-3}$
o-Coumaric acid	–	–	–
m-Coumaric acid	–	–	–
Ferulic acid	23.0 ± 4.0	7.0 ± 0.03	0.3 ± 0.05
Sinapic acid	24.0 ± 6.0	20.1 ± 0.5	0.9 ± 0.03
Hydroxybenzoic acids			
Gallic acid	590.0 ± 50.0	22.0 ± 2.0	$(4.0 \pm 0.2) \cdot 10^{-2}$
Syringic acid	45.0 ± 5.0	5.0 ± 0.5	0.1 ± 0.02
Flavonoids			
Quercetine	9.5 ± 0.5	11.0 ± 0.5	1.1 ± 0.1
Fisetine	13.5 ± 2.0	8.0 ± 0.5	0.6 ± 0.05
Catechin hydrate	120.0 ± 20.0	2.3 ± 0.4	$(2.0 \pm 0.4) \cdot 10^{-2}$

Ferulic and synapic acids were observed to be most efficiently oxidized by L95-1 laccase (0.3 and 0.9 μM^{-1} s^{-1}, respectively), comparing the k_{cat}/K_M values determined by oxidation of hydroxycinnamic acids. The minimum catalytic efficiency of L95-1 was determined by oxidizing p-coumaric acid ($1 \cdot 10^{-3}$ μM^{-1} s^{-1}). Moreover, L95-1 did not oxidize other coumaric acid isomers.

The highest affinity of L95-1 among hydroxybenzoic acids was determined for syringic acid. Among flavonoids, the highest affinity was determined for quercetin (9.5 μM).

The effect of pH on the rate of oxidation reaction

The optimum pH of the oxidation reactions of substrates catalyzed by laccases depends not only on the substrate but also on the nature of the enzyme. Because of a difference in the redox potential of type I copper of laccase and the substrate, the optimum pH of enzyme activity varies with the substrate used [27]. The effect of pH on the activity of laccase with different substrates (A–B are typical laccase substrates, B is hydroxycinnamic acid and D is hydroxybenzoic acid) was presented in Fig. 3. The bell-shaped dependence of the enzyme activity on the buffer solution pH was observed mostly during the oxidztion of typical laccase substrates (AMB, DMP, SYR). But the dependence was sigmoidal when laccase oxidizes ABTS. The optimum pH obtained for L95-1 laccase was 6.0 with AMB and DMP as substrates. During laccase catalyzed DMP oxidation only slight changes in the activity were observed in the pH interval from 4.0 to 5.0, whereas the activity decreased with both increasing and

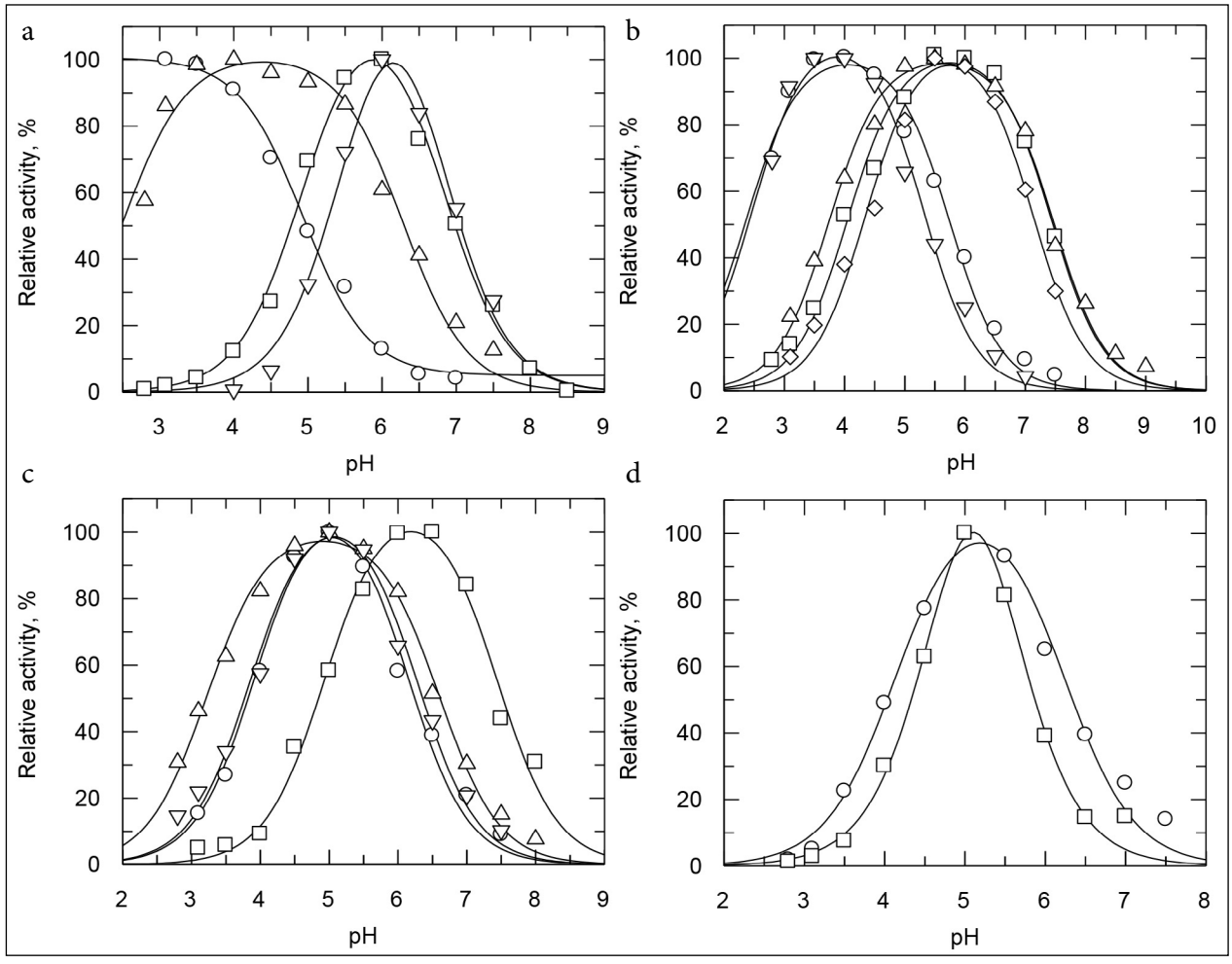

Fig. 3. Effect of different pH on the L95-1 activity using standard (a–b), hydroxycinnamic acid (c) and hydroxybenzoic acid (d) substrates. a: ABTS (○), AMB (□), DMP (△), SYR (▽); b: PPA (○), PZ (□), CAT (△), FERO (▽), HQ (◇); c: caffeic acid (○), p-coumaric acid (□), sinapic acid (△), ferulic acid (▽); d: gallic acid (○), syringic acid (□). Conditions: 60 mM Britton–Robinson buffer, 25°C. The highest activity was equated to 100%

decreasing the pH of the buffer solution. The analysis of literature data showed that the pH dependencies of phenolic compounds such as DMP and SYR were predominantly bell-shaped with activity peak values in a pH range from 4.0 to 7.0 [28].

The optimum pH of the oxidation of ABTS catalyzed by L95-1 was at pH 3.0. Laccases from *T. versicolor*, *T. hirsute*, *T. villosa*, *P. ostreatus* also have their optimum pH around pH 3.0 and show no significant activity above pH 6.5–8.0 when substrate ABTS was used [29].

Effects of metal ions

Metal ions are known to bind to the enzyme and alter its activity by stabilization or destabilization of the protein conformation [30]. The effects of some metal salts on the activity of L95-1 were shown in Table 5 and Fig. 4.

Table 5. **Influence of various metal salts on the activity of L95-1 laccase**

Metal salts	Concentrations, mM	Residual activity, %
NaN_3	0.1	10 ± 1
$Hg(CH_3COO)_2$	1	10 ± 1
$AgNO_3$	1	20 ± 2
NaF	1	30 ± 2
AlF_3	1	95 ± 2
KI	10	23 ± 1
CdI	10	25 ± 2
$Cu(CH_3COO)_2$	10	55 ± 5
$CaCl_2$	20	10 ± 3
$CoCl_2$	20	20 ± 1
$MgCl_2$	20	40 ± 2
KCl	20	40 ± 2
$AlCl_3$	20	30 ± 1

Table 5. **(Continued)**

Metal salts	Concentrations, mM	Residual activity, %
$NiCl_2$	20	35 ± 2
$CuCl_2$	20	50 ± 1
$MnCl_2$	20	55 ± 5
NaCl	20	60 ± 5
$Zn(CH_3COO)_2$	20	60 ± 4
$ZnSO_4$	20	60 ± 2
$CuSO_4$	20	80 ± 2
$Co(NO_3)_2$	20	80 ± 3
KNO_3	20	85 ± 5
$CoSO_4$	20	97 ± 4
Li_2SO_4	20	95 ± 5
$MnSO_4$	20	100 ± 4
$MgSO_4$	20	100 ± 8
KBr	100	45 ± 2
CH_3COONa	100	80 ± 5
Na_2SO_4	100	98 ± 4

Salts with Cu^{2+} ions weakly reduced the activity of L95-1 laccase. Similar results were observed for *Cerrena* sp. laccase [31]. The weakly effect of Cu^{2+} on the activity of laccase can be explained due to the filling of type I copper-binding site by Cu^{2+} ions [32]. Sodium azide and fluoride inhibited the activity of L95-1, the residual activity was 10 and 30%, respectively (when 0.1 mM NaN_3 or 1 mM NaF were used).

The inhibition by NaN_3 and NaF can be explained by the binding of azide or fluoride to the trinuclear copper centre, that affects internal electron transfer, which ultimately affects the overall oxidation process catalyzed by laccase [33, 27]. Also Cl^- and I^- strongly inhibited the activity of laccase L95-1. The inhibition of laccase activity by sulphate salts was so slight and cation dependent.

Immobilization of laccase

Laccase L95-1 was immobilized by applying adsorption. Adsorption is a fast and usual method of

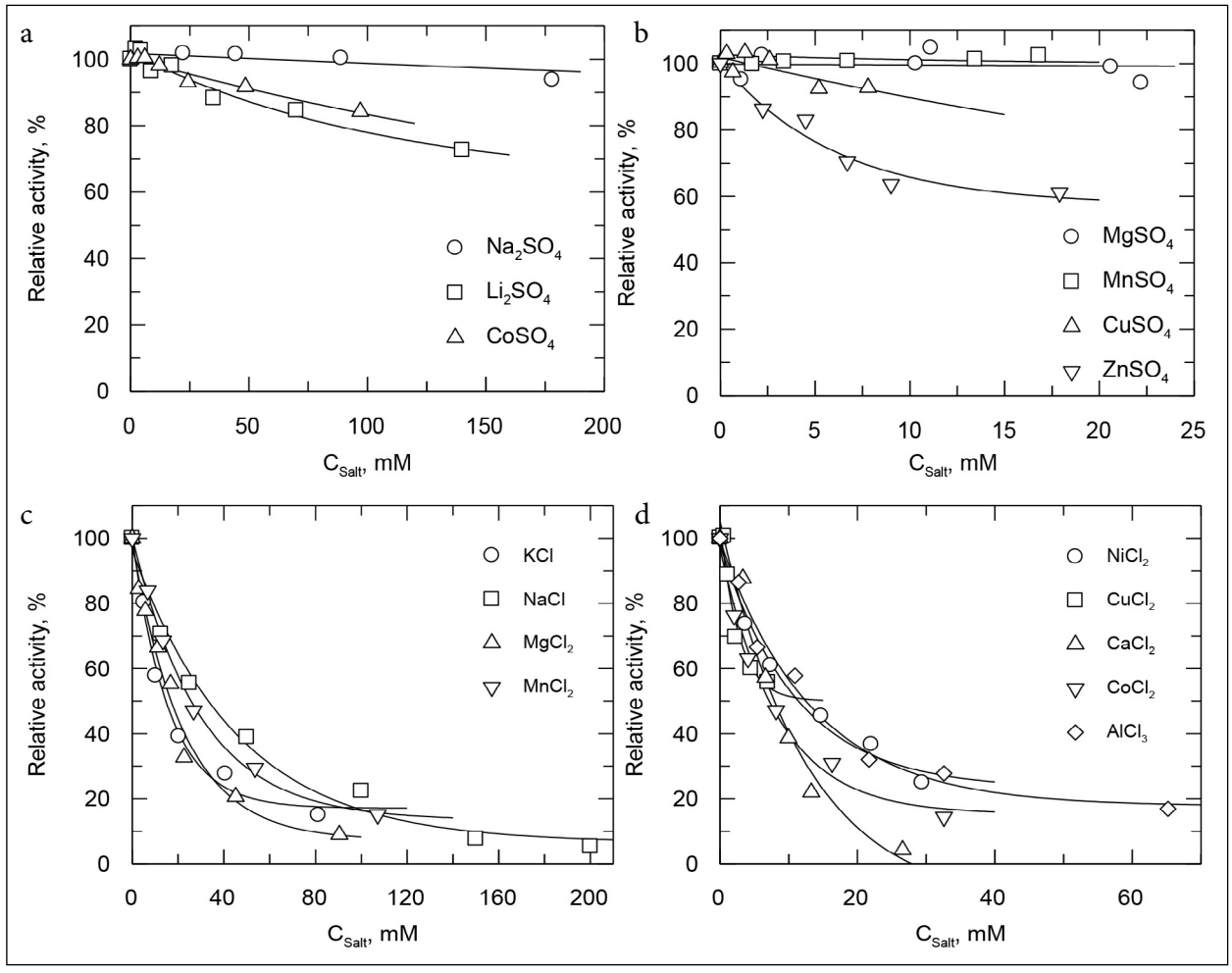

Fig. 4. Effect of different sulphate and chloride salts on the activity of L95-1. Conditions: 50 mM acetate buffer solution, pH 5.5, at 25°C, 100 µM of ABTS as substrate. The activity of enzyme without salt was equated to 100%

immobilization, which practically does not modify the natural conformation of an enzyme [34]. The synthesized magnetic nanoparticles used in this study were characterized previously [21]. Immobilization of laccase on the Fe_3O_4 nanoparticles can be affected by various factors. In the present study, the enzyme concentration which was used for immobilization and adsorption time was optimized.

The effect of laccase concentration used for immobilization (interval from 7 to 22 nM) on the relative activity of immobilized L95-1 was investigated and shown in Fig. 5. The immobilized laccase L95-1 showed the maximum activity at the enzyme concentration of 10.8 nM, whereas the enzyme activity in the supernatant at this enzyme concentration was low.

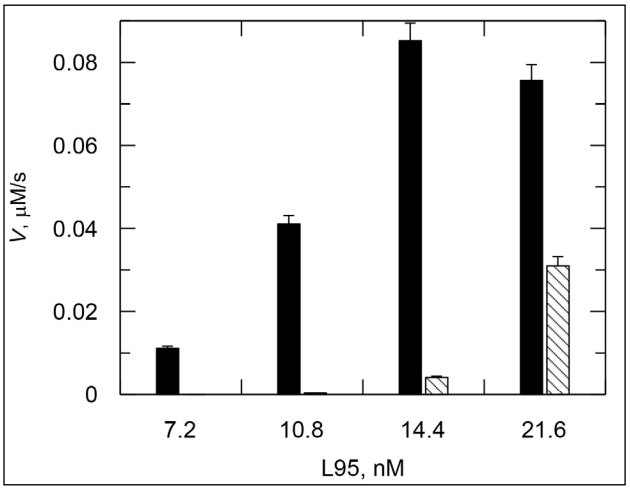

Fig. 5. The effect of L95-1 concentration used for immobilization on the immobilized laccase activity (■) and laccase activity in supernatant (▨). Conditions: activity was determined in 50 mM acetate buffer solution, pH 5.5, at 25°C, 100 μM of ABTS as substrate

Adsorption time is also a key factor during the enzyme immobilization process [35]. The effect of adsorption time on the immobilized laccase L95-1 enzymatic activity was shown in Fig. 6. The relationship of adsorption time to laccase activity displayed a dynamic equilibrium. The relative activity increased when the adsorption rate was greater than the desorption rate and decreased when the adsorption rate was slower than the desorption rate.

As can be seen in Fig. 6, the relative activity decreased after each incubation cycle. The adsorption time of 10 min was chosen for further immobilization. The activity of immobilized laccase decreased with increasing the incubation time. After 24 h

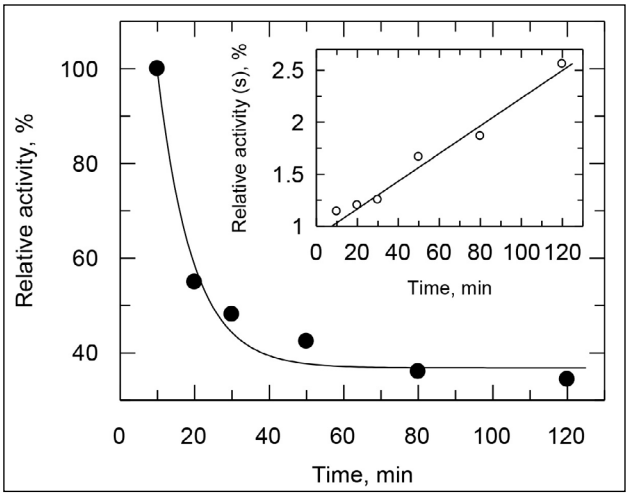

Fig. 6. The effect of adsorption time on the immobilized laccase (●) and laccase in supernatant (○) activity. Conditions: activity was determined in 50 mM acetate buffer solution, pH 5.5, at 25°C, 100 μM of ABTS as substrate

activity of immobilized enzyme remained similar to the activity after 1 h. The activity of enzyme increased in the supernatant with increasing the time of incubation.

According to the calculations, about 40% of native L95-1 was adsorbed on the Fe_3O_4 nanoparticles.

Reusability of the immobilized laccase

The reusability of immobilized laccase was studied. 10 consecutive cycles of substrate oxidation/magnetic separation/redispersion were used for reusability determination (Fig. 7). After 5 cycles of reuse,

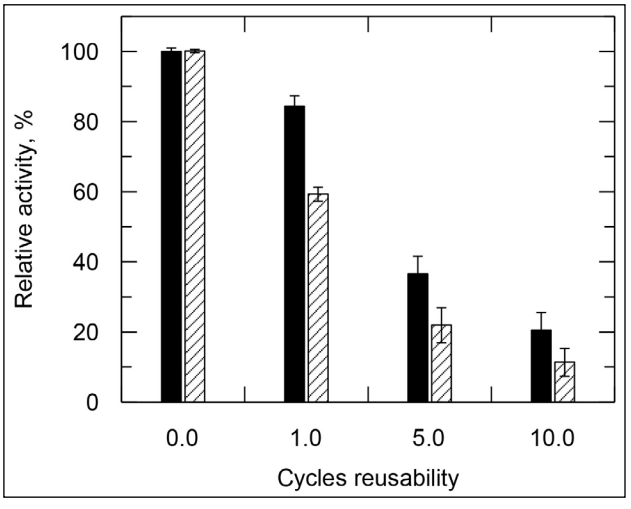

Fig. 7. Cycles of the reusability of L95-1 laccase in 50 mM sodium acetate buffer solution, pH 5.5 (■), and in DD water (▨). Conditions: activity was determined in 50 mM acetate buffer solution, pH 5.5, at 25 °C, 100 μM of ABTS as substrate

60 and 80% of the initial laccase activity were lost in the buffer solution and DD water, respectively. The enzymatic activity of immobilized laccase represented approximately 20% of the initial enzyme activity after 10 consecutive reutilization cycles in the buffer solution and DD water. The decrease was attributed to the stress suffered by the laccase after each magnetic separation because of the formation of compact aggregates. The amount of desorbed laccase depends on the re-suspending media and the number of repeated procedures [36].

Kinetic parameter study

The apparent Michaelis–Menten constants (K_M) for both the free and immobilized L95-1 were determined and compared (Table 6). The higher K_M value for the immobilized laccase indicates its lower affinity for the substrate compared to that of the free laccase. The higher catalytic efficiency constant was determined for the free laccase compared to the immobilized one (0.87 and 0.32 $\mu M^{-1} s^{-1}$, respectively). The effectiveness factor for the immobilized laccase was determined and was equal to 1.45. The value of effectiveness factor above 1 shows that the immobilization does not affect the diffusion of the substrate and the product. Generally, enzymes lose some of their catalytic power when they are immobilized as the apparent K_M of immobilized enzymes is higher, and V_{max} values are lower than those for free enzymes [37]. The observed changes of laccase L95-1 in the catalytic properties upon immobilization are the apparent K_M and V_{max} values increase, whereas k_{cat} and k_{cat}/K_M decrease.

A possible application of the immobilized laccase could be oxidation of the phenolic compounds, such as 2,6-DMP, in wastewater. The structure of such chromogenic phenolic laccase substrates is similar to that of certain target pollutants (such as industrial chemicals, nonylphenol and bisphenol A) [38, 39]. Therefore, we studied how the immobilized L95-1 laccase on magnetic nanoparticles was able to oxidize 2,6-DMP. The kinetic parameters were presented in Table 6. The apparent K_M of the immobilized laccase for DMP oxidation was relatively low compared to K_M values of the free L951-1. The maximum reaction rates of free and immobilized laccase were similar, 0.009 and 0.008 μM/s, respectively. Whereas the values of catalytic (k_{cat}) and catalytic efficiency (k_{cat}/K_M) constants decreased after laccase immobilization.

Effect of pH

The pH profile of native laccase L95-1 activity upon the oxidation of ABTS was sigmoidal, whereas the pH profile of this laccase immobilized on magnetic nanoparticles was bell-shaped, as shown in Fig. 8.

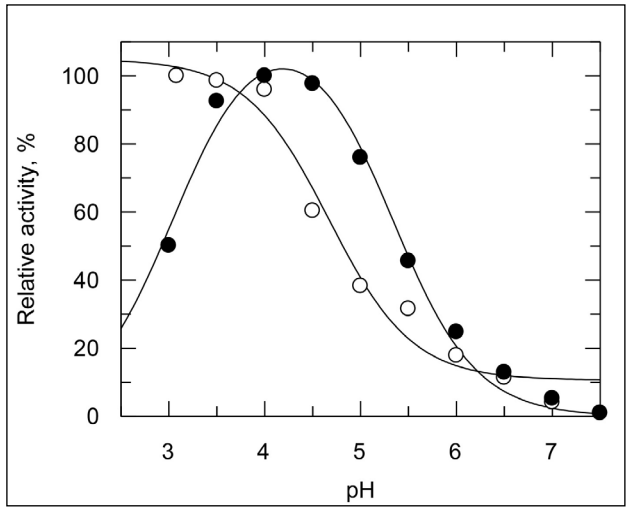

Fig. 8. The effect of buffer solution pH on the free (○) and immobilized (●) laccase activity. Conditions: 60 mM Britton–Robinson buffer, 25°C. The highest activity was equated to 100%

The optimum pH of immobilized laccase activity was found to be 4.0–4.5 and the optimum pH of free laccase was 3.0. The immobilized laccase L95-1 action was significantly better than that of the free laccase in a pH range from 4.5 to 6.0.

Hence, this could be due to the protective effect provided by the laccase fixation on the nanoparti-

Table 6. **Kinetic constants of the ABTS and DMP oxidation catalysed by free and immobilized L95-1 laccase**

Substrate	Enzyme	K_M, μM	V_{max}, μM/s	k_{cat}, s^{-1}	k_{cat}/K_M, $\mu M^{-1} s^{-1}$
ABTS	Free L95-1	14.9 ± 1.6	0.029 ± 0.001	13.0 ± 0.46	0.87 ± 0.09
	Immobilized L95-1	27.9 ± 3.7	0.042 ± 0.0015	8.9 ± 0.3	0.32 ± 0.04
DMP	Free L95-1	70.0 ± 15.0	0.009 ± 0.0005	4.4 ± 0.5	0.064 ± 0.01
	Immobilized L95-1	35.3+7.4	0.008 ± 0.0005	1.7 ± 0.1	0.046 ± 0.01

cles during adsorption at room temperature. A shift of the optimum pH after laccase immobilization was also reported in a number of previous studies [40, 41].

Effect of metal salts

The effect of metal salts on the activity of immobilized laccase was studied and compared with the results obtained with free laccase (Fig. 9).

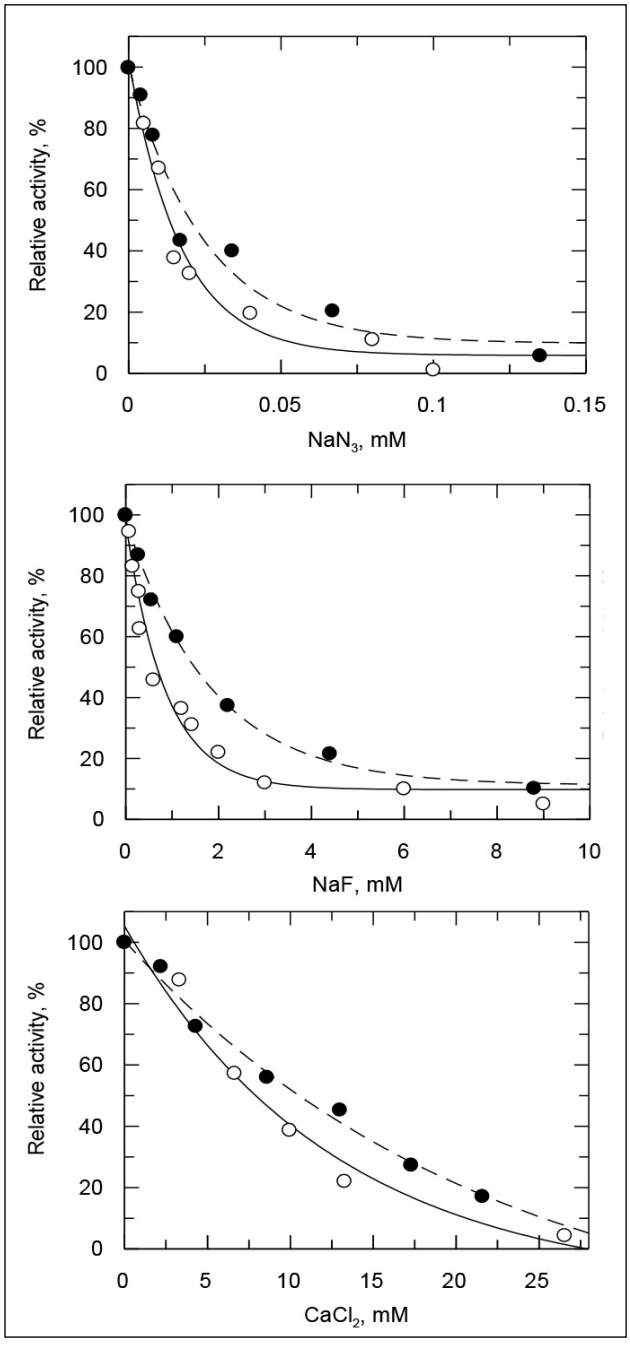

Fig. 9. The effect of metal salts on the free (○) and immobilized (●) activity of laccase L95-1. Conditions: 50 mM acetate buffer solution, pH 5.5, at 25°C, 100 μM of ABTS as substrate

The used salts inhibited the activity of the immobilized laccase L95-1 less than that of the free enzyme. The relative activity of immobilized laccase was higher about 15–20% with NaN_3, 20% with NaF and 10% with $CaCl_2$ compared to free.

CONCLUSIONS

In summary, laccase was purified and its properties were determined. The best substrate for purified laccase was syringaldazine. The bell-shaped dependence of the enzyme activity on the buffer solution pH was observed mostly during the oxidation of typical laccase substrates (AMB, DMP, SYR). But the dependence was sigmoidal when laccase oxidizes ABTS.

Copper salts weakly reduced activity, but azide, fluoride, chloride and iodide salts strongly inhibited the activity of laccase L95-1. The inhibition of laccase activity by sulphate salts was so slight and cation dependent.

Laccase was successfully immobilized on the Fe_3O_4 nanoparticles. According to the calculations about 40% of native L95-1 was adsorbed on the nanoparticles. The properties of immobilized laccase such as pH stability and resistance to various metal salts were significantly improved compared with those of the free enzyme. The results showed that the immobilized laccase L95-1 on magnetic nanoparticles have the potential of oxidation phenolic compounds.

ACKNOWLEDGEMENTS

We thank Liucija Marcinkevičienė from the Life Sciences Center of Vilnius University for her assistance with the biosynthesis and purification of laccase from *Lithothelium* sp. and for comments that greatly improved the manuscript.

Received 21 April 2020
Accepted 2 June 2020

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LAKAZĖS IŠ *LITHOTHELIUM* SP. BIOSINTEZĖ, GRYNINIMAS, CHARAKTERIZAVIMAS IR IMOBILIZACIJA

S a n t r a u k a

Nauji grybinės lakazės izofermentai (pavadinti L95-1 ir L95-2) produkuojami Askomicetų *Lithothelium* sp., išskirti iš miško dirvožemio ir išgryninti. Tačiau tik vienas iš jų charakterizuotas, nes kitas izofermentas gryninimo metu prarado savo aktyvumą. Ekstraląstelinė L95-1 lakazė buvo išgryninta 30 kartų naudojant jonų mainų ir hidrofobinės sąveikos chromatografiją, bendra išeiga 88 %. Nustatyta, kad išgrynintos L95-1 molekulinė masė yra 85 kDa, taikant SDS-PAGE analizę. L95-1 lakazė buvo stabili esant 4–22 °C temperatūrai ir pH 6,0–6,5. L95-1 lakazės substratinis specifiškumas ištirtas su įvairiais junginiais. Nustatytos gimiringumo konstantos (K_M) svyravo plačiame 3,7–2020 μM diapazone, o katalitinio efektyvumo konstantos (k_{cat}/K_M) varijavo 0,008–1,9 μM^{-1} s^{-1} intervale. Optimalus pH daugumai substratų svyravo nuo 5,0 iki 6,0. Natrio azidas ir fluoridas stipriai slopino L95-1 aktyvumą, o sulfato druskos – silpnai.

Lakazė buvo imobilizuota ant Fe_3O_4 nanodalelių ir charakterizuota. Nustatyta, kad išlieka 20 % pradinio fermentinio aktyvumo po dešimties reakcijų ciklų oksiduojant ABTS. Imobilizuota lakazė pasižymi didesne tolerancija įvairioms metalų druskoms. L95-1 lakazės savybės rodo potencialias galimybes panaudoti ją biotechnologiniuose procesuose.