Immobilization of Laccase on Magnetic Carriers and Its Use in Decolorization of Dyes

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Laccase from *Trametes versicolor* was immobilized on magnetic (Fe_3O_4) nanoparticles and microparticles. Free and immobilized laccase had activities 163 and about 4.6 nkat mg^{-1} respectively. Immobilized was 125.8 μg enzyme mg^{-1} of nanoparticles and 13 μg enzyme mg^{-1} of microparticles. The K_m were 10.55, 9.02 and 11.14 mmol L^{-1} for free laccase, immobilized on nanoparticles and immobilized on microparticles. The pH optimum after immobilization was about 0.5 pH less than before immobilization. Immobilized laccase retained 55 % (nano) and 47 % (micro) of initial activity after nine repetitions. Values of T_{50} were calculated respectively for free laccase, immobilized on nanoparticles and microparticles at 54.5 °C, 57.5 °C and 46 °C. Dyes Direct Blue 78, Acid Blue 225, Reactive Red 195, Acid Blue 74, and Phenol Red were used for decolorization by free and immobilized laccase. Laccase was able to decolorize most of the dyes well.

Key words:
laccase, immobilization, dyes, biodegradation, magnetic particles

Introduction

Oxidizers such as periodate or chromate are very common in biotechnological applications, however they represent an extensive burden on the environment. The use of enzyme reactions instead of chemical reactions allows this burden to be reduced. Laccase is an interesting alternative because of its broad substrate specificity. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2.) belongs to the family of so-called "blue multicopper oxidases". Substrates of laccase are a wide variety of phenolic and nonphenolic substances. The number of possible substrates further increases when using a substance that allows transfer of electrons between the laccase and the substrate. Laccase is present primarily in higher plants and filamentous fungi, but has been found in several species of bacteria[1]. Reactions catalysed by laccase can be divided into three types. The first type is direct oxidation of simple derivatives of phenol. The second type is oxidation of phenolic and nonphenolic substances by the presence of a mediator, and the third type is coupling of reactive radicals produced by laccase[2].

Laccase with its wide substrate specificity and oxidation properties is used in many industrial applications. It is used in the food and paper industry, synthetic chemistry, bioremediation and biodegradation of phenolic pollutants or in analytical applications[3,4]. Laccase also catalysed reactions with various kinds of textile dyes and degrades them[5]. The dyes are classified into several groups based on the structure of the chromophore. The largest and the most widespread group are probably azo dyes. Other groups can be anthraquinone, indigo, triphenylmethane, phthalocyanine or polyaromatic dyes[2,6]. The advantage of these dyes is endurance against bleaching by light, water or other chemical substances[4]. The disadvantage of using these dyes is in their possible carcinogenicity and mutagenicity[3]. Wastewaters from textile industry are colored by these substances. These dyes are difficult to degrade by microbial populations in common biological wastewater treatment plants. The dyes may be toxic for the microorganisms and can cause their death[7,8]. Adsorption and coagulation are commonly used to remove dyes. Adsorption and coagulation of dyes is effective, but produces a large amount of sludge, when substances are only transferred from wastewater to solid waste[7,9].

The wide use of enzymes in industrial applications can be limited for several reasons. Generally, enzymes have low stability. Low stability of laccase, when used in wastewater treatment, as well as the enzyme's price, discourage its use. Using relatively pure enzyme with good specific activity may lower the cost[10,11]. These problems can be resolved by immobilization of enzyme on a suitable carrier. Immobilization can improve or worsen the stability of the enzyme towards high temperature, acidity or

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exposure to organic solvents. Immobilization mostly results in a decrease of the enzyme's activity; the main advantage of immobilization of enzymes is the possibility of re-use and easy separation from the reaction mixture. The laccase was immobilized by different methods on many carriers. With extension of nanotechnology, the laccase was immobilized on silica nanoparticles[12], nanotubes[13], golden particles[14] or on magnetic particles coated with chitosan[15]. Handling of the enzyme is easier if magnetic materials are used, because it is possible to manipulate the enzyme using a magnetic field (etc. separation from reaction mixture).

Materials and methods

Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Metol (4-(methylamino)phenol hemisulfate), EDC. HCl (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride), NHS (N-hydroxysuccinimide), $NaIO_4$, Phenol Red were purchased from Sigma-Aldrich (St. Louis, MO, USA); Direct Blue 78 and Acid Blue 225 were provided from Synthesia a.s. (Pardubice, Czech Republic), Reactive Red 195 was provided from Textilcolor AG (Sevelen, Switzerland), Acid Blue 74 was purchased from Acros organics (Geel, Belgium); magnetic microparticles (Perlosa MG 100; average particle size 50–80 μm)[16] were obtained from Iontosorb (Ústí nad Labem, Czech Republic) and magnetic nanoparticles were synthesized and coated with chitosan in the Regional Centre of Advanced Technologies and Materials of the Palacký University, Olomouc, Czech Republic (average particle size 20–40 nm)[17,18].

Measurement of laccase activity

Determination of laccase activity was performed by the spectrophotometric method using metol as a substrate. Two milliliters of the reaction mixture were composed of buffer (0.1 mol L^{-1} Na-acetate, pH 5.0), 25 mmol L^{-1} metol and 10 μg of enzyme. Laccase from *Trametes versicolor* was used as the enzyme. The enzyme reaction was performed while stirring at room temperature. The reaction mixture with free enzyme was mixed by magnetic stirrer. A vortex was used for mixing reaction mixture of immobilized enzyme. The amount of dissolved oxygen depends on atmospheric pressure. 800 rpm was sufficient to achieve a stable concentration of dissolved oxygen in reaction mixture without enzyme denaturation. The reaction ran in an open cuvette to provide a free entry of oxygen. Reaction time was 10 minutes, and every minute absorbance of the product was measured at 540 nm on the Biochrom WPA spectrophotometer (Biochrom Ltd., Cambridge, United Kingdom). Activity of laccase was calculated based on the molar absorption coefficient of metol[19] $\varepsilon = 2000$ M cm^{-1}. The reaction mixture for immobilized enzyme contained magnetic nanoparticles or microparticles respectively with immobilized laccase, 25 mmol L^{-1} metol and buffer (0.1 mol L^{-1} Na-acetate, pH 5.0). The absorbance was measured as described above. Before the detection of absorbance, magnetic carrier with immobilized laccase was separated on the bottom of a cuvette using a magnetic separator (NdFeB magnet 15 x 15 x 15 mm; pull force ~10 kg) to no interference caused by magnetic particles.

Immobilization of laccase on magnetic nanoparticles

2 mg of magnetic nanoparticles coated with chitosan, 6.5 mmol L^{-1} EDC.HCl, buffer (0.1 mol L^{-1} Na-acetate, pH 5.0), 13 mmol L^{-1} NHS and 0.4 mg laccase from *Trametes versicolor* were used for immobilization. Final volume was 400 μL. The mixture was shaken 20 hours by a programmable rotator-mixer at 4 °C in a refrigerator. Magnetic nanoparticles were separated down by a magnetic separator. The residual solution was pipetted off and nanoparticles were washed fifteen times with buffer of 0.1 mol L^{-1} Na-acetate at pH 5.0 to remove the unbound enzyme. The supernatant was tested for activity of laccase as described above and also used to determine protein concentration using Bradford's method. For decolorization of dyes, laccase was immobilized in a different ration. 10 mg of magnetic nanoparticles coated with chitosan and 1 mg laccase were used. The rest of the procedure and the solutions were the same.

Immobilization of laccase on magnetic microparticles

15 mg of magnetic microparticles coated with cellulose and 0.02 mol L^{-1} $NaIO_4$ in deionized water were used for immobilization. The mixture was shaken 20 hours by a programmable rotator-mixer at 4 °C in a refrigerator. Magnetic microparticles were separated down by a magnetic separator. The residual solution was pipetted off and the microparticles were washed ten times with buffer of 0.1 mol L^{-1} Na-acetate at pH 5.0 to remove $NaIO_4$. 0.4 mg of laccase from *Trametes versicolor* in the same buffer was added to the washed microparticles. Final volume was 400 μL for both parts of the process. The mixture was shaken 20 hours by a programmable rotator-mixer at 4 °C in a refrigerator. Magnetic microparticles were separated down by a magnetic separator. The residual solution was pipetted off and the microparticles were washed fifteen times with buffer of 0.1 mol L^{-1} Na-acetate at pH 5.0 to re-

move the unbound enzyme. After washing, the activity of the enzyme was tested in the supernatant as described above, and the supernatant was also used to determine protein concentration using Bradford's method. For decolorization of dyes, laccase was immobilized in a different ration. 50 mg of magnetic microparticles coated with cellulose and 4 mg enzyme were used. The rest of the procedure was the same.

Determination of Michaelis constant (K_m) and V_{lim}

Free (0.005 mg enzyme mL^{-1}) and immobilized laccase in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0) with metol of various concentrations (5; 10; 15; 20; 25; 30 and 35 mmol L^{-1}) in total volume of 2 mL were used to determine K_m. Absorbance was measured as described above. The values were used to construct a LineweaverBurk plot and calculate K_m V_{lim}.

Operational stability of immobilized laccase

Laccase was immobilized on magnetic nanoparticles and microparticles. The activity of laccase was determined as described above. The first measurement was described as 100 %. After that, immobilized laccase was washed seven times to remove components of the reaction mixture, and used again to determine enzyme activity. This was repeated nine times.

Functional stability of free and immobilized laccase

Free and immobilized laccase was kept at room temperature for 48 hours. Activity of the laccase was measured at 5th, 24th, 29th and 48th hour. The activity of laccase was determined as described above.

Determination of thermal stability of free and immobilized laccase

Solutions of free and immobilized laccase in buffer were incubated for 45 minutes at different temperatures (from 0 to 75 °C with the interval of 5 °C) for free laccase and laccase immobilized on magnetic microparticles, and at temperatures (from 0 to 75 °C with the interval of 10 °C) for laccase immobilized on magnetic nanoparticles. After incubation, the enzyme was kept at a storage temperature of 4 °C. Activity of laccase was determined as described above. Value T_{50} was determined for comparison of thermal stability of free and immobilized laccase. Value T_{50} is temperature, when the enzyme retains 50 % of activity.

pH optimum

Activity of free and immobilized laccase was determined as described above. For this measurement, the buffer was changed to a sodium citrate-phosphate buffer of various pH values (2.6; 3.2; 3.8; 4.4; 5.0; 5.6; 6.2; 6.96).

Decolorization of dyes by laccase

For decolorization, several kinds of dyes were selected. As representatives of azo dyes, Direct Blue 78 and Reactive Red 195 were selected. As representative of anthraquinone dyes, Acid Blue 225 was selected. As representative of indigo dyes, Acid Blue 74 was selected. Also, triarylmethane dye Phenol Red was used. As enzyme, free (1.25 mg enzyme mL^{-1}) and immobilized laccase was used. Also, for some dyes, a different amount of free enzyme were used (for Acid Blue 74 2.5 mg enzyme mL^{-1}, for Reactive Red 195 5.0 mg enzyme mL^{-1} and for Acid Blue 225 0.125 mg enzyme mL^{-1}). The reaction mixture contained a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0), a solution of laccase with different amounts as described above or immobilized laccase, and a dye solution of a certain concentration (for Direct Blue 78 and Acid Blue 74 0.03 mg dye mL^{-1}, for Reactive Red 195 0.04 mg dye mL^{-1}, for Phenol Red 0.01 mg dye mL^{-1} and for Acid Blue 225 0.125 mg dye mL^{-1}). Total volume of the reaction mixture was 2 mL. Total time of decolorization was 60 minutes for free laccase and 120 minutes for immobilized laccase. For each dye, a calibration line was created to determine the amount of decolorized dye, and absorption spectrum was measured. Dye decolorization by empty carrier was also measured.

Results and discussion

Immobilization, enzyme activity, K_m and V_{lim} assay

On nanoparticles, 125.8 ± 4.8 μg enzyme mg^{-1} of particles (about 62.9 %) was immobilized, and on microparticles 13 ± 0.4 μg enzyme mg^{-1} of particles (about 48.7 %) was immobilized. The amounts of enzyme immobilized on carriers are different in accordance to the carrier used and its surface area. The amount of laccase immobilized on Fe_3O_4 particles ranged from 25 %[20] to 94 %[15]. Specific activities of free and immobilized laccases are summarized in Table 1. When we compare specific activities of free laccases and the immobilized forms, the immobilized forms of laccase show significantly decreased specific activity. For immobilized laccases from *T. versicolor,* 2.8 % of the activ-

Table 1 – *Values of specific activities, K_m and V_{lim} of free and immobilized laccase towards metol substrate*

Laccase form	Immobilization	Specific activity (nkat mg^{-1})	Michaelis constant (K_m) (mmol L^{-1})	V_{lim} (nkat)
free		163 ± 13.5	10.55 ± 0.77	1.79 ± 0.04
immobilized	nanoparticles	4.66 ± 0.28	9.02 ± 0.84	1.54 ± 0.05
	microparticles	4.58 ± 0.25	11.14 ± 0.92	1.42 ± 0.04

ity of free laccase was retained. Significant decrease in enzyme activity is common when the enzyme is immobilized[21]. For example, laccase activity after immobilization on EUPERGIT was 7 % activity of free laccase[22]. Most of the literature referred to usage of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and SGZ (syringaldazin) as substrates for laccase. When were compared Michaelis constants for ABTS[11,15] (34 – 57 μmol L^{-1}) and for SGZ[10;11] (9.4 – 33 μmol L^{-1}) for laccase from *T. versicolor*, laccase has higher affinity to ABTS and SGZ than metol, which was used. Metol was chosen because it costs significantly less than ABTS and SGZ. Michaelis constants and V_{lim} of metol as the substrate by the free and immobilized form of laccase from *T. versicolor* are listed in Table 1. Covalent[15] and noncovalent[10] immobilization of laccase on magnetic particles generally leads to an increase in K_m and thus to a decrease in their affinity to the substrate. The K_m of our samples remained the same after immobilization. Unfortunately, the Michaelis constant for our substrate (metol) was not found in literature, therefore the decrease or increase in K_m was compared only with K_m of ABTS and SGZ.

Operational and functional stability

The percentage change in activity of various immobilized laccases in relation to the number of repetitions is shown in Fig. 1. The immobilized enzyme on magnetic nanoparticles retained 55.1 % of initial activity after nine repetitions and error bars ranged between values of 4 % and 7 %. Immobilized enzyme on magnetic microparticles retained 47 % of initial activity after nine repetitions and error bars ranged between values of 4 % and 6 %. In other articles, laccase immobilized on magnetic nanoparticles had higher activity after nine repetitions (about 85 %)[10,15,23]. Immobilization depends on many things (carrier, carrier surface, immobilization method or structure of enzyme). Different methods of preparation and surface of magnetic particles may cause immobilization of different amounts of enzyme on particles. A good example of this can be 99 % of the initial activity after 24 repetitions[24], but this refers to usage of laccase from other organisms (*Paraconiothyrium variable*) and the enzyme was immobilized on glass beads. Laccase activity can

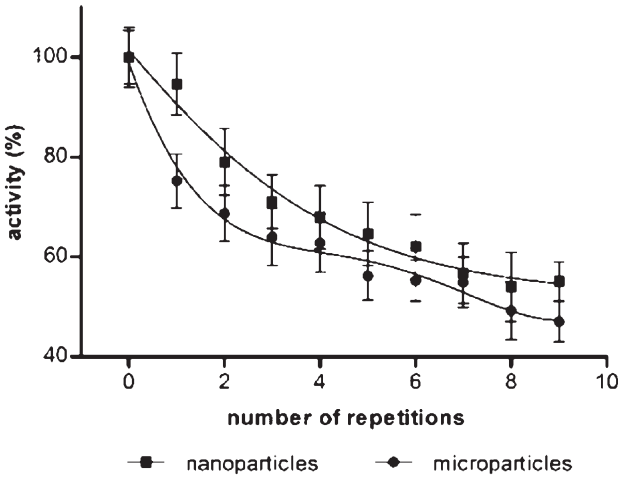

Fig. 1 – *Activity of various immobilized laccases in relation to the number of repetitions. Laccase from T. versicolor was used, and activity was measured in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0) and with 25 mmol L^{-1} metol, at room temperature. Laccase was immobilized using carbodiimide on magnetic nanoparticles coated with chitosan, and using periodate on magnetic microparticles coated with cellulose. Ten consecutive measurements were made with immobilized enzyme; this process was repeated three times. Error bars were less than 6 %.*

severely decrease after nine repetitions. Laccase immobilized on various types of polyacrylonitrile nanofibres showed 30 % to 50 % of initial activity after nine repetitions[25]. In the source cited in 26, immobilized laccase had higher functional stability than the free one. Our research shows that functional stability does not depend on whether or not the enzyme is immobilized. Both forms of laccase had 65 % activity after 48 hours.

pH optimum

Comparison of the activities of free and various immobilized laccases in relation to pH is shown in Fig. 2. The highest measured value of activity was marked as 100 %. pH optimums for laccase, which we used, were 5.1 for free laccase, 4.7 for laccase immobilized on nanoparticles and 4.5 for laccase immobilized on microparticles. From these values of pH, activity of laccase decreased on both sides. From pH 6.96, activity of laccase could not be measured by this method for determining activity, because decomposition of substrate and coloring occurred. This may have been caused by initiation of polymerization of metol products[19]. Error bars were,

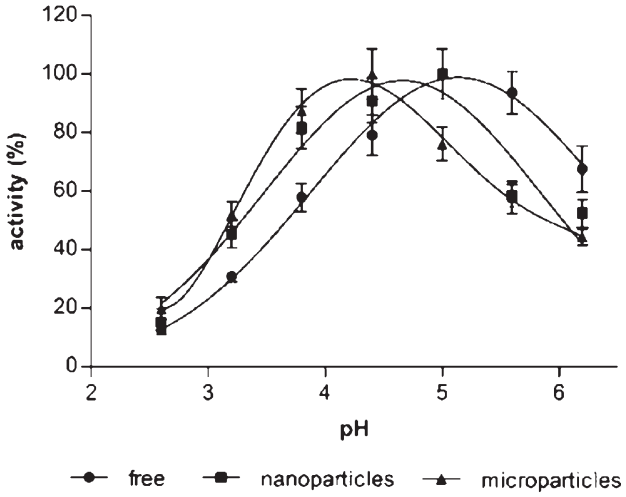

Fig. 2 – *Activity of free and various immobilized laccases in relation to pH. Laccase from T. versicolor was used, and activity was measured in a buffer (sodium citrate-phosphate buffer of various pH values (2.6; 3.2; 3.8; 4.4; 5.0; 5.6; 6.2; 6.96) and with 25 mmol L^{-1} metol, at room temperature. Laccase was immobilized using carbodiimide on magnetic nanoparticles coated with chitosan, and using periodate on magnetic microparticles coated with cellulose. Measurements were repeated three times. Error bars were less than 8.7 % at free and immobilized laccase.*

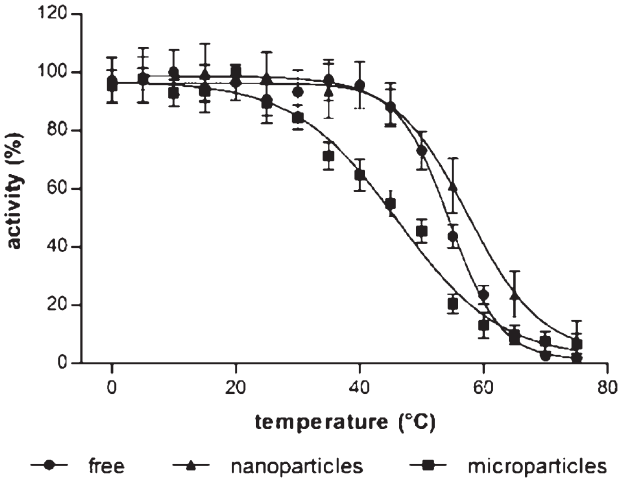

Fig. 3 – *Activity of free and various immobilized laccases in relation to temperature. Laccase from T. versicolor was used, and activity was measured in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0) and with 25 mmol L^{-1} metol at different temperatures (from 0 to 75 °C with the interval of 5 °C) for free laccase and laccase immobilized on magnetic microparticles, and at temperatures (from 0 to 75 °C with the interval of 10 °C) for laccase immobilized on magnetic nanoparticles. Laccase was immobilized using carbodiimide on magnetic nanoparticles coated with chitosan, and using periodate on magnetic microparticles coated with cellulose. Measurements were repeated three times for each temperature. Error bars were less than 9.7 % at free and immobilized enzyme.*

for free and immobilized enzyme, less than 8.7 %. pH optimums for free and immobilized laccase *T. versicolor* range from pH 3.0 to 5.5. In contrast to our results, immobilization mostly causes pH optimum to move about pH 0.5 higher[11,15,27,28]. Decrease in activity from pH optimum to pH 2.5 and 6.2 is similar as in articles[10,11]. An enzyme may slightly change conformation after immobilization, and this could be the reason the pH optimum changes. Immobilization on magnetic nanoparticles and microparticles therefore perhaps had an effect on the conformation of the laccase. Fungal laccases have pH optimum[29] from 3.0 to 6.0, which is in accordance with our results.

Thermal stability

The dependence of activities of free and various immobilized laccases on temperatures is shown in Fig. 3. The highest measured value was marked as 100 %. Value T_{50} was determined for free and immobilized laccase for comparison of thermal stability. T_{50} is a temperature at which the enzyme retains 50 % of its activity. Free laccase and laccase immobilized on nanoparticles retained almost 100 % of their activities up to 35 °C. Activities decreased at temperatures from 35 °C to 65 °C. No activity of laccase was measured from 65 °C upwards. This is similar as in articles[30,31]. For immobilized laccase on microparticles, activity decreased from 20 °C. Value T_{50} was calculated for free laccase at 54.5 °C, for laccase immobilized on nanoparticles at 57.5 °C, and for laccase immobilized on microparticles at 46 °C. Lower activity of free laccase (40 %) was also measured[32] at 50 °C. Thermal stability of laccases depends on glycosylation[33]. Immobilization may cause increased stability of laccase at high temperature. Laccase from *T. versicolor* can also show 70 % activity at 50 °C after immobilization on polymeric membrane[32]. More stable laccase also exists. Laccase from *Streptomyces lavendulae* was stable[34] up to 70 °C. Stability of our free laccase and laccase immobilized on nanoparticles remained similar, but the stability of laccase immobilized on microparticles decreased. This could be caused by the changing conformation of laccase after immobilization on microparticles[35].

Decolorization of dyes

Free and immobilized laccase from *Trametes versicolor* was used for decolorization of dyes. Decolorization of dyes in empty carriers was also measured. Comparison of the amounts of dyes decolorized by free laccase is shown in Fig. 4, for immobilized laccase on nanoparticles in Fig. 5 and for immobilized laccase on microparticles in Fig. 6. Wavelengths for measurements of decolorization of dyes were selected at 600 nm for Direct Blue 78, 540 nm for Reactive Red 195, 632 nm for Acid Blue 225, 610 nm for Acid Blue 74 and 433 nm for Phenol Red. The highest amount of decolorized

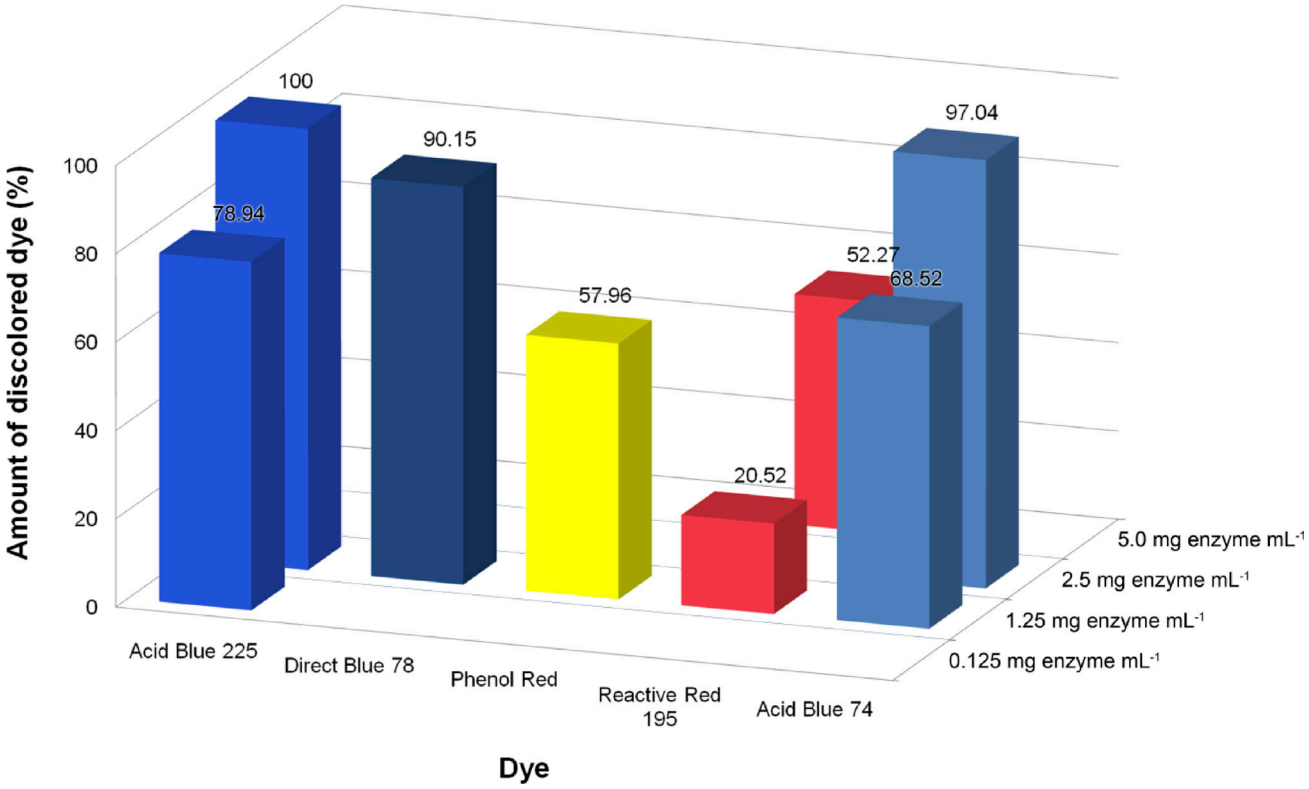

Fig. 4 – *Comparison of the amounts of various dyes decolorized by free laccase. Laccase from T. versicolor was used. Reactions were measured in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0). Dye concentrations were 0.03 mg dye mL^{-1} for Direct Blue 78 and Acid Blue 74, 0.04 mg dye mL^{-1} for Reactive Red 195, 0.01 mg dye mL^{-1} for Phenol Red and 0.125 mg dye mL^{-1} for Acid Blue 225. Measurements were repeated three times for each dye. Error bars were less than 8.7 %.*

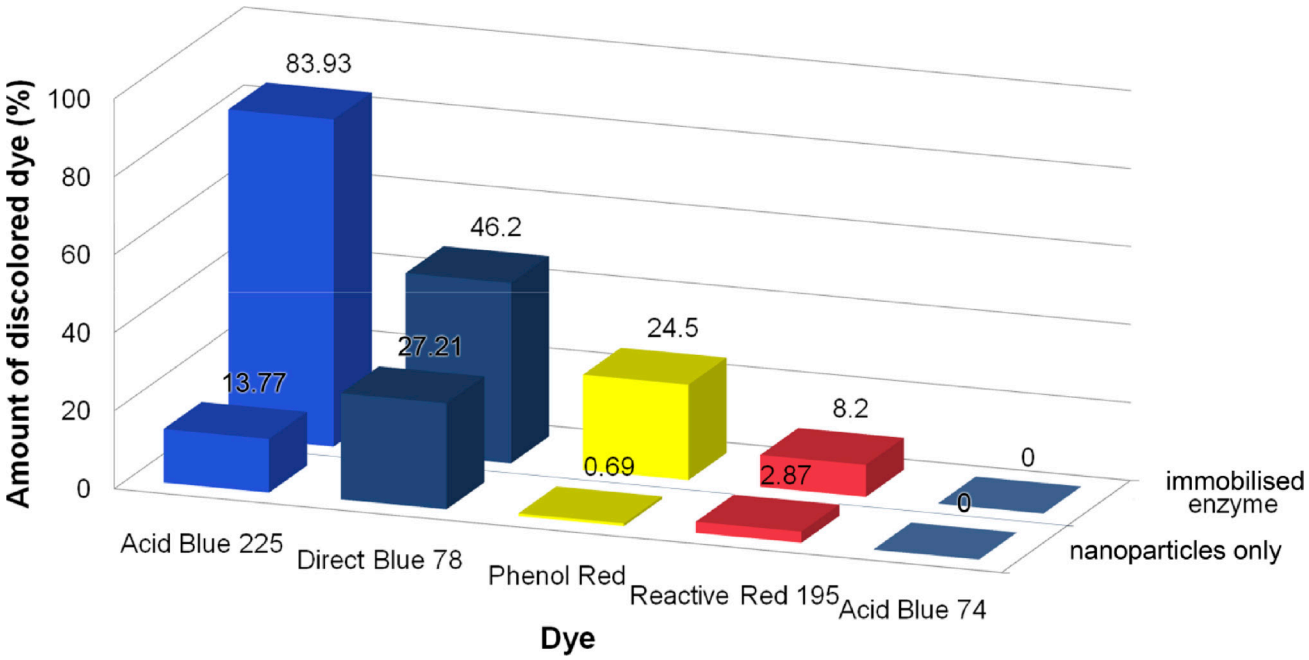

Fig. 5 – *Comparison of the amounts of various dyes decolorized by immobilized laccase on nanoparticles. Laccase from T. versicolor was used. Laccase was immobilized on nanoparticles coated with chitosan using EDC.HCl. Reactions were measured in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0). Dye decolorization by free carrier was also measured. Dye concentrations were 0.03 mg dye mL^{-1} for Direct Blue 78 and Acid Blue 74, 0.04 mg dye mL^{-1} for Reactive Red 195, 0.01 mg dye mL^{-1} for Phenol Red and 0.125 mg dye mL^{-1} for Acid Blue 225. Measurements were repeated three times for each dye. Error bars were less than 7.2 %.*

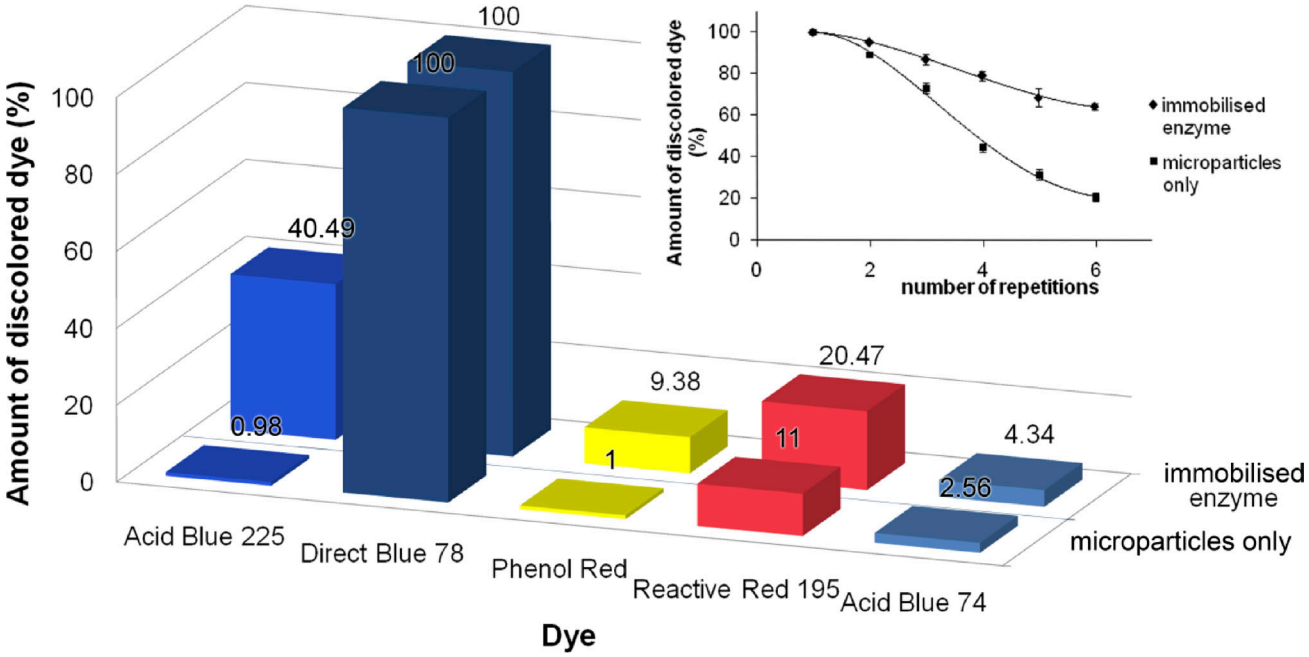

Fig. 6 – *Comparison of the amounts of various dyes decolorized by immobilized laccase on microparticles. Laccase from T. versicolor was used. Laccase was immobilized on microparticles coated with cellulose using $NaIO_4$. Reactions were measured in a buffer (0.1 mol L^{-1} Na-acetate, pH 5.0). Dye decolorization by free carrier was also measured. Inset of Fig. 6 shows decolorization of dye Direct Blue 78 on the number of repetitions for immobilized laccase and for microparticles only. Dye concentrations were 0.03 mg dye mL^{-1} for Direct Blue 78 and Acid Blue 74, 0.04 mg dye mL^{-1} for Reactive Red 195, 0.01 mg dye mL^{-1} for Phenol Red and 0.125 mg dye mL^{-1} for Acid Blue 225. Measurements were repeated three times for each dye. Error bars were less than 4.2 %.*

dyes by free laccase was calculated for the dye Acid Blue 225. In this case, for the successful measurement of the reaction, it was necessary to reduce the amount of laccase ten times compared to the comparative enzyme amount (1.25 mg enzyme mL^{-1}). This reaction thus corresponds to the argument that anthraquinone dyes are good substrates for laccase[36]. The structure of dyes strongly influences their degradability by laccase[37]. The chemical structure of dyes in general is comprised of a conjugated system of double bonds and aromatic rings[38]. Electron-donating groups enhance the susceptibility of the dye towards oxidative attack. This could be the reason why Acid Blue 225 was more degraded than the other dyes[39]. Weak degradation rate between diazoic compounds could rarely be imputed to the presence of the sulfonic and chloro substituents[40]. The second highest amount of dye decolorized by free laccase was calculated at dye Direct Blue 78. The azo dyes are substrates, which have disproportionately lower initial decolorization rate[36]. When this statement and our used azo dye Reactive Red 195 with Direct Blue 78 were compared, 90.15 % of decolorized azo dye Direct Blue 78 is quite effective. Azo dye Reactive Red 195 was decolorized, but only by 20.52 %. Other dyes (Acid Blue 74 and Phenol Red) were decolorized by more than half. The highest amount of decolorized dye was measured in decolorization by immobilized laccase on nanoparticles at anthraquinone dye Acid Blue 225. Other dyes were less than 50 % decolorized. The differences in decolorization of these dyes between nanoparticles and microparticles could be caused by the different methods of immobilization. Laccase was bound on nanoparticles by carboxyl group and on microparticles by amino group. This can lead to different enzyme orientation on nano- and microparticles. Adsorption of dye on an empty carrier also contributed to decolorization. The highest adsorption contributing to decolorization of dye on nanoparticles was calculated for dye Direct Blue 78. In this case, 27.21 % of dye was adsorbed on magnetic carrier. When testing decolorization by immobilized laccase on microparticles, complete adsorption of dye Direct Blue 78 on carrier occurred several times. Inset of Fig. 6 shows the dependence of the amount of decolorized dye (Direct Blue 78) on the number of repetitions. This dependence also shows the contribution of immobilized laccase to decolorization of this dye. Other dyes were not decolorized at half the amount of dye in the solution. In a number of publications, different dyes were tested for decolorization by laccase. Laccase for decolorization originated from different organisms and was immobilized on different carriers. Dyes identical to our dyes were[9,41,42] Acid Blue 225, Phenol Red and Acid Blue 74. With laccase from *T. versicolor*, only two dyes were decolorized: Phenol Red and Acid Blue 74. Phenol Red was decolorized by free laccase at 33 % after 120 hours. When me-

diator was used, the amount of decolorized dye increased to 43 % after 72 hours[9]. This result is very different from almost 58 % of decolorized dye after 60 minutes in our measurement. Indigoid dye Acid Blue 74, also known as Indigo carmine, was used for decolorization by immobilized laccase from *T. versicolor*. Laccase was immobilized on silica beads and was decolorized at 85 % the amount of this dye. It was only 34 % with free laccase. The large amount of dye decolorized by immobilized laccase was caused by adsorption of dye on the carrier. After standardization of the amount of used enzyme, the values were similar, about 70 % after 3 hours[42]. In our measurement, the dye Acid Blue 74 was decolorized at 68.5 % after 2 hours by free laccase. The immobilized laccase showed a significant loss in activity and it decolorized max. 2 % amount of dye. The amount of decolorized dye decreased with duration of the enzyme catalyse and the dependence on time had a hyperbolic character. This phenomenon could have been caused by accumulation of the product of the decolorizing reaction, which inhibited the laccase reaction. It could have been also caused by loss of concentration gradient of dye, therefore more difficult movement of dye to laccase[10]. Chitosan composites also adsorb metal ions[41]. After immobilization, this could lead to the release of copper atoms in laccase and decrease its activity. In our measurement, 14.9 – 2890 ng dyes mg^{-1} nanoparticles was adsorbed on nanoparticles (depending on the dye), and 3.96 – 1174 ng dyes mg^{-1} microparticles on microparticles (depending on the dye). Adsorption of dyes on carriers is different from used carrier and used dye. It depends on size of dye, its charge in different pH and size of pores and surface of carrier. On chitosan composites were adsorbed 30 – 1912 μg dyes mg^{-1} carriers[43]. In decolorization of dyes, mediators are often used. They mediate electron transfer between the dye and the enzyme. Using mediators can increase the amount of decolorized dye[7], can allow decolorization of other dyes[44] or possibly might not have any influence on the reaction[8]. In our measurement, a mediator was not used, because the use of a mediator in the decolorization process in textile industry wastewaters would not be appropriate due to the addition of other substances to this water.

Conclusion

Laccase from *T. versicolor* was immobilized on magnetic nano- and microparticles. Some properties of free and immobilized laccase were characterized (Michaelis constant, pH optimum and thermal stability or operational stability). Because immobilization did not significantly improve the attributes of the enzyme, the advantage of immobilization remains in the possibility of repeated use of the enzyme and easy handling of the enzyme. Laccase was able to decolorize some colors considerably. The amount of decolorization by the immobilized laccase was lower, and was also caused by partial adsorption of the dye on the carrier. Using immobilized laccase on magnetic carriers therefore significantly enhances manipulation of the enzyme when decolorizing wastewater.

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