Immobilization of laccase from *Trametes versicolor* on Lifetech™ supports for applications in degradation of industrial dyes

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Abstract

In this study, immobilization of laccase from *Trametes versicolor* on eight Lifetech™ supports, with different characteristics (pore size, length of the spacer arm and functional groups), was studied and optimized for intended use in bioremediation for decolorization of industrial wastewaters. Out of six tested amino-functionalized supports, the most promising carrier was proved to be porous Lifetech™ ECR8309F with primary amino groups and a C2 spacer arm. Onto this support, laccase is attached by forming electrostatic interactions so that the most active preparation has shown the activity of 66876 U/g support. On the other hand, during immobilization of laccase on epoxy-functionalized Lifetech™ ECR8285F, *via* hydrophobic interactions and covalent bonding confirmed by a desorption assay, immobilization yield of 60 % and the activity of 118929 U/g were accomplished. Furthermore, immobilized enzyme on this support showed high capacity for decolorization of dyes (Lanaset® Violet B, Lanaset® Blue 2R, bromothymol blue and bromocresol green), by combination of both adsorption and enzyme degradation. Decolorization was in the range of 88 to 96 % after 4 h, with more than 80 % achieved after only 45 min. Also, this preparation demonstrated high operational stability during seven consecutive reuses in all examined dye reaction systems.

Keywords: enzyme immobilization; bioremediation; textile dyes; decolorization, wastewater treatment

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1. INTRODUCTION

Laccases (EC 1.10.3.2) are enzymes, which catalyze oxidation of various organic substrates while reducing molecular oxygen to water by a one-electron transfer mechanism. These enzymes, known as blue copper oxidases, belong to the family of multicopper containing oxidoreductases [1,2]. Laccases are predominantly produced by wood-rotting fungi, such as *Trametes versicolor*, but they were also found in some lichens, bacteria, prokaryotes and higher plants, usually as extracellular enzymes [3].

Considering the great variety of substrates that laccase can transform, possibilities for their use in industrial processes include utilization in the paper industry for decolorization of the pulp by lignin degradation; removal of different xenobiotics and pollutants from waste waters originating from different industries including food industry (distillery, beverages, brewery); bioremediation of contaminated soil by dechlorination; degradation of polycyclic aromatic hydrocarbons (PAHs) or persistent organic pollutants (POPs); decolorization of industrial dyes in the textile industry, and many other applications [1,4,5].

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For elimination of synthetic, industrial dyes, like azo dyes, triphenylmethane, and anthraquinone, preferable processes are based on the biological method using microorganisms or their enzymes (laccases, peroxidases or azoreductases) [6]. Due to their complex aromatic structures, these dyes are resistant to light, moisture and oxidants, which is a favorable feature for industrial utilization, but they are also carcinogenic, mutagenic and toxic, therefore dangerous for the environment. Synthetic dyes present in water cause a decrease in aquatic bio-diversity by preventing the sunlight passing through the water, and also by creating obstacles for photosynthetic aquatic plants and algae. Dyes may also accumulate in sediments, and subsequently reach the groundwater system by leaching [7].

In order to develop sustainable biocatalytic processes for industrial applications, enzymes have to be immobilized on adequate supports. Immobilized enzymes exhibit increased stability at mostly non-favorable process conditions, and can be reused continuously and physically separated from the reaction media [1,8,9]. The enzyme immobilization process includes selection of the appropriate carrier and the immobilization technique that will provide high immobilized enzyme activity during prolonged application. Laccases have been, so far, successfully immobilized on different supports, such as: molecular sieves, silica gel, magnetic-chitosan, conjugated methacrylate-acrylate and kraft-lignin microspheres, glass beads, carbon nano-tubes, carbon paste, chitosan, graphite powder, modified fumed silica nanoparticles and others [10–17].

In this study, eight carriers from a Lifetech™ series (Table 1), with different polarities, porosities and functional groups, exhibiting good mechanical properties, chemical resistance and facilitated separation and regeneration after use, were investigated in immobilization of laccase from *T. versicolor*. Six amino-functionalized supports from this series were compared, which differ in pore diameters, types of amino groups (primary, tertiary, quaternary) and lengths of alkyl chains (spacer arms) to which the amino group is attached to the support surface. For the most promising support from this group, crucial immobilization factors, such as pH, enzyme/support ratio and immobilization time were optimized. Two porous supports, one hydrophobic and one epoxy-functionalized support from the Lifetech™ series, were also investigated, for possible hydrophobic and covalent binding immobilization, respectively, followed by application in degradation of industrial dyes. In order to optimize the covalent immobilization of laccase on the epoxy-functionalized support, activity of the immobilized enzyme, specific activity, protein loading and immobilization yield were investigated. Additionally, the type of enzyme-epoxy carrier attachment was also examined. The immobilized preparations with the highest enzyme activity were applied in decolorization of 4 selected dyes: Lanaset® violet B, Lanaset® blue 2R, bromothymol blue and bromocresol green. As it can be seen from Table 2, selected laccase substrates are representatives of two compound classes with substantially different chemical structures, that is Lanaset® dyes have an anthraquinone scaffold and the other two belong to sulfonephthalein dyes. Anthraquinone dyes (including the investigated Lanaset® violet B and Lanaset® blue 2R) are, after azo dyes, most commonly used dyes in textile industry. Sulfonephthalein dyes, including bromothymol blue and bromocresol green, have variety of uses - as pH indicators and textile dyes [18]. All the selected dyes, on one hand, have a great commercial importance while, on the other, they have a complex aromatic chemical structure, presenting organic pollutants in the environment. Degradation of these dyes was so far investigated by different methods, but, to the best of our knowledge, degradation by immobilized laccase, was not yet reported [19,20].

2. MATERIALS AND METHODS

2. 1. Materials

Laccase from *Trametes versicolor* (powder, ≥0.5 U/mg) obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) was applied in the present study. Syringaldazine (98 %, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a laccase substrate. Lifetech™ ECR resins (Table 1), which were kind donation from Purolite® (South Wales, UK), were used as immobilization supports.

Coomassie brilliant blue G-250 (Fluka Analytical, Switzerland) was used for preparation of the Bradford reagent, while bovine serum albumin (BSA, powder, ≥96 %, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a standard protein.

Table 1. Selected Lifetech™ supports and their main characteristics [21]

No.	Lifetech™ product	Type of support	Pore diameter, nm
1.	ECR8305F	Amino C2 methacrylate	30-60
2.	ECR8309F	Amino C2 methacrylate	60-120
3.	ECR8404F	Amino C6 methacrylate	30-60
4.	ECR8409F	Amino C6 methacrylate	60-120
5.	ECR1508F	Styrene tertiary amine	n.a.
6.	ECR1604F	Styrene quaternary amine	n.a.
7.	ECR8806M	Octadecyl methacrylate	50-70
8.	ECR8285F	Epoxy/butyl methacrylate	40-60

Anthraquinone dyes used in this work, Lanaset® Violet B and Lanaset® Blue 2R, were obtained from Huntsman Textile Effects (Langweid am Lech, Germany) while sulfonephthalein dyes, bromothymol blue and bromocresol green, were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2. 2. Methods

2. 2. 1. Enzyme immobilization procedure

At the beginning, Lifetech™ ECR carriers (Table 1) were preconditioned using the immobilization buffer in accordance to the manufacturer's recommendation. Laccase immobilization was conducted in 2 mL Eppendorf® tubes on a roller-shaker (Stuart, Paris, France) at 25 °C. For all experiments, 20 mg of carrier was incubated with 1 mL of the enzyme solution (predefined concentration of enzyme in the immobilization buffer). For all amino functionalized supports 0.1 M buffer was used while immobilization onto octadecyl and epoxy/butyl functionalized carriers (ECR8806M and ECR8285F, respectively, Table 1) was conducted in 1 M buffer. After a predefined time, supernatant was separated from the carrier particles and assayed for protein concentration, while immobilized laccase was washed twice with 1 ml of the immobilization buffer and subjected to enzyme activity determination. The experiments were performed in triplicates.

2. 2. 2. Determination of enzyme activity and protein concentration

Activity of free and immobilized laccase was determined spectrophotometrically (Ultrospec 3300 pro, Amersham Biosciences, Freiburg, Germany) and calculated in accordance to the manufacturer's standard procedure by using syringaldazine as a substrate [22]. The assay mixture consisted of 300 µL of substrate (0.216 mM syringaldazine dissolved in methanol), 2.2 mL of 0.1 M potassium phosphate buffer, pH 6.5 and 0.5 mL of freshly prepared enzyme solution in cold deionized water (25 U/mL - 50 U/mL) in the case of free laccase or an appropriate amount of the immobilized laccase. Distilled water was used as a blank. Oxidation of syringaldazine by free or immobilized laccase was performed at 30 °C and change in the absorbance relative to the blank sample was monitored at 530 nm every 30 s during the experimental period (3 to 10 min). One unit (U) will produce a change in absorbance at 530 nm of 0.001 per min at pH 6.5 at 30 °C in a 3 mL reaction volume using syringaldazine as the substrate. Immobilization yield was calculated as the percentage ratio of the amount of immobilized proteins and the initial protein amount in the solution. According to the established procedure, the Bradford method was used for determination of the protein concentration in the solution [23].

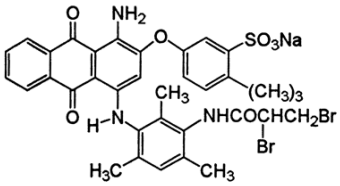
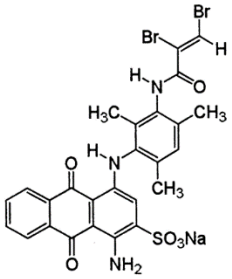
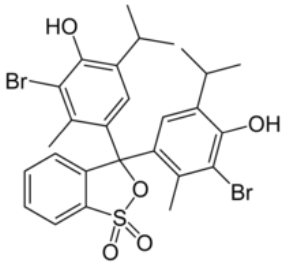
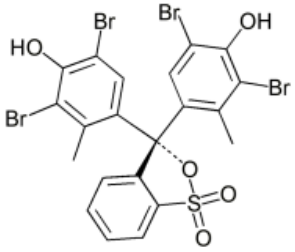
2. 2. 3. Desorption assay

Two step desorption of laccase immobilized onto the carrier with epoxy/butyl functional groups (ECR8285F, Table 1) was performed. At the beginning, the immobilized enzyme was incubated in 1 M $CaCl_2$ solution at 25 °C for 1 h after which period, the concentration of proteins in the supernatant was determined, while the carrier particles were washed two times with 1 mL of the immobilization buffer. Next, the immobilized laccase was treated with 1 % Triton™ X-100 solution at 25 °C for 1 h and thereafter the concentration of desorbed proteins was determined.

2. 2. 4. Dye decolorization

Dye decolorization was performed by laccase immobilized onto epoxy/butyl functionalized support (ECR8285F, Table 1). After the immobilization procedure described above and performed under optimal conditions, the supernatant was separated, and the immobilized enzyme was washed twice with 1 mL of distilled water. 1.5 mL of each dye solution (40 mg/L in 0.1 M citrate buffer pH 5.0) was added to each of the four tubes with immobilized laccase. Control samples containing the same amount of support without the enzyme and dye solution were prepared in order to investigate the effect of dye adsorption onto the carrier. At certain time intervals, the color change of the sample was monitored spectrophotometrically, measuring the absorbance at λ_{max} for each color, as given in Table 2. All experiments are performed in triplicates.

Table 2. Characteristics of the selected dyes

Dye	Type	Structure	λ_{max} / nm	Molar mass, g mol^{-1}
Lanaset® violet B	anthraquinone		590	823.52
Lanaset® blue 2R	anthraquinone		628	685.32
Bromothymol blue	sulfonephthalein		440	624.38
Bromocresol green	sulfonephthalein		614	698.01

The decolorization degree was calculated as a percentage ratio of the absorbance at the certain time and the initial absorbance of the dye solution.

In order to investigate immobilized laccase recycling potentials, the immobilized preparation was used in 7 consecutive reaction cycles (lasting 1 h) of dyes decolorization. After each cycle, the dye solution was removed and the absorbance was measured, while the immobilized enzyme was washed twice with the immobilization buffer. The same amount of dye stock solution was added to the tubes to initiate the next cycle. The experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

Enzyme immobilization onto solid supports is one of the most commonly used enzyme immobilization techniques, and depends on the available surface for enzyme attachment and on the type of interaction forces between introduced functional groups on the carrier surface and those on the surface of the enzyme molecule [24]. If the support is porous, the increased surface area can lead to higher protein loadings per unit mass of the support, in the case when immobilization occurs inside the pores as well [25]. In order to achieve unrestrained substrate diffusion to the enzyme active site and better enzyme flexibility within the pores, the pores should be sufficiently large [26]. Adsorption is the most commonly used, non-expensive and relatively simple method, which does not require enzyme modification. Interactions between the enzyme and the carrier can, in this case, be ionic or hydrophobic, depending on the chemical structure of the surface of the support and also of the enzyme [27]. A limitation of this technique is possible unfavorable enzyme leaching during immobilization and utilization, due to the above mentioned, weak forces of interaction, so that adsorption is used in large scale processes involving inexpensive enzymes [28]. In order to enhance the enzyme binding, the support surface can be functionalized with a variety of chemical groups, such as aldehyde, epoxide, carboxyl and amino groups, leading to immobilization by covalent binding [24,25]. For the optimal enzyme-support attachment, immobilization conditions, such as reaction time, pH value, temperature and the choice of buffers should be optimized [24].

3. 1. Immobilization onto amino-activated supports

Lifetech™ amino-activated supports that are selected in the present study for laccase immobilization are resins, solid spheres, with different pore sizes and functional groups (Table 1).

Activity of the immobilized laccase on these supports is presented in Figure 1. It could be concluded that there is a significant influence of the support functionality and polarity, as well as porosity on the immobilized enzyme catalytic activity.

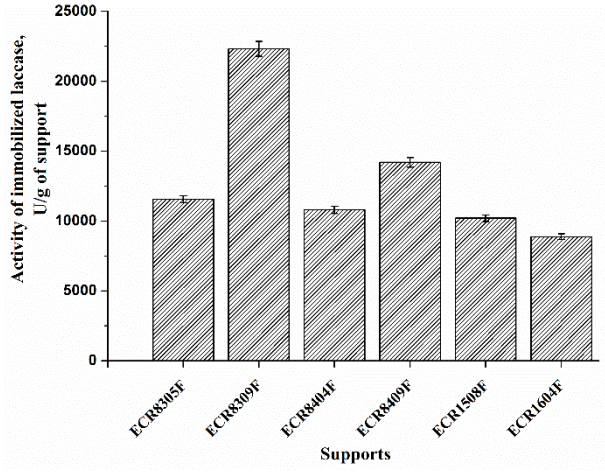


Figure 1. Comparison of various amino-functionalized Lifetech™ supports with respect to the activity of immobilized laccase from T. versicolor. The experimental conditions for laccase immobilization on all tested amino-functionalized supports: the initial protein concentration 2 mg/g, the immobilization time 1 h and the immobilization buffer 0.1 M, pH 5.0. (data are average of n=3).

The lowest activities were obtained with moderately hydrophobic non-porous styrene based supports with tertiary (Lifetech™ ECR1508) and quaternary (Lifetech™ ECR1604) amino groups. Regarding the porous supports, higher activities were obtained with porous hydrophilic methacrylate carriers with primary amino groups. Among them, based on the results presented, the pore size influence on the activity was more pronounced than the functional group spacer arm length (C2 and C6). Since laccase from *T. versicolor* is a protein of approximately 70 kDa and rather small dimensions, 6.5×5.5×4.5 nm [29], it can be assumed that the immobilization is carried out also within the pores, both in smaller and larger pores. Diffusion of substrate molecules into larger pores presumably led to the facilitated and more efficient access to the active sites of laccase molecules immobilized within the pores, since the highest activities were obtained for supports with larger pores in the size range 60 - 120 nm (Lifetech™ ECR8309F and ECR8409F). The highest activity of the immobilized preparations was determined for the porous support with larger pore size and shorter spacer arm C2

(Lifetech™ ECR8309F), which provided shorter distance between the carrier surface and the laccase molecule, resulting in the activity of 22320 U/g support. This value is ~40 % higher as compared to those of all other investigated preparations based on the amino-activated supports. Therefore, Lifetech™ ECR8309F was used for further examination, as the most promising support for laccase immobilization by adsorption.

For the selected support (Lifetech™ ECR8309F), the optimal immobilization parameters were next determined, namely the immobilization buffer pH, immobilization time and the initial protein concentration. Based on the available data in literature, it is known that fungal laccases such as this from *T. versicolor*, have the isoelectric point in the range 3.0 to 7.0, usually around 4.0 [30]. Moreover, if we have in mind the fact that the selected support (Lifetech™ ECR8309F) has primary amino groups on the surface, at suitable pH values, electrostatic interactions between negatively charged amino acid residues at laccase molecule surfaces and positively charged amino functional groups at the support could be expected. As a result of these assumptions, influence of pH on the immobilization process was studied in the same pH range, and the obtained results are shown in Figure 2A.

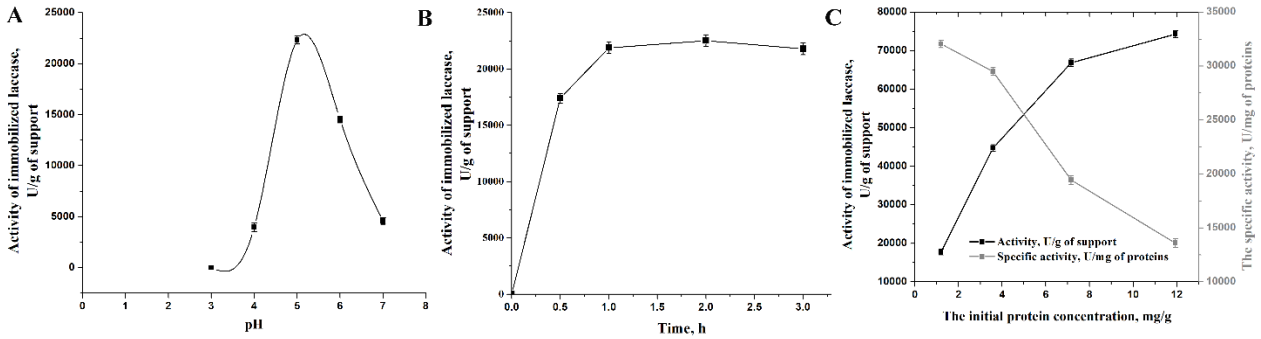


Figure 2. Optimization of laccase immobilization on the ECR8309F support regarding the obtained activity: A) the effect of pH; B) the effect of the immobilization time (at pH 5); C) the effect of the initial protein concentration (data are average of n = 3).

It can be seen that the immobilized laccase expressed the highest activity when the immobilization was performed at pH = 5.0, while changes from this value induced a steep drop in activity. Since it is well known that the enzyme surface is predominantly negatively charged at solution pH values that are above the isoelectric point (pI) of the enzyme, establishment of electrostatic interactions with positively charged primary amino groups at the carrier is enabled at certain suitable pH values, as well as attachment of enzyme molecules in their active conformation. For pH values below 5, very rapid activity decrease was detected, probably due to the dominant effect of repulsive electrostatic forces, which disabled formation of enzyme-support complexes. At pH values above 5, somewhat slower activity decrease was observed, which could be attributed to less favorable attachment of laccase molecules to the carrier surface as compared to that at pH = 5. Therefore, based on the shape of the immobilization pH profile, and under the assumption that electrostatic interactions adequately formed in the narrow pH range, only, the pH value of 5.0 was adopted for further studies.

The second investigated parameter was the immobilization time, and the obtained results are presented in Figure 2B. The maximal activity of immobilized laccase of 22320 U/g was achieved after 1 h, with 80 % reached after only 30 min, while further prolonging of the immobilization process did not result in higher activities. The immobilization time of 1 h was adopted as the optimum and used for further experiments.

For complete optimization of the immobilization process, the optimal initial protein concentration was evaluated by monitoring the activity of immobilized laccase and specific activity (Fig. 2C). The initial protein concentration was varied in a range of 1 - 12 mg/g of the support. The obtained results indicate that the increase in the initial enzyme concentration induces also the increase in the activity of immobilized laccase, which is linearly proportional up to the initial protein concentrations of 7 mg/g support. Above this value, the increase in activity was only 10 %, namely from 66876 U/g support to 74289 U/g support, indicating that further increase in the initial protein concentration does not lead to a proportional increase in activity. However, the specific activity (U/mg of bound proteins), as a parameter that best describes the catalytic efficiency of enzyme molecules attached to the support, decreased constantly with the

increase in the initial protein concentration (Fig. 2C). This result implies that at higher initial enzyme concentrations the enzyme molecules are immobilized deep inside the pores, and so less accessible to substrate molecules. These findings are in accordance with results published in literature, describing successful laccase immobilization onto porous and amino-modified supports by adsorption, such as siliceous ordered mesoporous materials (OMM), or modified fumed silica nanoparticles [14,31].

From all presented results, and having in mind that the industrial applications need immobilized preparations of the lowest possible cost, the initial enzyme concentration of 7 mg/g support was selected as the optimal for immobilization of laccase from *T. versicolor* on amino-activated Lifetech™ ECR8309F support.

3. 2. Immobilization onto hydrophobic and epoxy-activated supports

After the investigation and optimization of laccase immobilization on amino-activated supports, similar experiments were performed with two other types of Lifetech™ carriers, epoxy and octadecyl-activated (Lifetech™ ECR8285F and ECR8806M, respectively, Table 1). These two supports, in the form of porous solid spheres, differ from the previously investigated in the type of possible carrier-enzyme interactions. The octadecyl-activated support provides the possibility for enzyme attachment *via* hydrophobic interactions while the moderately hydrophobic epoxy-activated support, along with hydrophobic interactions, allows formation of covalent bonds, which is preferred over electrostatic interactions in adsorption. The results obtained for the immobilization of laccase onto these two supports are presented in Figure 3.

Expectedly, the preparation with laccase immobilized on epoxy/butyl methacrylate support (Lifetech™ ECR8285F) had more than three times higher activity than the preparation based on the octadecyl methacrylate support (Lifetech™ ECR8806M). Epoxy-functionalized supports allow multipoint covalent binding of enzymes, where covalent linkage can be established with different protein groups (amino, thiol, phenolic) [25]. The carrier Lifetech™ ECR8285F, however, has a unique combination of epoxy groups for covalent binding, and a very hydrophobic scaffold of butyl methacrylate.

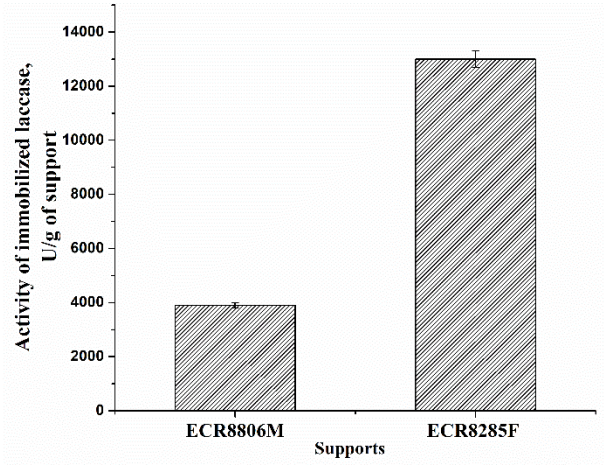


Figure 3. Activity of the immobilized laccase from T. versicolor on hydrophobic (Lifetech™ ECR8806M) and epoxy-functionalized (Lifetech™ ECR8285F) supports (data are average of n=3). The experimental conditions for laccase immobilization on hydrophobic and epoxy-functionalized support: the initial protein concentration 2 mg/g, the immobilization time 1 h and the immobilization buffer 1M, pH 5.0.

The main idea was to try to develop the preparation in which laccase will be covalently attached to the support with the enhanced activity while preventing the enzyme leaching. Also, these are the crucial features of an immobilized preparation for continuous industrial application. By comparing the results obtained during preliminary screening of Lifetech^{TM} supports which are presented in Figs. 1 and 3 it could be seen that the immobilized laccase on the epoxy/butyl methacrylate support Lifetech^{TM} ECR8285F retained high activity in comparison to the other tested amino-functionalized supports on which laccase was immobilized only by adsorption. Only in case of the amino-functionalized support Lifetech™ ECR8309F 1.5-folds higher activity than in case of laccase immobilized on Lifetech™ ECR8285F is obtained (Figs. 1 and 3). However, the possible covalent immobilization of laccase on the Lifetech™ ECR8285F support implies that this support could be promising which is the main reason that the epoxy/butyl methacrylate support Lifetech^{TM} ECR8285F was selected for further optimization of laccase immobilization. These results are also in accordance with other similar studies where laccase was covalently attached to the carrier [16,32]. In order to determine

optimal conditions for laccase immobilization onto epoxy/butyl methacrylate support LifetechTM ECR8285F, the optimal initial enzyme concentration was evaluated by monitoring the protein loading and protein immobilization yield as well as the activity and specific activity of the immobilized laccase, as in the previous experimental series when the optimal conditions for laccase immobilization on amino-functionalized support Lifetech™ ECR8309F were determined (Fig. 2), and the obtained results are presented in Figure 4.

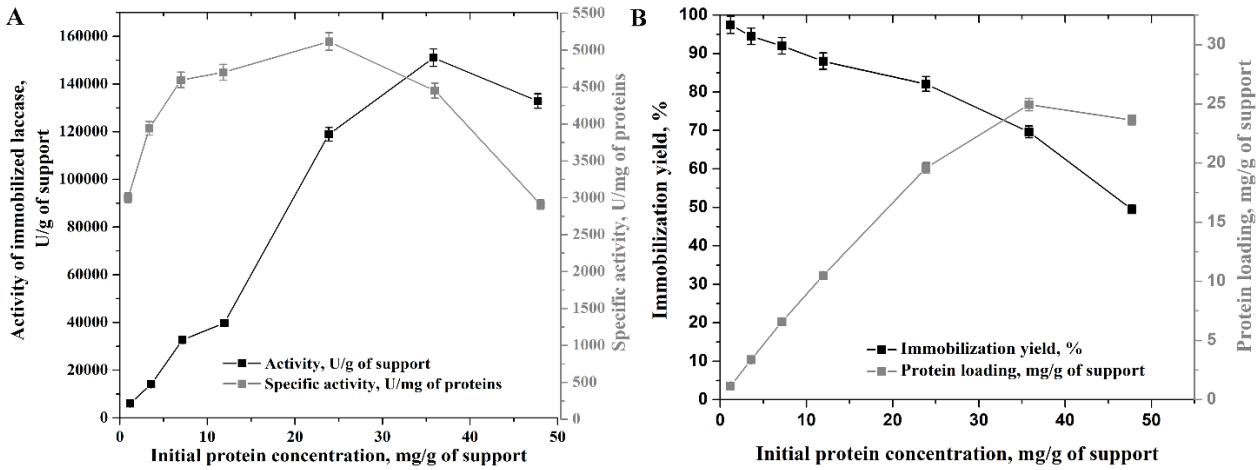


Figure 4. Optimization of laccase immobilization on Lifetech™ ECR8285F support: A) the effect of the initial protein concentration on the activity of immobilized laccase; B) the effect of the initial protein concentration on the immobilization yield and protein loading. (data are average of n=3).

The initial protein concentration was varied in a wide range of 1 - 48 mg/g of support. From the results obtained (Fig. 4A), a steep increase in activity of immobilized preparations can be observed for initial enzyme concentrations up to 24 mg/g support. Further increase in offered proteins (up to the enzyme concentration of 36 mg/g support) resulted in minor increase in activity of the immobilized laccase (up to 26 %). The initial protein concentration increases up to 48 mg/g support even led to a small decrease in preparations activity.

A similar trend can be observed for the specific activity, which is increasing with the increase in the initial protein concentration up to 24 mg/g support reaching the maximum after which the specific activity starts to decrease.

Influence of the initial protein concentration on the protein loading and the immobilization yield was also investigated in the same concentration range (1 - 48 mg/g support) (Fig. 4B). Increase in the initial protein concentration has an opposite effect on the immobilization yield as compared to the concentration of bound proteins. Up to 8 mg/g support of offered proteins the percentage of bound enzyme molecules on Lifetech™ ECR8285F was very high, over 90 % and then it starts decreasing down to 50 % (Fig. 4B). On the other hand, the protein loading increased continuously with the increase in the offered protein concentration up to 36 mg/g support, while the further increase to 48 mg/g did not bring any improvement. Therefore, it can be estimated that the maximum binding capacity for laccase from *T. versicolor* on Lifetech™ ECR8285F support was 25 mg/g of support.

In summary, after determination of optimal conditions for laccase immobilization on Lifetech™ ECR8285F (Fig. 4) , it can be concluded that the initial enzyme concentration of 24 mg/g support provides an optimal immobilized preparation with the activity of 118929 U/g support, which is significantly higher as compared to the maximal activity (66876 U/g of support) obtained with the amino functionalized Lifetech™ ECR8309F carrier at initial protein concentration of 7 mg/g (Fig. 2).

In order to confirm the assumed interaction type between the enzyme and support, a desorption experiment was consequently carried out by using two desorption solutions. By treating the immobilized preparation with 1 M $CaCl_2$ solution, all enzymes attached by electrostatic interactions will be removed, hence, the residual immobilized preparation activity will be the result of attached molecules *via* hydrophobic interactions and covalent bonds. The obtained results showed that after the treatment of the immobilized preparation with this solution, the activity remained constant while proteins remained immobilized, implying absence of electrostatic interactions between laccase and Lifetech™ ECR8285F support. On the other hand, treatment with the Triton™ X-100 solution, should provide

information on enzyme molecules attached *via* electrostatic interactions and covalent bonding, since hydrophobically attached enzyme molecules will be removed. After the treatment with this solution, 68 % of bound proteins were desorbed. Since the absence of electrostatic interactions was proven, it can be concluded that 68 % of laccase molecules were attached to the Lifetech™ ECR8285F support by hydrophobic interactions, while remaining 32 % of the enzyme molecules was covalently attached to the support surface.

Since establishment of hydrophobic interactions and covalent bonds was confirmed, which are both preferred over electrostatic interactions, due to higher stability at process conditions, and higher accomplished activity as compared to that of the best amino functionalized support (Lifetech™ ECR8309F), further experiments were conducted by using laccase immobilized onto epoxy-butyl methacrylic support - Lifetech™ ECR8285F.

3. 3. Decolorization of industrial dyes

After optimization of the immobilization process of laccase from *T. versicolor* on the Lifetech™ ECR8285F support, oxidative activity of the immobilized laccase was investigated in decolorization of different industrial dyes. Industrial dyes are usually released as effluents either from synthesis plants or from dye-using industries, such as textile factories. The estimation is that 10 % to 15 % of the total dye used in a dyeing process can be detected in wastewater, out of more than 7×10^5 tons of the total dyes produced per year [33]. Several of types of dyes are very stable exposed to temperature, light and microbial attack, making them recalcitrant compounds [34].

In the present work four textile dyes with diverse structures (Table 2) were used to investigate potentials of the immobilized laccase preparation for industrial dye decolorization. Decolorization was monitored over 4 h (Fig.5).

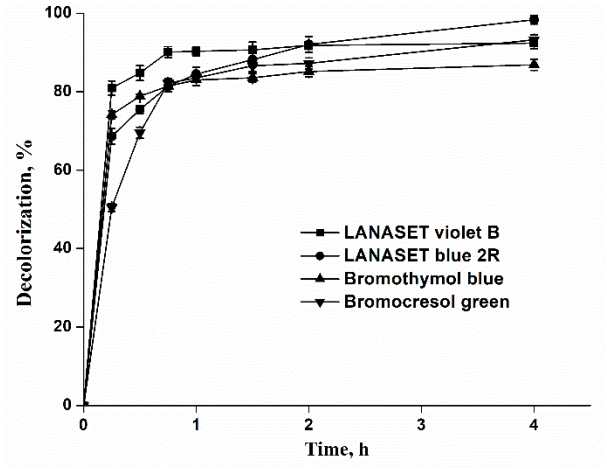


Figure 5. Application of laccase immobilized on the Lifetech™ ECR8285F support in decolorization of selected industrial dyes (data are average of n=3).

From the results shown, it can be seen that the immobilized preparation was very active, since the decolorization degree was in the range of 88 - 96 % after 4 h. More than 80 % decolorization is achieved after only 45 min, which confirms the assumption that the reaction was very rapid. Bleaching of dye samples by laccase immobilized on Lifetech™ ECR8285F support is presented in Figure 6. In order to compare effect of free laccase and immobilized preparation, the experiment in which the same activity (measured by syringaldazine assay) is introduced in reactions, was performed (Fig. S1). It is evident that, in both cases (free enzyme and immobilized preparation), similar decolorization profiles were obtained for each of four dyes. The immobilized preparation demonstrated even higher potency comparing to free enzyme (for example for bromocresol green), still this improvement does not originate only from laccase activation upon immobilization, but it could be attributed to the simultaneous effects of dyes adsorption onto free surface of the carrier particles and their enzymatic degradation.

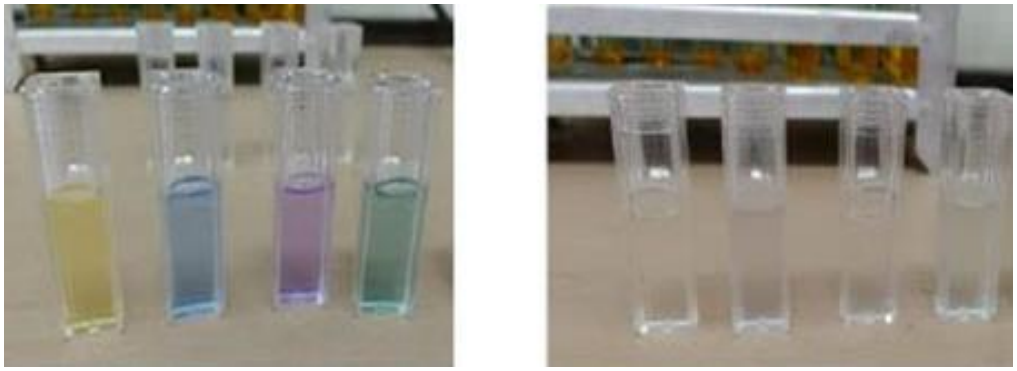


Figure 6. Complete decolorization of dyes bromothymol blue, Lanaset® blue 2R, Lanaset® violet B, and bromocresol green by laccase immobilized on the Lifetech™ ECR8285F support.

Since the obtained results suggest that the immobilized preparation can be successfully applied in decolorization of four selected dyes, possibility of its reuse in these reactions was further investigated in 7 reaction cycles, (Fig.7 A-D).

During the experiment it was confirmed that along with degradation of dyes by the immobilized preparation, adsorption of dyes on the Lifetech™ ECR8285F support occurs. Although adsorption process onto carrier particles with and without immobilized enzyme molecules is not completely identical, it could serve as an indicator of relative contributions of two processes to the overall decolorization effects. Therefore, both processes were monitored over time. The percentage of dye decolorization, as a result of both adsorption and degradation, after seven cycles remained very high, since the lowest decolorization was 58 % achieved for bromocresol green (Fig. 7D) and the highest was 80 % for Lanaset® violet B (Fig. 7A).

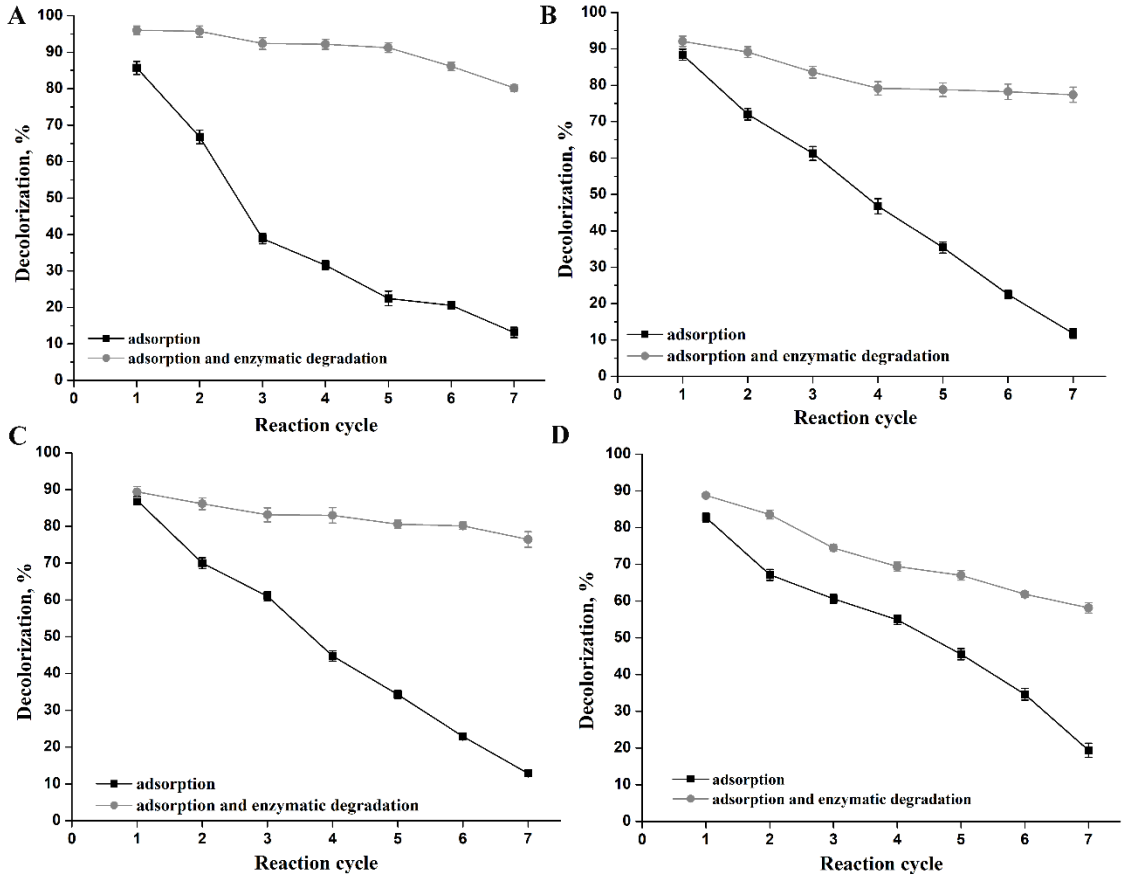


Figure 7. Use of the immobilized enzyme preparation on the Lifetech™ ECR8285F support in several reaction cycles of decolorization of: A) Lanaset® violet B, B) Lanaset® blue 2R, C) bromothymol blue, D) bromocresol green (data are average of n=3).

Adsorption of dyes onto the carrier was rapidly decreasing after each cycle, as a result of a finite carrier surface available for adsorption. When adsorption contribution was monitored during seven reuses in decolorization of Lanaset® violet B, Lanaset® blue 2R, bromothymol blue and bromocresol green decrease from 86, 88, 87 and 83 % to 13, 12, 13 and 19 % was detected in the four systems, respectively. Having in mind that the overall effect of adsorption and enzymatic degradation stayed high (58 to 80 % after the seventh reaction cycle), it is evident that the enzyme maintained high catalytic activity during the repetitive use. In fact, decolorization effect in all four systems during the prolonged application could be almost entirely attributed to enzymatic degradation and the future researches will be directed towards development of chromatographic methods for the quantitative analysis of degradation products which will give deeper insight into degradation mechanisms and kinetics. Good results for dye decolorization by either white-rot fungi or their enzymes, free or immobilized, can be found in literature, as well. Decolorization of malachite green dye by *T. versicolor* f. antarcticus was realized in a bioreactor and after 5 consecutive cycles, it reached 82 % [35]. With laccase immobilized onto porous PVA/HNTs hybrid beads and reactive blue as a model dye, decolorization due to adsorption and degradation was 41 % [36]. These results confirm that the obtained preparation has a significant operational stability and good prospects for economically sustainable use in the industrial wastewater treatment.

4. CONCLUSION

Immobilization of laccase from *T. versicolor* was successfully performed using six different amino-functionalized Lifetech™ supports, by the adsorption mechanism, and the highest activity of immobilized preparation was achieved for the porous Lifetech™ ECR8309F support with short C2 spacer arm and pore sizes of 60 - 120 nm. For this preparation, optimal parameters of the immobilization process were determined as the initial enzyme concentration of 7 mg/g support, 1 h immobilization period, and pH 5.0, resulting in the preparation activity of 66876 U/g support, with syringaldazine as a substrate. Furthermore, two more Lifetech™ supports, hydrophobic and epoxy-functionalized, were also investigated for possible hydrophobic and covalent enzyme binding. Laccase was successfully immobilized onto the Lifetech™ ECR8285F support, with the immobilization yield of 60 % and 32 % covalent binding and 68 % hydrophobic interactions proven by a desorption assay, at the optimal initial protein concentration of 24 mg/g support. This preparation exhibited the activity of 118929 U/g and was successfully used in decolorization of four selected structurally different industrial dyes, Lanaset® Violet B, Lanaset® Blue 2R, bromothymol blue and bromocresol green. By adsorption to the carrier and enzyme degradation, the solutions were decolorized in the range of 88 % to 96 % after 4 h, with more than 80 % decolorization happening in the first 45 min. After 7 reaction cycles, the immobilized laccase kept high catalytic activity indicating satisfying operational stability. Since the *T. versicolor* laccase immobilized onto the Lifetech™ ECR8285F support exhibited a high degradation rate and good reusability for selected industrial dyes, this preparation has a great potential for dye removal in wastewater.

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SAŽETAK

Imobilizacija lakaze producenta *Trametes versicolor* na Lifetech™ nosače u cilju primene u razgradnji industrijskih boja

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(Naučni rad)

U ovom radu je ispitivana i optimizovana imobilizacija lakaze producenta *Trametes versicolor* na osam *Lifetech*™ nosača, različitih karakteristika (kao što su veličina pora, dužina "nožice" i funkcionalne grupe), za predviđenu namenu u bioremedijaciji radi obezbojavanja industrijskih otpadnih voda. Od šest ispitanih amino-funkcionalizovanih nosača, kao najadekvatniji se pokazao porozni *Lifetech*™ ECR8309F nosač sa primarnim amino grupama i C2 "nožicom". Na ovaj nosač lakaza se vezuje formiranjem elektrostatičkih interakcija i dobijeni preparat je imao aktivnost od 66876 U/g nosača. Nasuprot tome, prilikom imobilizacije lakaze na epoksi/butil metakrilatni nosač *Lifetech*™ ECR8285F, hidrofobnim interakcijama i kovalentnim vezama, što je potvrđeno tretmanom imobilisanog preparata desorpcionim sredstvima, postignut je prinos imobilizacije proteina od 60 %, a dobijena je aktivnost od 118929 U/g. Pored toga, preparat ECR8285F-lakaza pokazao je veliku aktivnost u obezbojavanju boja (*Lanaset® Violet* B, *Lanaset® Blue* 2R, bromtimol plavo i bromkrezol zeleno) kombinacijom adsorpcije i enzimske razgradnje, u opsegu obezbojavanja od 88 % do 96 % nakon 4 h, sa više od 80 % obezbojavanja postignutog nakon samo 45 min. Takođe, ovaj preparat je pokazao veliku operativnu stabilnost tokom 7 uzastopnih ponovnih upotreba u svim ispitanim reakcionim sistemima.

Ključne reči: lakaza, imobilizacija enzima, bioremedijacija, industrijske boje