Electrospun biosystems made of nylon 6 and laccase and its application in dyes removal

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Highlights

- Production of nylon 6 electrospun material
- Adsorption and covalent immobilization of laccase
- Evaluation of effect of operational condition on textile dyes removal
- The highest removal efficiencies of RB5 and RB4 were 63% and 77%, respectively

Abstract

Electrospun materials, due to the possibility of design of their properties, are suitable as supports for enzyme immobilization. Produced biocatalytic systems might be then apply in various biocatalytic reactions, including conversion of pollutants. In our study, electrospun fibers made from nylon 6 was produced, modified and applied as a support for laccase immobilization by adsorption and covalent binding. The systems with immobilized laccase were used in decolorization process of selected dyes, azo dye Reactive Black 5 and the anthraquinone dye Reactive Blue 4. It was found that at from dye solution at concentration 1 mg/L at pH 5, temperature 25 ºC, after 24 h of process the efficiency of decolorization of Reactive Blue 4 and Reactive Black 5 reached 77% and 63%, respectively. The storage stability studies showed that after 30 days of storage, the relative activities were 60% and 95% for adsorbed and covalently bonded oxidoreductase respectively. Moreover, after 10 consecutive catalytic cycles adsorbed and covalently bonded laccase retained over 60% and 70% respectively, indicating the possibility of application of the obtained systems on a larger scale for removal of phenolic pollutants from wastewaters.

Keywords

Electrospinning, laccase, enzyme immobilization, dye removal, azo dyes, anthraquinone dyes

1. Introduction

Fibers produced by the electrospinning method are currently among the most promising materials in various fields of science and industry. First of all, ultrathin electrospun materials can be produced from a wide range of polymers, copolymers, composites, biopolymers and ceramics (Kijeńska and Swieszkowski, 2017; Xue et al., 2019). Furthermore, various additives, such as carbon nanotubes, nanoparticles of metal oxides and/or metals (Agyemang et al., 2018; Xi et al., 2015; Kadam et al., 2018; Cao et al., 2017), can be used to improve their specific properties. It should also be noted that the possibility of changing the electrospinning parameters, such as flow rate, distance between collector and nozzle, or process time, and of using various apparatus set-ups, provides an opportunity to design tailor-made materials with precisely defined diameters and durability, which determine the potential applications of the fabricated electrospun materials (Xue et al., 2019).

Beside applications in medicine, energy storage, air purification and many others (Mirjalili and Zohoori, 2016), materials produced by electrospinning might also be successfully used in water treatment and as supports for enzyme immobilization. The great advantages of the electrospun fibers used as materials for biomolecule immobilization, are their large surface area-to-volume ratio, the presence of specific moieties and possibility to full control of their process production (Wang et al., 2009), what facilitate the attachment of biomolecules not only onto the surface of the fibers (Taheran et al., 2017) but also between electrospun layers (Haider et al., 2018) and into the fibers (Wen et al., 2017). It should be added that besides a high enzyme affinity, the open structure of the electrospun materials and relatively long distances between fibers reduce diffusional limitations and enable effective transport of substrates and products (Herricks et al., 2005; Zdarta et al., 2019a).

The increasing content of phenolic compounds in water, such as chlorophenols, pharmaceuticals, dyes or pesticides, particularly in surface waters, is a still unsolved global

problem (Bilal et al., 2019a, 2019b). These hazardous compounds are released from various branches of industry, such as pharmaceutical, wood, automotive or even cosmetic industries. Among these pollutants, effluents from the textile industry are the most serious threats to the environment due to the release of huge amounts of hazardous wastewaters after dyeing processes, since according to a 2019 European Parliament report, the production of 1 kg of clothes requires about 150 liters of water (Sajn, 2019). It should be noted that most textile materials and clothes are imported from Asian countries, where wastewaters are not properly treated, and hazardous compounds such as dyes and/or microplastic find their way into river and sea waters (Sajn, 2019). This affects not only the environment, but also animal and human health, causing gene mutations, cancers and various other diseases (Rawat et al., 2016; Tavares et al., 2019). Among the wide range of dyes used in the textile industry, azo and anthraquinone dyes deserve special attention. They are used on a large scale, and azo dyes are among the most frequently applied colorized chemicals, with annual use exceeding 50% of that of all textile dyes (Fernandes et al., 2015). Hitherto various methods of dyes removal were developed. One of the mostly used is adsorption due to its ease of carrying out and economic reasons (Forgacs et al., 2004). What is more, the systems with adsorbed dyes can be also applied as reusable pH sensors, what was presented by Silina et al. (2015). Nowadays various types of adsorbent can be distinguished, such as oxides, various polymers and biopolymers. However, in case of adsorption, the efficiencies of dyes decolorization strictly depend on maximum adsorption capacity of selected adsorbent and specificity of adsorbed pollutant (Uddin et al., 2019; Jiang et al., 2020). The other method dyes removal is photocatalysis. This method is expensive and required specific catalysts. Guang et al. (2020) used $LeFeO_3$/BiOBr as photocatalysts in decolorization process of Rhodamine B, and reached 98% of dyes removal. Despite high removal efficiency, this method is hard to use in industry due to complicated synthesis of photocatalysts and high cost of this process, its industrial application is limited. As alternative

for the above-mentioned methods, biological approach should be considered. There are reports concerning the removal of phenolic compounds, including dyes using biomethods, such as enzymatic decolorization and degradation (Bilal et al., 2019c; Bilal et al., 2020; Legerska et al., 2018). In our previous study, almost 100% of Alizarin Red S was removed from aqueous solution using laccase immobilized onto TiO_2-ZrO_2-SiO_2 oxide system. It clearly shows that laccase immobilized onto various supports can be effectively used as a tool for removal of phenolic compounds from aqueous solutions (Morsi et al., 2020; Barrios-Estrada et al., 2018). Further, it should be clearly stated that immobilized laccase could be also used for removal of other phenolic pollutants, including, phenols, bisphenols and pharmaceuticals. Liu et al. (2020), used laccase immobilized onto 3-D printing system made from sodium alginate-acrylamide-hydroxyapatite in removal of p-chlorophenol from aqueous solutions, whereas Qiu et al. (2020) used laccase immobilized onto magnetic nanoparticles modified by amino-functionalized ionic liquid for removal of phenol, 4-chlorophenol and 2,4-dichlorophenol with almost 100% efficiencies.

In view of the significant problems with the efficient removal of dyes from wastewaters, we decided to focus on enzymatic decolorization processes. Therefore, in the present study, for the first time, laccase from *Trametes versicolor* was immobilized on nylon 6 electrospun support material by means of separate adsorption and covalent binding with the use of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). The obtained biocatalytic systems were used in the removal of azo and anthraquinone dyes from model aqueous solutions. The effectiveness of fabrication of the electrospun material and attachment of the enzyme to both unmodified and modified electrospun material was investigated. Nevertheless, the most important part of the study was a detailed investigation of the effect of various process parameters, such as dye concentration, pH, temperature and process duration, on the efficiency of decolorization of the azo dye Reactive Black 5 and the

anthraquinone dye Reactive Blue 4 representing a novel scientific contribution of the work presented.

2. Materials and methods

2.1. Chemicals and materials

Nylon 6, laccase from *Trametes versicolor* (EC 1.10.3.2), the azo dye Reactive Black 5 (RB5), the anthraquinone dye Reactive Blue 4 (RB4), sodium acetate, phosphate and ammonium buffer solutions, 2,2-azinobis-3-ehylbenzothiazoline-6-sulfonate (ABTS), Bradford reagent, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (USA). 1,1,1,3,3,3-hexafluoro-2-propanol (≥99%) (HFP) was purchased from Flurochem Ltd. (UK). Characteristics of the textile dyes used in the study are given in Table 1.

Table 1

2.2. Fabrication of nylon 6 electrospun material

To produce electrospun fibers from nylon 6, pellets were dissolved in HFP to obtain 10% (w/v) solution and then mixed at room temperature for 24 h. After this time, the solution of nylon 6 was placed into a 5 mL syringe and electrospun under an applied voltage of 15 kV, with a feed rate of 1 mL/h and a distance of 150 mm from the tip to the aluminum foil-covered steel plate collector for 30 min. The process was carried out using a NANON-01A apparatus (MECC Co., Ltd., Japan). The nylon 6 electrospun materials were collected in the form of fibrous mats and dried in a vacuum drier (Memmert, Germany) at 25 °C, 50 mb for 24 h.

2.3. Laccase immobilization

For the immobilization of laccase by a covalent bond, a modification of nylon 6 was performed. This was done using EDC/NHS, according to the methodology described in our previous study,

with slight modification (Zdarta et al., 2019a). In this case *N*-(-3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in a molar ratio of 1:2 were dissolved in a phosphate buffer at pH 7. Next, nylon 6 electrospun materials were immersed in previously prepared solution and mixed for 3 h. In the next step, to perform adsorption and covalent immobilization of the enzyme, respectively, unmodified and modified pieces of nylon 6 material, 1 cm x 1 cm in size, were placed separately into a beaker with laccase solution at concentration 1 mg/mL, at pH 5. The immobilization process was carried out at 25 ºC for 24 h using a KS4000i incubator (IKA Werke GmbH, Germany). After this time the electrospun fibers with immobilized laccase were washed three times using distilled water to remove unbound enzyme. The biocatalysts prepared in this way were used in the next step of investigation.

2.4. Effect of pH and temperature on the activity of free and immobilized laccase and the quantity of immobilized enzyme

To investigate the effect of pH and temperature on the relative activity of native and immobilized laccase, experiments were carried out in the pH range 3–10 at 30 ºC and in the temperature range 10–70 ºC at pH 5, with measurements made every 10 ºC. The activity of the immobilized laccase was examined based on the model reaction with ABTS. Each experiment was conducted for 1 h using an ABTS as a substrate at concentration 0.05 mM, using free laccase and the products after immobilization, each containing 1 mg of enzyme. The relative activity of free and immobilized laccase was calculated based on spectrophotometric measurements. The changes in the absorbance of the solution before and after reaction were measured at wavelength 420 nm, using a UV-Vis Jasco V-750 spectrophotometer (Japan). 100% relative activity was taken to be the highest activity exhibited by free or immobilized enzymes.

To calculate the quantity of laccase immobilized on the electrospun fibers, the Bradford method was used (Bradford, 1976). The enzyme solutions after immobilization processes were mixed with Bradford reagent in a 1:1 ratio. After 5 min, the absorbance at 595 nm was measured and the quantity of immobilized enzyme was calculated using the bovine serum albumin calibration curve. The enzyme content was determined as the difference in the initial concentration of enzyme solution and the concentration of protein present in the supernatant after immobilization.

2.5. Decolorization of textile dyes

Decolorization of azo and anthraquinone dyes was carried out at selected dye concentrations (1, 5, 10 mg/L at pH 5 and 25 ºC), pH values (3–9 at concentration 5 mg/L and 25 ºC) and temperatures (5–45 ºC at pH 5 and concentration 5 mg/L), for various reaction times (1–24 h). To each dye sample 1 mg of laccase immobilized by adsorption or covalent binding was added, and the mixtures were placed in an IKA KS 260 Basic shaker (IKA Werke, Germany) for 24 h. In case of decolorization of Reactive Black 5, ABTS at concentration 0.05 mM was used as a mediator. The decolorization efficiency (*DE*, %) was calculated based on spectrophotometric measurements at wavelength 597 nm and 595 nm for RB5 and RB4 respectively, as the difference between selected dye concentration before (C_B) and after decolorization (C_A) and considering the initial dye concentration (Equation 1).

$$DE\ (\%) = \frac{C_B - C_A}{C_B} \cdot 100\% \quad (1)$$

2.6. Storage stability and reusability

To investigate the storage stability of the obtained biocatalytic systems and free laccase, they were stored for 30 days at 4 ºC in buffer solution at pH 5. The relative activity was studied using an above-presented model reaction with ABTS as a substrate every single day.

The reusability of laccase adsorbed or covalently bonded onto nylon 6 electrospun material was investigated over 10 consecutive catalytic cycles, based on the above-mentioned model reaction with ABTS. Each catalytic cycle means a reaction carried out for 1 h, at pH 5 and temperature 25 ºC. After each catalytic cycle, the obtained biocatalytic systems were separated from the reaction solution, washed several times with acetate buffer at pH 5 and placed into a new ABTS solution at an initial concentration of 0.05 mM. Moreover, after 10 catalytic cycles the amount of immobilized enzyme was measured by Bradford method. Each experiment was performed three times in a batch mode and from each one representative samples were taken. Results are presented as a mean value ±SD from three experiments.

2.7. Analytical procedures

Fourier transform infrared spectra (FTIR) of electrospun material before and after laccase immobilization were obtained using a Bruker Vertex 70 spectrometer (Bruker, Germany) in attenuated total reflectance (ATR). The mass contribution of selected elements present in nylon 6 and the obtained biocatalytic systems was analyzed by energy dispersive X-ray spectroscopy (EDS) using a Princeton Gamma-Tech unit with a prism digital spectrometer (UK). Scanning electron microscopy (SEM) photographs, obtained using an EVO40 scanning electron microscope (Zeiss, Jena, Germany), enabled determination of the morphology of nylon 6 with and without immobilized laccase. Furthermore, the SEM photographs were used for calculation of the average diameters of electrospun fibers. For this purpose, Image J analysis software (National Institute of Health, USA) was applied. The spectrophotometric measurements for calculation of the activity of immobilized enzyme, quantity of immobilized laccase, efficiency of decolorization of Reactive Black 5 and Reactive Blue 4, and storage stability and reusability of the immobilized enzyme were carried out using a JASCO V-750 spectrophotometer.

3. Results and discussion

3.1. Characterization of nylon 6 electrospun fibers before and after laccase immobilization

In the FTIR spectrum of nylon 6 (Fig. 1), bands with maxima at 3300 cm^{-1}, between 1700–1600 cm^{-1}, and 680 cm^{-1} corresponding to stretching vibrations of –NH, C=O and bending vibration of C–C bonds respectively, confirm the effective fabrication of electrospun fibers. This is in agreement with FTIR results obtained by Granato et al. (2009), who prepared nylon 6/polypyrrole nanofibers and noticed similar observations. Moreover, a broad peak between 3300–3000 cm^{-1} indicates the presence of stretching vibrations of –NH and –OH bonds in the spectra after laccase immobilization. The stretching vibration of –NH are related to used support, but also biomolecule attached to the nylon 6 electrospun fibers, whereas –OH bonds are associated with the enzyme structure. Although signals attributed to stretching vibrations of OH bonds might indicate the presence of water in the samples in Fig. 1, we cannot see the peak in 1630 cm^{-1}, characteristic for bending vibration of H_2O molecules, indicating the lack of physically adsorbed water in the systems with immobilized laccase. Nevertheless, it is worth adding that the presence of water in the reaction system has a large impact on the activity of laccase. As presented by Zaks and Klibanov (1988,1997), the activity of laccase increases in reaction environment where amount of water increases relative to organic solvent, what causes lack of diffusional limitation of substrates. This fact corresponds with high activity of laccase immobilized used in aqueous solutions in the presented work. Furthermore, the FTIR spectra of materials with immobilized laccase contain characteristic signals attributed to the biomolecule with maxima at 1645 cm^{-1}, 1545 cm^{-1}, 1250 cm^{-1} and 800 cm^{-1} corresponding to stretching vibrations of amide I, amide II, amide III and bending vibration of C–H bonds respectively (Zdarta et al., 2018).

The results of FTIR analysis indicating the effective immobilization of laccase on nylon 6 fibers are complemented by the results of EDS microanalysis of surface composition,

presented in Table 2. The higher mass contribution of elements such as oxygen, sulfur, chlorine, nitrogen and copper in the samples after immobilization, as compared to the nylon 6 material, proves unambiguously that laccase was successfully immobilized onto nylon 6 electrospun fibers. Similar results and conclusions were presented by Das et al. (2017), where laccase was effectively immobilized onto iron nanoparticles, after which a significant increase in amounts of nitrogen and sulfur was observed.

The results obtained unquestionably confirm the suitability nylon 6 electrospun material as a support for laccase immobilization. This is mainly due to the presence of $–NH_2$ and C=O groups on the electrospun support, which facilitate attachment of the enzyme to the nylon 6 fiber and prone its functionalization.

Figure 1

Table 2

The SEM photographs enabled determination of the average diameters of fibers of nylon 6 material and confirmed the effective immobilization of the enzyme on the fibers (Fig. 2). It is observed that fibers without immobilized laccase have diameters less than 1 μm; this corresponds with the results of the calculation of average fiber diameters of nylon 6 electrospun material, which gave a value of 784 ± 215 nm. For the nylon 6 fibers on which laccase was immobilized by adsorption or covalent binding, an increase in the average fiber diameter was observed, and the calculated values of the parameter were 1124 ± 239 nm and 1599 ± 850 nm respectively. Further, in the study the amount of immobilized enzyme was calculated. Laccase loading capacity for adsorbed enzyme was 282 mg/g, whereas for biomolecule covalently bonded it was 423 mg/g. In another study laccase was immobilized by adsorption onto electrospun fibers made from poly(l-lactic acid)-co-poly(ε-caprolactone). Similarly, as in this study, after enzyme attachment the average diameter of the fibers increased from 373 ± 127 nm to 430 ± 143 nm (Zdarta et al., 2019b). It should also be noted that not only

enzyme deposition increases the diameter of fibers. As reported by Liu et al. (2012), the modification of a silk surface with EDC and NHS linkers unquestionably affected the size of the fibers, as after functionalization the average fiber diameter increased by 200 nm. Furthermore, it should be considered that in the case of various supports, the area available for biomolecule immobilization may be located on the internal or external surface, depending on the porosity of the material. The structure of the support material also affects diffusion transport of biomolecules between pores and the external surface, and in consequence may affect biocatalyst binding (Datta et al., 2013).

Figure 2

3.2. Effect of pH and temperature on the activity of free and immobilized laccase

In our study, two types of immobilized laccase were used: laccase attached to electrospun nylon 6 fibers by adsorption and by covalent bonding. The type of interactions involved in attachment of an enzyme to a support can play a crucial role in enzyme activity and stability (Mehta et al., 2016). The activity of both immobilized systems and the native enzyme was compared under wide range of pH and temperature conditions, with the aim of selecting the best biocatalytic system for application in dye decolorization and to find suitable parameters for the catalytic reaction, and thereby to reduce future process costs (Patel et al., 2014).

Figure 3 presents graphs showing the effect of pH and temperature on the relative activity of immobilized laccase and its free form. It can be seen that the enzyme immobilized by both methods, adsorption and covalent binding, had higher relative activities than the native laccase over the whole investigated pH and temperature range. At the extreme pH values of 3 and 10, the native biomolecule exhibited relative activity of 62% and 14% respectively, compared with 86% and 38% for the adsorbed enzyme, and 92% and 55% for the biomolecule connected to nylon 6 by the EDC/NHS method. Besides the relatively good results for the

catalytic activity of immobilized laccase at extreme pH values, in the pH range 3–7 the biomolecule immobilized by both methods retained over 70% of its enzymatic activity. For instance, the native laccase exhibited only 40% relative activity at pH 7, whereas the forms immobilized by adsorption and covalent binding achieved relative activities of 70% and 87% respectively. These results show clearly that immobilization of the enzyme—regardless of the chosen method—improves its stability in harsh reaction conditions, compared with the free enzyme. This may be due to conformational changes in the biomolecule occurring after connection of laccase to the support. In the case of the adsorption method, non-specific forces such as hydrophobic or van der Waals interactions are involved, whereas in the covalent binding method the formation of covalent bonds is observed that stabilizes structure of the biomolecules, which in consequence leads to an increase in enzyme activity over a wide pH range, as compared to the native form (Mohamad 2015; Chen et al., 2020). In testing of the effect of temperature on the relative activity of the investigated forms of laccase, the highest relative activity of both the immobilized enzyme and its native form was recorded at 30 ºC. In the temperature range 20–60 ºC, laccase immobilized by covalent binding retained over 80% of its catalytic activity, whereas the adsorbed oxidoreductase exhibited over 50%. The most interesting finding is that the covalently bonded enzyme displayed higher catalytic activity than the adsorbed laccase over the whole pH and temperature range. The greatest differences, of about 20%, between the relative activities of adsorbed and covalently bonded laccase were observed at pH 8, 9 and 10. In the case of elevated temperature, at 60 ºC and 70 ºC the differences were about 30%. The laccase immobilization by covalent binding on the Fe_3O_4-NH_2@MIL-101(Cr) support modified by –NH_2 groups was shown by Wu et al. (2019). They concluded that the presence of –NH_2 groups have a protective effect on the secondary and tertiary structure of laccase, resulting in better stability and higher activity of the enzyme.

The results presented here show that laccase immobilized on nylon 6 electrospun material had higher relative activity than the native enzyme over a wide range of pH and temperature. What is more, the enzyme immobilized by the covalent binding method exhibited higher catalytic activity than the enzyme immobilized by adsorption. This may be explained by the different type of interactions between the biomolecule and the support, as these play a crucial role in the retention of catalytic activity and the stability of immobilized laccase.

Figure 3

3.3. Effect of process parameters on decolorization of Reactive Black 5

As mentioned above, the selection of suitable parameters for decolorization of the azo dye Reactive Black 5 is important for the efficiency of the remediation process. In this study, the effect of parameters such as initial dye concentration, pH, temperature and process duration was investigated (Fig. 4).

As shown in Fig. 4a, the highest decolorization efficiencies for native, adsorbed and covalently bonded laccase were obtained using dye solution at the lowest concentration, 1 mg/L. These efficiencies were 100%, 65% and 87% respectively. Increasing the dye concentration to 5 and 10 mg/L caused a significant decrease in the decolorization efficiencies. Nevertheless, over the whole analyzed concentration range the values were higher for native laccase than for the covalently bonded and adsorbed enzyme. This reduction in activity is probably due to conformational changes of the laccase structure upon immobilization (Das et al., 2020). However, partial elution of the enzyme from nylon 6 fibers, accompanied by loss of activity of the biocatalytic system, should not be excluded. In the next steps of the investigation (effect of pH, temperature and reaction time) a solution at concentration 5 mg/L was used, due to the fact that at this concentration the changes occurring were most pronounced, which is important for better understanding of the decolorization process.

In testing of the effect of pH on the efficiency of decolorization of Reactive Black 5, the highest values, 83%, 45% and 72% for free, adsorbed and covalently bonded enzyme respectively, were obtained at pH 5 (Fig. 4b). The results may be related to the pK_a value of Reactive Black 5, which is between 3.8 and 6.9 (Saroyan et al., 2019), while the optimal pH for laccase activity is slightly acidic, around pH 5 (Antecka et al., 2018; Khlifi et al., 2010). In other work, Daassi et al. (2014) obtained the highest decolorization efficiency at pH 5, however it was only 30%. The most significant observations were that laccase immobilized on nylon 6 modified by the EDC/NHS method was most efficient in the decolorization process at pH values ranging from 3 to 9, excluding 5. Due to the formation of covalent bonds as result of the use of EDC and NHS salts, stabilization of the enzyme structure occurred, and this led to a higher decolorization efficiency than with the other analyzed systems. The increase in enzyme stability upon covalent immobilization has been also confirmed in previous reports (Lassounane et al., 2019; Zhou et al., 2015).

To investigate the effect of temperature on the efficiency of decolorization of RB5, degradation processes were carried out at temperatures ranging from 5 to 45 ºC (with measurements made every 10 ºC). The wide range of temperatures was dictated by the fact that many synthetic dyes, including Reactive Black 5, are resistant to biodegradation at various temperatures (Murugesan et al., 2007). The highest efficiencies of decolorization were obtained at 25 ºC for each of the biocatalytic systems used (Fig. 4c), which correlates with our previous findings (Antecka et al., 2018). However, it should be noted that at temperatures 5, 15, 35 and 45 ºC the decolorization efficiencies were the highest for laccase immobilized by covalent binding. At the highest studied temperature (45 ºC), the efficiencies of decolorization of RB5 were 17%, 24% and 32% using free, adsorbed and covalently bonded laccase respectively. This indicates that laccase was stabilized and better protected against heat inactivation by the formation of bonds between the enzyme and a support modified with EDC and NHS, as

reported also in previous studies (Zdarta et al., 2019a; Wickramathilaka and Tao, 2019). The presented results could be contrasted with study published by Othman et al. (2016). Laccase immobilized onto multiwalled carbon nanotubes decolorized around 60% of Reactive Black 5 from aqueous solution. Although immobilized laccase was characterized by relatively good thermal stability, it needed relatively high temperature, which was 50 ºC, to obtained presented efficiency.

The next analyzed parameter, process duration, is crucial for the potential application of the obtained biocatalytic systems on an industrial scale, where time is an extremely important factor (Fig. 4d). The decolorization efficiency gradually increased over the first 12 h of the process, and after 24 hours the efficiencies of decolorization of RB5 were 45%, 72% and 83% for adsorbed, covalently bonded and free laccase respectively. After this time (24 h) a plateau was reached, and further investigation had no practical justification. By contrast, Martinez-Sanches et al. (2018) immobilized laccase from *Trametes versicolor* by an adsorption method in polyurethane foam cubes and used the system in a decolorization process of Reactive Black 5 at concentration 200 ppm, obtaining 85% efficiency only after 144 h.

To briefly conclude, it was found that the highest decolorization efficiencies were obtained in a process using the native form of the studied oxidoreductase under the following conditions: RB5 concentration 5 mg/L, pH 5, temperature 25 ºC, process time 24 h. Notwithstanding, the adsorbed and covalently bonded laccase possessed an advantage over the free enzyme in case of decolorization over a wider range of pH and temperature.

Figure 4

3.4. Effect of process parameters on decolorization of Reactive Blue 4

The next stage of the study was an investigation of the effect of process parameters on decolorization of the anthraquinone dye Reactive Blue 4. Comparison of the decolorization

399 efficiencies obtained for Reactive Blue 4 with those for Reactive Black 5 is important in terms
400 of the possible application of the biocatalytic systems in the removal of dyes from mixed
401 solutions containing various groups of dyes with different chemical structures. RB5 is
402 characterized by the presence of azo chromophores and bonds between aromatic rings, whereas
403 RB4, as an anthraquinone dye, possesses =C=O and =C=C= chromophore groups (Costa et al.,
404 2012). These functional moieties are responsible for the color of the dye solution (Jamal et al.,
405 2011) and play a crucial role in decolorization processes.

406 In the case of every studied parameter, similar trends were observed in the
407 decolorization processes of Reactive Blue 4 and Reactive Black 5 by the obtained biocatalytic
408 systems and native laccase. For RB4 the decolorization efficiencies were the highest when the
409 dye concentration was 1 mg/L. However, in the decolorization of RB4 from solution at
410 concentration 10 mg/L the efficiencies were 34%, 51% and 54% using adsorbed laccase,
411 covalently bonded laccase and the native form respectively; this is about 20% higher than the
412 removal rate of Reactive Black 5 dye. The observed differences in the decolorization of RB5
413 and RB4 may be due to different mechanisms of the degradation of the dyes by laccase. As
414 reported by Legerska et al. (2016), a possible pathway of RB5 degradation includes steps such
415 as cleavage of bis azo bonds, deamination, hydroxylation and sulfonation, whereas the pathway
416 of anthraquinone dye degradation consists of reduction, hydroxylation, deamination and
417 oxidation reactions. Oxidation, a significant step from the point of view of the catalytic activity
418 of laccase, does not occur in the degradation pathway of Reactive Black 5 unless a mediator
419 compound, such as ABTS, is added to the reaction environment. Although the presence of
420 mediator enhances the degradation efficiency, its concentration must be relatively high. What
421 is more, RB5 has a conjugated structure, similarly to other azo dyes, which is a further
422 impediment to its catalytic decolorization by laccase (Jamal et al., 2011; Soares et al., 2001).

In the investigation of the effect of pH, similar findings were made. The highest decolorization efficiencies at the extreme pH values of 3 and 9 were 68% and 63% respectively, for covalently bonded laccase. When the free form of laccase was used the decolorization efficiencies at those pH values were 24% and 4% respectively, whereas oxidoreductase adsorbed onto nylon 6 material decolorized over 50% and 20% of the RB4 dye solution at pH 3 and 9 respectively. At pH 5 the decolorization efficiencies were 63%, 77% and 91% respectively for the adsorbed, covalently bonded and free enzyme. The results clearly indicate that laccase immobilized on nylon 6 electrospun fibers is a more efficient tool for the removal of textile dyes, as compared to the free enzyme, over a wider pH range which may be encountered in industrial scenarios (Yaseen and Scholz, 2019).

The temperature of dye solution is important from the point of view of its decolorization process. Wastewaters produced by various textile dyeing processes may reach temperatures up to 100 ºC. However, after cooling processes, when the temperature attains lower values, efficient tools for decolorization are being sought (Attéké et al., 2013). The investigation of the effect of temperature on the decolorization efficiency of Reactive Blue 4 showed that the highest efficiencies were obtained at 25 ºC, both for free laccase and for its adsorbed and covalently bonded forms. It is notable that the results are higher by 8%, 5% and 18% respectively than those obtained for Reactive Black 5. In contrary to other work, laccase immobilized onto cross-linked magnetic chitosan beads can remove 59% of RB4 from aqueous solution at 30 ºC (Bayramoglu et al., 2010).

The greatest differences in the removal rates of the two dyes—about 20% for both immobilized enzymes—were observed at 45 ºC. In the case of RB4, the relatively high decolorization efficiencies (54% and 36%) obtained using laccase immobilized by covalent binding at temperatures of 35 ºC and 45 ºC unquestionably confirmed that this system may be used efficiently in a process performed at high temperature.

Comparing the efficiencies of decolorization of Reactive Blue 4 and Reactive Black 5, it is seen that after 24 hours of the process, the decolorization efficiencies for RB4 were greater than for RB5, using both immobilized laccases and the native enzyme. However, for each of the biocatalytic systems used, the process took place gradually. For instance, after 1 h of decolorization of Reactive Blue 4, the efficiencies were 6%, 8% and 12% using adsorbed, covalently bonded and native laccase respectively, whereas after 12 h of the process these values reached 60%, 75% and 86%, finally rising to 63%, 77% and 91% after 24 h.

The results obtained for the decolorization of Reactive Blue 4 showed that laccase immobilized by adsorption and covalent binding decolorized the dye solution with higher efficiencies over a wider range of pH and temperature conditions than the free enzyme. Despite that, free form of the used oxidoreductase in selected conditions degraded dyes with higher efficiencies than immobilized laccase. However, the enzyme attached to nylon 6 fibers could decolorize dyes over broader range of process condition and is characterized by reusability, what is a significant advantage over a native biomolecule. Therefore, it can be concluded that systems based on electrospun nylon 6 and immobilized laccase might be used in textile dye decolorization processes under various conditions with relatively high efficiency. What is more, all of the described patterns and differences between the decolorization of Reactive Black 5 and Reactive Blue 4 result mainly from differences in the dye structures, as immobilized laccase is capable of providing efficient conversion of both dyes.

Figure 5

3.5. Storage stability and reusability of the biocatalytic system nylon 6/Lac

The next stage of the study consisted of an investigation and comparison of the storage stability of the produced biocatalytic systems and native laccase, and the possibility of reuse of the immobilized biomolecules (Fig. 6). After 30 days of storage, the relative activities of the studied

systems were 31%, 60% and 95% for native, adsorbed and covalently bonded oxidoreductase respectively. This confirms that laccase immobilized by both methods retained higher catalytic activity than native laccase. Moreover, the enzyme covalently bonded to nylon 6 retained 95% of its initial activity, indicating improvement of the stability of laccase upon covalent immobilization. Similar conclusions were reached by Tavares et al. (2015), who immobilized laccase by covalent binding on multi-walled carbon nanotubes (MWCNTs). The difference between adsorbed and covalently bonded laccase should also be emphasized. The adsorbed laccase exhibited lower catalytic activity after 30 days of storage, compared with the covalently bonded laccase, by about 35%. This may be due to the stabilizing effect of covalent bonds on the immobilized biomolecule and multiple enzyme attachment to the nylon 6 fibers. The lower activity of adsorbed laccase may also be caused by the fact that this immobilized enzyme is less protected than the covalently bonded biomolecule.

The next investigated property of the produced systems was their reusability. The study was conducted over 10 consecutive catalytic cycles. After 10 cycles the relative activities of the immobilized enzyme were 61% and 70% for adsorbed and covalently bonded oxidoreductase respectively. The results show that the obtained biosystems, supported by nylon 6 electrospun fibers, offer relatively good reusability. The connection of the immobilized enzyme may prevent leakage of laccase from the support during successive catalytic cycles. However, the higher values of relative activity obtained with the biomolecule immobilized by the covalent binding method may be related to a more stable connection between the laccase and the electrospun support, compared with the case of the enzyme adsorbed onto nylon 6 fibers, where the physical interactions are much weaker. It was shown that after 10 catalytic cycles amount of immobilized laccase was 234 mg/g and 389 mg/g for biomolecule adsorbed and covalently bonded, respectively. These results are lower, as compared to amount of enzyme attached to nylon 6 fibers in first catalytic cycle, which was 282 mg/g and 423 mg/g for adsorbed and

covalently bonded laccase. It shows that partial elution of the enzyme occurred, that affects the relative activity of immobilized enzyme. What is more, the decreasing activity of adsorbed laccase may be caused by the blocking of active sites by molecules of substrate during each catalytic cycle (Mohamad et al., 2015). These findings may be important from the point of view of industrial applications, as they may facilitate cost reduction and improvement of efficiency.

Figure 6

4. Conclusions

In the present work, the nylon 6 electrospun material was produced, characterized, and used as a support for enzyme immobilization by adsorption and covalent binding. The important aspect of the presented study is application of the nylon 6, which is not widely described as a support for laccase immobilization in recently published articles. Effective immobilization of laccase was confirmed by FTIR spectra, EDS results and SEM images. The laccase attached to modified and unmodified fibers demonstrated high relative activity over wide pH and temperature ranges, and was capable of efficiently catalyzing the decolorization of two dyes from different groups, the azo dye Reactive Black 5 and the anthraquinone dye Reactive Blue 4, which are frequently used in textile coloration, and thus are present in wastewaters. Optimal parameters of decolorization, enabling total removal of the dye, were determined for both dyes; these were: dye concentration 1 mg/L, pH 5, temperature 25 ºC, reaction time 24 h. In both cases, the best decolorization efficiencies were obtained in a process catalyzed by laccase covalently immobilized on modified nylon 6 fibers. The results obtained and the phenomena described allow the conclusion that biocatalytic systems based on electrospun material made from nylon 6 might be successfully used in the catalytic decolorization of textile dyes. Moreover, to highlight the importance of presented study, comparison of the obtained data with previously published is shown in Table 3.

Table 3

Despite some previously published research on dye decolorization by immobilized laccase, this is still a relevant and overlooked topic, which requires constant and continued research. This is a consequence of the increasing quantities of pollutants being released by the textile industry and the emergence of *fast fashion,* which imposes a need for the continuous production of new textiles.

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828 **Table 1.** Characteristics of the dyes RB5 and RB4 subjected to decolorization
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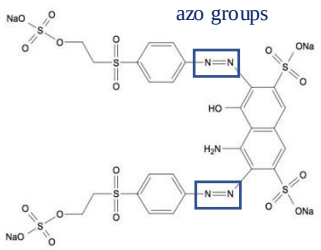
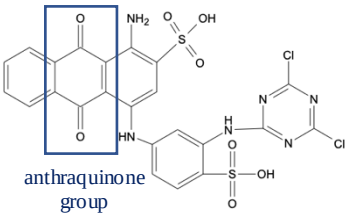
Name	Type of dye	Color index number	Molecular formula	Chemical structure with marked chromophore groups	Molecular weight (g/mol)	λ_{max} (nm)
Reactive Black 5 (RB5)	Azo	20505	$C_{26}H_{21}N_5Na_4O_{19}S_6$		991.82	597
Reactive Blue 4 (RB4)	Anthraquinone	61205	$C_{23}H_{12}Cl_2N_6Na_2O_8S_2$		681.39	595

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Table 2. Results of EDS analysis of nylon 6 electrospun material, nylon 6 with adsorbed laccase and nylon 6 with covalently bonded laccase

Element	Mass contribution (%)		
	nylon 6	**nylon 6/adsorbed laccase**	**nylon 6/covalently bonded laccase**
C	94.74	92.15	91.56
O	4.42	5.86	6.02
S	0.47	1.11	0.72
Cl	0.14	0.20	0.76
N	0.23	0.24	0.25
Cu	0.00	0.44	0.69

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837 **Table 3.** Comparison of obtained results with other published studies regarding use of
838 immobilized laccase in decolorization of Reactive Black 5 and Reactive Blue 4 from aqueous
839 solutions
840

Degraded dye	Immobilization method	Laccase support	Removal conditions	Removal efficiency	References
1. Reactive Black 5 2. Reactive Blue 4	covalent binding	nylon 6 electrospun fibers	T=25 ºC pH=5 time 24 h	1. 72% 2. 77%	presented study
Reactive Black 5	adsorption	polyurethane foam cubes	room temperature pH=5 time 144 h	85%	(Martinez-Sanchez et al., 2018)
Reactive Black 5	covalent binding	multiwalled carbon nanotubes	T=50 ºC pH=5 time 120 min	60%	(Othman et al., 2016)
Reactive Black 5	entrapment	Ca-alginate beads	T=30 ºC pH=5 time 24 h	around 30%	(Daassi et al., 2014)
Reactive Blue 4	covalent binding	magnetic chitosan beads	T=30 ºC pH=5.5 time 18 h	59%	(Bayramoglu et al., 2010)

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