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A green approach combining polyelectrolyte-based core-shell microparticles and laccase for indigo carmine degradation

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ABSTRACT

Highly effective composite biocatalysts were developed by the immobilization of laccase (LAC), a copper oxidoreductase, on core-shell microparticles obtained by the layer-by-layer deposition of polyelectrolytes, SP/(PEI/PAA)_{4,5} and SP/(PEI/PMANa)_{4,5}. The relation design – properties – applications was successfully elucidated by a comprehensive characterization performed using a combination of scanning electron microscopy (SEM), atomic force microscopy (AFM), potentiometric titrations, energy dispersive X-ray (EDX) spectroscopy and catalytic activity assays. The core-shell composite microparticles were able to immobilize more than 50 % of the enzyme, conserving a satisfactory enzymatic activity. LAC@SP/(PEI/PAA)_{4,5} and LAC@SP/(PEI/PMANa)_{4,5} were further employed in the degradation of indigo carmine as a model pollutant under various experimental conditions. The influence of the amount of enzyme immobilized, the pH, the mediator and the dye concentration were assessed, observing that LAC_n@SP/(PEI/PAA)_{4,5} and LAC_n@SP/(PEI/PMANa)_{4,5} performed better when syringaldehyde was used as a mediator. The obtained immobilized LAC achieved 100 % discoloration efficiency, demonstrating their potential as green catalysts for water cleaning applications. Additionally, the immobilized LAC exhibited significantly enhanced stability upon reuse, being able to completely discolor the dye sample up to 4 reuse cycles.

1. Introduction

Enzymatic catalysts are of high interest to both industry and research due to their versatility and increased selectivity. The particularities of this type of catalysts, including their high specificity and targeting for precise substrates, as well as their natural origin made enzymes ideal candidates for the catalysis of industrial and laboratory processes [1]. Nevertheless, enzymatic catalysts exhibit low stability in industrial processes, being easily denatured at high temperatures, at extreme pH values or in the presence of organic solvents. Also, enzymes are difficult to be recovered and reused and, consequently, expensive to use in large-scale processes. To overcome these limitations, enzyme immobilization has been imposed as an efficient method to increase the stability of the biocatalysts to process parameters and to make their reuse easier [2].

Among the methods used for enzyme immobilization, the most common ones are chemical bonding, physical adsorption, and

entrapment on/ to solid matrixes. The use of support materials for the immobilization of enzymes ensures increased stability of the biocatalyst, leading to the fabrication of composite materials with enhanced properties, such as better stability at pH or temperature [3,4]. The characteristics of the obtained composite biocatalysts can be easily tuned by carefully choosing the support material; the use of organic supports usually ensures better availability of the enzymatic site and favours interaction with a large number of chemical compounds while the use of inorganic supports leads to composites with a higher stability [5]. The use of organic/inorganic hybrids as support for the immobilization of enzymes guarantees both stability and availability of the enzymatic catalysts.

Core-shell composites can be obtained by the deposition of a single organic layer or by the alternating deposition of polymeric layers of opposite charge [6] on the surface of an inorganic core. The organic/inorganic hybrids fabricated by the layer-by-layer (LbL) deposition of polyelectrolytes on inorganic support materials arouse a high scientific

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interest due to their tuneable properties and enhanced affinity for charged compounds such as proteins and enzymes. LbL composites are stable and resistant to mechanical stress, have a selective permeability and can be adjusted to become stimuli responsive. Also, polyelectrolyte-based composites have many functional groups able to interact with charged compounds, such as enzymes, favouring their immobilization by electrostatic interactions. Several authors proposed the use of LbL matrices for the immobilization of enzymes such as pepsin [7], glucose-oxidase [8], lysozyme [9], lipase [10], or horseradish peroxidase [11], obtaining stable catalysts that maintained a satisfactory enzymatic activity. Such composites were successfully used in water treatment [12], CO₂ capturing [13], as biosensors [14], or as surfaces with antimicrobial properties [15]. Among the applications of immobilized enzymes, their use for the decontamination of wastewater has garnered considerable attention due to the efficiency of some enzymes, such as horseradish peroxidase or laccase, in oxidizing emerging pollutants such as pharmaceutical compounds [16,17], phenol [18], or dyes [19,20].

In this study we propose an innovative method for the immobilization of *Trametes versicolor* laccase (LAC), an oxidoreductase enzyme, on LbL composite materials fabricated with poly(ethyleneimine) (PEI) and poly(acrylic acid) (PAA) or poly(sodium methacrylate) (PMANa) deposited on silica microparticles (SP). The composite materials used in this study were stabilized by an unconventional method, employing the step-by-step crosslinking of the polycation chains coupled with an efficient method for the removal of weakly-bounded polyelectrolyte chains that emphasized better stability of the polymeric architecture and increased the porosity of the obtained materials. The samples with immobilized LAC (LAC_n@SP/(PEI/PAA)_{4,5} and LAC_n@SP/(PEI/PMANa)_{4,5}) were employed in the degradation of indigo carmine (IC), selected as a model pollutant, in the presence of syringaldehyde (SG) as mediator. To the best of our knowledge, the proposed combination of support material – enzyme – target pollutant – mediator was not reported in the literature. The degradation of the dye was studied in various experimental conditions, striving to show how this complex system can act as a green catalyst for water-cleaning applications. The results reported in this study highlight a very high efficiency of the immobilized LAC in dye degradation, suggesting that such biocatalysts can find important applications in the development of emerging water treatment technologies.

2. Materials and methods

2.1. Materials

Silica microparticles of Daisogel type (SP) with a mean diameter of 40–60 μm and pores of 100 nm were purchased from Daiso Co., Japan. The used polyelectrolytes are branched poly(ethyleneimine) (PEI), weight molar mass $M_w = 25,000$ g/mol (Sigma – Aldrich, Germany), poly(acrylic acid) (PAA), $M_w = 100,000$ g/mol, 35 % aqueous solution (Sigma – Aldrich, Germany) and poly(sodium methacrylate) (PMANa), $M_w = 9500$ g/mol, 30 % aqueous solution (Sigma-Aldrich, Germany). The used cross-linking agent was glutaraldehyde (Sigma – Aldrich, Germany). LAC from *Trametes versicolor* and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were acquired from Sigma – Aldrich (Germany), SG from Alfa Aesar and indigo carmine (IC), from VWR Chemicals.

2.2. Fabrication of the composite microparticles

The fabrication of the core-shell composite microparticles was ensured by the LbL deposition of positively (PEI, 10⁻² M) and negatively charged polyelectrolytes (PMANa or PAA, 10⁻² M), on SP microparticles, following a methodology previously established [21]. Briefly, the alternate deposition of positively and negatively charged layers was achieved by immersing the silica microparticles in PEI solution, under

stirring, for one hour, followed by decantation and intensive washing with distilled water. After washing, the PEI chains were chemically cross-linked with GA 2.5 %, the ratio between the aldehyde functional groups of GA and the NH₂ functional groups of PEI being 1:10. The amount of GA necessary for the crosslinking of the PEI chains was calculated based on the deposited amount of PEI assessed from polyelectrolyte titrations, taking into consideration the fact that each PEI structural unit contains one -NH₂ functional group. The cross-linking step aimed to increase the stability of the polymeric architecture and to ensure a better control of the amount of PEI deposited [22]. After the cross-linking step, the microparticles were subjected to the deposition of the polyanion layer, using PAA or PMANa solutions. The microparticles were immersed in the polyanion solution for one hour under stirring, then decanted, washed, and subjected to the deposition of the next PEI layer, following the same procedure as described above. The alternate deposition of the PE chains was continued until the formation of core-shell composite materials with five layers of PEI and four layers of PAA or PMANa (the first and the last layer being cross-linked PEI), the number of deposited layers being chosen in agreement with previous works of our group [23]. The alternating deposition of a layer of PEI and a layer of PAA or PMANa was defined as a bilayer. The excess of deposited organic material was removed by washing the composite microparticles in acidic solution (to extract the weakly bounded PEI chains) and in basic solution (to extract the excess of polyanion), a method that ensures a good stabilization of the LbL architecture as reported elsewhere [7]. After the stabilization/flexibilization treatment, the samples were washed with distilled water to remove the traces of solvents. The resulting materials codes are SP/(PEI/PAA)_{4,5} and SP/(PEI/PMANa)_{4,5}, respectively depending on the used polyanion (PAA or PMANa) for LbL deposition, where the subscript 4.5 stands for the number of PEI/polyanion bilayers deposited on the SP.

2.3. LAC immobilization

The immobilization of LAC on the core-shell composite microparticles was achieved by keeping in contact 50 mg of composite microparticles with 20 mL of enzyme solution, under stirring and at room temperature. Various pH and enzyme concentrations were tested to optimize the immobilization process. The selected pH interval was 3–5.5 (chosen based on the optimal activity of LAC) and the LAC concentration range was 1 – 8 mg/mL. The amount of immobilized enzyme was determined from the supernatant fraction separated after centrifugation, by measuring the absorbance at 280 nm using a Genesys 150 spectrophotometer (Thermo Scientific). Based on the results obtained from the assay, the immobilization yield (YI, %) (Eq. 1) was calculated:

$$YI (\%) = (1 - C_s / C_{LAC}) \cdot 100 \quad (1)$$

where: C_s – concentration of protein in the supernatant fraction and C_{LAC} – concentration of protein in the initial LAC solution, both determined by UV-Vis measurements (mg/mL).

Codes of the samples after LAC immobilization are LAC_n@SP/(PEI/PAA)_{4,5} and LAC_n@SP/(PEI/PMANa)_{4,5}, respectively, n representing the initial concentration of LAC.

2.4. Characterization of the core-shell composite materials

The LbL deposition was assessed by the *polyelectrolyte titration method*, using a Particle Charge Detector (Mütek PCD 3.0, Weßling, Germany). The method allows the calculation of the concentration of polyelectrolytes in aqueous solution, initially and after the deposition on the support microparticles, by titrating the solutions with oppositely charged standard solutions of 10⁻³ M poly(sodium ethylenesulfonate) or poly(diallyldimethylammonium chloride). The concentration of the examined solution was calculated from the volume of standard solution needed to compensate the opposite charges in the aqueous medium, by

reaching the zero value of the streaming potential. The amount of deposited organic material after each step was calculated using Eqs. (2) and (3):

$$C_{PE} = (C_t \cdot V_t) / V_{PE} \quad (2)$$

$$C_{ads} = (C_i - C_{PE}) \cdot M_{US} \cdot V / m_{SP} \quad (3)$$

where: C_{PE} – concentration of PE in the supernatant fraction (mol/L); C_t – titrant concentration (mol/L); V_t – volume of titrant (mL); V_{PE} – volume of PE solution (mL); C_{ads} – amount of adsorbed PE (mg/g); C_i – initial PE concentration (mol/L); M_{US} – mass of structural unit of the PE (g/mol.); V – volume of PE used for deposition (mL); m_{SP} – mass of silica micro-particles used as support for deposition (g).

The morphology of the core-shell composite microparticles surface, before and after the immobilization of LAC was observed using the Verios G4 UC scanning electron microscope (SEM) (Thermo Scientific, Czech Republic) equipped with Energy Dispersive X-ray (EDX) spectroscopy analyser (Octane Elect Super SDD detector, USA). To avoid electrostatic charging, the samples were coated with a platinum layer using a Leica EM ACE200 Sputter coater.

The NTEGRA Scanning Probe Microscope (NT-MDT, Zelenograd, Moscow, Russia) was used to conduct Atomic Force Microscopy (AFM) research. The AFM data were collected under ambient settings using a NSG10 cantilever supplied by TipsNano OÜ, based in Tallinn, Estonia. The tapping mode was employed, and the data acquisition was performed using the Nova software 1.0.26.1443 (developed by NT-DMT, Zelenograd, Moscow, Russia). The probe had a resonance frequency of 169 kHz and a normal spring constant of 7.4 N/m, which was determined by the Sader's method using the length and width of the cantilever (125 µm and 27 µm, respectively).

X-ray photoelectron spectroscopy (XPS), a surface-sensitive technique, implied an Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical, Manchester, U.K.). The spectrometer was equipped with a monochromatic Al Kα ($h\nu = 1486.6$ eV) X-ray source of 300 W at 15 kV. The kinetic energy of photoelectrons was determined with a pass energy of 160 eV for wide-scan spectra. Quantitative elemental compositions were determined from peak areas using experimentally determined sensitivity factors and the spectrometer transmission function.

The degradation of the dye was assessed by UV-Vis spectrophotometry, by studying the decrease in absorbance at $\lambda = 610$ nm, corresponding to the dye absorption maxima, using Genesys 150 equipment (Thermo Scientific). The amount of IC degraded over time was assessed based on the calibration curve of the dye (Figure S1).

The activity of the free or immobilized LAC was determined by following the conversion of a 1 mM ABTS solution, at the selected temperature and pH. If not stated otherwise, the activity assays were performed at pH = 4.5 and 25 °C. The amount of formed product was detected by measuring the change in absorbance at 420 nm using a SpectraMax ABS Plus spectrophotometer (Molecular Devices, LLC, Munich, Germany). One unit of activity was defined as the amount of enzyme needed to oxidize 1 µmol ABTS in 1 min. Additionally, the relative activity of the biocatalysts (free and immobilized form) in similar experimental conditions was calculated. For the biocatalysts obtained by the immobilization of LAC on the same support material (SP/(PEI/PAA)_{4.5} or SP/(PEI/PMANa)_{4.5}), the maximum activity reached in the selected experimental conditions (eg. for pH stability) was defined as 100 % and the relative activity registered in the other assays was calculated accordingly.

2.5. Dye degradation study

The use of the LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5} in water cleaning applications was assessed by studying the degradation of IC, a negatively charged dye, as a target pollutant. The degradation of the dye was tested in the presence of ABTS or SG as mediators, at an IC initial concentration of 50 mg/L and pH = 7, using

either free LAC or the LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5}. The influence of the pH on the enzymatic degradation of the dye was performed in similar conditions, using 50 mg/L dye solutions prepared in buffers of various pHs. The influence of the dye concentration was assessed using solutions of 50 mg/L, 100 mg/L and 150 mg/L prepared in distilled water.

To study the influence of potential dye sorption by the support on the degradation process, an experimental methodology was designed, consisting of the comparative study of the discoloration process using *active* and *thermally inactivated* biocatalysts. To denature the enzyme, the biocatalysts were thermally treated at 80 °C, for one hour, a treatment that will prevent further enzymatic activity. In these conditions, it is expected that the discoloration of the dye sample will be solely the result of the sorption of the dye on the surface of the inactive biocatalyst. The experiments were performed in similar conditions employing active and inactive biocatalysts, using a dye concentration of 50 mg/L at pH = 7. The kinetics of the sorption and degradation processes were studied employing the first-order kinetic model graphically represented by plotting the variation $\ln[A]/[A_0] = f(t)$, where $[A_0]$ is the initial concentration of the dye and $[A]$ is the concentration of the dye at moment t (sec).

3. Results and discussions

3.1. Substrate characterization

The successful LbL deposition on the SP was assessed by performing polyelectrolyte titrations after the deposition of each layer (Fig. 1a). The use of PAA as a polyanion (Fig. 1a) led to the deposition of a higher amount of organic material taking into account its high molar mass and the partial ionization of the carboxylic groups in the deposition conditions (Supplementary material, Table S1 and Figure S2). The partial ionization of the functional groups leads to a coiled conformation of the macromolecular chain, resulting in a ladder-like shape of the LbL deposition. On the other hand, the use of PMANa (Fig. 1a) led to the deposition of a thinner organic layer. The presence of Na⁺ ions confers to the charged functional groups of the PMANa the strong polyelectrolyte character, increasing the chance of forming electrostatic interactions with the ionized primary amino groups of PEI, its stretched conformation enabling a continuous and uniform increase of each deposited layer.

The surface charge of the composite material plays an important role in the subsequent immobilization of enzymes by adsorption. Indeed, enzyme immobilization is the result of the formation of weak interactions between the support material and the enzyme, with electrostatic, hydrogen bonding and hydrophobic interactions being the most important types of forces involved in immobilization [2], as represented in Scheme 1. The surface charge of the core-shell composites was analysed by potentiometric titration, in the pH interval 3–10 (Fig. 1b). The isoelectric points (IEPs) experimentally determined were ~5.75 for SP/(PEI/PAA)_{4.5} and ~5.5 SP/(PEI/PMANa)_{4.5}, respectively. By analysing the variation of the potential as a function of pH, it was observed that both types of composites are positively charged at pH < 5, confirming that the microparticles could potentially be used as support for the immobilization of negatively charged laccase at pH < 5, in relation with its IEP ≈ 3 (experimentally determined by potentiometric titration, as presented in Supplementary material, Figure S3), which is in agreement with the findings of Call et al. [24]

The obtained composite microparticles were subjected to extraction of the excess of deposited organic material by washing in acidic solution (to extract the non-cross-linked PEI chains) and basic solution (to extract the excess of polyanion). The extraction process has been evaluated by means of the XPS (Fig. 2). The chemical composition of the materials before and after the extraction of the chains was studied based on the ratios of the constituent elements, calculated from the XPS data. Fig. 2a shows that the SP/(PEI/PAA)_{4.5} composite material contains important amounts of N (from PEI), C (from both PEI and PAA), but also O (from

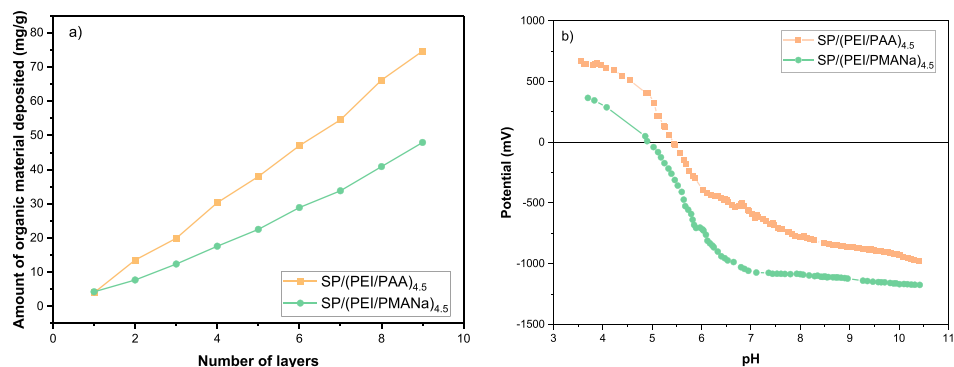
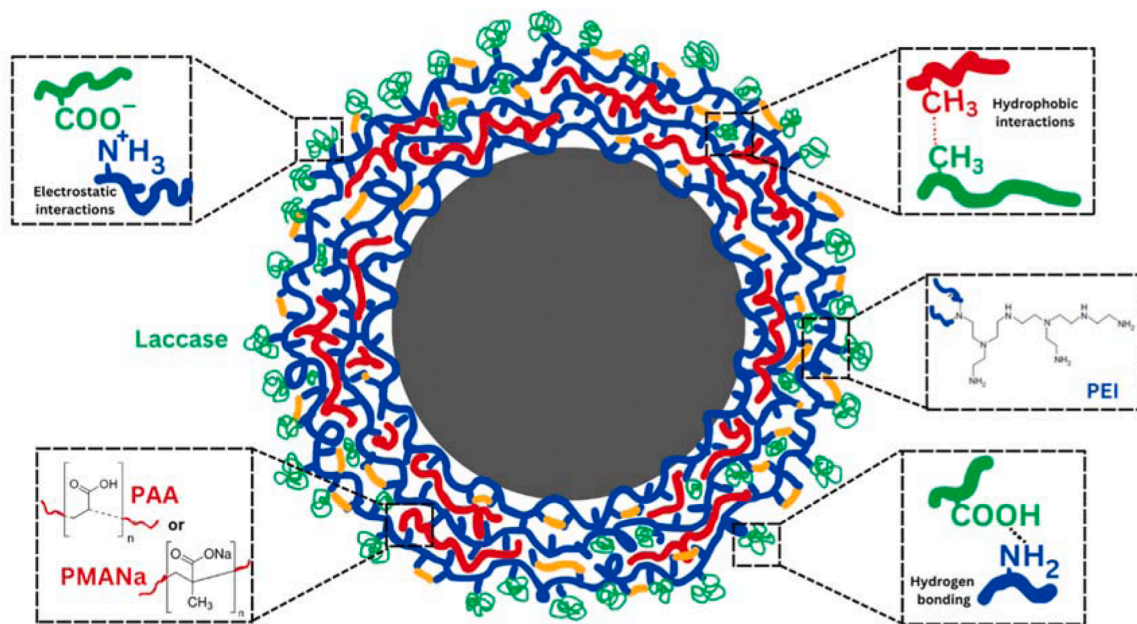


Fig. 1. The deposition of polyelectrolyte layers on SP microparticles(a) and the variation of potential as a function of pH for the composite materials for SP/(PEI/PAA)_{4.5} and SP/(PEI/PMANa)_{4.5} (b).



Scheme 1. The main types of forces involved in the immobilization of LAC on the core-shell composite microparticles.

PAA and the silica used as a support). After the extraction of the PAA chains, the intensity of the peak corresponding to the N atoms remains almost constant, while the intensity of the peaks corresponding to C and O decreases, as a result of the removal of the excess PAA chains from the material. Also, in the spectra of the extracted material the appearance of peaks corresponding to Cl 2 s, Cl 2p_{3/2} and Cl 2p was found, which may be the result of traces of HCl, used as a solvent in the stabilization process. The SP/(PEI/PMANa)_{4.5} composites show an almost constant amount of N before and after the extraction of the free PMANa chains (Fig. 2b), suggesting that the PEI chains deposited on the microparticles of silica are stable. On the other hand, the intensity of the O and Si peaks increased after polyanion extraction, suggesting the thinner organic material on the silica surface. Correlating this observation with the mass of organic material deposited on the support (much lower than in the case of the PEI/PAA polyelectrolyte pair), we can assume that the formed organic shell is thin but stable.

By determining the [N]/[Si] and [C]/[Si] ratios (Fig. 2, c and d), before and after the extraction steps, information about the amount of organic material deposited on the silica microparticles can be obtained as well as about the stability of the coating after the extraction treatment. For SP/(PEI/PAA)_{4.5} composite material, analysing the [N]/[Si] ratios for the composites, an important decrease after the extraction

steps can be observed, as a result of the partial delamination of PEI. A decrease in [C]/[Si] ratios can also be observed, which also suggests on the one hand the extraction of polyanion and on the other hand a possible loss of PEI chains. The composites with PMANa have a lower amount of organic material deposited, but are more stable to the extraction treatment, as also suggested by the small variation of [N]/[Si] ratios before and after the extraction steps (Fig. 2c).

3.2. LAC immobilization

3.2.1. Influence of the pH of LAC solutions

Subsequently, the immobilization of LAC, a negatively charged enzyme, with an IEP at pH ≈ 3 was studied. The effect of the pH on the immobilization yield was studied in the pH range of 3.5–5.5, as presented in Fig. 3a, using a LAC solution of 1 mg/mL. The best results in terms of immobilization yield were found at pH = 4.5 for both types of support microparticles, with more than 50 % immobilization which is most likely the result of a good ionization degree of both the enzyme and the support material. Also, the immobilization yields obtained in the pH range of 4.5–5.5 do not differ very much, suggesting that a small variation in the pH would not affect strongly the immobilization process. Moreover, the enzymatic activity of the LAC1@SP/(PEI/PAA)_{4.5} and

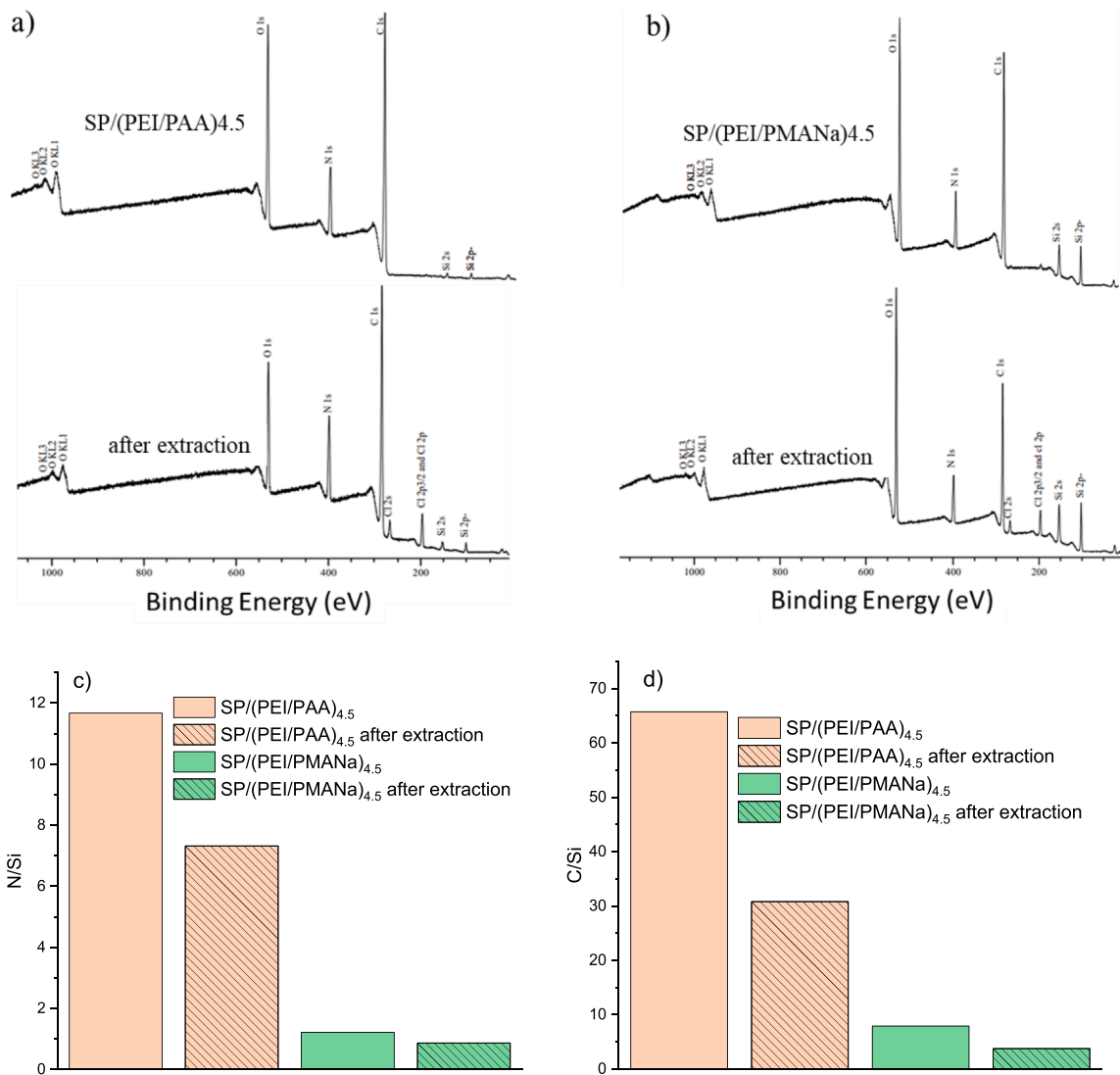


Fig. 2. XPS spectra of (a) SP/(PEI/PAA)_{4.5} and (a) SP/(PEI/PMANa)_{4.5} composites and atomic ratios (c) [N]/[Si] and (d) [C]/[Si], before and after extraction steps.

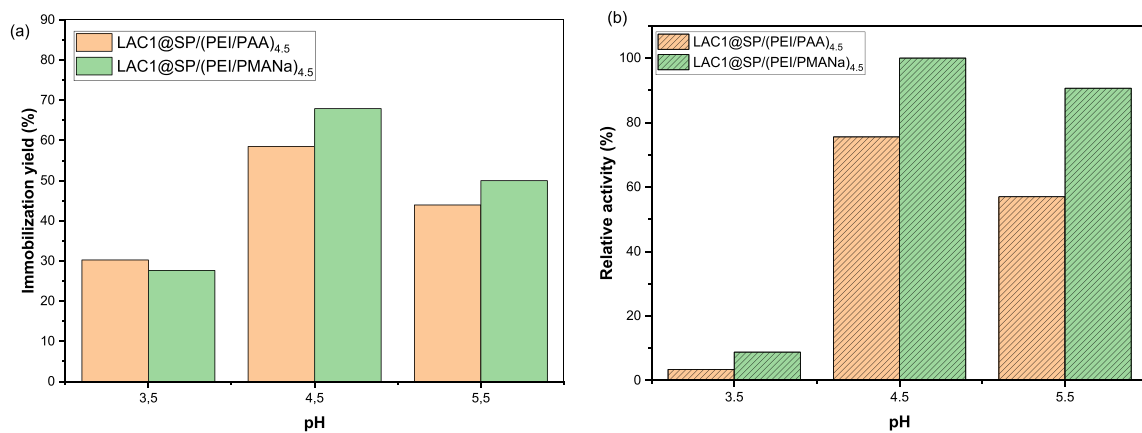


Fig. 3. The influence of pH on (a) immobilization yield and (b) relative catalytic activity of the LAC1@SP/(PEI/PAA)_{4.5} and LAC1@SP/(PEI/PMANa)_{4.5} at various pH values.

LAC1@SP/(PEI/PMANa)_{4.5} biocatalysts was studied (Fig. 3b), observing that the immobilization of LAC at pH = 4.5 preserved the best catalytic activity (defined as 100 % for the best obtained results, the other values

being related to the highest value), a pH value that ensures a satisfactory degree of ionization of the composite material's functional groups. Additionally, it can be expected that this pH value conserved better the

conformation of the enzyme, favouring its immobilization without affecting the active site of the enzyme.

The relative activity of the immobilized LAC was satisfactory, a better activity being observed for the composite material fabricated with PMANa. In the case of this material, the immobilization yield was also better, suggesting a higher affinity of the carrier towards the enzyme, most likely due to a better interaction with the charged support material, based on both electrostatic and hydrophobic interactions. Similar results were reported by other researchers who studied enzyme immobilization on methacrylate-based supports [25]. For example, Chen et al. fabricated poly(methyl methacrylate)-based nanofibrous membranes that were later used for the immobilization of *Candida rugosa* lipase with the immobilization of 332 mg enzyme/g support material [25]. Benitez-Mateos and Contente compared the immobilization of enzymes on agarose or methacrylate-based commercially available microparticles, observing that the hydrophobicity of the support material plays a vital role in the immobilization and enzyme activity recovery [26].

3.2.2. Influence of LAC initial concentration

Another important parameter in the efficiency of the enzyme immobilization process is the initial concentration of the enzyme which is related to diffusional constraints of reagents during the process. The use of higher concentrations of enzyme solution can lead to two opposite effects: (i) increase in the immobilization yield since more concentrated solutions contain more molecules that can bind to the support material (increased chance of interaction enzyme/support) and (ii) decrease in immobilization yield due to support saturation. In this regard, the effect of the concentration of the enzyme on the immobilization step was studied in the 1 – 8 mg/mL concentration range, Fig. 4a presenting the immobilization yields and Fig. 4b the relative enzymatic activities as a function of initial enzyme concentration.

As presented in Fig. 4a, the highest enzyme immobilization yield was achieved at an initial concentration of 1 mg/mL and corresponds to $59.02 \pm 0.074\%$ for the SP/(PEI/PAA)_{4,5} and to $78.9 \pm 5.53\%$ for the SP/(PEI/PMANa)_{4,5} support materials, respectively to 238.33 mg/g and 318.23 mg/g. A satisfactory immobilization efficiency was maintained within the 2–8 mg/mL LAC concentration range, while for higher enzyme concentrations, surface saturation could occur. While an increase in concentration can lead to enhanced immobilization due to enzyme aggregation on the material surface generated by homophilic enzyme-enzyme interactions, the stability and the catalytic activity of biocatalysts obtained with higher concentrations of enzymes can be affected by the weak interactions between the enzyme aggregates and the support material and by the diffusional limitations and hindering of the active sites that appear when the enzyme layer is thicker. Thus, by analysing the enzymatic activities for the LACn@SP/(PEI/PAA)_{4,5} and LACn@SP/(PEI/PMANa)_{4,5} composite biocatalysts obtained at initial

enzyme concentrations 1 – 8 mg/mL (Fig. 4b), it can be observed that, in general, the relative enzyme activity decreases as the enzyme concentration increases, with the best activity being retained at an initial concentration of 1 mg/mL for both SP/(PEI/PAA)_{4,5} and SP/(PEI/PMANa)_{4,5} materials. This behaviour suggests either a decrease in the accessibility of the active site when larger quantities of enzymes are immobilized, or the formation of enzyme-support interactions leading to conformational changes and a reduction in the number of accessible active sites as the amount of immobilized enzyme increases.

The morphology of the composite supports, before and after the immobilization of the enzyme was studied by SEM (Figure S4) and AFM (Fig. 5), the main characteristics of the surface topology, being included in Table 1. AFM images in Fig. 5a and c confirm the results in Figs. 1a and 2, showing a smoother surface when PMANa was used in LbL deposition, suggesting a uniform compensation of complementary ionic sites. By comparing Fig. 5a and b, and Fig. 5c and d, respectively a difference in porosity can be detected between the composite microparticles before and after enzyme immobilization, the immobilization of LAC reducing the presence of pores at the composite surface but also forming some aggregates, suggesting that the immobilization of the enzyme takes place probably inside the pores of the polymeric network but mainly at the surface of the material.

The conformational state of a multilayer surface can be explored indirectly via measurements of the surface roughness of dried films. As Table 1 shows, the surface characteristics are influenced by the polyanion involved in the multilayer preparation. Thus, the higher roughness (Sa) value was found for the SP/(PEI/PAA)_{4,5} sample which resulted most probably due to the deposition of thicker PAA layers (as suggested by polyelectrolyte titration, Fig. 1a), its further removal from the shell conducting to higher pores than in the case of thinner PMANa layers which adopted a flat conformation during multilayer assembly. The immobilization of LAC determines the roughness modification on both analysed surfaces, most probably due to the reduction of the pores at the composite surface. Other important parameters are surface skewness (Ssk) and coefficient of kurtosis (Ska). Ssk is related to the surface symmetry, with negative skew being assigned to the predominance of valleys, while positive skew suggests a peaky surface. Thus, the previous observations are sustained by Ssk negative values for the initial samples and positive values for LAC6@SP/(PEI/PAA)_{4,5} and LAC6@SP/(PEI/PMANa)_{4,5}, confirming the pores covering during the LAC immobilization. Ska is a measure of the randomness of profile heights of a surface, a perfectly random surface having a value of 3, spiky surfaces with high values, and bumpy surfaces with low values. As Table 1 shows, except for SP/(PEI/PAA)_{4,5}, all samples present a spiky surface (Ska > 3), which confirms the highest porous surface of SP/(PEI/PAA)_{4,5}. The most random surface was obtained for LAC6@SP/(PEI/PMANa)_{4,5} (Ska = 3.678) which also confirms that, due to the thinner surface and lower

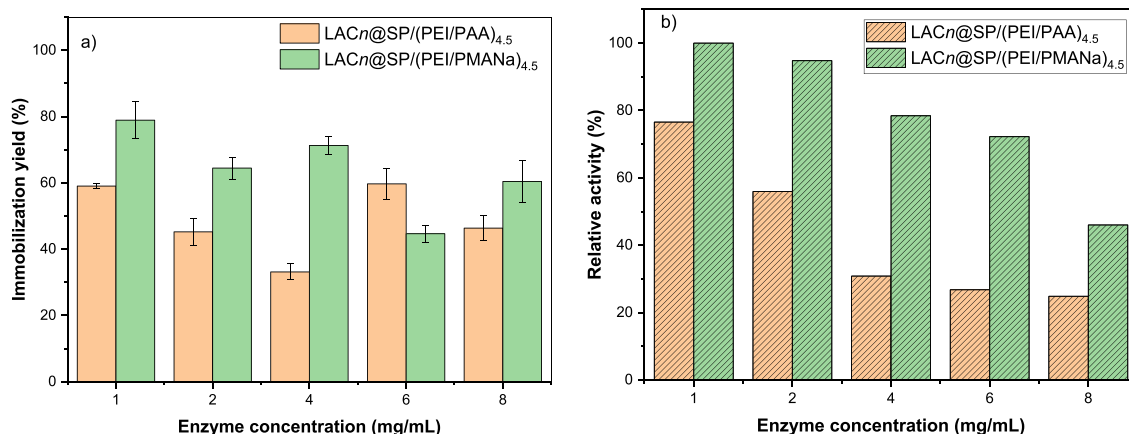


Fig. 4. The immobilization yield (a) and the relative enzyme activity (b) obtained at various LAC concentrations.

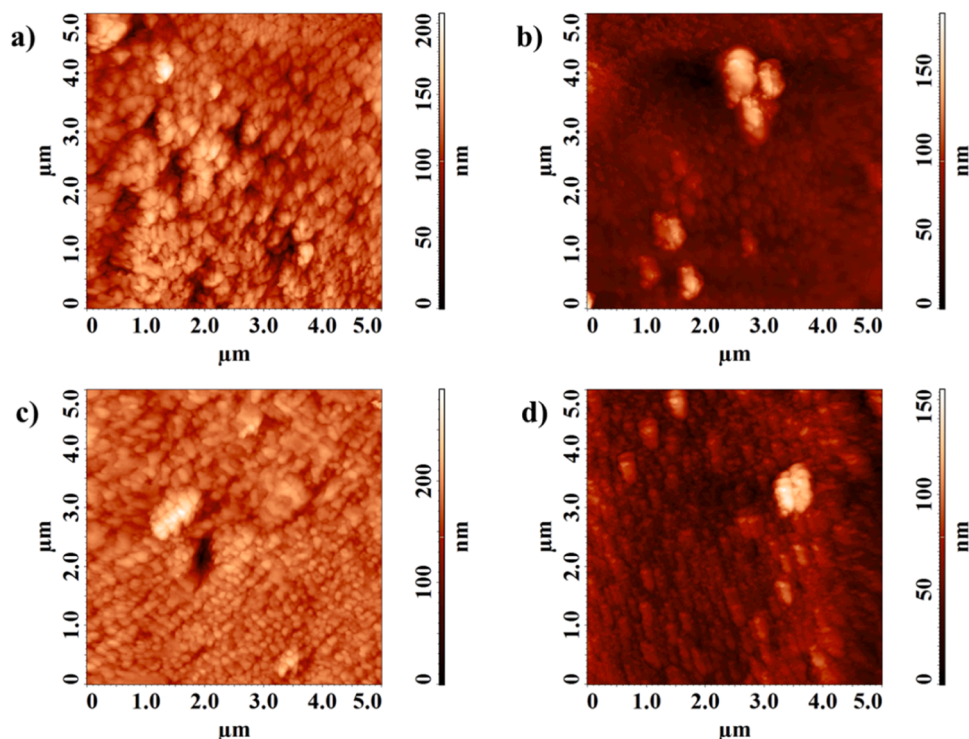


Fig. 5. The AFM images of the SP/(PEI/PAA)_{4.5} (a and c) and SP/(PEI/PMANa)_{4.5} (b and d) composites, before (a, c) and after (b, d) the immobilization of LAC6.

Table 1

The main characteristics of the surface topology of SP/(PEI/PAA)_{4.5} and SP/(PEI/PMANa)_{4.5} composites, before and after the immobilization of LAC6: average roughness (Sa), root mean square, (Sq), surface skewness (Ssk), coefficient of kurtosis (Ska).

Sample	Sa (nm)	Sq (nm)	Ssk	Ska
SP/(PEI/PAA) _{4.5}	15.31	20.33	-0.507	1.473
LAC6@SP/(PEI/PAA) _{4.5}	10.87	17.53	+1.820	6.462
SP/(PEI/PMANa) _{4.5}	14.22	19.64	-0.551	6.292
LAC6@SP/(PEI/PMANa) _{4.5}	12.06	16.17	+1.185	3.678

porosity, the LAC sorption in this case is located mostly on the surface, forming numerous aggregates.

The successful immobilization of LAC on the surface of the composite microparticles was confirmed by EDX (Fig. 6), where C and N can be associated with the presence of organic components such as the

polyelectrolytes or the enzyme, while the Si detected during the analysis belongs to the inorganic core. By analysing the [C]/[Si] and [N]/[Si] mass ratios before and after enzyme immobilization, information about the increase in organic material content generated by the immobilization of LAC can be obtained. As observed in Fig. 6, the immobilization of LAC resolves in an increase in both [C]/[Si] and [N]/[Si] mass ratios, as compared to initial composites. As expected, the increase in the [C]/[Si] ratio is larger since the C content of the enzyme (belonging to its backbone) is higher compared to the N content (belonging to the -NH₂ functional groups).

3.3. Indigo carmine degradation

3.3.1. Influence of the composite support

Subsequently, the potential use of LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5} in dye degradation was assessed, using IC as a model dye. Firstly, the degradation of the dye in the presence of free

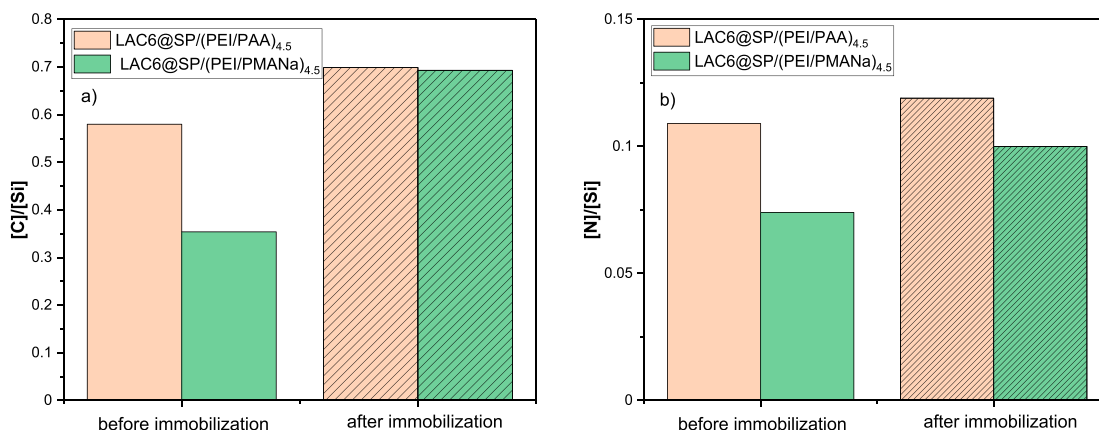


Fig. 6. The [C]/[Si] (a) and [N]/[Si] (b) ratios, before and after the immobilization of LAC for the LAC6@SP/(PEI/PAA)_{4.5} and LAC6@SP/(PEI/PMANa)_{4.5}.

LAC and ABTS or SG as mediators was studied, as the degradation of such molecules is achieved by a two-step electron transfer consisting of (i) the enzymatic oxidation of the mediator and (ii) the oxidation of the target molecule (IC) by the oxidized mediator [27,28]. The tests performed highlighted a very good degradation capacity irrespective of the used mediator (Fig. 7a). Nevertheless, in the presence of SG, the complete discoloration of the sample was achieved in 12 min while the use of ABTS as a mediator led to a slower, but complete discoloration of the sample within 20 min. As reported in the literature, various natural or synthetic mediators can potentially facilitate the LAC-catalysed degradation of IC, ABTS and SG being widely recognized as efficient mediators, as reflected in Table 2.

Thus, the degradation of the dye was successful in the presence of both mediators, but the faster discoloration observed in the presence of SG confirmed the increased feasibility of using LAC/ SG system for pollutants degradation as found also in similar studies [33,34].

The use of the composites with immobilized LAC in the degradation of IC was tested in the presence of the same mediators, using the LAC1@SP/(PEI/PAA)_{4.5} and LAC1@SP/(PEI/PMANa)_{4.5} (Fig. 7b). Thus, both immobilized LAC performed well in the experimental conditions, being able to lead to complete sample discoloration, in the presence of either ABTS or SG as mediators. The use of SG as a mediator led to a faster degradation in the case of the LAC1@SP/(PEI/PAA)_{4.5} but had a lower impact in the case of the LAC1@SP/(PEI/PMANa)_{4.5}, suggesting a better interaction between the mediators and LAC1@SP/(PEI/PMANa)_{4.5}. Most likely, the faster degradation of the dye sample in the presence of SG is favoured by the lower molar mass of the mediator which can diffuse more easily towards the active site, even when using immobilized LAC. While SG is oxidized more slowly by the enzyme, its oxidized form is more stable, favouring the transformation of the dye in its degradation products [27]. It was also observed that when using the composite material fabricated with PMANa as support for LAC immobilization, the degradation of the dye sample was faster, due to both a higher amount of immobilized LAC and its better stability, as observed previously from the enzyme activity tests (Fig. 4). The results obtained suggest a good affinity between the immobilized LAC and the tested mediators. To provide a broader context, the results of this study were compared with relevant literature data (Table 3).

As evidenced in Table 3, the enzymatic degradation of IC using immobilized LAC is possible in the presence of various mediators such as ABTS and SG, but the efficiency of the process is strongly dependent on the concentration of the dye, but also on the nature of the support material used of enzyme immobilization.

Additionally, it was observed that the use of ABTS as a mediator can lead, during the process, to the accumulation of ABTS⁺ on the surface of the material which will eventually be released in the reaction media, interfering with the detection method used to assess the dye degradation as the oxidized mediator adsorption spectra partially overlap with the dye adsorption spectra [41].

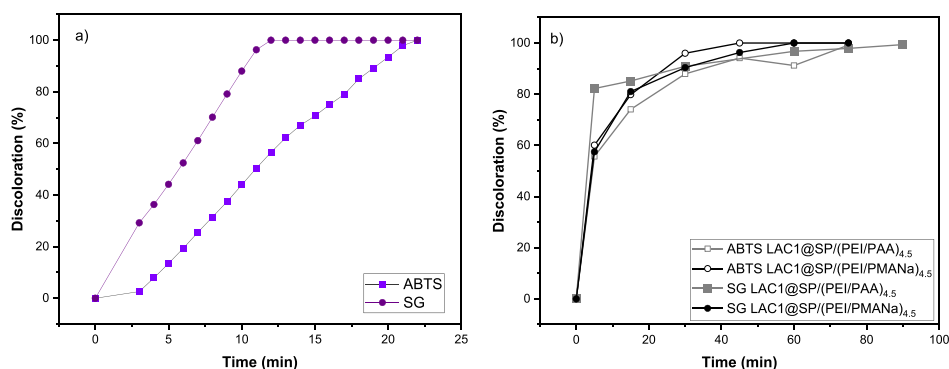


Fig. 7. IC discoloration efficiency in the presence of (a) free LAC or (b) LAC1@SP/(PEI/PAA)_{4.5} and LAC1@SP/(PEI/PMANa)_{4.5}, using SG or ABTS as mediators.

Table 2
Decolorization of IC in the presence of LAC and various mediators.

Enzyme source	Mediator	Dye concentration	Discoloration efficiency (%)	Reference
<i>T. versicolor</i>	ABTS	50 mg/L	100 % after 19 min	This study
<i>T. versicolor</i>	SG	50 mg/L	100 % after 12 min	This study
<i>T. versicolor</i>	ABTS-modified silica	20 mg/L	95 % after 15 min	[29]
<i>Peniophora sp. CBMAI 1063</i>	SG	50 mg/L	93 % after 2 h	[30]
<i>B. safensis HL3 spores</i>	acetosyringone	47 mg/L	97 % after 2 h	[31]
<i>B. velezensis</i>	ABTS	50 mg/L	90 % after 6 h	[32]
<i>B. velezensis</i>	SG	50 mg/L	45 % after 6 h	[32]

Further tests were directed towards the assessment of IC sorption and discoloration by the composites, as possible processes. For this, the discoloration of IC in the presence of active or thermally inactivated immobilized LAC was studied, as presented in Fig. 8. The thermal treatment proposed for preparing the inactive biocatalysts had the role of denaturing the enzyme, preventing eventual catalytic activity and directing the discoloration process towards sorption.

By analysing the discoloration rate as a function of initial enzyme concentration, it can be observed that independent of the type of support microparticles used for immobilization, the discoloration rate increases with enzyme concentration, suggesting that the main process driving the discoloration is the enzymatic degradation of the dye. On the other hand, when using the thermally inactivated biocatalysts LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5}, the discoloration rate was not influenced by the amount of enzyme immobilized, demonstrating that the inactivated enzyme is unable to either sorb or degrade the dye. Nevertheless, the sorption of the dye on the surface of the LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5} is slightly possible, even if its effect is negligible compared to the enzymatic discoloration. Moreover, by analysing the kinetics of the dye discoloration process in the presence of active or inactive LACn@SP/(PEI/PAA)_{4.5} and LACn@SP/(PEI/PMANa)_{4.5} (Supplementary material, Figure S5), it was observed that in the case of the enzymatic degradation, the correlation coefficients are lower, highlighting a more complex kinetic behaviour that might suggest a competition between sorption and degradation. As the amount of enzyme present in the system increases, the equilibrium is switched towards enzymatic degradation, an expected result considering that when using lower enzyme concentration, the saturation of both SP/(PEI/PAA)_{4.5} and SP/(PEI/PMANa)_{4.5} surfaces is not reached, so other small molecules, such as IC, can still interact with the support composite, being sorbed on its surface. Nevertheless, the results confirm that the contribution of enzymatic degradation to the overall discoloration process is much more important than the dye sorption. While the

Table 3

Decolorization of IC in the presence of immobilized LAC and various mediators.

Enzyme source	Immobilization support	Mediator	Dye concentration	Discoloration efficiency (%)	Reference
<i>T. versicolor</i>	SP/(PEI/PMANa) _{4,5}	ABTS	50 mg/L	100 % after 60 min	This study
<i>T. versicolor</i>	SP/(PEI/PAA) _{4,5}	ABTS	50 mg/L	100 % after 90 min	This study
<i>T. versicolor</i>	SP/(PEI/PMANa) _{4,5}	SG	50 mg/L	100 % after 45 min	This study
<i>T. versicolor</i>	SP/(PEI/PAA) _{4,5}	SG	50 mg/L	100 % after 45 min	This study
<i>T. versicolor</i>	CHI beads	ABTS	0.5 mg/mL	100 % after 14 min	[35]
papaya	CHI beads	Not mentioned	50 µg/mL	100 % after 8 h	[36]
<i>B. amyloliquefaciens</i>	Calcium alginate beads	acetosyringone	25 mg/mL	96 % after 1 h	[37]
<i>E. coli</i>	Magnetic zeolitic imidazolate framework–8 nanoparticles	acetosyringone	25 mg/mL	100 % after 15 min	[38]
<i>T. versicolor</i>	Lysine functionalized cellulose	Not mentioned	50 mg/L	99 % after 7 h	[39]
<i>Ganoderma sp. KU-alk4</i>	Cu-alginate beads	Not mentioned	25 mg/L	100 % after 2 h	[40]

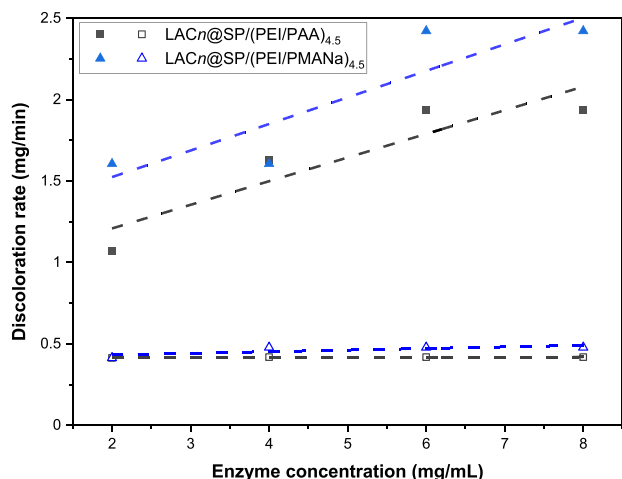


Fig. 8. The effect of initial enzyme concentration on the discoloration rate of the active (full symbols) and inactivated (empty symbols) LACn@SP/(PEI/PAA)_{4,5} and LACn@SP/(PEI/PMANa)_{4,5}.

competition between the two phenomena is clearly balanced in favour of enzymatic degradation, it is important to mention that the sorption process could complete the degradation, supplementing an eventual loss in enzymatic activity or the desorption of enzyme in repeated discoloration cycles.

3.3.2. Influence of LAC initial concentration

The following tests focused on the study of the influence of the initial concentration of LAC used in the biocatalysts fabrication in the efficiency of dye degradation. For this, the LACn@SP/(PEI/PAA)_{4,5} and

LACn@SP/(PEI/PMANa)_{4,5} obtained by the immobilization of LAC at 1–8 mg/mL were tested in the presence of SG as mediator (Fig. 9).

The study of degradation efficiency using the ten biocatalysts revealed two main patterns. Firstly, the use of LACn@SP/(PEI/PMANa)_{4,5} resulted in higher efficiencies (smaller degradation times) in all cases, demonstrating that the presence of PMANa chains in the structure of the composite material used as support for enzyme immobilization led to a better interaction with the enzyme, preserving the structure of the active site and maintaining the enzyme's catalytic activity even after immobilization. Secondly, it can be observed that regardless of the type of support material, increasing the amount of immobilized enzyme results in a higher degradation capacity of the sample, confirming that the main process is the enzymatic degradation of dye molecules. Moreover, all LACn@SP/(PEI/PAA)_{4,5} and LACn@SP/(PEI/PMANa)_{4,5} tested samples were able to lead to 100 % dye degradation in reasonable time intervals (between 15 and 90 min, depending on the amount of immobilized LAC), suggesting that these biocatalysts could represent a good alternative for an environmentally friendly wastewater treatment method.

3.3.3. Influence of pH

The influence of pH on the activity of the immobilized LAC was tested in the presence of ABTS as substrate (Fig. 10a) and in the discoloration of the dye sample in the presence of SG as mediator (Fig. 10b), using LAC2@SP/(PEI/PAA)_{4,5} and LAC2@SP/(PEI/PMANa)_{4,5}.

In enzymatic processes, the variation of pH is a crucial parameter since it influences not only the enzyme's activity, but also the stability of the substrate or the oxidized mediator. As observed in Fig. 10, the pH has a significant impact on both the LAC activity and its ability to degrade the IC molecule. As presented in Fig. 10a, the free enzyme has an optimum pH of around 4.5, while the immobilized enzyme has a better catalytic activity at pH = 5.5. Moreover, it can be observed that as the

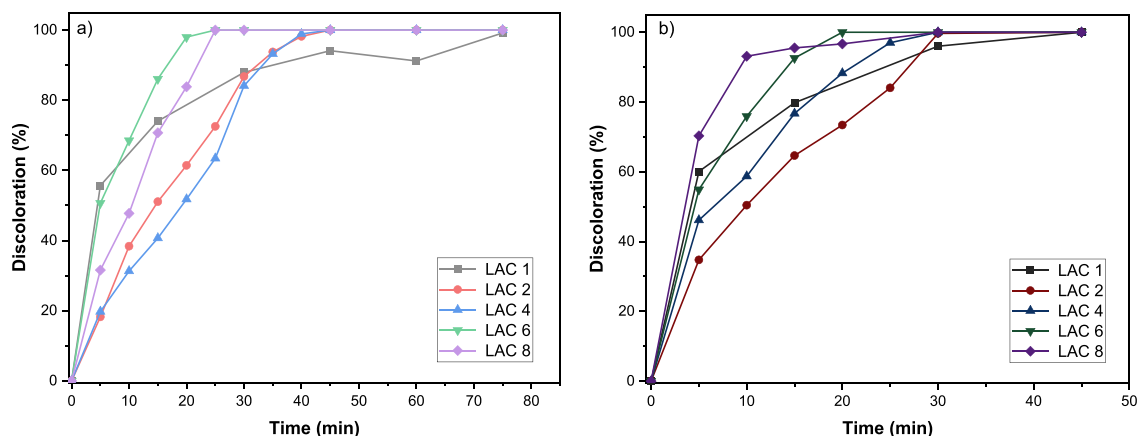


Fig. 9. The IC sample discoloration efficiency using the composite (a) LACn@SP/(PEI/PAA)_{4,5} and (b) LACn@SP/(PEI/PMANa)_{4,5}.

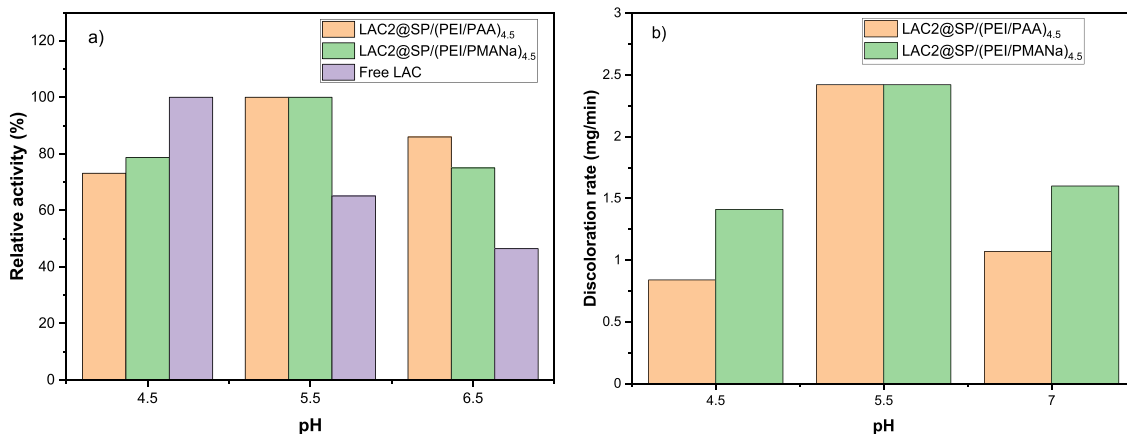


Fig. 10. Enzymatic activity as a function of pH (a) and the dye discoloration rate as a function of pH (b).

pH increases, the activity of the free LAC decreases strongly, while in the case of the immobilized LAC, the influence is weaker, suggesting that the immobilization of the enzyme increases its stability to pH variations. In the case of the dye degradation process, analysed by calculating the discoloration rate at different pH values, it was observed that at lower pH values, the discoloration rate was lower, due to the decrease in enzymatic activity but also because of the aggregation of the dye molecules due to the stronger attraction generated by the ionization of the dye functional groups. Increased protonation of the dye molecule could potentially lead to either electrostatic repulsions between the charged dye and the biocatalyst or to the formation of dye aggregates which will be less prone to enzymatic degradation due to diffusional limitations. On the other hand, increased electrostatic attraction between the dye and the biocatalyst can generate an increase in the local dye concentration in the proximity of the biocatalyst and enhance its interaction with the oxidized mediator, leading to dye degradation. Also, the high protonation degree of IC as the pH decreases will lead to enhanced electrostatic repulsions between the negatively charged dye and the negatively charged LAC2@SP/(PEI/PAA)_{4.5} or LAC2@SP/(PEI/PMANa)_{4.5}, preventing their interaction. At higher pH values, the discoloration rate increases strongly for both LAC2@SP/(PEI/PAA)_{4.5} and LAC2@SP/(PEI/PMANa)_{4.5}, with the fastest discoloration being observed at pH = 5.5 which can be attributed to a satisfactory enzymatic activity (as it was observed that the optimal activity of the immobilized LAC is at pH = 5.5), correlated with good stability of the SG radicals that are able to oxidize the dye solution at this pH value. This observation is in agreement with other studies that present the enzymatic degradation of

pollutants, such as amoxicillin, with the best results being obtained at pH = 5.5 [42].

3.3.4. Influence of dye concentration

The concentration of the dye can strongly influence the effectiveness of the degradation process, higher concentrations of dyes requiring, in general, higher enzyme concentrations for complete discoloration. As observed in Fig. 11, for the immobilized LAC, a faster discoloration rate can be observed as the dye concentration increases, probably due to the synergy between the enzymatic degradation of the dye and its sorption on the surface of the composite material. At the same time, it can be observed that LAC6@SP/(PEI/PAA)_{4.5} and LAC6@SP/(PEI/PMANa)_{4.5} are able to fully discolor IC samples, even at higher concentrations, demonstrating their good catalytic capacity.

3.3.5. Reusability

In terms of reusability, the LAC_n@SP/(PEI/PAA)_{4.5} and LAC_n@SP/(PEI/PMANa)_{4.5} were successfully tested in 4 dye degradation cycles, with 100 % discoloration efficiency. An interesting observation was that as LAC_n@SP/(PEI/PAA)_{4.5} and LAC_n@SP/(PEI/PMANa)_{4.5} are reused, the time necessary for the full discoloration of the sample is slightly increasing, but nevertheless, the full discoloration of the dye sample can be achieved. For this reason, the discoloration efficiency was calculated for each sample after a time interval corresponding to the minimum time needed for the full discoloration of the sample (corresponding to the first cycle of use of each LAC_n@SP/(PEI/PAA)_{4.5} and LAC_n@SP/(PEI/PMANa)_{4.5}) (Fig. 12).

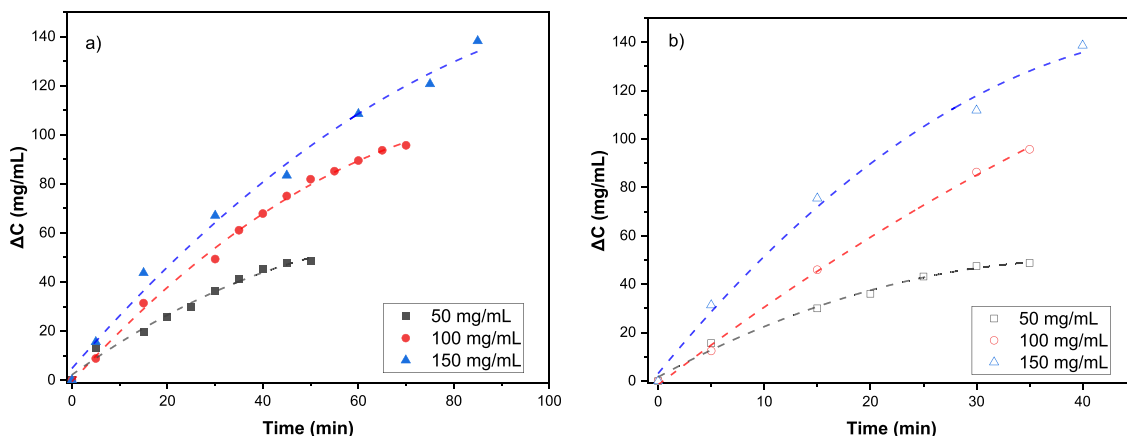


Fig. 11. Dye discoloration as a function of dye concentration using (a) LAC6@SP/(PEI/PAA)_{4.5} and (b) LAC6@SP/(PEI/PMANa)_{4.5}.

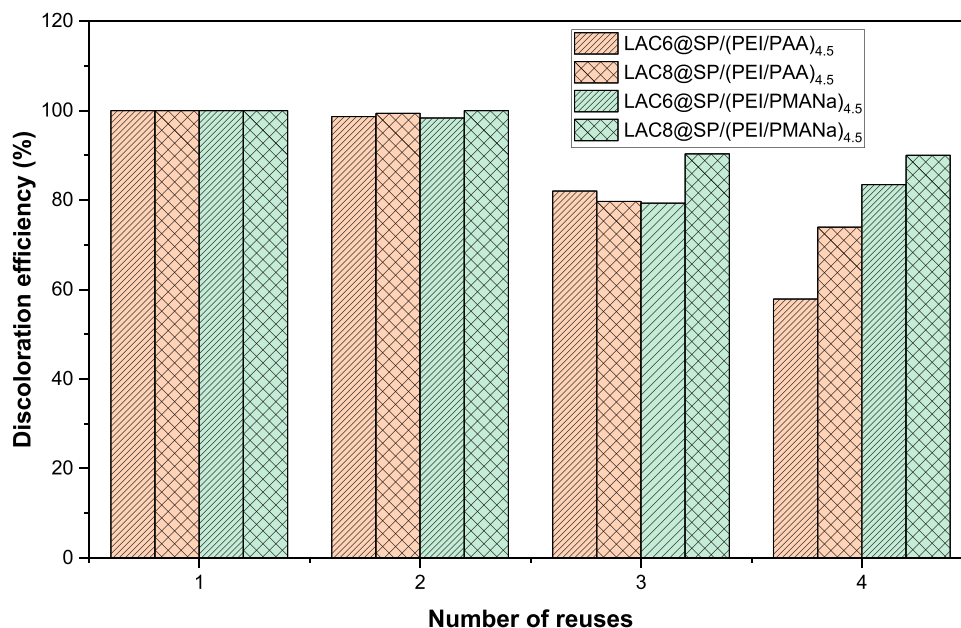


Fig. 12. The discoloration efficiency of LAC6@SP/(PEI/PAA)_{4.5}, LAC8@SP/(PEI/PAA)_{4.5}, LAC6@SP/(PEI/PMANa)_{4.5} and LAC8@SP/(PEI/PMANa)_{4.5}, calculated after the minimum time interval needed for full discoloration.

The decrease in degradation efficiency with increasing the number of reuses of the LAC n @SP/(PEI/PAA)_{4.5} and LAC n @SP/(PEI/PMANa)_{4.5} (by increasing the degradation time) is a normal phenomenon that can be associated with the accumulation of some impurities on the surface of the biocatalysts, reducing the diffusion of the reagents and therefore the immobilized LAC efficiency. Nevertheless, the dye degradation observed in this study was satisfactory even after 4 reuse cycles, demonstrating the enhanced stability of the immobilized enzyme.

4. Conclusions

This study presents a comprehensive examination of the development of polymer/enzyme composite materials obtained by the immobilization of LAC onto polyelectrolyte-based core-shell microparticles and their employment in water-cleaning applications. The LAC was successfully immobilized on the composite supports with immobilization yields ranging from 30 % to 80 %. The innovative composites with immobilized LAC exhibited remarkable catalytic activity and stability in various process conditions and were successfully employed in the degradation of IC, a negatively charged pollutant which was completely degraded under the action of the immobilized LAC/ mediator tested systems. All the tested LAC n @SP/(PEI/PAA)_{4.5} and LAC n @SP/(PEI/PMANa)_{4.5} biocatalysts were able to degrade 100 % of the IC sample in time intervals ranging from 15 to 90 min at concentrations of dye 50 mg/L. Globally, the immobilized LAC represent a promising alternative to classical wastewater treatment methods, presenting an efficient, robust, and environmentally friendly alternative to classical water cleaning methods.

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CRedit authorship contribution statement

Rénato Froidevaux: Validation, Supervision, Funding acquisition, Conceptualization. **Froidevaux Renato:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Funding acquisition, Conceptualization. **Mihai Marcela:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Stoica Iuliana:** Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation, Conceptualization. **Bucatariu Florin:** Writing – original draft, Validation, Software, Formal analysis, Data curation. **Petrila Larisa-Maria:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Froidevaux Renato reports financial support was provided by University of Lille. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jece.2025.115631](https://doi.org/10.1016/j.jece.2025.115631).

Data Availability

No data was used for the research described in the article.

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