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CHEMISTRY Field

ADVANCES IN POLYMER/ENZYMES COMPOSITES FOR APPLICATIONS IN CATALYSIS

SUMMARY OF THE DOCTORAL THESIS

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ROMANIAN ACADEMY

Petru Poni Institute of Macromolecular Chemistry

Mrs./Ms.....

The public defense of the thesis "***Advances in polymer/enzymes composites for applications in catalysis***", authored by **eng. Larisa-Maria PETRILA,** will take place on **20th of November, 2025**, **10^{00}**, in the Conference Room of Petru Poni Institute of Macromolecular Chemistry, for the purpose of conferring the scientific title of Doctor.

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In accordance with the Regulation on the organization and conduct of the doctoral studies for the awarding of scientific titles in the Romanian Academy, we are sending you the summary of the doctoral thesis, kindly requesting your appreciations and observations. On this occasion, we invite you to participate in the public defense of the doctoral thesis.

Director PPIMC,
CS I dr. Valeria HARABAGIU

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I am deeply grateful to ***my family****, the best supporters I could have wished for. To my brothers* ***Mădălin****,* ***Valentin****, and* ***Ovidiu****, my sister-in-law* ***Iulia****, and my nephew* ***Tudor****, I am grateful for their unconditional love, unwavering support, and unshakeable faith in me. I thank my boyfriend,* ***Roland****, my safe haven in the storm, for his patience, support, and encouragement in all the important moments. I thank* ***all my friends*** *for their constant presence, for the optimism and balance they offered me when I needed it most.*

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With gratitude,

Drd. Eng. Larisa-Maria PETRILA

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LIST OF ABBREVIATIONS

ABTS – 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AFM – atomic force microscopy

ALG – alginate

ASG – syringaldehyde

CHI – chitosan

CHI-*g*-PNIPAM – chitosan grafted with poly(*N*-isopropylacrylamide)

CR – Congo Red

DLS – dynamic light scattering

EDAX – energy-dispersive X-ray spectroscopy

ESI – MS – electrospray ionization mass spectrometry

GA – glutaraldehyde

HPLC-MS – high-performance liquid chromatography coupled with mass spectrometry

IC – Indigo Carmine

LAC – laccase

LbL – layer-by-layer

LCST – low critical temperature solution

MS – mass spectrometry

PAA – poly(acrylic acid)

PEI – poly(ethyleneimine)

PEI_M – poly(ethyleneimine) with high molar mass

PEI_m – poly(ethyleneimine) with low molar mass

PEP – pepsin

PMA – poly(methacrylic acid)

PNIPAM – poly(*N*-isopropylacrylamide)

RM – mass ratio

RMSD – root mean square deviation

SEM – scanning electron microscopy

STEM – scanning transmission electron microscopy

TGA – thermogravimetric analysis

XPS – X-ray photoelectron spectroscopy

INTRODUCTION

General conceptual references. Importance, relevance, and motivation of the doctoral thesis

Enzymes, considered the "catalysts of life", are naturally occurring protein structures involved in a multitude of biological processes. They accelerate the biological processes essential to life, in their absence, metabolic reactions being too slow to sustain it. Enzymes are characterized by extraordinary specificity and catalytic efficiency, contributing to food digestion and metabolism regulation, DNA synthesis, and immune response determination. In addition to their primary biological role, enzymes are also increasingly important in other fields such as industry, medicine, and environmental protection. Depending on their origin and catalytic specificity, enzymes can catalyse processes of industrial interest, serve in the diagnosis or treatment of diseases, or catalyse the degradation of environmental pollutants.

Although the catalytic properties of enzymes are extremely promising, their use in large-scale applications is limited by their low stability in conditions other than their natural environment. To overcome these limitations, enzymes are often immobilized to improve their stability under various conditions. Enzyme immobilization is a topic of growing interest in scientific research, motivated by the desire to increase the stability and performance of enzymatic catalysts, particularly regarding the development of biocatalysts with industrial applicability. From this perspective, the literature describes some methods of enzyme immobilization as well as different types of materials that can serve as supports for their immobilization.

Although the subject of enzyme immobilization has already been studied in recent years, it is far from exhausted, presenting a high interest for the development of new support materials for enzyme immobilization, the study of interactions between enzymes and support materials that modulate the catalytic activity of immobilized enzymes, or the development of new directions of use of the immobilized enzymes.

Current studies on enzyme immobilization are also of interest from the point of view of developing innovative catalytic systems, such as enzyme-polymer composite materials. The use of polymers in obtaining such materials is justified by their structural and functional versatility, as polymers of natural or synthetic origin with different physical or chemical properties, biodegradable polymers, stimuli-sensitive polymers, or combinations of polymers can be used. Careful selection of polymers allows for the adaptation of the

properties of the materials obtained, directly influencing the efficiency and/or stability of the biocatalysts.

In this context, **the motivation** for developing **polymer/enzyme composite materials** is supported by both theoretical aspects and considerations of applicability. The development of this field can make significant contributions to understanding the interactions between enzymes and polymers, identifying the effect that enzyme immobilization can have on their catalytic properties, optimizing the conditions for immobilizing different enzymes, and designing efficient biocatalysts for different types of applications. This approach is particularly relevant in view of the potential applications of the materials obtained, as their design can be directed towards various applications such as biosensors, surfaces with antimicrobial properties, catalysts for industrial, environmental, pharmaceutical or food applications.

An application with a significant impact on society is the use of polymer/enzyme composite materials as **catalysts for the degradation of certain pollutants from water**, a wastewater treatment method that responds to current environmental protection challenges. The motivation for designing polymer/enzyme composite materials for such applications is also supported by the need to develop sustainable and environmentally friendly depollution solutions, as well as the possibility of using naturally sourced polymers, which justifies their choice.

Thesis objectives and experimental methodology

Based on the considerations presented above, the main objective of the doctoral thesis was formulated, consisting of ***the design, obtaining, and physicochemical and biochemical characterization of new composite materials based on enzymes and natural or synthetic polymers, intended for catalytic applications***.

Four secondary objectives were derived from this main objective:

- Optimization of the fabrication of support materials for enzyme immobilization by layer-by-layer deposition of polyelectrolytes on silica microparticles;
- Immobilization of enzymes on core-shell support materials obtained by layer-by-layer deposition of polyelectrolytes on silica microparticles;
- Preparation of enzyme/polymer nanostructures in the form of interpolyelectrolyte complexes;
- The use of composite materials obtained by laccase immobilization as catalysts for the degradation of pollutants in wastewater.

Content of the doctoral thesis

The doctoral thesis entitled "***Advances in polymer/enzymes composites for applications in catalysis***" spans 195 pages and comprises seven chapters, including 100 figures, 21 tables, 12 equations, and 310 bibliographic references. The thesis is structured in two main sections: Part I - Current state of knowledge, which comprises two chapters and presents the scientific context that motivates the choice of research topic, materials, and experimental methods, and Part II - Original contributions, which is structured in five chapters and presents the original results obtained during the doctoral studies.

Chapters 1 and **2** present the current state of knowledge and describe the general context of enzyme immobilization, the main immobilization methods reported in the literature, as well as six main types of polymer-based materials that are used in enzyme immobilization, respectively the main directions of use of polymer/enzyme composite materials. A thorough analysis of research trends in this field has allowed the identification of research directions that have been explored in the experimental studies carried out during the preparation of this doctoral thesis, taking into account the expertise of the department and the possibilities for developing the selected research directions.

Chapter 3 refers to the materials used, including a detailed description of the experimental methods used to obtain polymer/enzyme composite materials. The chapter describes a comprehensive methodology for the characterization of materials and the evaluation of the catalytic properties and potential applications of the composite materials obtained, using modern characterization methods and established biochemical techniques.

Chapter 4 presents the preparation of composite materials designed to serve as a support for enzyme immobilization using the layer-by-layer deposition of polyelectrolytes on silica microparticles. The chapter includes details on the preparation of composite materials and their characterization using various experimental techniques, identifying the main aspects that influence the properties of the materials and selecting the most efficient materials for further testing.

Chapter 5 describes the immobilization of two enzymes - pepsin and laccase - on some core-shell composite materials selected from those presented in Chapter 4. The results presented in this chapter aim to optimize the enzyme immobilization process, characterize the physicochemical properties of the materials after enzyme immobilization, and, in the case of materials with immobilized laccase, evaluate their catalytic properties, demonstrating the feasibility of using these materials as a support for enzyme immobilization. At the same time, the studies carried out have allowed the identification of the most promising composite

materials with immobilized enzymes in terms of catalytic performance, which have been selected for testing in catalytic applications.

Chapter 6 presents the obtaining of nanostructures based on laccase and chitosan and chitosan grafted with poly(*N*-isopropylacrylamide). The methodology proposed in this chapter aims to investigate the mechanism of interaction between the enzyme and polysaccharides through various experimental methods and to confirm it through molecular dynamics simulations, as well as to highlight the main characteristics of the formed nanostructures and to evaluate the catalytic activity of the nanostructures formed under different experimental conditions. The chapter contributes to the understanding of the interaction mechanisms between proteins and polysaccharides and proposes the preparation of innovative nanostructures formed with laccase, an enzyme of industrial interest, and a modified polysaccharide sensitive to external stimuli.

Chapter 7 presents the potential applications of polymer/enzyme composite nanostructures in the catalytic degradation of certain dyes in aqueous media, highlighting their potential for practical application. The studies presented examine the capacity of some of the selected materials to degrade dyes under different experimental conditions, as well as identifying the mechanism of enzymatic degradation of one of the dyes tested. Finally, the chapter highlights an unconventional solution for wastewater treatment, namely the use of enzyme/polysaccharide nanostructures as catalysts for the degradation of certain dyes.

The doctoral thesis finishes with a series of general conclusions and perspectives for the development of the proposed studies, accompanied by two Annexes organized as follows: Annex 1 – Dissemination of scientific results and Annex 2 – Copies of published articles.

The results presented in the doctoral thesis entitled "***Advances in polymer/enzymes composites for applications in catalysis***" presents the obtaining of composite materials based on polymers and enzymes. The experimental methods used have enabled the preparation of composite materials with practical applications, both in the form of inorganic core microparticles with polymeric coating and immobilized laccase, and in the form of laccase/polysaccharide nanostructures. The two types of composite materials obtained demonstrated satisfactory catalytic activity, tested under various experimental conditions, and an overall improvement in enzyme properties, demonstrated by an increase in its stability under various conditions. The two types of materials with immobilized laccase were also successfully used in the degradation of dyes in an aqueous environment, demonstrating

their feasibility as catalysts for the degradation of organic pollutants in an aqueous environment.

PART II

ORIGINAL CONTRIBUTIONS

CHAPTER 4

OBTAINING COMPOSITE SUPPORT MATERIALS FOR ENZYME IMMOBILIZATION

The results presented in this chapter have been the subject of the following publications:

1. ***Petrila, L. M.**, Bucatariu, F., Stoica, I., Mihai, M., Froidevaux, R. (2025a). J Environ Chem Eng, 13(2), 115631. https://doi.org/10.1016/j.jece.2025.115631*
2. *Bucatariu, F., **Petrila, L.,** Teodosiu, C., & Mihai, M. (2022). CR Chim, 25, 95–108. DOI: 10.5802/crchim.171*
3. ***Petrila, L. M.**, Bucatariu, F., Mihai, M., (2025b). Rev Roum Chim, 70, 465-473.https://doi.org/10.33224/rrch.2025.70.7-8.10*

4.2. Obtaining composite materials by layer-by-layer deposition of polyelectrolytes

4.2.1. Layer-by-layer deposition of polyelectrolytes and cross-linking of the resulting composite materials

For the preparation of the composite materials, a series of ionic polymers classified as weak polyelectrolytes was employed. These polymers exhibit specific characteristics, particularly sensitivity to variations in pH and ionic strength (Petrila, 2021). Moreover, the selected polyelectrolytes differ in terms of molecular weight and chain arrangement (linear/branched). Consequently, the degree of ionization of the polyelectrolytes, and thus their conformation, is expected to be influenced by the experimental working conditions.

For the formation of polyelectrolyte multilayers, combinations of polycations (PEI_m, PEI_M) and polyanions (PAA, PMA) were employed, while silica microparticles differing in porosity (SP1k and SP2k) were used as supports. The deposition of the organic shell via multilayer formation was monitored through polyelectrolyte titrations, which allowed the indirect determination of the amount of organic material deposited after each step. The multilayer deposition was carried out to obtain materials with an increasing number of

bilayers, where a bilayer is defined by the alternate deposition of one polycation layer and one polyanion layer. Figure 4.2 illustrates the variation in the amount of organic material deposited on the inorganic support as a function of the number of bilayers deposited and of the type of inorganic support used.

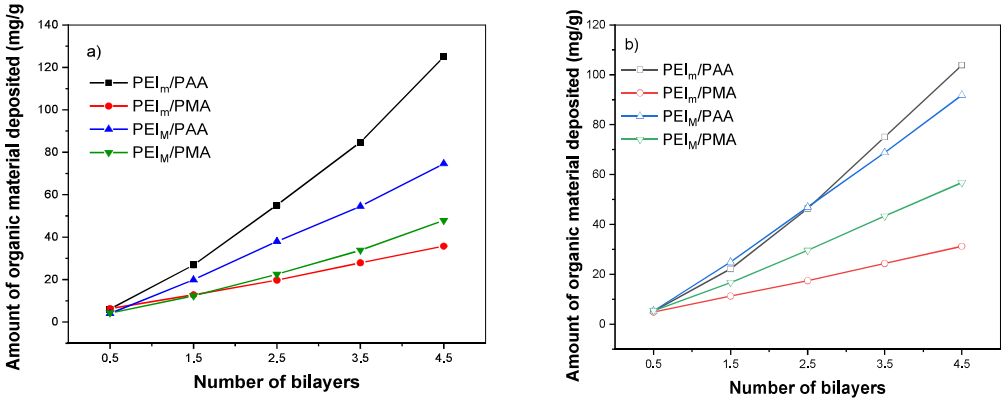

Figure 4.1: Variation of the amount of organic material deposited on Daisogel SP1k (a) and SP2k (b) microparticles as a function of number of bilayers (results published partially in ***Petrila, 2025a****)*

Based on the graphical representations in Figure 4.2, it can be observed that, regardless of the porosity of the silica used as a deposition support, the amount of deposited organic material follows the same approximate trend of linear increase with the increase in the number of deposited layers. It is also noteworthy that the composite materials obtained with PAA, for the same polycation and regardless of the inorganic support employed, contain a larger amount of organic material, which suggests the deposition of a thicker polymeric film on the support compared to the materials obtained with PMA. This behaviour results from the conformation of PAA: at a solution pH of ~3, the functional groups along the PAA chains are only partially ionized, and the high-molecular-weight chains adopt a coiled conformation, forming globules that lead to the deposition of a thicker organic layer. On the other hand, PMA is strongly ionized at pH ~8, and its chains adopt an extended conformation; under these conditions, the deposited layer is thinner than in the case of PAA deposition. With respect to the influence of the polycation, it can be observed that, for the same polyanion and irrespective of the inorganic support employed, the use of the lower-molecular-weight polycation, PEI_m, results in the deposition of a thicker layer.

To ensure better cohesion of the deposited layers, chemical crosslinking of the PEI chains was carried out after each deposition step, using an amount of crosslinker that provided a 10:1 ratio between the carbonyl groups of the crosslinker and the amine groups of the polycation. The selected ratio had been optimized in previous studies conducted within

the research team, with the required amount of crosslinker being calculated based on the amount of deposited polycation, indirectly determined through polyelectrolyte titrations (Bucatariu, 2022). Crosslinking of the PEI chains provides greater stability to the deposited layers and facilitates the subsequent use of the composite materials in applications involving multiple reuse cycles or hydrodynamic pressure conditions, as suggested by the relevant literature (Ghiorghita, 2019).

4.4. Partial conclusions

In this chapter, the preparation of inorganic core-organic shell composite materials was investigated through the layer-by-layer deposition of polyelectrolytes onto silica microparticles, the main objective of the study being the optimization of the polyelectrolyte deposition process in order to obtain stable core-shell microparticles. Several experimental combinations were examined, including two types of silica supports, two polycations, and two polyanions. Additionally, composite materials were obtained by depositing multiple polyelectrolyte layers, yielding materials with 0.5, 1.5, 2.5, and 4.5 bilayers. To improve the characteristics of the resulting materials, an acid-base treatment was applied. The obtained materials were extensively characterized using a combination of modern physicochemical methods to investigate their chemical composition, surface charge, and morphology.

The results highlighted several important aspects:

- the porosity of the silica support does not significantly influence the deposition tendency of the polyelectrolyte multilayers, nor the total amount of organic material deposited;
- the molecular weight of the polyelectrolytes employed affects the formation and stability of the multilayers, with the materials obtained using PEI_m being characterized by the lowest stability;
- the use of PEI_M led to the formation of more stable organic coatings as a result of improved interactions between the two polyelectrolytes.

Based on these observations, **the materials selected as supports for enzyme immobilization were the composites obtained through the layer-by-layer deposition of the PEI_M/PAA and PEI_M/PMA polyelectrolyte pairs**.

CHAPTER 5
ENZYME IMMOBILIZATION ON CORE-SHELL COMPOSITE MATERIALS

The results presented in this chapter have been the subject of the following publications:

1. ***Petrila, L. M.**, Bucatariu, F., Stoica, I., Mihai, M., Froidevaux, R. (2025a). J Environ Chem Eng, 13(2), 115631. https://doi.org/10.1016/j.jece.2025.115631*
2. ***Petrila, L. M.**, Bucatariu, F., Mihai, M., (2025b). Rev Roum Chim, 70, 465-473. https://doi.org/10.33224/rrch.2025.70.7-8.10*

5.3. Studies on the immobilization of laccase

Influence of the pH on the immobilization of LAC

pH plays a crucial role in the successful immobilization of enzymes, as it influences both the ionization of functional groups that may participate in enzyme immobilization and the conformation and stability of the enzymes themselves. The effect of pH on the immobilization yield was studied in the range of pH = 3.5-5.5, to preserve both satisfactory enzyme activity and the stability of the polymer chains in the composite material. The analysis of the immobilization yields obtained, shown in Figure 5.12, indicates that the best results are achieved at pH = 4.5 for both types of microparticle supports.

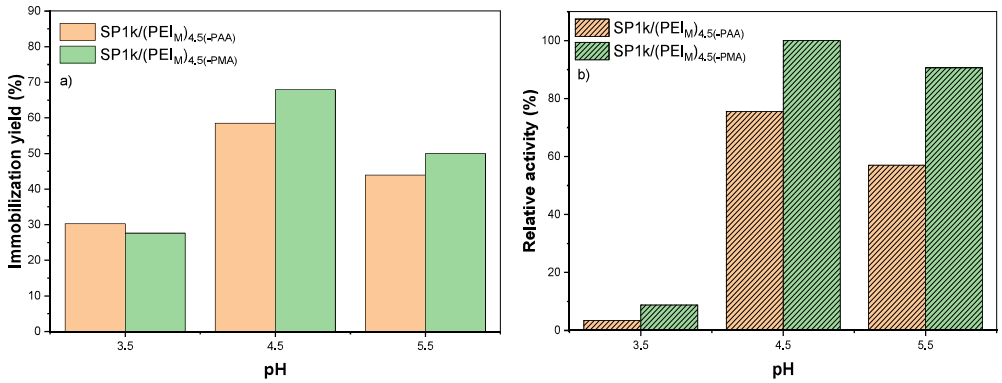

Figure 5.1: Immobilization yield (a) and relative enzymatic activity (b) of immobilized LAC as a function of immobilization pH ***(Petrila, 2025a)***

As shown in Figure 5.12, at low pH, the immobilization is reduced, most likely due to the ionization of the functional groups of the polyanion remaining in the core-shell composite structure, which generates electrostatic repulsions between the composite and the enzyme. The immobilization yields obtained in the pH range 4.5-5.5 vary only slightly, suggesting that minor pH fluctuations do not strongly affect the immobilization process.

Furthermore, the enzymatic activity of the composite materials LAC1@SP1k/$(PEI_M)_{4.5(-PAA)}$ and LAC1@SP1k/$(PEI_M)_{(-PMA)4.5}$ (Figure 5.12 b) was studied, showing that LAC immobilization at pH = 4.5 retained the highest catalytic activity (defined as 100% for the best results obtained, with other values reported relative to the maximum activity). This pH value ensures a satisfactory degree of ionization of the functional groups in the composite material. Moreover, it can be inferred that at this pH the enzyme conformation was appropriate, favouring immobilization without affecting the enzyme's active site.

It is also noteworthy that both the immobilization yield and the relative enzymatic activity are higher when LAC is immobilized on the composite material obtained with PMA, suggesting a greater affinity of the support for the enzyme, most likely due to stronger interactions with the support material, involving both electrostatic and hydrophobic interactions.

Influence of concentration on LAC immobilization

Another important parameter influencing the efficiency of the enzymatic immobilization process is the initial enzyme concentration, which significantly affects its diffusion during immobilization. In this context, the effect of enzyme concentration on the immobilization step was studied in the range of 1-8 mg/mL. Figure 5.13 presents the immobilization yield and the amount of immobilized enzyme (mg/mg) as a function of the initial enzyme concentration.

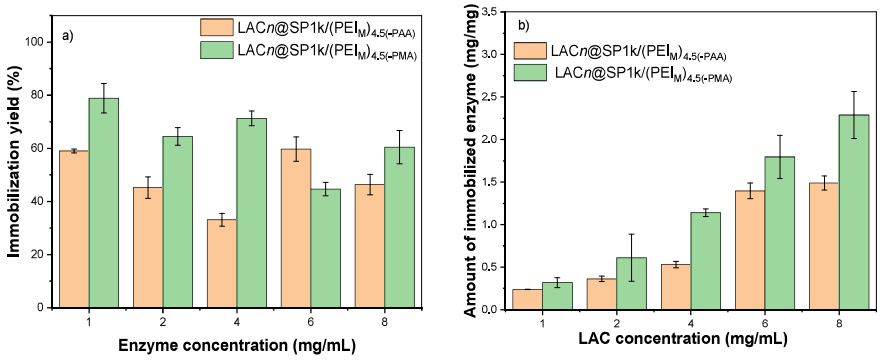

Figure 5.2: LAC immobilization yield (a) and amount of immobilized enzymes (b) as a function of initial enzyme concentration ***(Petrila, 2025a)***

As shown in Figure 5.13 a, the highest enzyme immobilization yield was obtained at an initial concentration of 1 mg/mL, corresponding to 59.02 ± 0.07% for the SP1k/$(PEI_M)_{4.5(-PAA)}$ support materials and 78.90 ± 5.53% for the SP1k/$(PEI_M)_{4.5(-PMA)}$ support materials. A satisfactory immobilization efficiency was maintained in the concentration range of 2-8 mg/mL, whereas at higher enzyme concentrations, surface saturation may occur. While

increasing the concentration can lead to a higher amount of immobilized enzyme due to enzyme aggregation on the material surface, the stability and catalytic activity of a biocatalyst prepared at higher enzyme concentrations may be compromised by weak interactions between enzyme aggregates and the support material, as well as by diffusion limitations and steric hindrances that reduce the likelihood of substrate molecules interacting with the enzyme's active site. Thus, it can be inferred that using lower enzyme concentrations results in better immobilization yields, even though the greatest total amount of immobilized enzyme is found at the highest tested concentration for LAC (Figure 5.13 b).

5.3.4. Evaluation of the catalytic properties of the composite materials with immobilized LAC

Determination of the catalytic activity of the composite materials with immobilized enzyme

By far, the most important parameter to monitor in the preparation of biocatalysts via enzyme immobilization is the preservation of satisfactory enzymatic activity. For this reason, determining the catalytic activity of biocatalysts obtained through enzyme immobilization is essential for establishing the optimal parameters for producing efficient biocatalysts. The enzymatic activity of the composite materials after LAC immobilization was studied under conditions similar to those used for determining LAC activity in solution, specifically by monitoring the conversion of ABTS at pH = 4.5 and a temperature of 25 °C. The enzymatic activity of the composite materials obtained through LAC immobilization at initial concentrations of 1-8 mg/mL was expressed as relative activity, with the maximum activity achieved being set as 100% (regardless of the type of microparticles used as immobilization support) and the other activity values reported relative to this maximum.

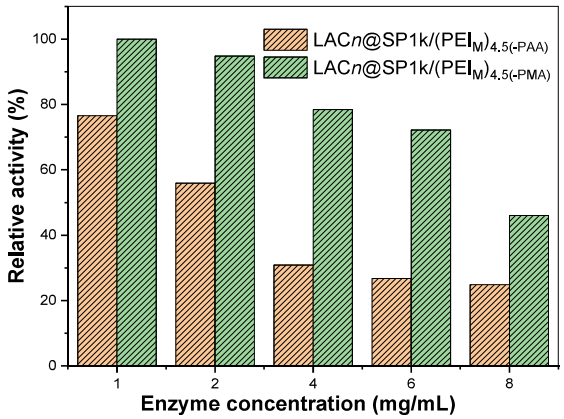

Figure 5.3: Relative enzymatic activity of the composite materials with LAC immobilized at concentrations n = 1-8 mg/mL and pH = 4.5 ***(Petrila, 2025a)***

The analysis of the enzymatic activity of the LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-PAA})}$ and LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-PMA})}$ materials obtained after LAC immobilization at initial enzyme concentrations of 1-8 mg/mL and pH 4.5 (Figure 5.17) shows that, in general, the relative enzymatic activity decreases with increasing enzyme concentration. The highest activity was maintained at an initial concentration of 1 mg/mL for both the SP1k/$(PEI_M)_{4.5(\text{-PAA})}$ and SP1k/$(PEI_M)_{4.5(\text{-PMA})}$ composites.

Reusability of the composite materials with immobilized LAC

To evaluate the reusability of the composite materials with LAC immobilized at a concentration of 2 mg/mL, the same method for determining ABTS conversion under optimal temperature and pH conditions was employed. After each cycle of use, the composite materials with immobilized LAC were separated, washed with acetate buffer, and reused for ABTS conversion with a fresh substrate solution.

The analysis of the reusability of the composite materials with immobilized LAC (Figure 5.20) indicates that they can be successfully reused for seven cycles, with less than a 5% loss of activity during the first three reuse cycles and approximately 50% of the initial activity retained after seven cycles.

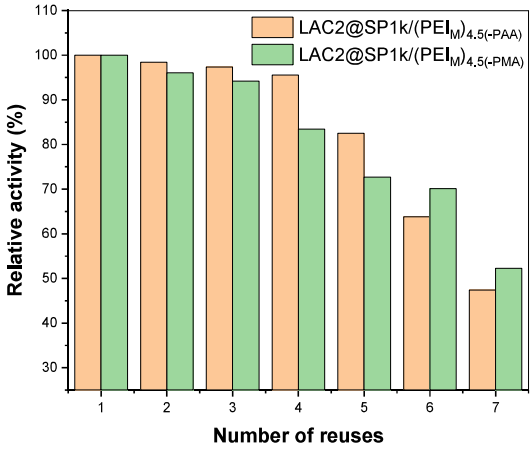

Figure 5.4: Relative enzymatic activity upon reusing the composite materials with immobilized LAC

These results confirm that LAC immobilization provides significant economic advantages compared to the use of the enzyme in its native state, as immobilization facilitates the recovery and reuse of the biocatalysts, thereby reducing both the costs associated with enzymatic catalyst use and the environmental impact.

5.5. Partial conclusions

This chapter investigated enzyme immobilization on core-shell composite materials obtained through the layer-by-layer deposition of polyelectrolytes. In the first stage, the immobilization of PEP as a model enzyme on the two types of materials was studied, focusing on key parameters such as the pH and initial enzyme concentration, the amount of organic material deposited on the microparticle supports, and the application of acid-base treatments for flexibilization and/or chemical crosslinking of the organic coating. Following the optimization of these parameters, LAC immobilization was studied under the optimized conditions to obtain biocatalysts with potential catalytic applications.

For the composite materials prepared via layer-by-layer polyelectrolyte deposition, the studies revealed several conclusions:

- the main characteristics influencing enzyme immobilization are the stability of the organic shell, its porosity, hydrophilic/hydrophobic nature, and the availability of functional groups;
- enzyme properties have a significant influence on the immobilization process, with the optimization requiring the determination of the optimal pH and enzyme concentration;
- for the materials with immobilized LAC, the catalytic activity was studied, and the results showed that satisfactory catalytic activity is maintained after enzyme immobilization, accompanied by improved stability of the immobilized enzyme over a broader pH range and at elevated temperatures compared to free LAC;
- the composite materials with immobilized LAC offer a considerable advantage over the free enzyme, being successfully reused for at least seven catalytic cycles.

Overall, the best results were achieved when enzymes were immobilized at pH = 4.5 on microparticles prepared via layer-by-layer deposition of 4.5 bilayers of polyelectrolytes and subjected to the acid-base treatment. Since the optimal immobilization pH for PEP may induce denaturation, its catalytic properties were not studied. In contrast, the results obtained for LAC immobilization demonstrated favourable interactions between the core-shell composite materials and the negatively charged enzyme, suggesting that these composite supports can be successfully used for enzyme immobilization while maintaining satisfactory enzymatic activity. It was found that **the best immobilization outcomes occur at pH = 4.5 and at an enzyme concentration in the range of 1–6 mg/mL**.

CHAPTER 6
OBTAINING OF NANOSTRUCTURES BY FORMING INTERPOLYELECTROLYTIC COMPLEXES BETWEEN LACCASE AND NATURAL POLYMERS

The results presented in this chapter have been the subject of the following publications:

1. **Petrila, L. M.**, Karayianni, M., Vasiliu, T., Puf, R., Mihai, M., Pispas, S. (2025c). *Int J Biol Macromol., 322(2), 146754.* https://doi.org/10.1016/j.ijbiomac.2025.146754
2. **Petrila, L. M.**, Ciobanu, T.A., Vasiliu, T., Pispas, S., Mihai, M. (2025d). *Biomacromolecules, 26,* 6244-6257. https://doi.org/10.1021/acs.biomac.5c01217

6.2. Preparation of laccase/chitosan complexes

6.2.2. Study of the interaction between LAC and CHI

The interaction between LAC and CHI was investigated by fluorescence spectroscopy, based on fluorescence quenching studies. Due to the presence of aromatic amino acid residues such as tryptophan, tyrosine, and phenylalanine, LAC exhibits intrinsic fluorescence. The role of tryptophan in LAC fluorescence emission is significant, as LAC contains seven tryptophan residues (Saoudi, 2017). Linear regression was used to determine K_{SV}, and the values of K_{SV} and k_q at each temperature are presented in Table 6.1.

Table 6.1: Stern-Volmer constant (K_{SV}), bimolecular quenching constant (k_q), apparent binding constant (K_a) and number of binding sites (n) upon interaction of LAC with CHI, at different temperatures. R^2 – correlation coefficient. ***(Petrila, 2025d)***

Temperature (K)	K_{SV} (M^{-1})	k_q ($M^{-1} \cdot s^{-1}$)	R^2	K_a (M^{-1})	n	R^2
298	$1.56 \cdot 10^5$	$1.56 \cdot 10^{13}$	0.991	$6.30 \cdot 10^4$	0.93	0.987
303	$1.73 \cdot 10^5$	$1.73 \cdot 10^{13}$	0.985	$6.45 \cdot 10^3$	0.76	0.989
308	$1.90 \cdot 10^5$	$1.90 \cdot 10^{13}$	0.980	$4.78 \cdot 10^3$	0.73	0.990

As presented in Table 6.1, K_{SV} increases slightly with temperature, suggesting more efficient quenching, as previously reported for the interaction of CHI with bovine serum albumin (Bekale, 2015), as well as for the interaction of CHI and CHI/graphene oxide nanocomposites with β-galactosidase (Li, 2025). The increase in K_{SV} can be associated with the enhanced molecular motion induced by temperature, which facilitates collisions between

molecules in the system, but does not necessarily imply stronger interactions between them. The decrease in fluorescence intensity observed with increasing temperature may also result from partial thermal denaturation of LAC, leading to the exposure of tryptophan residues located within the internal structure of the enzyme, thereby increasing their likelihood of interacting with CHI.

The quenching mechanism can be elucidated based on the bimolecular quenching constant, k_q, considering that for dynamic quenching processes, the maximum value of k_q for various quenchers is around 2×10^{10} ($M^{-1} \cdot s^{-1}$) (Antonov, 2024; Lakowicz, 2006). The calculated k_q values for the LAC/CHI systems (Table 6.1) are considerably higher than this limit, which strongly suggests that the observed quenching mechanism is static (Lakowicz, 2006). A similar effect has been reported for the quenching of intrinsic fluorescence in different proteins in the presence of CHI or CHI derivatives, including sodium caseinate (Antonov, 2024), casein (Ma, 2023), ovalbumin (Leite Milião, 2022), β-lactoglobulin (Agudelo, 2013), and both bovine (Bekale, 2015; Liu, X., 2015) and human serum albumins (Bekale, 2015).

The effect of temperature on the interaction between LAC and CHI can be evaluated based on the apparent binding constant (K_a) and the number of binding sites, which were calculated (Table 6.1) from the double logarithmic Stern-Volmer plot. As shown by the values in Table 6.1, at ambient temperature, there is approximately one binding site for CHI. The binding constant K_a decreases with increasing temperature, suggesting that binding becomes less favourable and the stability of the formed complex diminishes at higher temperatures, consistent with observations reported for other protein-based systems (Asemi-Esfahani, 2021; Dehdasht-Heidari, 2021; Habibian Dehkordi, 2021). Such behaviour is expected, since elevated temperature accelerates Brownian motion, favouring collisions between molecules but not their binding. In addition, temperature may induce conformational changes in biomolecules, thereby either increasing or decreasing the accessibility of binding sites.

To gain a deeper understanding of the interaction mechanism between LAC and CHI, molecular dynamics simulations were performed to identify the amino acid residues in the enzyme structure that interact with CHI. In addition, simulations conducted at two different temperatures were used to assess the potential protective effect of CHI on the enzyme. The initial and final conformations of LAC, in the absence and presence of CHI, obtained from the molecular dynamics simulations, are presented in Figure 6.5 a, where the enzyme is displayed in three-dimensional representation with highlighted secondary structure

elements, and in Figure 6.5 b, which primarily illustrates the outer surface of the enzyme (*quicksurface representation*).

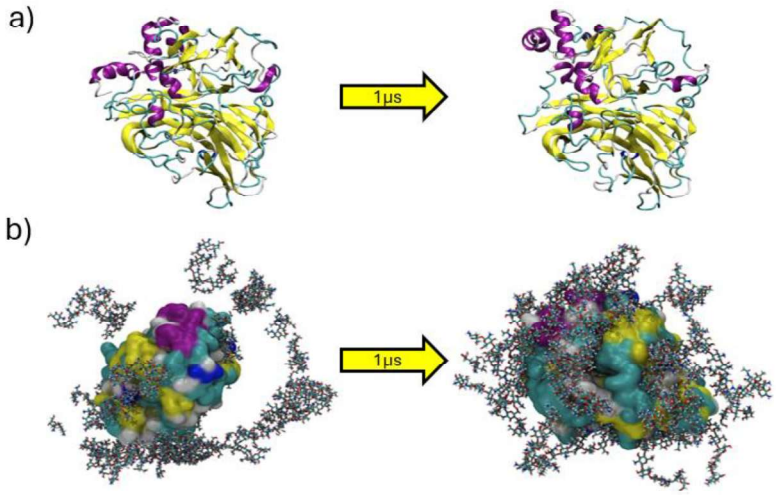

Figure 6.5: Initial and final conformation of LAC (a) and initial and final conformation of LAC upon interaction with CHI (b). Each structural region of the enzyme is coloured as follows: α helix in purple, β-sheets in yellow, and random coils in teal and white. CHI chains are represented as liquorice, and the atoms are coloured as follows: carbon in teal, nitrogen in blue, oxygen in red, and hydrogen in white ***(Petrila, 2025d)***

As shown in Figure 6.5 a, the enzyme structure undergoes only minor changes during the molecular dynamics simulation. Furthermore, Figure 6.5 b highlights that the interaction with CHI occurs at the enzyme surface, while the catalytic center of the enzyme remains conformationally unaffected. Thus, the interaction with CHI leads to the formation of a polymeric layer at the enzyme surface, which may play an important role in stabilizing the enzyme molecule.

6.2.3. Formation and characterization of interpolyelectrolyte complexes

The formation of LAC/CHI complexes was carried out by adding variable volumes of a 1 mg/mL CHI solution prepared in acetate buffer at pH = 4.5 to a 0.5 mg/mL LAC solution prepared in the same solvent, following the method described in Chapter 3, Section 3.4.1. The samples were coded according to the model $(LAC/CHI)_{RM}$, where RM represents the mass ratio between the components. The formation of LAC/CHI complexes was monitored by DLS, analysing changes in scattered light intensity, polydispersity index, and size distribution of the resulting complexes.

An initial confirmation of the formation of LAC/CHI interpolyelectrolyte complexes was obtained by analysing the variation in scattered light intensity as a function of the mass

ratio between the components (Karayianni, 2024). As shown in Figure 6.7 a, the scattered light intensity increases with the amount of CHI used in complex formation, demonstrating the successful formation of nanostructures and suggesting that the polysaccharide plays a key role in defining the characteristics of the complexes. Furthermore, the polydispersity index exhibits relatively low values for all samples (Figure 6.7 b), indicating that the formed nanostructures have relatively homogeneous dimensions.

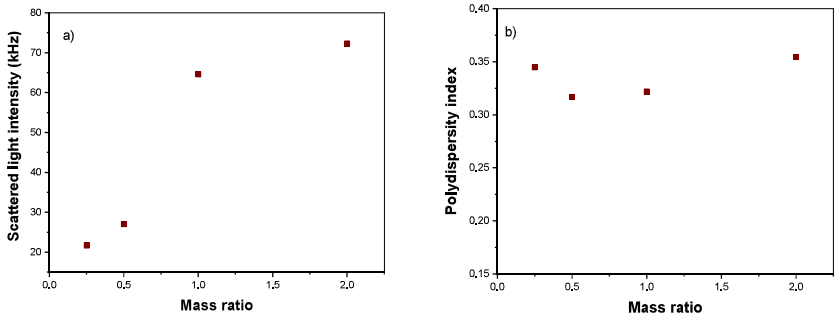

Figure 6.6: Variation of the scattered light intensity (a) and polydispersity index (b) as a function of mass ratios used for complexes prepared

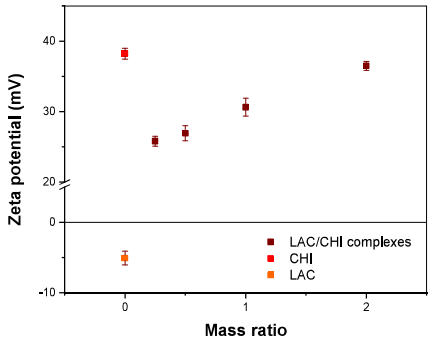

Figure 6.7: Zeta potential values for LAC/CHI nanostructures as compared with the values for LAC and CHI

Zeta potential measurements (Figure 6.9) indicate that all formed complexes carry a positive ionic charge, suggesting that the enzyme surface is partially coated by CHI chains, as also evidenced by molecular dynamics simulations (Figure 6.5). A similar variation in zeta potential was reported by Li and collaborators, who studied the formation of interpolyelectrolyte complexes between CHI and the whey protein α-lactoglobulin (Li, 2018), and by Bourouis and collaborators, who investigated CHI complexes with soy proteins (Bourouis, 2025). Both studies concluded that in complex formation, the protein is surrounded by CHI chains, with the positive ionic charge of CHI determining the overall charge of the resulting nanostructures. The measured zeta potential values of the formed

nanostructures are slightly lower than that of CHI in solution, demonstrating that an important role in complex formation is played by the partial neutralization of opposite charges on the CHI and LAC chains, as well as by the establishment of electrostatic interactions between the two macromolecules.

6.2.5. Evaluation of the catalytic properties of LAC/CHI nanostructures

A critical aspect in the case of enzyme-containing nanostructures is the effect of the complexation process on the tertiary structure of the enzyme and, consequently, on its catalytic activity. The catalytic activity of the LAC/CHI nanostructures was evaluated by monitoring the oxidation of ABTS and compared with that of the initial LAC solution, with the results presented in Figure 6.11.

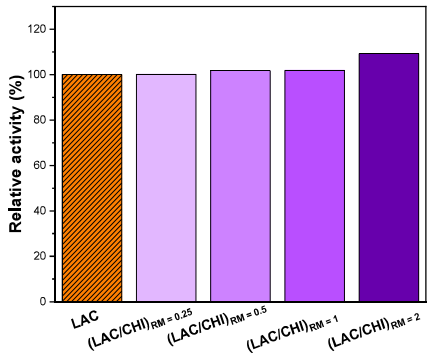

Figure 6.8: Relative enzymatic activity of the LAC/CHI nanostructures compared with the catalytic activity of LAC solution ***(Petrila, 2025d)***

As shown in Figure 6.11, the enzymatic activity of LAC does not vary significantly after its incorporation into the interpolyelectrolyte complexes, demonstrating that interaction with CHI does not affect the catalytic properties of the enzyme. However, a slight increase in catalytic activity of approximately 3% was observed for complexes prepared at RM = 0.25-1, and around 10% for complexes prepared at RM = 2. This suggests that CHI may stabilize the enzyme structure while still allowing access to its active site. The enhanced catalytic activity observed for LAC/CHI complexes may also result from changes induced by the introduction of nanostructures into the reaction medium, which could shift the pH towards the optimal value for the enzyme, reduce electrostatic repulsion between the enzyme and substrate, or enhance secondary interactions that facilitate substrate binding to the active site.

Another important aspect related to the catalytic activity of enzymes is their stability under various experimental conditions. One key factor to consider is the pH of the medium,

which can influence both the enzyme-substrate interaction and the stability of the nanostructures in which LAC is incorporated. To investigate this, the effect of pH on the catalytic activity of both free LAC and LAC/CHI complexes was studied using ABTS solutions prepared at different pH values.

As illustrated in Figure 6.12 a, variations in pH strongly influence the catalytic activity of both free LAC and LAC incorporated into nanostructures, confirming that the formation of LAC/CHI complexes does not alter the catalytic characteristics of the enzyme. It is evident that the highest enzymatic activity was obtained at pH = 3.5 for both free enzyme and LAC/CHI nanostructures, this pH value representing the optimum for the enzyme, as previously shown in Chapter 5. Regarding the catalytic activity of the nanostructures, it is notable that at low pH (pH = 3.5), the activity remains high, similar to that of the free enzyme. In contrast, increasing the pH leads to a pronounced decrease in catalytic activity, a trend observed for both the free enzyme and the nanostructured systems.

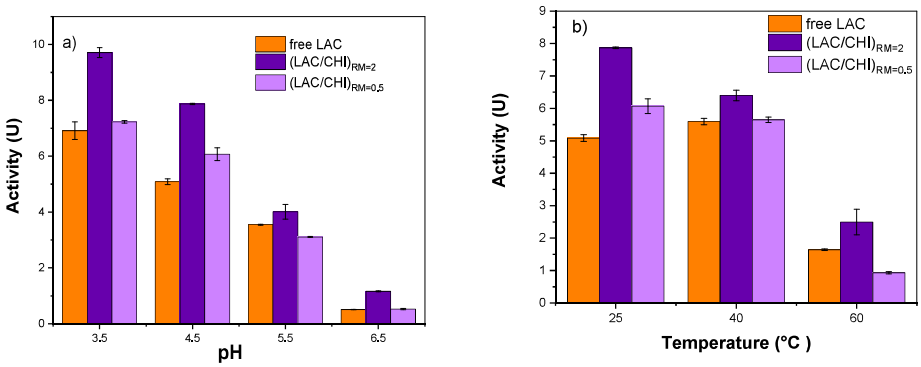

Figure 6.9: Influence of pH (a) and of temperature (b) on the catalytic activity of LAC in solution and LAC/CHI nanostructures

Notably, at higher pH values, the catalytic activity of the nanostructures is slightly higher than that of the free enzyme solution under similar conditions, suggesting that, at least for nanostructures formed at higher mass ratios, complex formation exerts a protective effect on the enzyme. Moreover, the variation of catalytic activity with pH follows a similar trend for both free and nanostructure-embedded enzyme, confirming that complex formation does not significantly affect the enzyme's structure or kinetic parameters.

A similar effect is observed regarding enzyme stability at elevated temperatures. Analysis of the enzymatic activity of LAC and LAC/CHI complexes after incubation at different temperatures shows a considerable decrease in catalytic activity above 40 °C, likely due to thermal degradation of the enzyme (Figure 6.12 b). However, for the LAC/CHI

nanostructures, higher catalytic activities are observed compared to the free enzyme after thermal incubation, suggesting better preservation of the structural integrity relative to the native enzyme. Thus, in terms of both pH and temperature variations, LAC/CHI nanostructures prepared with higher CHI content (RM = 2) better retain their properties compared to samples prepared with lower CHI content (RM = 0.5), indicating that the protective effect of CHI is also dependent on the amount of polymer used.

6.3. Preparation of LAC/CHI-*g*-PNIPAM nanostructures

6.3.2. Formation and characterization of LAC/CHI-g-PNIPAM complexes

The formation of LAC/CHI-*g*-PNIPAM nanostructures was carried out following the method described in Chapter 3, Section 3.4.2. The samples were coded according to the model $(LAC/CHI\text{-}g\text{-}PNIPAM)_{RM}$, where RM represents the mass ratio between the components. The formation of LAC/CHI-*g*-PNIPAM nanostructures was confirmed by an increase in scattered light intensity, as shown in Figure 6.18 a, similarly to the case of LAC/CHI nanostructures.

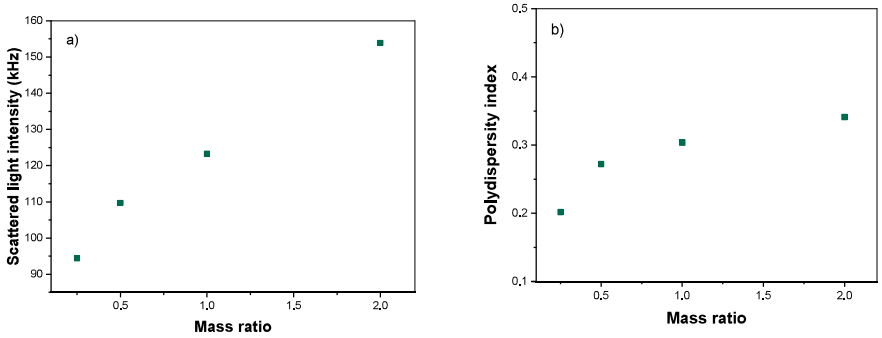

Figure 6.10: Variation of the scattered light intensity (a) and polydispersity index (b) with the mass ratio for the nanostructures prepared at pH = 4.5
*(results partially published in **Petrila, 2025c**)*

Similarly, in this case, the scattered light intensity increases with increasing amounts of polysaccharide used, suggesting the formation of nanostructures with larger size or higher density. Regarding the polydispersity of the formed nanostructures (Figure 6.18 b), it is noteworthy that the polydispersity index values are relatively low, confirming the formation of nanostructures with relatively homogeneous dimensions.

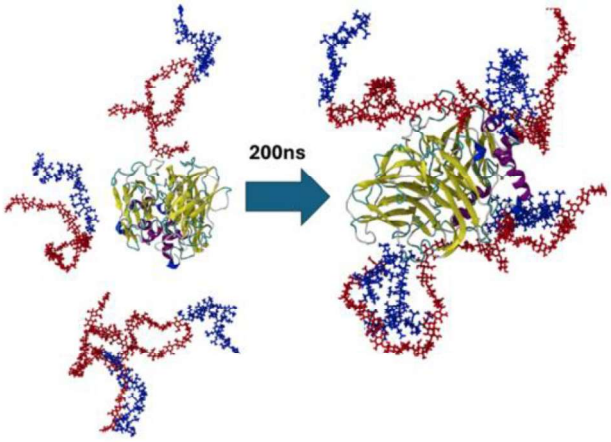

Figure 6.11: Sequences of simulation representing the initial state of the enzyme-copolymer and of the complex LAC/CHI-g-PNIPAM obtained after 200 ns of interaction. CHI is coloured in red and PNIPAM is coloured in blue ***(Petrila, 2025c)***

The study of the interaction between LAC and CHI-*g*-PNIPAM, conducted through molecular dynamics simulation, corroborates these observations. The simulation, starting from four CHI-*g*-PNIPAM molecules placed in proximity to the enzyme, shows that the interaction between the protein and the polysaccharide is rapid, leading to the formation of nanostructures, as illustrated in Figure 6.20.

The simulations lead to two main findings: (1) both the CHI and PNIPAM chains of the copolymer interact with the enzyme, and (2) these interactions occur at the enzyme surface, as more clearly shown in Figure 6.21.

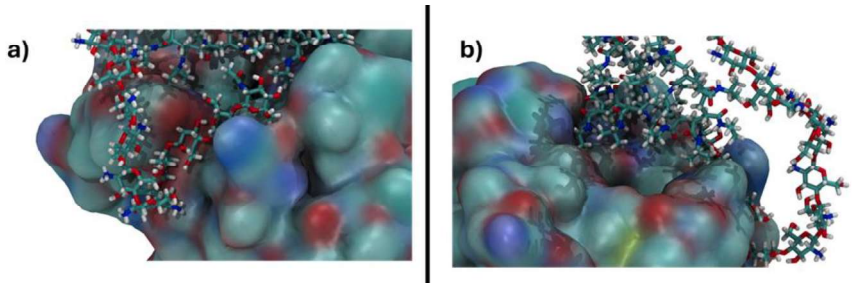

Figure 6.12: Detail depicting the interaction between CHI and LAC (a) and LAC and PNIPAM (b). For the copolymer, the colouring scheme of the atoms is as follows: N – blue, O – red, C – teal, H – white. The surface of the enzyme follows the same colouring scheme

As shown in Figure 6.23, all formed nanostructures carry a positive ionic charge, with zeta potential values ranging from +16 to +26 mV. The zeta potential slightly increases with higher LAC/CHI-*g*-PNIPAM mass ratios, indicating that the positive charge of the copolymer dictates the properties of the resulting nanostructures, while also confirming that

the copolymer is positioned at the surface of the nanostructures. This is further supported by Figures 6.21 and 6.22, which present molecular dynamics simulation results clearly showing that the polysaccharide is electrostatically bound to the enzyme surface, leading to a net positive ionic charge. Moreover, the decrease in zeta potential of the nanostructures compared to the initial CHI-*g*-PNIPAM solution demonstrates partial charge neutralization upon complexation of CHI-*g*-PNIPAM with LAC.

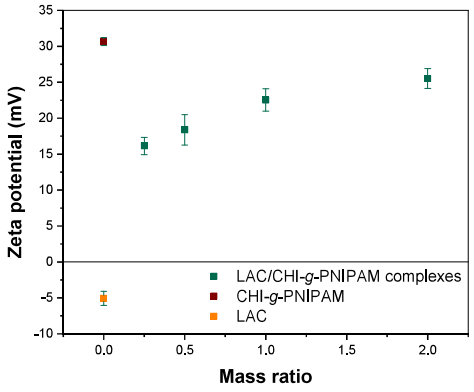

Figure 6.13: Variation of the zeta potential as a function of mass ratio for the nanostructures prepared at pH = 4.5 ***(Petrila, 2025c)***

6.3.5. Evaluation of the catalytic properties of LAC/CHI-g-PNIPAM nanostructures

The assessment of the catalytic activity of LAC/CHI-*g*-PNIPAM nanostructures is crucial for determining their potential applications and was evaluated via ABTS oxidation, with the results presented in Figure 6.29.

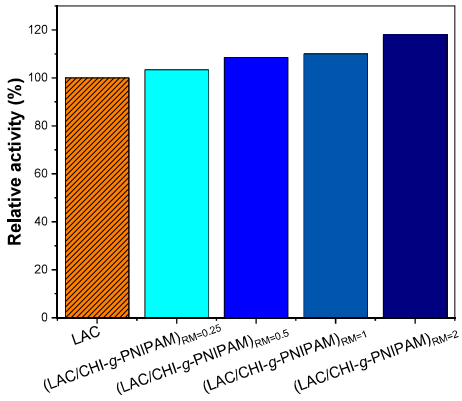

Figure 6.14: Relative activity of the LAC/CHI-g-PNIPAM compared with the activity of LAC in solution ***(Petrila, 2025c)***

As observed for LAC/CHI complexes, complexation with CHI-*g*-PNIPAM induces a slight increase in the catalytic activity of LAC. These results confirm that the presence of the copolymer does not lead to enzyme denaturation; on the contrary, it appears to enhance

either the accessibility of the active site or the affinity between the substrate and the enzyme, likely due to improved adsorption of ABTS on the complex surface. Additionally, the increase in enzymatic activity may also result from the creation of a more favourable microenvironment for the catalytic reaction.

Equally important is the stability of the catalytic activity of LAC/CHI-*g*-PNIPAM complexes under different experimental conditions. Variations in pH can drastically influence enzymatic activity, as they may affect both the ionization state of the substrate and its interaction with the enzyme. The effect of pH on the catalytic activity of LAC and LAC/CHI-*g*-PNIPAM complexes was evaluated over the range 3.5-6.5, using ABTS oxidation as a model catalytic reaction, with the results presented in Figure 6.31 a.

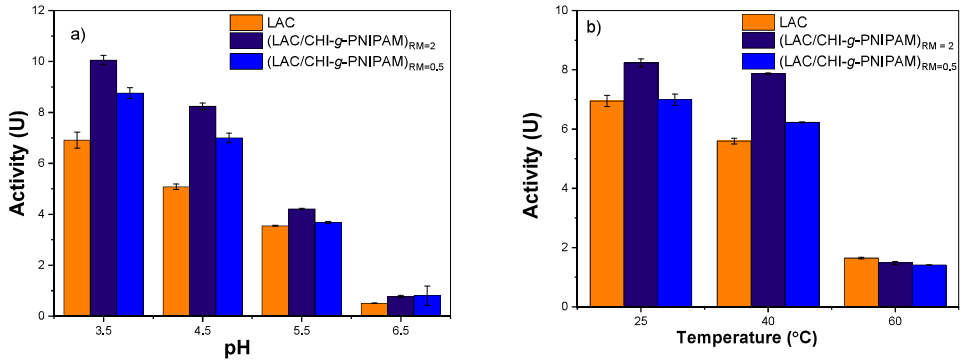

Figure 6.15: The effect of pH (a) and of temperature (b) on the enzymatic activity of the LAC/CHI-g-PNIPAM complexes ***(Petrila, 2025c)***

As shown in Figure 6.31 a, pH strongly affects the catalytic activity of both free LAC and LAC/CHI-*g*-PNIPAM complexes. The highest enzymatic activity was recorded at pH = 3.5 for both free LAC and LAC incorporated in nanostructures, demonstrating that complexation with CHI-*g*-PNIPAM does not significantly alter the enzyme's characteristics. Increasing the pH results in a progressive decrease in enzymatic activity, likely due to a reduced ability of the enzyme to interact with the substrate. Moreover, higher pH values may induce structural changes in both free LAC and the nanostructures, leading to a significant loss of enzymatic activity. Nevertheless, it is noteworthy that the catalytic activity of the nanostructures is higher at all tested pH values compared to that of the free enzyme, suggesting that complexation with CHI-*g*-PNIPAM exerts a protective effect against the adverse impact of environmental variations.

Temperature is another parameter that can influence the enzymatic activity of both free and immobilized LAC. The thermal stability of LAC in solution and of the nanostructures was evaluated by measuring enzymatic activity after incubation at different temperatures for

15 minutes (Figure 6.31 b). Incubation of LAC and LAC/CHI-*g*-PNIPAM complexes at elevated temperatures results in a significant decrease in enzymatic activity, most likely due to perturbation of the enzyme's tertiary structure and alteration of the active site under thermal stress. This effect is expected, considering that the enzyme was exposed to temperatures at which a phase transition occurs in the PNIPAM side chains, potentially causing rearrangements of the copolymer chains surrounding the enzyme. Nevertheless, the catalytic activity of LAC/CHI-*g*-PNIPAM nanostructures incubated at temperatures ≤ 40 °C remains higher compared to the activity of LAC in solution under similar conditions, highlighting the beneficial effect of CHI-*g*-PNIPAM complexation on enzyme stability.

6.4. Partial conclusions

This chapter presents the formation of nanostructures through the interaction between LAC and two polysaccharides – CHI and CHI-*g*-PNIPAM. The first study focused on the formation of LAC/CHI nanostructures, with particular attention given to elucidating the mechanism of interaction between the enzyme and the polysaccharide. Accordingly, a series of fluorescence studies is presented, serving as a starting point for analysing LAC/CHI interactions through established theoretical models. The results obtained from this analysis were further confirmed by molecular dynamics simulations, which highlighted the types of interactions and the binding modes in the LAC/CHI system. Special emphasis was placed on characterizing the nanostructures in terms of preservation of the catalytic activity of the embedded enzyme. The main conclusions drawn from these studies are as follows:

- LAC and CHI are capable of interacting under the analysed conditions, with the primary types of interactions being electrostatic, hydrogen bonding, and van der Waals forces;
- The formation of LAC/CHI nanostructures depends on the mass ratio between the enzyme and the polysaccharide, with the resulting nanostructures exhibiting an overall positive charge;
- Embedment of LAC within CHI nanostructures does not induce enzyme denaturation or significant conformational changes, but leads to a slight increase in enzymatic activity due to the stabilization of the enzyme structure and the promotion of substrate interaction;
- CHI plays a stabilizing role for the enzyme under variations in temperature and pH, as well as during storage, ensuring better preservation of catalytic activity compared to the enzyme solution under similar conditions.

The second study investigated the formation of nanostructures between LAC and CHI-*g*-PNIPAM, considering the thermosensitive properties of the grafted polysaccharide. The results demonstrated successful formation of the nanostructures, along with several key observations:

- The mass ratio between components influences the size and properties of the resulting nanostructures, similarly to CHI-based nanostructures;
- The use of CHI-*g*-PNIPAM in forming composite nanostructures promotes a more homogeneous self-assembly, yielding a better-defined, approximately spherical conformation compared to the LAC/CHI system;
- The incorporation of the modified polysaccharide confers temperature-responsive properties to the nanostructures;
- The nanostructures exhibited a significant increase in the enzymatic activity of LAC, as well as improved stability under variations in temperature and pH and during storage, compared to the native enzyme.

Overall, the results presented in this chapter highlight the ability of enzymes to interact with polysaccharides, leading to the formation of nanostructures that enhance the catalytic properties of the enzyme. The resulting nanostructures are stable under various environmental conditions, may respond to external stimuli, and can successfully catalyse specific reactions.

CHAPTER 7
USE OF COMPOSITE MATERIALS OBTAINED BY IMMOBILIZING LACCASE IN THE CATALYTIC DEGRADATION OF DYES

The results presented in this chapter have been the subject of the following publications:

1. **Petrila, L. M.**, Bucatariu, F., Stoica, I., Mihai, M., Froidevaux, R. (2025a). *J Environ Chem Eng*, *13*(2), 115631. https://doi.org/10.1016/j.jece.2025.115631
2. **Petrila, L. M.**, Karayianni, M., Vasiliu, T., Puf, R., Mihai, M., Pispas, S. (2025c). *Int J Biol Macromol., 322(2), 146754.* https://doi.org/10.1016/j.ijbiomac.2025.146754
3. **Petrila, L. M.**, Ciobanu, T.A., Vasiliu, T., Pispas, S., Mihai, M. (2025d). *Biomacromolecules, 26,* 6244-6257.https://doi.org/10.1021/acs.biomac.5c01217

7.3. Degradation of IC under the action of LAC immobilized on composite microparticles obtained by layer-by-layer deposition of polyelectrolytes

7.3.1. Evaluation of the ability of the enzyme/mediator system to degrade dyes using immobilized enzyme

The use of the composites with immobilized LAC in the degradation of IC was tested in the presence of the same mediators, employing the composite materials LAC1@SP1k/$(PEI_M)_{4.5(\text{-PAA})}$ and LAC1@SP1k/$(PEI_M)_{4.5(\text{-PMA})}$. As shown in Figure 7.3, it can be observed that both composite materials retain their ability to degrade the dye in the presence of the two mediators tested.

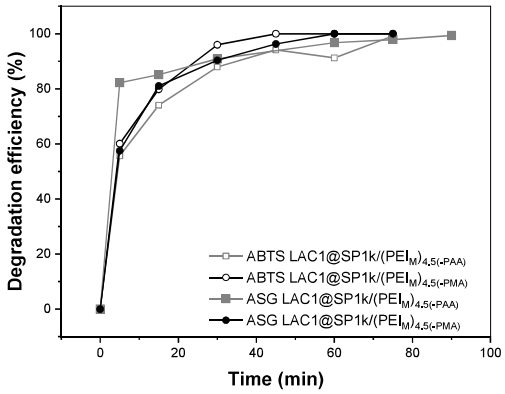

*Figure 7.1: IC degradation efficiency in the presence of the composite materials LAC1@SP1k/$(PEI_M)_{4.5(\text{-PAA})}$ and LAC1@SP1k/$(PEI_M)_{4.5(\text{-PMA})}$, using ABTS or ASG as mediator (**Petrila, 2025a**)*

The use of ASG as a mediator resulted in faster degradation, reaching up to 80%, for the LAC1@SP1k/$(PEI_M)_{4.5(\text{-PAA})}$ sample, but had a less pronounced effect on LAC1@SP1k/$(PEI_M)_{4.5(\text{-PMA})}$, suggesting a stronger interaction between the mediator and the latter composite material. The more rapid degradation of the dye in the presence of ASG is likely facilitated by the mediator's lower molecular weight, which allows it to diffuse more easily to the active site, even when the enzyme is immobilized. Although ASG is oxidized more slowly by the enzyme, its oxidized form is more stable, promoting the transformation of the dye into its degradation products (Cañas, 2010). It was also observed that when LAC is immobilized on the composite obtained with PMA, dye degradation occurs more rapidly, due both to the higher amount of immobilized LAC and its greater stability, as previously demonstrated in enzymatic activity tests (Figure 5.5 b).

7.3.6. Reusability of the composite materials

Regarding the reusability of the composite materials LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PAA)}$ and LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PMA)}$, they were successfully tested over four dye degradation cycles, achieving 100% degradation efficiency. An interesting observation was that, as the LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PAA)}$ and LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PMA)}$ materials were reused, the time required for complete degradation of the dye slightly increased; nevertheless, full IC degradation could still be accomplished. For this reason, the degradation efficiency was calculated for each type of composite material tested, considering the shortest time necessary for complete dye degradation (corresponding to the first cycle of use) (Figure 7.8).

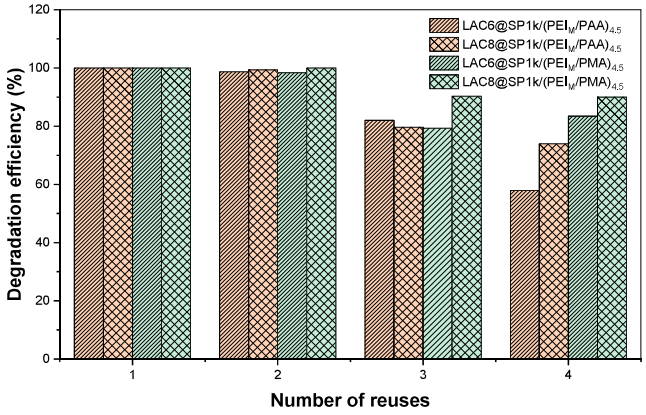

*Figure 7.2: IC degradation efficiency using LACn@SP1k/$(PEI_M)_{4.5(\text{-}PAA)}$, and LACn@SP1k/$(PEI_M)_{4.5(\text{-}PMA)}$, (**Petrila, 2025a**)*

The decrease in degradation efficiency with the increasing number of reuse cycles of LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PAA)}$ and LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-}PMA)}$ (evidenced by the longer time required for complete dye degradation) is a normal phenomenon that can be associated with the accumulation of impurities on the surface of the composite materials, which reduces the diffusion of reactants and, consequently, the efficiency of the immobilized enzyme. Nevertheless, the dye degradation observed in this study remained above 60% even after four reuse cycles, demonstrating the enhanced stability of the immobilized enzyme.

7.3.7. Mechanism of enzymatic degradation of IC

Elucidating the enzymatic degradation mechanism of the dye is extremely important for evaluating the efficiency and safety of water depollution processes, as the cleavage of the dye molecule can lead to the formation of potentially more toxic metabolites than the parent compound. In this context, various analytical methods can be employed to identify reaction intermediates and to assess the efficiency of the enzymatic degradation process. The degradation mechanism of IC under the action of LAC was investigated using two

complementary analytical methods, namely ESI-MS and HPLC-MS. Dye degradation was tested using a 50 mg/L IC solution in the presence of ASG, at room temperature, using LAC4@(SP1k/$(PEI_M)_{4.5(-PMA)}$), and, for comparison, a 1 mg/mL LAC solution at pH = 4.5. To better highlight the compounds formed through enzymatic degradation, two experimental methodologies were proposed (described in Chapter 3, subchapter 3.7.9): (i) the ESI(+)-MS and ESI(−)-MS method, consisting of sampling the reaction medium every 5 minutes and analysing the samples directly via ESI-MS, using both positive and negative ionization modes; and (ii) the HPLC-MS method, involving sampling every 10 minutes, separating the molecules on a C18 chromatographic column, and analysing the compounds directly via ESI-MS.

Figure 7.13 presents the mass spectra obtained from samples collected from the reaction medium and recorded in negative ionization mode. In the spectra, several signals are observed at higher *m/z* ratios (*m/z* 263, 305, 341, 387, 427, 469), which can be attributed to the presence of the solvent and the adducts it forms during ionization.

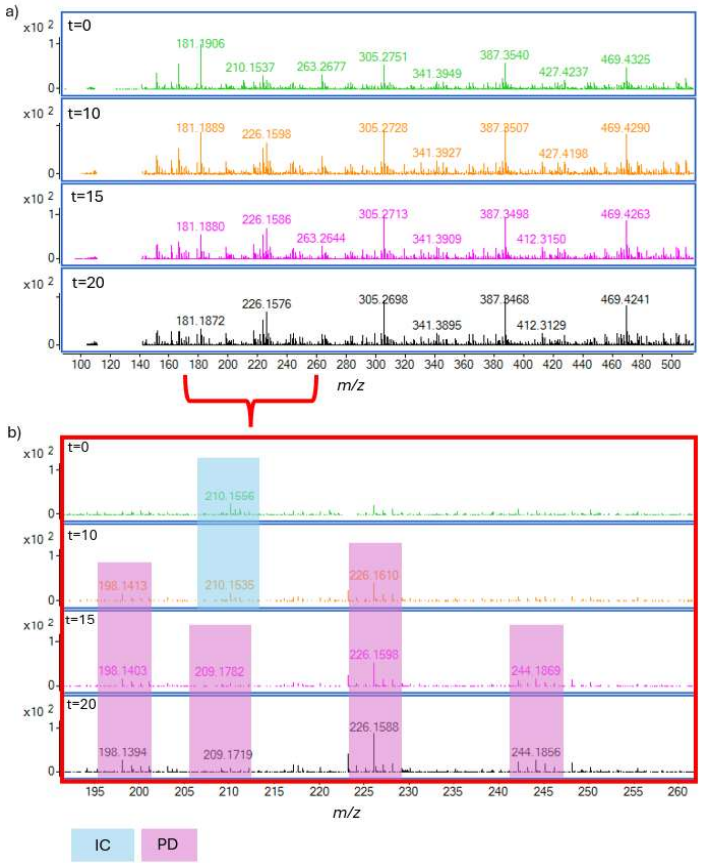

Figure 7.3: Mass spectra registered in ESI (–) mode at the enzymatic degradation of IC using LAC4@(SP1k/$(PEI_M)_{4.5(-PMA)}$ and section representing a region of interest of the same spectra (b) (PD – product of degradation)

For a more accurate identification of possible degradation products, a section of interest from the recorded spectra was selected and is shown at a larger scale in Figure 7.13 b. Thus, the presence of the mediator at *m/z* 151, 166, and 181 is highlighted, as also identified in the mass spectrum of the initial ASG solution (Figure 7.10.). At the same time, the presence of the initial dye molecule is highlighted by the m/z 210 signal, as identified in the literature (Wang, 2022) and confirmed in the mass spectrum of the dye solution (Figure 7.9 a). It should be noted that the signal recorded for the dye molecule decreases in intensity during degradation and disappears completely after about ten to fifteen minutes of reaction, suggesting the cleavage of the dye molecule. Corroborated with the visually observed disappearance of the sample colour after about 10 minutes, it can be concluded that IC degradation occurs through a mechanism that causes, in the first stage, the cleavage of the dye's chromophore groups.

Regarding the potential degradation products of IC, two main signals are highlighted: one at *m/z* 226, associated with the presence of isatin 5-sulfonic acid, and another at *m/z* 198, associated with the presence of indoline 5-sulfonic acid. These are the primary metabolites generated during the enzymatic degradation of IC, as also reported by other researchers (Ahlawat, 2022; Leontieș, 2022; Wang, 2022). It is noteworthy that the signals corresponding to these compounds are already observed in the sample collected at 10 minutes, suggesting that the enzymatic degradation reaction occurs very rapidly.

7.4. Degradation of IC and CR dyes under the action of LAC/CHI and LAC/CHI-g-PNIPAM nanostructures

7.4.1. Evaluation of the ability of LAC/CHI nanostructures to catalyse the degradation of certain dyes

The conditions selected for the dye degradation studies were established based on those presented in Section 7.3. The tests were conducted using LAC/CHI nanostructures, evaluating their ability to degrade aqueous solutions of IC or CR at an initial concentration of 50 mg/L. The results obtained are presented in Figure 7.18.

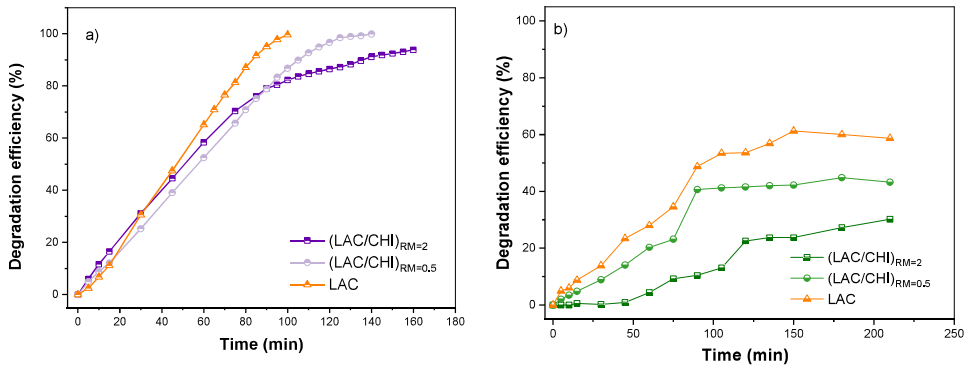

Figure 7.4: Degradation efficiency of IC (a) and CR (b) using LAC or LAC/CHI nanostructures ***(Petrila, 2025d)***

As shown in Figure 7.18, the incorporation of LAC into CHI-based nanostructures affects their ability to degrade dyes, influencing the kinetics of the degradation process. In the case of IC degradation (Figure 7.18 a), complete degradation of the dye is observed in the presence of LAC/CHI nanostructures, although at a slower rate compared to free LAC. It is noteworthy that during the first 20-30 minutes of contact, the degradation rate remains nearly constant for both LAC in solution and LAC/CHI nanostructures, suggesting that during this initial phase, the interaction between the enzyme, mediator, and dye molecules occurs efficiently, and the catalytic sites are likely saturated, allowing degradation at near-maximal rates. In the case of LAC/CHI nanostructures, this effect may also be enhanced by the adsorption of IC molecules onto the positively charged surface of the nanostructures, thereby facilitating interaction with the enzyme. As the reaction progresses, differences in the enzymatic degradation profiles of IC become evident. Specifically, the degradation rate gradually decreases for the LAC/CHI nanostructures, indicating that CHI plays a critical role in the catalytic efficiency of these systems. The dye degradation rate is lower for the $LAC/CHI_{RM=2}$ complexes, a behaviour consistent with the fact that enzymatic degradation of the dye is diffusion-limited. The presence of a higher number of CHI chains in the LAC/CHI complexes reduces the accessibility of the enzyme's active site or slows the expulsion of degradation products. This effect is particularly important in the context of IC degradation, as the dye's more complex chemical structure hinders its accessibility to the enzyme's catalytic center, while the presence of a conjugated system in its structure imparts enhanced stability and resistance to enzymatic degradation compared to simpler molecules. Nevertheless, complete dye degradation is achieved with both free LAC and LAC/CHI nanostructures, demonstrating that these systems retain the catalytic activity of LAC even for more complex compounds. Although degradation is slower in the presence of LAC/CHI

nanostructures, their higher stability under environmental factors may present advantages compared to free enzyme when degrading dyes in more complex media. In the case of CR, degradation is incomplete in both free enzyme solution and nanostructures, as this dye exhibits higher stability toward enzymatic degradation. Free LAC demonstrates a higher degradation capacity, facilitating the removal of approximately 55% of the dye at an initial concentration of 50 mg/L, compared to only 20-25% for the LAC/$CHI_{RM=2}$ complexes. The lower degradation efficiency for CR can be attributed to a combination of factors, including lower affinity between the dye and enzyme, higher molecular weight, and a more complex structure, which impede mass transfer and make enzymatic degradation of CR more challenging (Stoilova, 2010).

7.4.2. Evaluation of the ability of LAC/CHI-g-PNIPAM nanostructures to catalyse the degradation of certain dyes

The results obtained for the LAC/CHI nanostructures confirm that they retain the catalytic activity of the enzyme, acting as effective catalysts for the degradation of environmental pollutants. Subsequently, the catalytic performance of LAC/CHI-*g*-PNIPAM nanostructures was evaluated for the degradation of both dyes at an initial concentration of 50 mg/L, using ASG as a mediator, with the results presented in Figure 7.20.

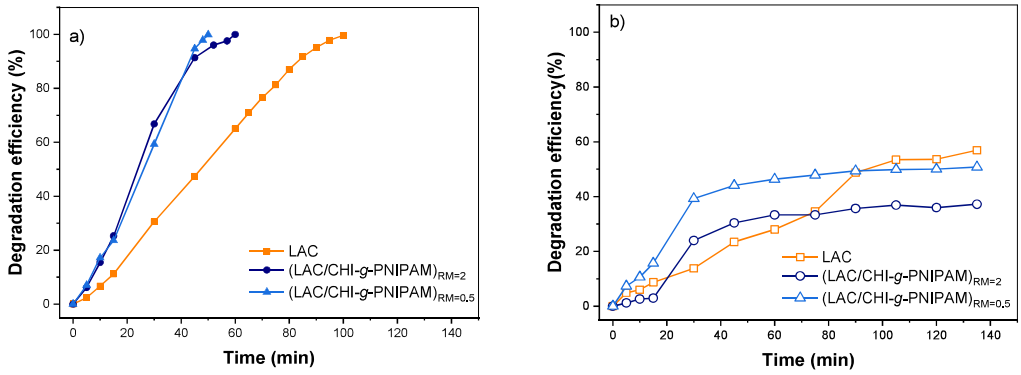

Figure 7.5: Degradation efficiency of IC (a) and CR (b) using LAC or LAC/CHI-g-PNIPAM ***(Petrila, 2025c)***

For comparison, Figure 7.20 also includes the degradation curves of the two dyes in the presence of free LAC, previously presented in Figure 7.18. As shown in Figure 7.20, the degradation of IC is complete both in the presence of the LAC solution and the LAC/CHI-*g*-PNIPAM nanostructures. Degradation of IC with free LAC was achieved in approximately 80-90 minutes, whereas the use of LAC/CHI-*g*-PNIPAM nanostructures led to an increased degradation rate, with complete IC removal occurring in about 45 minutes for the

(LAC/CHI-*g*-PNIPAM)$_{RM=0.5}$ complexes and 60 minutes for the (LAC/CHI-*g*-PNIPAM)$_{RM=2}$ complexes. These results highlight the higher efficiency of LAC/CHI-*g*-PNIPAM nanostructures in IC degradation, correlating with the significant increase in enzymatic activity (Figure 6.29 in Chapter 6). The enhanced dye degradation efficiency of these nanostructures compared to LAC/CHI nanostructures may result from interactions between CHI-*g*-PNIPAM and IC, which appear to facilitate the interaction between the dye and the enzyme-mediator pair. Notably, the degradation rate is three times higher for the nanostructures compared to free enzyme. Furthermore, the increased catalytic efficiency may also be attributed to better accessibility of the LAC active site after complexation with CHI-*g*-PNIPAM, as well as improved stability of the enzyme. Similar observations were reported by Zhang and colleagues, who studied the enzymatic degradation of Malachite Green and Acid Orange 7 dyes in the presence of nanostructures prepared with LAC and PEI (Zhang, 2015). The authors highlighted the increased efficiency of nanostructures in degrading Acid Orange 7, with a threefold increase in degradation rate compared to the free enzyme. On the other hand, the degradation rate of Malachite Green was lower, probably due to structural differences between the two dyes.

In the case of CR degradation, the degradation efficiency was lower for both free LAC and LAC/CHI-*g*-PNIPAM nanostructures, confirming the behaviour observed with LAC/CHI complexes. The degradation efficiency recorded for CR was approximately 55% with the LAC solution and around 25-40% with the LAC/CHI-*g*-PNIPAM complexes.

These results confirm that LAC retains its catalytic capability toward the oxidation of more complex substrates after incorporation into complex nanostructures, suggesting that these systems can serve as effective catalysts for the degradation of dyes in aqueous environments.

7.5. Partial conclusions

The studies presented in this chapter focused on the enzymatic degradation of dyes using LAC immobilized on composite microparticles obtained through layer-by-layer deposition of polyelectrolytes or in the presence of complex nanostructures, namely LAC/CHI and LAC/CHI-*g*-PNIPAM.

In the first study, the composite materials obtained via LAC immobilization were tested for the degradation of a model pollutant, the sulfonic dye IC. Dye degradation by the biocatalysts was investigated under various conditions, including the use of two mediators,

different pH values and pollutant concentrations, as well as materials with varying amounts of immobilized enzyme. The main conclusions drawn are as follows:

- All tested composite materials were able to achieve 100% degradation of the dye in the presence of both mediators, with higher efficiency observed using ASG compared to ABTS;
- LAC-immobilized composites successfully degraded IC at different pH values and dye concentrations, achieving complete degradation under all tested conditions;
- The composites could be reused multiple times without a significant decrease in catalytic efficiency, achieving complete dye degradation even after four reuse cycles;
- Investigation of the IC degradation mechanism in the presence of free LAC or immobilized LAC composites demonstrated that enzyme immobilization does not alter the degradation profile;
- Analysis of the degradation products allowed the identification of the main metabolites formed during enzymatic degradation of IC, including isatin-5-sulfonic acid, 4-amino-3-(carboxycarbonyl) benzenesulfonate, 2-amino-5-benzenesulfonic acid, 3-oxoindoline-5-sulfonic acid, and indoline-5-sulfonic acid.

The second study evaluated the ability of LAC/CHI and LAC/CHI-*g*-PNIPAM nanostructures to facilitate the degradation of IC and CR dyes. The findings confirmed their catalytic activity and highlighted several key points:

- Both LAC/CHI and LAC/CHI-*g*-PNIPAM nanostructures enabled complete degradation of IC under all tested conditions;
- For LAC/CHI nanostructures, the dye degradation rate was slightly lower than that of free LAC, suggesting possible diffusion limitations in the interaction between the dye, enzyme, and mediator;
- LAC/CHI-*g*-PNIPAM nanostructures exhibited higher catalytic efficiency than free LAC in IC degradation, likely due to improved accessibility to the enzyme's active site, consistent with previous enzymatic activity tests;
- For both CR and IC degradation, higher degradation rates were observed for nanostructures prepared at RM = 0.5 compared to RM = 2, confirming that dye degradation is a diffusion-limited process, with higher amounts of CHI or CHI-*g*-PNIPAM in the nanostructures limiting mass transfer.

The results presented in this chapter emphasize the efficiency of using immobilized enzymes for the development of effective, innovative, and environmentally friendly wastewater treatment methods. The tested composite materials demonstrated high efficiency

in degrading the target pollutant, increased stability under process conditions, and the ability to be reused without significant loss of catalytic activity. Moreover, the findings confirm the feasibility of using LAC/polysaccharide nanostructures for the degradation of environmental pollutants. Overall, the studies included in this work propose an innovative and effective approach for the treatment of wastewater contaminated with organic compounds, aligning with current trends in the development of green technologies with high efficiency and low environmental impact.

GENERAL CONCLUSIONS

The doctoral thesis entitled "***Advances in polymer/enzymes composites for applications in catalysis***" aimed to develop innovative materials based on natural or synthetic polymers and enzymes that could be used as catalysts in various processes. The thesis spans 195 pages and comprises seven chapters, including 100 figures, 21 tables, 12 equations, and 310 bibliographic references. The work is organized into two main sections: Part I, entitled "Current State of Knowledge," consists of two chapters and presents a synthesis of the scientific context motivating the choice of materials and experimental methods used and Part II, entitled "Original Contributions," is structured in five chapters and presents the original results obtained from the conducted studies.

A detailed analysis of the experimental results revealed a series of key findings for each of the proposed studies, as follows:

1. Optimization of the obtaining of support materials for enzyme immobilization by layer-by-layer polyelectrolyte deposition:

- The porosity of the silica used as a support for polyelectrolyte deposition does not influence the properties of the obtained material;
- During acid-base treatment, the use of a low molecular weight polycation results in lower stability of the multilayers compared to the use of a higher molecular weight polycation;
- The molecular weight and conformation of the polyanion influence the properties of the composite material, including the thickness and stability of the organic shell under acid-base treatment;
- Crosslinking of polycation chains stabilizes the organic shell, while acid-base treatment increases the flexibility of the polymer network;

- Physicochemical characterization confirmed the successful deposition of the organic shell and highlighted the influence of the polycation/polyanion pair on the properties of the composites, including morphology, surface charge, and chemical composition;
- The best results in terms of stability were obtained for the composites SP2k/$(PEI_M)_{4.5(\text{-}PAA)}$ and SP2k/$(PEI_M)_{4.5(\text{-}PMA)}$.

2. Pepsin immobilization on core-shell supports obtained by layer-by-layer polyelectrolyte deposition:

- Enzyme immobilization was not influenced by the porosity of the silica support, occurring mainly at the surface of the material;
- In materials with a small number of deposited double layers, enzyme adsorption occurred predominantly at the material's surface;
- Higher amounts of immobilized enzyme were observed for materials subjected to acid-base treatment, which increased polymeric shell flexibility, with the highest pepsin loadings on SP2k/$(PEI_M)_{4.5(\text{-}PAA)}$ and SP2k/$(PEI_M)_{4.5(\text{-}PMA)}$ composite materials;
- The highest enzyme loadings were achieved using the PEI_M/PMA polyelectrolyte pair, which favoured the formation of weak interactions between the organic shell and enzyme, reaching approximately 266 mg/g for SP2k/$(PEI_M)_{4.5(\text{-}PMA)}$ and 271 mg/g for SP2k/$(PEI_M)_{2.5(\text{-}PMA)}$;
- Optimal immobilization was achieved at pH = 4.5 and an initial pepsin concentration of 1 mg/mL;
- Despite satisfactory immobilization, the conditions used did not maintain enzymatic activity.

3. Laccase immobilization on core-shell supports obtained by layer-by-layer polyelectrolyte deposition:

- LAC immobilization was successfully achieved on the tested composite microparticles, with the highest immobilization yields at pH = 4.5 and an initial enzyme solution concentration of 1 mg/mL;
- LAC immobilization yields were higher for composites prepared with the PEI_M/PMA pair compared to those prepared with PEI_M/PAA, confirming trends observed with pepsin;
- Maximum LAC immobilization reached ~80% for SP1k/$(PEI_M)_{4.5(\text{-}PMA)}$ and ~60% for SP1k/$(PEI_M)_{4.5(\text{-}PAA)}$;

- Immobilization preserved significant catalytic activity, with higher activity for LAC immobilized on SP1k/$(PEI_M)_{4.5(\text{-PMA})}$;
- Immobilization enhanced enzyme stability under varying experimental conditions, including elevated temperatures and pH changes, shifting the optimal pH from 3.5 (native enzyme) to 5.5 (immobilized enzyme);
- Enzyme reuse was feasible for at least seven catalytic cycles, with less than 5% activity loss in the first three cycles and retention of over 50% of initial activity after seven cycles;
- Successful enzyme immobilization was confirmed through characterization methods, which revealed changes in morphology, chemical composition, and surface charge.

4. Enzyme/polysaccharide nanostructures formation:

- Fluorescence quenching studies of LAC-CHI interaction confirmed their ability to interact, with interaction decreasing at higher temperatures; hydrogen bonding and van der Waals forces were identified as the predominant interactions;
- Molecular dynamics simulations corroborated these findings, elucidating interaction types and self-assembly mechanisms;
- LAC/CHI nanostructures showed a slight catalytic activity increase compared to free enzyme (3% for RM = 0.25-1, ~10% for RM = 2);
- LAC/CHI-*g*-PNIPAM nanostructures were also successfully formed, confirmed experimentally and through simulations;
- Embedding LAC in these nanostructures increased enzymatic activity up to 20% (RM = 2), likely due to improved active site accessibility, increased substrate affinity, or local microenvironment changes;
- LAC/CHI-*g*-PNIPAM nanostructures were temperature-sensitive due to synthetic polymer chains;
- Both types of nanostructures exhibited nanoscale size and positive surface charge (confirmed by DLS and zeta potential), with morphology confirmed by STEM;
- Embedding LAC improved stability against pH changes and temperature, suggesting a protective effect of the polysaccharides.

5. Use of immobilized laccase composites for dye degradation in aqueous solutions:

- Core-shell LAC composites catalysed complete IC degradation in the presence of both chemical mediators;
- Dye degradation rate was higher for LAC*n*@SP1k/$(PEI_M)_{4.5(\text{-PMA})}$ due to better catalytic properties;

- Tests with active and thermally inactivated enzyme confirmed that the enzymatic degradation;
- Complete degradation occurred across different pH and dye concentrations, with optimal rate at pH = 5.5;
- Composites were successfully reused for at least four cycles;
- MS and HPLC-MS analyses elucidated the enzymatic degradation mechanism, identifying main products: isatin-5-sulfonic acid, 4-amino-3-(carboxycarbonyl) benzenesulfonate, 2-amino-5-benzenesulfonic acid, 3-oxoindoline-5-sulfonic acid, and indoline-5-sulfonic acid;
- LAC/CHI and LAC/CHI-*g*-PNIPAM nanostructures also catalysed IC and CR degradation;
- Complete IC degradation was achieved with both nanostructures using ASG as mediator;
- Degradation rate depended strongly on enzyme active site accessibility, with faster degradation for LAC/CHI-*g*-PNIPAM than LAC/CHI;
- Lower polysaccharide content in nanostructures enhanced degradation kinetics by reducing diffusion limitations;
- Fastest IC degradation occurred in 45 minutes with $(LAC/CHI\text{-}g\text{-}PNIPAM)_{RM=0.5}$.
- CR degradation was lower for both nanostructures (25-40% efficiency), suggesting lower affinity between dye and enzyme-mediator system;
- CR degradation profiles confirmed diffusion-limited enzymatic processes.

The results obtained have allowed the identification of valuable research perspectives that can be further developed in future studies, some of which are:

- Using core-shell microparticles for immobilizing other industrially relevant enzymes (glucose oxidase, lipase, catalase);
- Testing immobilized LAC composites for degradation of other pollutants (pharmaceuticals, dyes, emerging contaminants) and elucidating degradation mechanisms via HPLC-MS;
- Evaluating immobilized materials under dynamic conditions (fixed-bed or fluidized columns);
- Immobilizing LAC/CHI or LAC/CHI-*g*-PNIPAM nanostructures on metal nanoparticles for easier separation;

- Studying interactions between other enzymes and natural or synthetic polymers, including stimuli-responsive polysaccharides, to create new enzyme/polymer nanostructures for catalytic, biomedical, or environmental applications.

The original results of this doctoral thesis led to six peer-reviewed publications in ISI journals (three in Q1 quartile), supported by 14 oral presentations and 5 poster presentations at national and international conferences, with five awards received. Additionally, six scientific articles and five oral presentations not included in the thesis were produced. Funding was provided through three national research projects, two research stages, and participation in three seasonal schools.

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ANNEX 1: DISSEMINATION OF RESULTS OBTAINED DURING THE DOCTORAL STUDIES

1. Scientific papers published in ISI journals (results included in the thesis):

1.1. **Petrila L.M.**, Grădinaru R., Bucatariu F., Mihai M. (2022). Polymer/enzyme composite materials – versatile catalysts with multiple applications. *Chemistry*, *4*(4), 1312-1338. (FI_{2024} = 2,4, Q3| $FI_{publishing}$ = N/A; **article on the cover**)

1.2. Bucatariu F., **Petrila L.M.**, Mihai M., Teodosiu C. (2022). Versatile nanostructured SiO_2/cross-linked polyelectrolyte composites for emergent pollutants removal from contaminated waters. *Comptes Rendus Chimie*, *25* (S3), 1-14. (FI_{2024} = 0,6, Q3| $FI_{publishing}$ = 2,55, Q3; **article on the cover**)

1.3. **Petrila L.M.**, Bucatariu F., Stoica I., Mihai M., Froidevaux R. (2025a). A green approach combining polyelectrolyte-based core-shell microparticles and laccase for indigo carmine degradation. *Journal of Environmental Chemical Engineering*, *13*(2),115631. (FI_{2024} = 7,4, Q1| $FI_{publishing}$ = 7,4, Q1)

1.4. **Petrila L.M.**, Bucatariu F., Mihai M. (2025b). Optimization of Pepsin Sorption on Core-Shell Composite Materials for Enhancing Enzyme Retention. *Revue Roumaine de Chimie, 70,* 465-473. (FI_{2024} = 0,6, Q4).

1.5. **Petrila, L. M.**, Karayianni, M., Vasiliu, T., Puf, R., Mihai, M., Pispas, S. (2025c). Stimuli-responsive laccase/chitosan-*g*-PNIPAM complexes: a sustainable strategy for biodegradation of organic pollutants. *International Journal of Biological Macromolecules, 322*, 146754. (FI_{2024} = 8,5, Q1).

1.6. Petrila, L.M., Ciobanu, T.A., Vasiliu, T., Pispas, S., Mihai, M. (2025d). Chitosan/*Trametes versicolor* Laccase Nanostructures with Modulated Catalytic Activity. *Biomacromolecules, 26*, 6244–6257 (FI_{2024} = 5,5, Q1)

2. Scientific papers published in ISI journals (results not included in the thesis):

2.1. Bucatariu F., Teodosiu C., Morosanu I., Fighir D., Ciobanu R., **Petrila L.M.**, Mihai M. (2021). An overview on composite sorbents based on polyelectrolytes used in advanced wastewater treatment. *Polymers*, *13*, 3963. (FI_{2024}= 4,9, Q1| $FI_{publishing}$ = 4,967, Q1)

2.2. Bucatariu F., Zaharia M.M., **Petrila L.M.**, Simon F., Mihai M. (2022a). Sand/polyethyleneimine composite microparticles: Eco-friendly, high selective and efficient heavy metal ion catchers. *Colloids and Surfaces A:*

Physicochemical and Engineering Aspects, *649*, 129540. (FI_{2024} = 5,4, Q2 | $FI_{publishing}$ = 5,518, Q2)

2.3. Bucatariu F., **Petrila L.M.**, Zaharia M.M., Simon F., Mihai M. (2022b). Sand/ polyethyleneimine composites with enhanced pollutants sorption/ desorption properties. *Water*, *14*(23), 3928. (FI_{2024} = 3, Q2| $FI_{publishing}$ = 3,4)

2.4. Bucatariu F., **Petrila L.M.**, Ciobanu, T.A., Zaharia M.M., Mihai M. (2025). Dynamic ultra-fast sorption/desorption of Indigo carmine onto/from versatile core-shell composite microparticles. *Applied Sciences, 15*(19), 10725 (FI_{2024} = 2,5, Q2)

2.5. Bucatariu F., **Petrila L.M.**, Zaharia M.M., Pispas S., Mihai M. (2024). Complexation of human serum albumin with thermoresponsive Chitosan-*g*-PNIPAM graft copolymer. Journal of Molecular Liquids (FI_{2024} = 5,2, Q2). ***Under review.***

3. Scientific papers published in extenso in the proceeding of scientific conferences:

3.1. **Petrila, L.M.**, Bucatariu F., Zaharia M.M., Mihai M. Separation and water cleaning by composites of polyelectrolytes and inorganic microparticles (2023). 12th International Conference on Environmental Engineering and Management, ISSN 2457-7049, ISSN – L 2457-7049, 195-196, 2023.

3.2. **Petrila L.M.**, Zaharia M.M., Bucatariu F., Mihai M., Pispas S. (2023). Exploring the remarkable properties of water soluble chitosans. Proceedings of International Conference Progress in Organic Macromolecular Compounds, ISSN 2810 – 2347 ISSN – L 2810 – 2126, 102-105, 2023.

3.3. **Petrila L.M.**, Karayianni M., Pispas S., Mihai M. (2024). Laccase/ Chitosan-g-PNIPAM hybrid nanostructures for potential environmental applications. 7th Edition International Conference EmergeMAT, ISSN 2602-0424 ISSN-L 2602-0416, 154, 2024.

3.4. **Petrila L.M.**, Karayianni M., Vasiliu, T., Pispas S., Mihai M. (2025). Novel biocatalysts as laccase/polysaccharide nanoassemblies. Proceedings of International Conference Progress in Organic Macromolecular Compounds, ISSN 2810 – 2126 ISSN – L 2810 – 2126, 134-136, 2025.

4. ***Oral communications at national and international scientific conferences (results included in the thesis):***

4.1. **Petrila L.M.***, Bucatariu F., Mihai M. Composite materials based on polyelectrolytes used as sorbents in batch and column studies. *MacroYouth' ICMPP – Open Door to the Future. Scientific Communications of Young Researchers*, 19 November 2021, Iasi, Romania.

4.2. **Petrila L.M.***, Bucatariu F., Mihai M. Silica/polyelectrolyte sorbents for the removal of organic pollutants. *1st Edition Ph.D. Students' Days*, 26 November 2021, Arad, Romania.

4.3. Bucatariu F., **Petrila L.M.**, Zaharia M.M., Mihai M. Core-shell polyelectrolyte composites with versatile properties in pollutants removal from contaminated waters. *Congresul Internațional al Universității „Apollonia" din Iași*, 28 February – 2 March 2022, Iasi, Romania.

4.4. **Petrila L.M.***, Bucatariu F., Mihai M. Composite materials employed in the removal of emerging pollutants from contaminated waters. *12th International Conference on Materials Science and Engineering,* 9-12 March 2022, Brasov, Romania.

4.5. **Petrila L.M.***, Bucatariu F., Mihai M. Composite materials with immobilized pepsin for water cleaning. *NeXT-Chem: Innovative Cross-Sectoral Technologies Exploratory Workshop*, 19-20 May 2022, Bucharest, Romania.

4.6. **Petrila L.M.***, Bucatariu F., Simon F., Mihai M. Core-shell microparticles for enzyme immobilization. ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 18 November 2022, Iasi, Romania.

4.7. **Petrila L.M.**, Bucatariu F.*, Zaharia M., Mihai M. Separation and water cleaning by composites of polyelectrolytes and inorganic microparticles. *12th International Conference on Environmental Engineering and Management*, 13-16 September 2023, Iasi, Romania.

4.8. **Petrila L.M.***, Bucatariu F., Mihai M. Catalase/polymer composite microparticles for environmental applications. ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 17 November 2023, Iasi, Romania.

4.9. **Petrila L.M.***, Froidevaux R., Mihai M. Harnessing immobilized laccase for sustainable water remediation. *Exploratory Workshop NeXT-Chem: Innovative Cross-Sectoral Technologies*, 21-22 March 2024, Bucharest, Romania.

4.10. **Petrila L.M.***, Froidevaux R., Mihai M. Polyelectrolytes/ laccase composite biocatalysts for water cleaning applications. *International Conference of the Doctoral School at the "Gheorghe Asachi" Technical University of Iasi (TUIASI)*, 15-17 May 2024, Iasi, Romania.

4.11. **Petrila L.M.***, Karayianni M., Pispas S., Mihai M. Green hybrid nanostructures for efficient degradation of water pollutants. International Symposium *„Priorieties of Chemistry for a sustainable development"*, 16 – 18 October 2024, Bucharest, Romania.

4.12. **Petrila L.M.***, Karayianni M., Pispas S., Mihai M. Laccase/ chitosan-*g*-PNIPAM hybrid nanostructures for potential environmental applications. *7th Edition International Conference EmergeMAT*. 30-31 October 2024, Bucharest, Romania.

4.13. **Petrila L.M.***, Karayianni M., Pispas S., Mihai M. Laccase/ polysccharide hybrid nanostructures for efficient dye degradation. ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 15 November 2024, Iasi, Romania.

4.14. **Petrila L.M.***, Karayianni M., Vasiliu, T., Pispas S., Mihai M. Novel biocatalysts as laccase/polysaccharide nanoassemblies. *Progress in organic and macromolecular compounds*, 30th edition, 23-26 September 2025, Iasi, Romania.

5. Poster communications at national and international scientific conferences (results included in the thesis):

5.1. **Petrila L.M.***, Zaharia M.M., Bucatariu F., Mihai M., Pispas S. Exploring the remarkable propeties of water soluble chitosans. *Progress in organic and macromolecular compunds*, 29th edition, 4-6 October 2023, Iasi, Romania.

5.2. **Petrila L.M.***, Bucatariu F., Froidevaux R., Mihai M. Polyelectrolyte layer-by-layer nanoarchitectures – versatile materials with various applications. *NATO ASI Summer School Nanomaterials and Nanoarchitectures II. Composite Materials & Their Applications*, 28 June – 5 July 2024, Smolenice, Slovakia.

5.3. Marandiș C.G.*, Zaharia M.M., **Petrila L.M.**, Lotos E.D., Bucatariu F., Mangalagiu I., Mihai M., Pispas S. Chit-*g*-PNIPAM: a versatile pH/temperature multi-responsive copolymer in aqueous environment. *PolyChar World Forum on Advanced Materials 30th Edition*, 11-13 September 2024, Iasi, Romania.

5.4. **Petrila L.M.***, Karayianni M., Pispas S., Mihai M. Laccase/ polysccharide hybrid nanostructures for efficient dye degradation. ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 15 November 2024, Iasi, Romania.

5.5. **Petrila L.M.***, Karayianni M., Ciobanu, A.T., Vasiliu, T., Pispas S., Mihai M. Enzyme/polysaccharide nanoassemblies: preparation, characterisation and potential applications. *Progress in organic and macromolecular compounds*, 30th edition, 23-26 September 2025, Iasi, Romania.

6. Oral communications at national and international scientific conferences (results not included in the thesis):

6.1. Lotos E.D.*, Karayianni M., Vasiliu A.L., **Petrila L.M.**, Mihai M., Pispas S., Simionescu B. Interaction between water-soluble chitosan and thermo-responsive poly(*N*-isopropylacrylamide). ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 17 November 2023, Iasi, Romania.

6.2. **Petrila L.M.***, Ciobanu T.A., Bucatariu F., Mihai M. Unlocking Innovative Solutions for Polycation/Polyanion/Catalase Bi- and Tri-Component Interpolyelectrolyte Complexes. *EPF European Polymer Congress*, 22-27 June 2025, Groningen, the Netherlands.

6.3. Ciobanu T.A.*, **Petrila L.M.**, Bucatariu F., Zaharia M.M., Mihai M. Sand/polyelectrolyte composites for wastewater treatment. *Applications of Chemistry in Nanosciences and Biomaterials Engineering (NanoBioMat),* 25 – 27 June 2025, online.

6.4. Ciobanu T.A.*, **Petrila L.M.**, Bucatariu F., Zaharia M.M., Mihai M. Core/shel composites based on sand and weak polyelectrolytes for water cleaning. *13th International Conference on Environmental Engineering and Management*, 17-20 September 2025, Iasi, Romania.

6.5. Bucatariu F.*, Zaharia M.M., **Petrila L.M.**, Mihai M., Pispas S. Interaction studies of chitosan-*g*-PNIPAM multiresponsive chains with a model protein.

Progress in organic and macromolecular compounds, 30th edition, 23-26 September 2025, Iasi, Romania.

7. Member in the implementation team of national research:

7.1. Ph.D. student (period 01.02.2022 – 31.10.2022) within the project *Quartz sand/polyelectrolyte composite microparticles with high loading/release of some inorganic/organic compounds from polluted waters*, project code PN-III-P2-2.1-PED-2019-1996, acronym POLYSAND.

7.2. Ph.D. student (period 01.07.2023 – present) within the project *Polysaccharide based (bio)hybrid nanostructures*, project code PNRR-III-C9-2022 – I8, acronym HYBSAC.

7.3. Ph.D. student (period 08.01.2025 – present) within the project *Polyelectrolytes/enzymes architectures constructed on inorganic microparticles for static/dynamic water cleaning by sorption/catalysis*, project code: PN-IV-P1-PCE-2023-1545, acronym PolyEnzIM.

8. Research and professional development stages:

8.1. Summer School „*Technological challenges of transformation to renewable energy*", University of Aalborg, Danmark, 29 July – 8 August 2022, self-financed.

8.2. Research stage at Leibniz Institute for Polymer Research (IPF), Dresden, Germany, 16 August – 6 November 2022, financed by IPF.

8.3. Spring School on Project Management BioNanoTech, Lacul Roșu, Romania, 24 – 28 April 2023, financed by *BioNanoTech – Centru Suport pentru Managementul Proiectelor Orizont 2020,* MySmis 107524.

8.4. Research stage at Charles Viollette Institute of the University of Lille, France, 1 October 2023 – 31 May 2024, financed by the Ministry of Education and Research within the scholarship HG 118/2023 and by the University of Lille within the mobility scholarships MobLilex 2023.

8.5. NATO ASI Summer School Nanomaterials and Nanoarchitectures II. Composite Materials & Their Applications, 28 June – 5 July 2024, Smolenice, Slovakia, financed by *Polysaccharide based (bio)hybrid nanostructures (HYBSAC),* PNRR-III-C9-2022 – I8.

9. Awards obtained in scientific conferences:

9.1. **Best Oral Presentation** for the presentation *Silica/polyelectrolyte sorbents for the removal of organic pollutants*, within 1st Edition Ph.D. Students' Days, 26 November 2021, Arad, Romania.

9.2. **„Sorin I. Roşca" Prize of the Romanian Chemical Society** for the presentation *Catalase/polymer composite microparticles for environmental applications* within ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 17 November 2023, Iasi, Romania.

9.3. **Innovation Award** for the presentation *Harnessing immobilized laccase for sustainable water remediation* within *Exploratory Workshop NeXT-Chem: Innovative Cross-Sectoral Technologies*, 21 – 22 March 2024, Bucharest, Romania.

9.4. **The Prize of the Romanian Chemical Society** for the presentation *Green hybrid nanostructures for efficient degradation of water pollutants* within the International Symposium *„Priorieties of Chemistry for a sustainable development*", 16 – 18 October 2024, Bucharest, Romania.

9.5. **First Prize for the best oral presentation** for the presentation *Laccase/ polysccharide hybrid nanostructures for efficient dye degradation* within ICMPP – *Open door to the future scientific communications of young researchers MacroYouth*, 15 November 2024, Iasi, Romania.