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**Rational Integration of Nanomagnetism and
Biocatalysis for Sustainable Circular Polymer Systems**

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Abstract

The thesis advances a holistic framework for circular polymer processing by integrating enzymatic catalysis, magnetic nanoparticles-based immobilization and functionalized bioplastic interfaces. The resulting approach aspires to contribute to next-generation technologies by developing sustainable and efficient solutions for biocatalytic processes and polymer material management, both during synthesis and in a recycling-oriented perspective, where biocatalysis and nanomagnetism operate in harmony to close the polymer lifecycle within a fully circular bioeconomy.

First, a rational Biocatalyst Magnetic Nanoarchitecture (BMN) was designed using spinel iron oxide nanoparticles with optimized morpho structural and textural properties. These tailored nanomaterials served as support for the immobilization of *Candida antarctica* Lipase B (CaLB), enabling the formation of a stable biocatalyst that could be magnetically recovered for several cycles of reactions. CaLB, immobilized on magnetic nanoparticles, retained high activity in polycondensation reactions using renewable monomers; it exhibited >87% monomer conversion over multiple cycles yielding polyesters with number-average molecular weight (M_n) between 4.2 and 5.6 kDa. The efficient magnetic recycling demonstrated the robustness of the BMN platform.

In parallel, the synthetic potential of three novel thermophilic α/β hydrolases, LCC (leaf-branch compost cutinase), its variant form LCC^{ICCG} and Thb from *Thermoanaerobacterales* bacterium, was assessed to widen the range of biocatalysts beyond the well-established use of CaLB. A design of experiments (DoE) approach allowed to precisely evaluate their thermostability and substrate selectivity in esterification and transesterification reactions. By achieving up to 90% of conversion and producing polyesters with molecular weights of ~3.6-3.8 kDa, the study demonstrated the versatility of these thermostable hydrolases as biocatalysts for sustainable polymer synthesis.

The concept of magnetically responsive system was further extended to recycling by integrating the concept of nanomagnetism to the end-of-life management of bioplastics. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) films were functionalized with spinel iron oxide nanoparticles via a Layer-by-Layer (LbL) assembly using bio-derived polymers (i.e., DNA and chitosan) as dispersing agents. This coating imparted magnetic properties without compromising polymer integrity, enabling magnetic separation from mixed plastic waste with potential nanoparticles recovery and reuse after enzymatic degradation.

Preface

The originality of this PhD research lies in combining the combination of three complementary but distinct scientific disciplines (**physical chemistry**, **organic chemistry** and **biotechnology**) into a unified strategy aimed at advancing sustainable polymer science and contributing to the circular bioeconomy.

This interdisciplinary approach reflects my personal academic evolution: starting from a background in biotechnology during my Bachelor's and Master's studies, I progressively integrated concepts from nanomagnetism, polymer chemistry and enzymatic biocatalysis during the doctoral research.

During my Master's thesis, I was first introduced to the world of nanomagnetism, which allowed me to explore the physical properties of nanoscale materials and their potential applications in catalysis and material science. This experience provided a solid foundation for understanding the physicochemical mechanism supporting enzyme immobilization and nanostructured systems, concepts that would later play a pivotal role in my PhD project.

Based on my academic background, the PhD research within the “Sciences and Technologies of Chemistry and Materials” curricula initially focused on the enzymatic synthesis of bio-based polymers. The research line explores a novel magnetic nanoarchitecture for enzyme immobilization that allows polyester synthesis under mild conditions enabling the biocatalyst to be easily recovered and reused through magnetic separation. This approach avoids the use of solvents and other harsh processing conditions both highlighting the versatility of enzymes as green catalysts and demonstrating how polymer science could directly benefit from biocatalytic methodologies to achieve both efficiency and environmental sustainability.

My international research period at the Department of Agrobiotechnology, IFA-Tulln (Institute of Environmental Biotechnology, Universität für Bodenkultur Wien (BOKU)), represents a turning point for my PhD project since I expanded my scientific work with biotechnology and molecular biology. I spent six months working with recombinant DNA (rDNA) techniques to produce *de novo* enzymes for polymer synthesis. This experience allowed me to reconnect with my background in biotechnology, shifting the focus from just using enzymes as catalytic tools to their engineering and production. This effectively closed the loop between my biotechnological roots and my current focus on materials chemistry. In addition, it was a great opportunity to broaden the range of enzymatic tools by testing and characterizing alternative hydrolases for their synthetic capabilities, paving the way for future developments beyond CaLB-based systems.

Beyond biocatalysis, this research proposes a surface modification strategy for Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) through the Layer-by-Layer (LbL) assembly of bio-based polyelectrolytes. The co-dispersion of DNA and chitosan with spinel iron oxide nanoparticles allows the development of a functional coating that impart magnetic responsiveness to the polymer without compromising its biodegradability. This modification is particularly significant for end-of-life management; it enables the use of magnetic field based sorting to facilitate post-consumer recovery. By addressing the persistent challenges of contamination and the complexity of separating mixed plastic waste, this approach offers a viable pathway toward the improvement of both efficiency and purity of the plastic recycling stream.

The culmination of these efforts is the BIO-MAG-LOOP (BIOcatalytic and MAGnetic strategies for Closed-LOOP Polymer Systems) project, which combines the three disciplines (**Figure 1**) with the common goal of developing sustainable and efficient solutions for biocatalysis and polymer management:

1. From **physical chemistry**, it proposes a magnetic nanoarchitecture as supports for enzyme immobilization, enabling solvent-free polyester synthesis with magnetically recoverable catalysts. Additionally, it introduces a smart platform for magnetically sortable bioplastics.
2. From **organic chemistry**, it incorporates enzymatic polymerization to produce bio-based polymers under mild, sustainable conditions.
3. From **biotechnology**, it integrates rDNA-based enzyme engineering to broaden the variety of biocatalysts used in polyester synthesis beyond the well-established CaLB.

This research provides an innovative and comprehensive response to the pressing challenges of sustainable polymer production and their end-of-life management. It makes a significant contribution to the circular bioeconomy, demonstrating how interdisciplinary approaches can reveal new opportunities in green chemistry and materials science. By combining biotechnology, organic chemistry and physical chemistry in a single project, it exemplifies how integrated scientific strategies can enhance resource efficiency, minimize environmental impact and waste, and promote the development of truly closed-loop polymer systems.

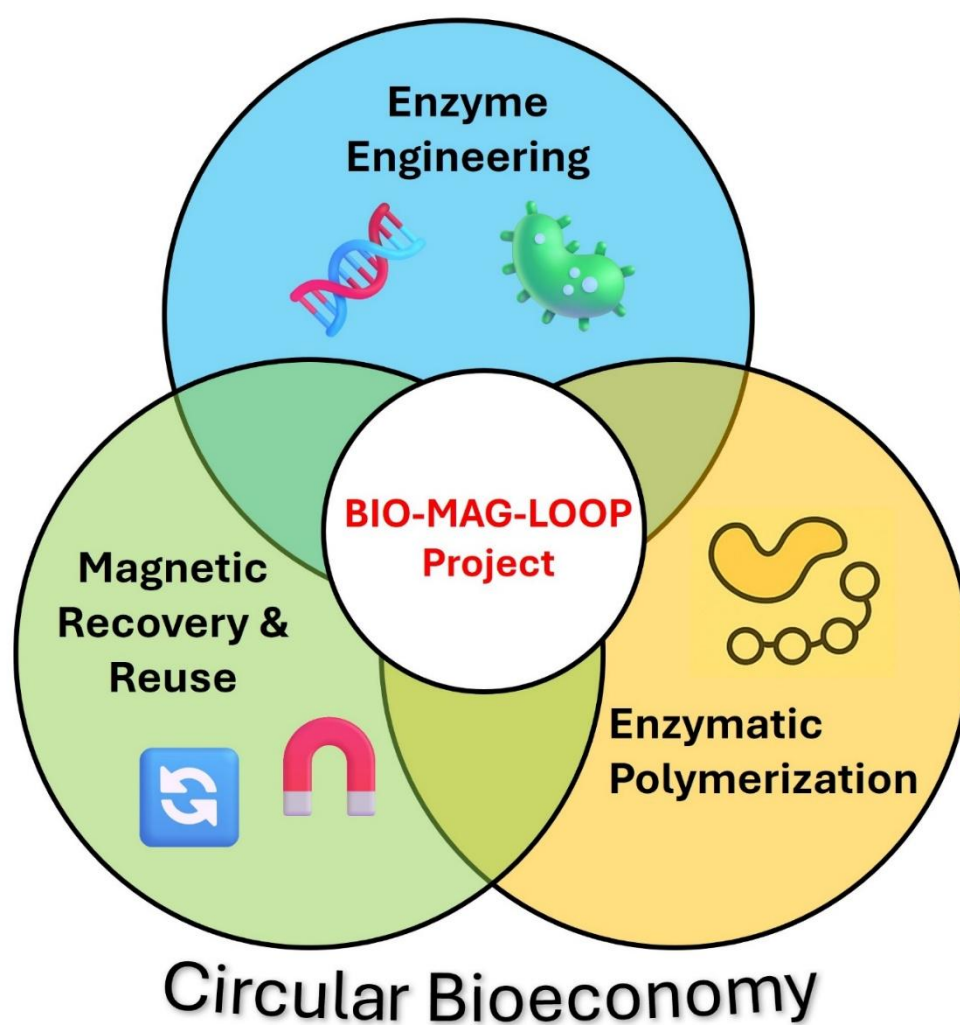


Figure 1: Schematic representation of the interdisciplinarity activities carried out during the BIO-MAG-LOOP project.

Summary

The growing demand for sustainable materials, in line with environmental policies such as the European Green Deal, has intensified research into bio-based and biodegradable polymers. Among the most promising strategies to meet this demand is the use of enzymes as biocatalysts, which offer a green alternative to traditional chemical synthesis. Enzymes enable the formation of polyesters under mild, environmentally friendly conditions, avoiding the use of toxic metal catalysts and reducing energy consumption. Hydrolases, such as lipases, esterases and cutinases have shown great potential in polyester synthesis, exploiting their reversible catalytic mechanism to perform esterification and transesterification reactions in low-water or solvent-based systems. However, while enzymatic catalysis has proven effective, several limitations still prevent its broader industrial application, most notably the difficulty in recovering and reusing enzymes, their limited operational stability and high costs.

To address these challenges, enzyme immobilization has emerged as a key enabling technology. Recent advances in nanotechnology have opened new avenues for immobilization using magnetic nanoparticles (MNPs), which offer high surface area, tunable surface chemistry and superparamagnetic behaviour. Therefore, Chapter 1 introduces MNPs as versatile platform for enzyme immobilization. After a brief introduction to the fundamentals of nanomagnetism, this Chapter examines the design of magnetic materials with tailored magnetic properties and outlines contemporary strategies for enzyme immobilization. The overview is set within the context of condensation reactions, highlighting how the magnetic response of the system facilitates separation and improves recyclability. Building on this concept, the following chapter moves from theory to practice, demonstrating how magnetic nanoarchitectures can be effectively integrated in real biocatalytic processes.

Chapter 2 explores the use of a biocatalyst magnetic nanoarchitecture to make enzymatic polyester production more sustainable. The focus here is on how these hybrid magnetic biocatalytic systems can be effectively recovered and reused across multiple catalytic cycles. Thanks to their magnetic properties, the enzyme-MNP conjugate can be recovered from the reaction mixture using an external magnetic device, eliminating the need for organic solvents or complex filtration systems.

Moreover, moving toward more robust and circular processes calls for addressing several open challenges, among which the need to expand the enzymatic toolbox beyond CaLB stands out as a crucial step. While CaLB remains a reliable and well-characterized biocatalyst, its dominance has potentially limited the exploration of alternative that could offer improved selectivity, stability or

broader substrate scope. Chapter 3 have begun to address this gap by investigating other enzymes within the α/β -hydrolase fold superfamily, particularly cutinases such as newly characterized polyester hydrolases like LCC (leaf-branch compost cutinase), its engineered thermostable variant LCC^{ICCG} and Thb, a promising enzyme from *Thermoanaerobacterales*, with high homology to known PET hydrolases. These enzymes, traditionally used in polyester depolymerization processes, are now used to catalyze the reverse reaction (polyester synthesis), showing promising activity and selectivity in esterification reactions. However, their overall potential, particularly with respect to recyclability and long-term operational stability, has yet to be fully investigated.

In parallel with the advances in enzymatic catalysis, an increasing attention has also been directed toward the improvement of the recyclability and functional performance of bio-based polymers. One such polymer, poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH), has gained commercial interest due to its biodegradability, tunable mechanical properties and microbial production from renewable resources. However, as with many bioplastics, the efficient separation and recovery of PHBH in recycling streams remains a critical challenge. To address this issue, Chapter 4 explores a surface functionalization strategy based on Layer-by-Layer (LbL) assembly, aimed at introducing magnetic responsiveness while preserving the polymer's biodegradability and enhancing the magnetic field sorting procedure.

Finally, Chapter 5 (Conclusion section) highlights how the synergistic design of magnetic supports and enzymes can lead to advanced biocatalytic magnetic nanoarchitectures. Remaining challenges in stability and loading point towards future work with tunable MOF-based supports, enabling improved activity, recyclability and rationally engineered hybrid systems.

Chapter 1: State of the art

1.1 Introduction

Biocatalysis stands as a sustainable and inherently interdisciplinary technology, historically shaped by the convergence of biochemistry, organic chemistry, microbiology and molecular biology [1]. Biocatalytic approaches to polymer synthesis represent a rapidly developing field with significant scientific and technological potential, as well as clear environmental relevance. Although the chemical and economic benefits of biocatalysis over conventional synthetic methods have been acknowledged for decades, large-scale industrial implementation remained limited until recent advances in modern biotechnology, such as efficient protein expression platforms and directed enzyme evolution, transformed the landscape. Within this context, enzymatic polymerization has emerged as a particularly powerful strategy [2]. It not only offers a competitive alternative to traditional chemical and physical methods for producing established polymeric materials but also enables the creation of entirely new macromolecular structures that cannot be readily obtained through conventional synthetic routes [3]. In recent years, however, the development of engineered supports for enzyme immobilization has introduced new opportunities [4]: this allows materials scientists, particularly those working in physical chemistry, to contribute more directly to this area. Among these emerging platforms, magnetic nanoparticles (MNPs) have gained particular interest due to their tunable surface chemistry and the advantage of being easily manipulated and recovered using external magnetic fields [5].

At the same time, the research recognizes that sustainable polymer science requires attention not only to the synthesis of materials, but also to their end-of-life management. The expanding deployment of biodegradable polymers derived from renewable resources demands for innovative approaches to refine and expand their functional performance. To become fully compatible with a circular bioeconomy, these materials must be designed in a way that facilitates their identification and separation from conventional, fossil-based plastics within recycling systems. However, such enhancements must not compromise their biodegradability, which remains one of the key environmental benefits of bioplastics. In this context, MNPs offer an interesting and versatile solution. They can serve not only as support for immobilized enzymes in sustainable polymer synthesis, but also as functional components that give to bioplastics magnetic responsiveness, simplifying their recovery. By carefully engineering the surface of magnetic particles without altering the polymer's biodegradability, these particles can enable rapid magnetic field-based separation, improving recovery efficiency and ensuring sustainability. Together, these considerations make magnetic nanoparticles as a unifying platform bridging biocatalytic production and environmentally

responsible material recovery, reinforcing the importance of integrated design across synthesis, function and end-of-life pathways.

1.2 Principles of Magnetism at the Nanoscale

The tendency of systems to minimize their energy is a fundamental principle used to predict how physical processes evolve in the universe. In ferromagnetic materials, for example, the reduction of magnetostatic energy leads to the formation of multiple small regions known as magnetic domains. These domains are uniformly magnetized areas in which the atomic moments are aligned in the same direction, but they can vary considerably in shape and size [6] [7]. In addition, the physical properties of a material can change significantly with size, especially when its dimensions approach the characteristic length scales relevant to the property that are examined [8].

In magnetic systems, among relevant dimensions are the magnetic domains that typically fall within the 10-1000 nm range, depending on the material's magnetic properties [9]. This explains why nanoscale magnetic nanoparticles behave so differently from their larger bulk counterparts. When the dimensions of a magnetic particle are reduced below a certain critical size, it becomes energetically preferable for the particle to exist as a single magnetic domain rather than splitting into multiple domains. A magnetic single-domain nanoparticle, that is in a state of uniform magnetization at any field, behaves like a “superspin” with a large magnetic moment (μ_p) generally ranging from 10^3 to 10^5 Bohr magneton (μ_B) [10]. If interactions between particles are significant, the ensemble of MNPs may exhibit collective magnetic behaviour that can dominate the anisotropic properties of individual particles. Under sufficiently strong interparticle interactions, the system can develop a superspin glass (SSG) state, analogous to the behaviour observed in atomic spin glass systems in bulk. With a further increase in interaction strength, the system may even transition to a superferromagnetic (SFM) state [11]. Conversely, for non-interacting single-domain nanoparticles, with uniaxial magnetic anisotropy, the energy landscape (**Figure 1a**) features an energy barrier (ΔE_a) separating two stable orientations of magnetic moment, parallel or antiparallel to the anisotropy axis. This energy barrier is directly proportional to the particle volume (V_p) and the magnetic anisotropy constant (K). When the temperature drops below a characteristic value known as the blocking temperature (T_B), such as $k_B T \ll \Delta E_a$, the magnetic superspins are confined within these two minima. Under these conditions, the system exhibits a non-reversible magnetization response to an applied external magnetic field, resulting in a hysteresis loop. For sufficiently small particles, where magnetic moments rotate coherently, this behaviour is well described by the Stoner-Wohlfarth model [12]. As an example, **Figure 1b** illustrates the magnetization curve measured at 5K for a sample of ~ 5 nm CoFe_2O_4 nanoparticles[13].

Above the blocking temperature, when $k_B T \gg \Delta E_a$, the particle's magnetic moment can undergo thermally induced transitions between the two easy magnetization orientations separated by the energy barrier. For an isolated spherical nanoparticle, this manifests as spontaneous flipping of the superspin direction over time. From a macroscopic perspective, this resembles paramagnetic behaviour, characterized by zero coercivity ($H_c=0$) and no remanent magnetization ($M_r=0$). However, because the fluctuating units are superspin instead of individual atomic moments, the phenomenon occurs on different magnetization and time scales and is therefore termed superparamagnetism [14]. For the CoFe_2O_4 nanoparticles referenced earlier, the blocking temperature is approximately 150 K. **Figure 1c** displays the magnetization curve recorded at 300K (i.e., $T \gg T_B$), where the M vs. H behaviour is fully reversible ($H_c=0$ and $M_r=0$), despite the saturation magnetization remains relatively high [14]. The key benefit of dealing with superparamagnetic material is that, once the external magnetic field is switched off, it no longer retains magnetization ($M_r=0$). This prevents particles from aggregating and eliminates residual local magnetic fields, which can be problematic in certain applications. It is important to highlight that the magnetic behaviour of a nanoparticle ensemble is strongly influenced by both physico-chemical factors (such as structure and chemical composition) and morphological parameters (including particle size and size distribution) [15]. Therefore, the distinctive structural features and versatile crystal chemistry of spinel materials allow precise control of their magnetic behaviour, making them highly attractive for several biomedical applications, including biosensing [16], cell separation [17], magnetic hyperthermia [18], targeted drug delivery [19], use as contrast agent in magnetic resonance imaging [20] and magnetic hyperthermia[21].

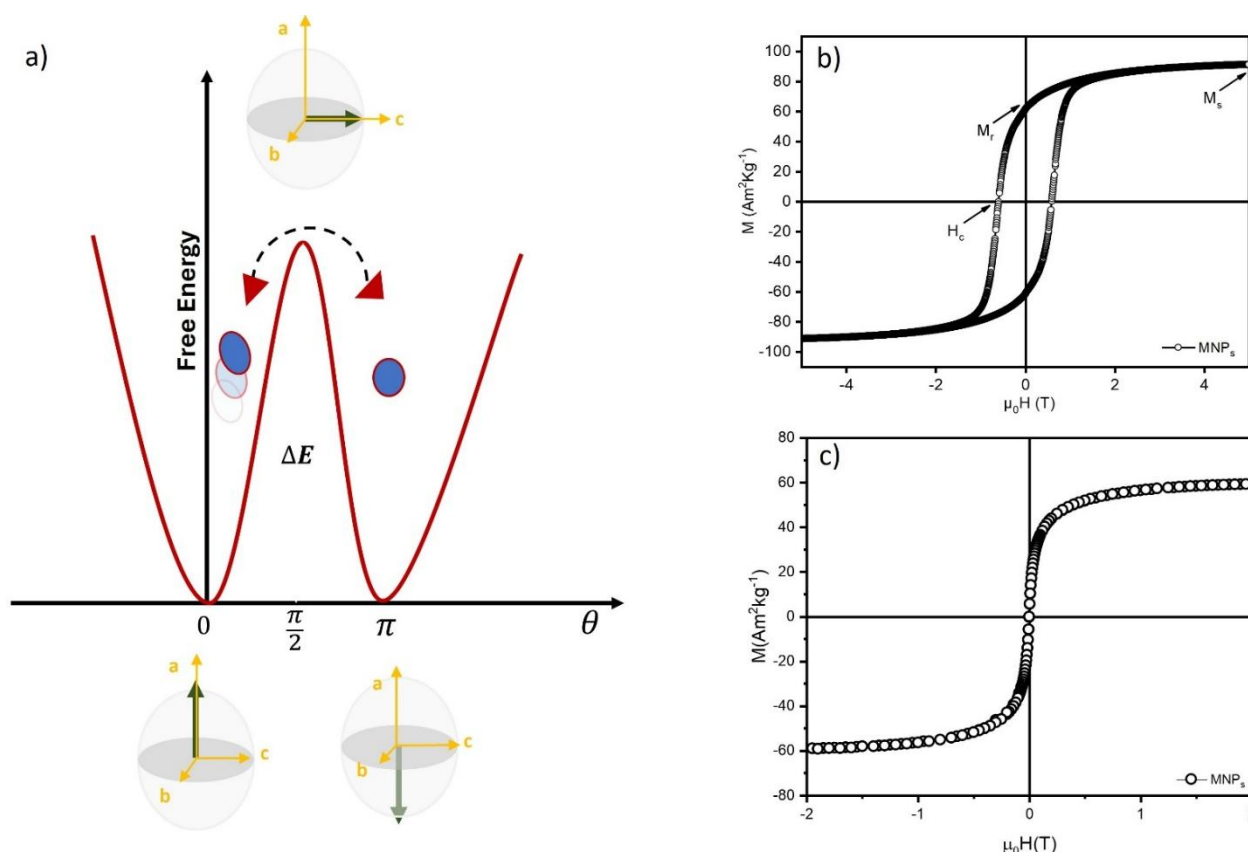


Figure 1: a) Schematic illustration of the anisotropy energy barrier in magnetic nanoparticles; b) Main parameters describing hysteresis: Saturation magnetization (M_s), the magnetization retained at zero field (M_r), often expressed as the reduced remanence M_r/M_s (ranging from 1 for a perfectly square loop to 0 for fully reversible behaviour), and the coercive field (H_c), the field required to reduce the remanence magnetization to zero. In general, H_c increases with magnetic anisotropy (K) and decrease with M_s ; c) Example of a M vs. H curve characteristic of superparamagnetism, showing $H_c=0$ and $M_r=0$.

1.3 Magnetic nanoparticles: a smart platform for enzyme immobilization

Significant progress in nanotechnology over the last two decades has facilitated the creation and deployment of various nano-carriers for anchoring enzymes. Among these, MNPs have become highly favoured immobilization supports due to their unique magnetic and physiochemical characteristics. These supports offer several advantages, including biocompatibility, low toxicity, cost-effectiveness and the capacity for precise surface functionalization. A key benefit of using MNPs aligns with the principles of green chemistry: their inherent responsiveness to an external magnetic field allows for the simple, rapid and eco-friendly recovery of immobilized enzymes without the need for conventional separation steps. This facilitates the practical handling of MNPs based biocatalyst during reaction processes reducing processing time and operational costs compared to traditional purification and filtration methods.

Another strength of MNPs is their high surface area to volume ratio, which provides many binding sites for enzyme attachment contributing to high enzyme loading capacity. Furthermore, surface modification strategies enable precise adjustment of enzyme attachment, improving catalytic performance, stability and substrate specificity. The excellent recyclability of MNPs is another major advantage, maintaining enzyme activity over multiple reaction cycles with minimal loss of activity, which enhances sustainability and industrial scalability.

MNPs have been successfully employed as enzyme supports in several fields, including biosensing [22], immunoassays and organic chemistry [23] [24]. In many cases, enzymes immobilized on MNPs demonstrate increased stability and higher catalytic efficiency compared to free enzyme [25] [26]. For example, Huang et al. (2003) immobilized *Candida rugosa* lipase on ~13 nm Fe₃O₄ and observed improved enzymatic activity towards p-nitrophenyl palmitate, as well as enhanced thermal and pH stability. This stabilization was attributed to the support surface helping to preserve the native structure of the enzyme [27].

However, the performance of immobilized enzymes is highly dependent on the density of enzymes on the nanoparticle surface. When enzymes are packed too closely, unfavourable interactions between protein molecules can lead to conformational distortions and reduced biological activity. Therefore, supports with large surface help minimizing these effects by reducing steric crowding while maximizing enzyme loading.

Although magnetic supports provide promising platforms for enzyme immobilization, biocatalyst' activity on MNPs surface can be significantly influenced by how the active sites are oriented on the surface, as well as by possible mass transfer constraints [28]. To address these issues, ongoing research explores surface functionalization using biocompatible molecules and the application of physical stimuli to enhance enzymatic activity. In particular, the use of rotating magnetic fields (RFM) has shown promising results. RMF can influence enzyme conformation and reaction kinetics. For example, Wasak et al. (2019) demonstrated that RFM exposure enhances enzyme activity, broadens the effective pH range and shifts the optimal operating pH [29].

Other researchers have indicated that applying a static DC magnetic field can similarly increase the activity of immobilized enzymes [30] [31]. This effect is attributed to changes in the energy states of unpaired electrons in metal ions exposed to the magnetic field, which can promote free radical formation and influence reaction pathways [32]. Moreover, increasing the gradient of the magnetic field can enhance the separation efficiency of immobilized enzymes from reaction mixture [33] [34].

1.4 Overview on hydrolases' structure, catalytic reaction and mechanism

Hydrolases represent one of the largest enzyme classes, encompassing more than 200 distinct proteins. According to the Enzyme Commission (EC) classification system, they are grounded under EC 3, a category that can be further divided into subclasses depending on the specific type of bond cleaved, such as ester, glycosidic, peptide or phosphodiester bonds. Each hydrolase family has evolved to fulfil a unique biochemical role, reflecting the extraordinary versatility of this enzyme class across all forms of life [35].

Despite the structural diversity among hydrolases, the overall chemical transformation they catalyze follows a common principle: the cleavage of a covalent bond through the addition of a water molecule. Interestingly, as visible in **figure 2**, the mechanism can be virtually reversed under specific reaction conditions, leading to condensation reaction, in which two starting substrates are combined with the elimination of water or another small by-product. This reversible nature represents one of the most remarkable features of hydrolases catalysis underscoring their immense potential as biocatalysts in both degradative and synthetic processes. Depending on the reaction medium and thermodynamic equilibrium, certain hydrolases can promote the cleavage of chemical bonds in aqueous systems, while in organic medium or solventless condition, they can drive the reverse reaction, promoting bond-formation [2].

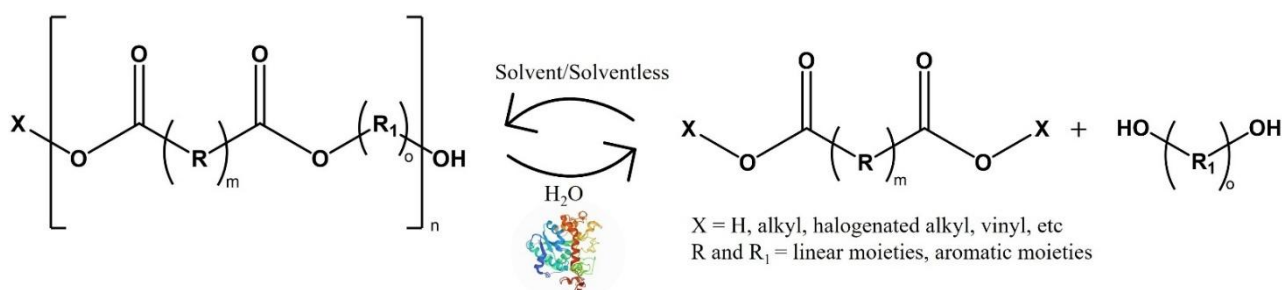


Figure 2: Cleavage of a chemical bond by hydrolytic enzymes in presence of H_2O and its reverse condensation reaction in the absence of H_2O .

Hydrolases can be obtained from a variety of biological sources, including plants, animals and microorganism. Modern biotechnology tools have further expanded their accessibility, allowing the recombinant expression of hydrolases in microbial systems for large-scale applications. They are among the most widely used enzymes in research and industry, contributing to different fields such as biomass conversion, polymer degradation, waste valorization, food technology and fine chemical synthesis [36] [37].

Among the structural families of hydrolases, the α/β hydrolases fold stands out as one of the most evolutionarily conserved and functionally versatile architectures. Enzymes adopting this fold include,

lipases (EC 3.1.1.3), proteases (EC 3.4), cutinases (EC 3.1.1.74), esterases (EC 3.1.1.1), epoxide hydrolases (EC 3.3.2.3), dehalogenases (EC 3.8) and glycosidases (EC 3.2.1). Despite catalyzing a wide range of reactions, these enzymes share a common structural core composed of 6 to 8 parallel β strands surrounded by α -helices, forming a stable scaffold that houses the Ser-His-acid catalytic triad [38]. The nucleophile (normally a Ser residue) is positioned on a conserved “nucleophilic elbow” motif (Gly-X-Ser-X-Gly), immediately after β -sheet 5. The acidic residue, usually aspartate or glutamate, follows β -sheet 7, while the histidine residue is located after the final β strand. This conserved arrangement ensures optimal geometric and electronic alignment for catalysis and has been maintained throughout divergent evolution [39]. The catalytic triad remains the central feature responsible for the hydrolytic activity of most α/β hydrolases. The reaction mechanism typically proceeds via the acylation-deacylation cycle, in which the serine attacks the carbonyl carbon of the substrate forming a tetrahedral intermediate stabilized by the oxyanion hole. This latter is a pocket composed of backbone amides or side chains (often Thr and Gln) that donate hydrogen bonds to stabilize the negatively charged oxygen atom. After intermediate collapse and product release, the enzyme is regenerated by water-mediated deacylation [39]. Consistent to what discussed for hydrolysis, also the catalytic mechanism in polymer synthesis proceeds through these two steps [40]. Regarding a model transesterification reaction model involving a diester and a diol, the Ser reacts with the diester to form an acyl-enzyme intermediate, which is subjected to a nucleophilic attack by the diol. This attack results in the release of Ser in its free form, making it available again to participate in the next cycle of polymer elongation.

An illustrative example of the dual catalytic nature and of this mechanism is provided by *Candida antarctica* Lipase B (CaLB), one of the most extensively studied and employed biocatalysts. CaLB, a globular enzyme composed of 317 amino acids, displays the typical α/β hydrolases fold with 7 β strands and 10 α -helices, and is characterized by the Ser₁₀₅-His₂₂₄-Asp₁₈₇ catalytic triad and an oxyanion hole defined by Thr₄₀ and Gln₁₀₆ [41], as visible in **figure 3**. In aqueous media, lipases catalyze the hydrolysis of triglycerides into glycerol and fatty acids. However, under non-aqueous conditions (solventless or in classical, petrol-derived organic solvents such as toluene, benzene and cyclohexane) [42] or in green solvents such as ionic liquids and supercritical CO₂ [43], or the new emerging 2,2,5,5- tetramethyl-oxolane [44], lipases can catalyze the reverse reactions (esterification, transesterification, amidation and transamidation) leading to the synthesis of valuable flavour esters, polyesters and polyamides [45].

Another important class within the hydrolase family is represented by proteases (EC 3.4), which catalyze the cleavage of peptide bonds linking amino acids in proteins [46]. Proteases are traditionally divided into six major subclasses according to their catalytic residues: serine, threonine, cysteine,

aspartate, glutamate and metallo-proteases. Like lipases, proteases can act as synthetic catalysts under low-water content, promoting peptide bond formation through condensation or transamidation. Peptide synthesis via protease catalysis can proceed through two distinct mechanisms: a thermodynamically controlled process, in which synthesis is in equilibrium with hydrolysis, and a kinetically controlled process, where the reaction is governed by the relative rates of acylation and deacylation steps [47]. Despite their efficiency, proteases generally show limited ability to form high-molecular weight polymers because the competing hydrolysis reaction imposes a natural limit on polymer growth [48].

More recently, another group of hydrolases, the cutinases (EC 3.1.1.74), has attracted growing attention due to their unique catalytic versatility. Cutinases play a central role in the hydrolytic degradation of polyester [49] as well as natural polymer such as cutin resulting in the formation of alcohol and carboxylic acid monomer. However, as it will be discussed in Chapter 3, their catalytic mechanism can be exploited in the reverse direction similarly to lipases and proteases, enabling esterification and polymerization reactions under suitable conditions.

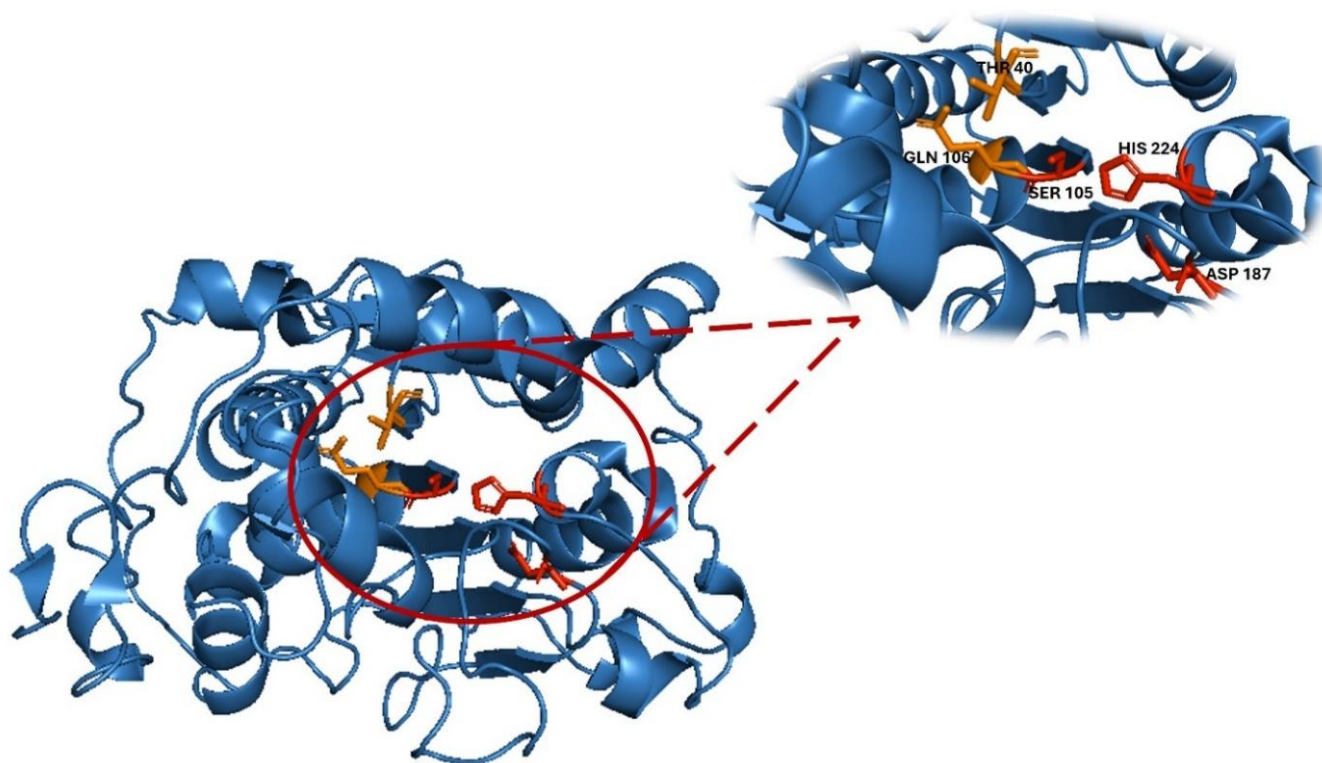


Figure 3: 3D structure of CaLB with a zoom to catalytic triad highlighted in red and the oxyanion hole highlighted in orange.

1.5 Enzyme immobilization

Over the past thirty years, biocatalysis has developed into a key technology that supports the growing need for greener and more sustainable chemical processes, particularly in the manufacture of pharmaceuticals, flavours and other fine chemicals [50]. Advances in enzyme discovery, protein engineering and large-scale production have expanded the applications of biocatalysts, enabling the synthesis of complex molecules under mild and eco-friendly reaction conditions [51]. However, industrial use of enzymes is still often limited by their insufficient operational stability, difficulties in separation from the reaction mixture and the inability to reuse these relatively expensive catalysts. Therefore, enzyme immobilization strategies can help overcome many of these constraints. The primary aim of immobilizing enzymes is to preserve or even improve their functional properties, including activity, stability and selectivity. Immobilized enzymes can be easily separated from reaction products, which allows catalyst recovery and reuse, an essential requirement for achieving economically competitive biocatalytic processes [5]. In addition, immobilization generally enhances enzyme stability during storage and operation, improving tolerance to heat and enabling use in non-aqueous environments [4]. Nevertheless, immobilization can also lead to partial loss of catalytic efficiency due to structural modifications or mass-transfer limitations.

1.5.1 Immobilization methods

A variety of immobilization techniques have been developed to address different applicational needs, including adsorption or covalent attachment to solid supports, entrapment within polymeric matrices and direct cross-linking [52] (**Figure 4**).

Depending on the method used, the enzyme may be held either permanently or only temporarily on the support. The overall performance of the immobilized biocatalyst depends on both the enzyme and the characteristics of the support material, which must provide mechanical resistance, stability, hydrophilicity, biocompatibility and affordability. Supports can be commonly grouped into four categories:

1. Natural polymers (e.g., alginate, chitosan, dextran, gelatine, cellulose, pectin and collagen);
2. Synthetic polymers, often porous or resin-like materials (e.g., DEAE-cellulose, PEG-based materials, polystyrene, polyacrylates, polypropylene and polyamides);
3. Conductive polymers such as polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) [53];
4. Inorganic supports, including zeolites, silica, ceramics, activated carbon and glass.

Recently, increasing interest has been placed on sustainable supports derived from renewable biomass (e.g., bamboo [54] and rice husks [55]) as well as inorganic materials like magnetic nanoparticles, which offer high surface area and biocompatibility [56].

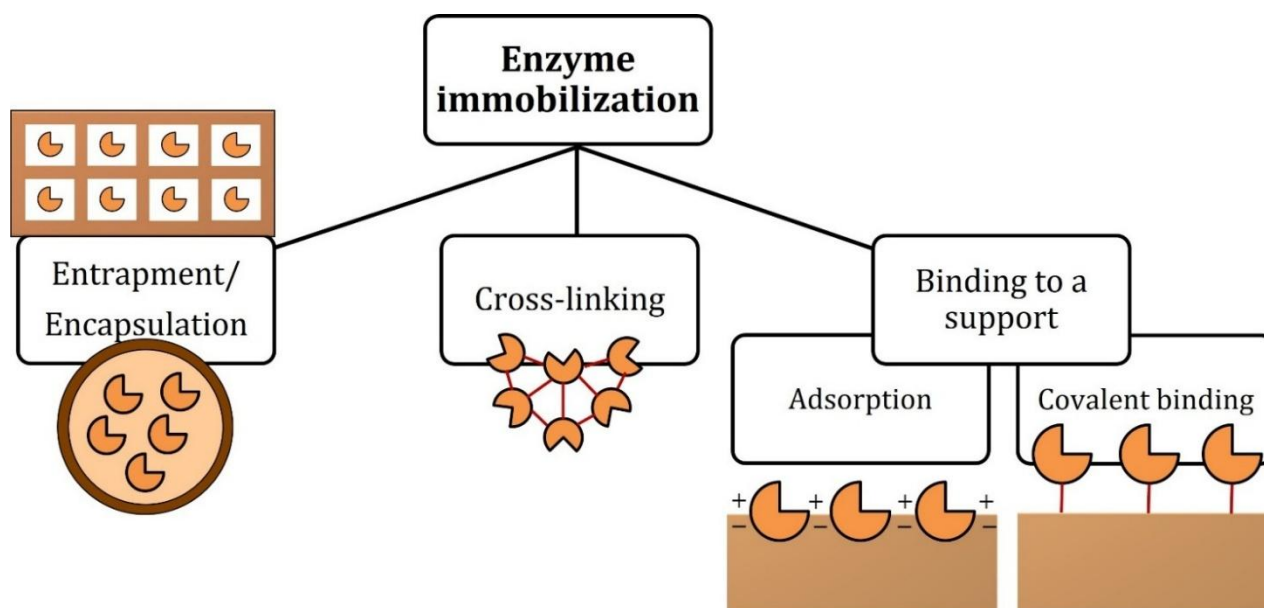


Figure 4: Schematic representation of enzyme immobilization strategies.

1.5.1.1 Binding to a support

Enzymes may be immobilized on solid materials either by physical adsorption or covalent attachment.

- Physical adsorption is reversible and driven by weak forces (ionic interactions, hydrogen bonding, hydrophobic interactions, Van der Waals forces). Although this method avoids structural modification and minimizes diffusion limitations, the weak binding often results in enzyme desorption under industrial conditions.
- Covalent attachment forms strong, irreversible linkages between the enzyme and the support, preventing catalyst leaching [57]. However, the chemical reaction involved may lead structural alteration of the enzyme and potential loss of catalytic activity.

1.5.1.2 Cross-linking

In cross-linking strategies, enzymes are interconnected through bifunctional reagents such as glutaraldehyde and diazonium salts, producing cross-linked enzyme aggregates (CLEAs) or cross-linked enzyme crystals (CLECs) [58]. These immobilization systems avoid the use of external carriers and generally provide high enzymatic activity and low production cost. However, they often suffer from limited mechanical stability, handling difficulties and reduced reproducibility due to enzyme

denaturation during cross-linking. To address these challenges, research has shifted towards carrier-bound immobilization methods, which have become the most widely adopted strategy for enzyme stabilization.

1.5.1.3 Entrapment (Encapsulation)

In entrapment methods, enzymes are physically confined within porous polymeric matrices, like polyacrylamide gels or silica sol-gel networks. The matrix protects the enzyme from direct exposure to the reaction medium while permitting diffusion of substrates and products [59]. Because the physical interactions alone are often insufficient to ensure stability, covalent bonding is sometimes introduced to reinforce the system and minimize enzyme leakage. Enzyme entrapment is mostly achieved by incorporating the biocatalyst within a gel matrix, embedding it in fibrous materials, or enclosing it in microcapsules formed from polymers such as polyamines or calcium alginate. Although simple to perform and useful in applications such as biosensors [60], this method may suffer from enzyme leakage and mass-transfer limitations due to poorly controlled pore size.

1.5.2 Magnetic nanoparticles as versatile carrier for enzyme immobilization

To further improve catalyst recovery, magnetic nanoparticles have emerged as effective supports for enzyme immobilization [61]. These materials enable the rapid separation of the biocatalyst, aligning with sustainability criteria and the principles of modern green chemistry [62] [63]. The most used MNPs are spinel ferrites (MeFe_2O_4 , where $\text{Me}=\text{Fe}^{2+}$, Co^{2+} , Mn^{2+} , Zn^{2+}), which combine high magnetic responsiveness with low toxicity. Their catalytic and magnetic behaviour depends strongly on cation distribution between tetrahedral and octahedral sites in the spinel structure [64] [65].

The morphological and structural characteristics of MNPs, which directly influence their magnetic behaviour, are highly dependent on the synthesis route employed. As a result, different applications require tailored synthetic protocols to obtain nanoparticles with specific properties. Over the last thirty years, a wide range of preparation methods has been developed, including micellar techniques [66], sol-gel synthesis [67], hydrothermal synthesis [68], aerosol and vapor-phase approaches [69], and surfactant assisted high temperature decomposition route [70]. Among the others, surfactant-mediated solution-phase techniques, such as micellar synthesis and high-temperature thermal decomposition, have gained particular relevance due to their capacity to provide fine control over particle size, morphology, crystallinity and chemical composition [7].

Although these synthetic strategies allow fine control of the nanoparticles' structural and magnetic features, the use of naked MNPs in biocatalytic systems is limited by their tendency to aggregate,

minimizing surface energy, and by their high surface reactivity which can lead to oxidation and loss of magnetic performance. Hence, surface functionalization is a key step in designing stable magnetic supports for enzymes. Surface coating not only improve colloidal and chemical stability but can also introduce reactive groups that enable subsequent enzyme attachment, thus facilitating the fabrication of magnetically recoverable biocatalysts (**Figure 5**). Various functionalization methods are currently employed. Polymer coatings based on biocompatible and non-toxic materials such as chitosan [71], polyvinyl alcohol (PVA) [72], polyethylene glycol (PEG) [73] or polyacrylic acid (PAA) [74] are widely used. Alternatively, silica coatings represent another effective strategy: this approach not only prevent aggregation and enhance chemical stability but also improve biocompatibility while rendering the MNPs surface hydrophilic, thereby favouring enzyme binding in aqueous media [75]. Furthermore, silanization using metal alkoxides such as tetraethyl orthosilicate (TEOS) enables the incorporation of amino-functional groups onto the nanoparticle surface [76]. Another employed surface modification approach involves molecular coating with biocompatible α -hydroxy acids (i.e., citric acid or dimercaptosuccinic acid, DMSA) which anchor to the nanoparticles surface via carboxylate coordination, leaving functional groups available for further bio-coupling [77].

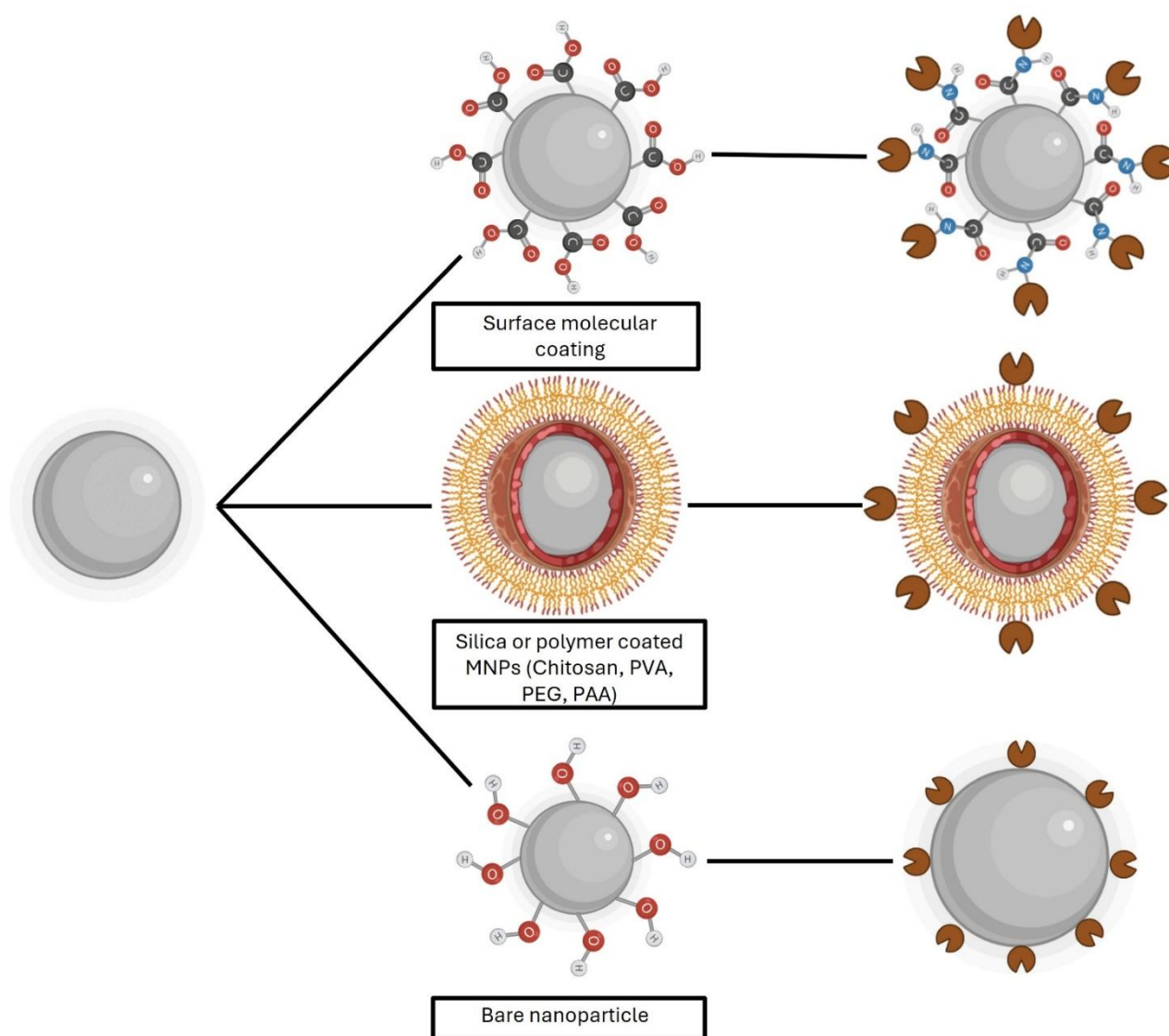


Figure 5: Schematic representations of naked nanoparticles and those with organic or inorganic surface layers, illustrating how surface modification introduces functional groups that enable enzyme attachment, either through covalent bonding or adsorption, resulting in magnetically recoverable and reusable biocatalysts.

1.6 Hydrolytic enzymes for esterification reactions

Biocatalysis has increasingly become gained prominence as a key approach in the chemical industry, especially for producing compounds of pharmaceutical interest. Progress in this area has enabled the development of tailored enzymes and optimized reaction conditions suitable for synthesizing a wide range of macromolecular materials. These materials are of growing interest across various technological sectors where highly sophisticated materials are required, such as electronics, mechanical engineering, communications, transportation, pharmaceuticals and medicine [3]. The evolution of polymer science has been driven by innovative production strategies and catalytic

systems, including the development of Ziegler-Natta catalysts [78], the introduction of the living polymerization [79], the discovery of conductive polymers [80] and the emergence of metathesis catalysts [81]. Since the early development of polymer science in the 1920s, polymerization processes relied primarily on traditional catalytic systems, including Brønsted and Lewis acids, various cationic species, Lewis bases, different anions and radical initiators. In the 1950s, the field underwent a major transformation with the introduction of transition-metal catalysts, most notably those used in Ziegler-Natta polymerization, followed later by catalysts for metathesis reactions and by the employment of rare-earth metals. By the 1980s, an alternative strategy for polymer synthesis began to emerge through the use of biocatalytic agents, giving rise to what is now known as enzymatic polymerization [2]. This approach, defined as “the *in vitro* chemical synthesis of polymers through non biosynthetic routes catalyzed by an enzyme” [82] opened new possibilities for more selective and sustainable polymer production. Enzymatic catalysis provides several benefits within preparative chemistry: owing to their excellent chemo-, regio- and enantioselectivity, enzymes can achieve high substrate conversion efficiencies and offer superior control over reaction outcomes. As discussed before, when immobilized, enzymes can be recycled and reused, making them particularly attractive as catalysts in bulk reaction systems where organic solvents and toxic metal-based catalysts can be avoided. Among the various enzyme classes, hydrolases (particularly lipases, cutinases and esterases) have become the most frequently employed biocatalysts for polyester production; their versatility and suitability for ester-forming reactions have established hydrolases as essential tools in this field [3].

The industrial relevance of enzyme-mediated processes expanded rapidly after the pioneering work of Zaks and Klivanov, who demonstrated that enzymes could retain catalytic activity even in organic media [83]. Nowadays, enzyme play a central role not only in textile treatments but also in several processes involving polymer breakdown. While they can hydrolyze polyesters, certain biocatalysts can also promote the reverse reaction, enabling the synthesis of aliphatic polyesters, branched oligomers and aromatic-aliphatic copolymers. Lipases catalyze the majority of these reactions, and among them, *Candida antarctica* Lipase B (CaLB) is the most widely employed due to its commercial accessibility in both free and immobilized forms [84]. The first demonstration of lipase-catalyzed synthetic reaction was reported in 1984 by Okamura and colleagues, who showed how a lipase from *Asperigillus niger* could mediate the polycondensation of various dicarboxylic acids with polyols to produce short-oligomers [85]. A few years later, Gutman et al. (1987) described the first example of enzymatic polymerization involving A-B type monomers, representing another key milestone for the field [86]. These initial findings sparked growing interest in the use of lipases as biocatalyst for polyester synthesis, encouraging Linko and co-workers to explore different lipase families and compare their catalytic performance. Experimental studies identified *Mucor miehei* lipase as a

particularly effective catalyst, and in the late 1990s, the immobilized form of CaLB on acrylic resin, commercially known as Novozym 435, had become the most prominent and widely employed biocatalyst for synthetic polymer applications [87].

Early investigations into the synthetic use of hydrolytic enzymes primarily focused on generating small molecular products. Klibanov, for instance, demonstrated that proteases could catalyze synthetic reactions in organic solvents such as pyridine [88]. During the 1980s, extensive efforts were devoted to exploring proteases-mediated formation of polypeptides and polyamides. These studies showed that proteases were effective for producing oligomeric species; however, their usefulness diminished when high-molecular weight polymers were required. In contrast, lipases proved significantly more adaptable. They have been successfully applied to the synthesis of both oligoamides and polyamides, and under appropriate reaction conditions they can efficiently yield high-molecular weight polymers through either polycondensation or ring-opening polymerization routes [47]. As a result, scientific interest gradually shifted away from proteases, and over the past twenty years lipases have increasingly become the preferred class of enzymes for polymer synthesis. Today, effective polymer synthesis demands more than selectivity and catalytic performance; it also requires that enzymes be readily recoverable and reusable to support sustainable processes. For this reason, as outlined in the paragraph 1.5, immobilization is essential. Robust immobilization strategies are needed to prevent enzyme detachment, which can contaminate the final product, and to avoid irreversible deactivation of the catalytic system.

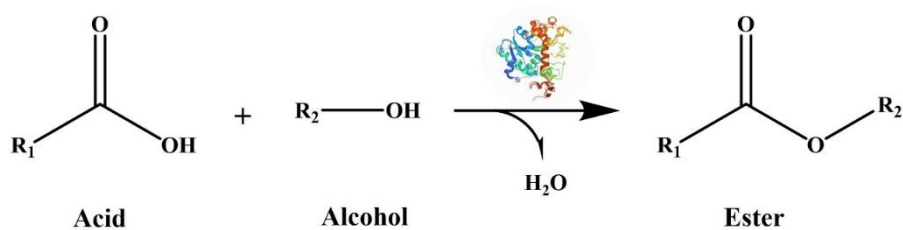
1.6.1 Short-esters synthesis

During the 1980s, the expanding use of biocatalysts in non-aqueous media opened new avenues for both fundamental research and industrial application in organic synthesis [89] [90]. Among the wide range of target molecules, short chain alkyl esters of organic acids represent some of the most valuable and versatile constituents of natural flavour and fragrance formulations. Their growing commercial relevance stems from their extensive use across the food, beverage, cosmetics and pharmaceutical sectors, where they contribute characteristic sensory notes, stabilize aromatic profiles or mask undesirable odours [91]. Recognized for their distinct fruity aromas, these esters are commonly incorporated as additives into confectionery, jams, beverages, baked products and dairy items. Acetate esters, for example, impart key flavour attributes in grape-derived wines and other alcoholic beverages, whereas ethyl and methyl esters play analogous roles in food such as cheese [92]. Cyclic esters further broaden the sensory palette, yielding coconut-like, creamy, nutty, sweet or buttery notes [93]. While these compounds are traditionally obtained via chemical synthesis, the growing consumer

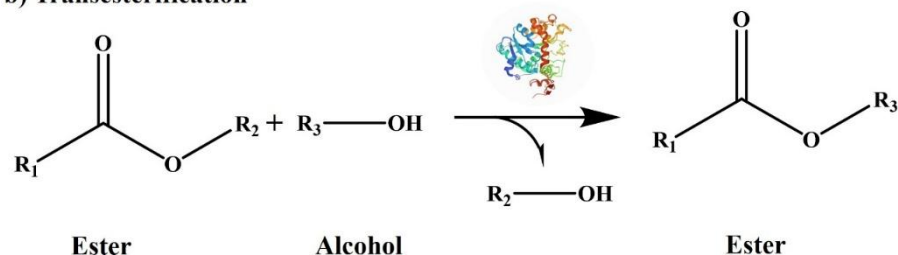
preference for naturally derived additives has intensified interest in alternative production methods. Conventional chemical routes often suffer from limited environmental compatibility, poor-regio- and enantioselectivity and the formation of undesirable by-products that diminish overall yields [94]. In contrast, flavour and fragrance esters produced through enzymatic processes can be labelled as “natural”, like plant-derived extracts [95]. As a result, biocatalytic synthesis, using either free or immobilized enzymes, has become an increasingly attractive strategy [96]. Following numerous reports identifying lipases as highly effective catalysts for short-chain ester formation [97], both academic and industrial efforts have focused extensively on their application, in response to consumer demand for naturally processed ingredients. Immobilized lipase from *Mucor miehei*, for instance, has been employed to synthesize low molecular weight esters derived from propionic, butyric or hexanoic acids in combination with ethyl or hexyl alcohols in organic media [98]. Given their broad catalytic versatility in organic transformation [99], lipases facilitate the formation of numerous flavour esters under mild conditions than those typically used in the chemical industry. A notable example is CaLB, which exhibits excellent catalytic performance in short-esters synthesis through esterification, transesterification and acidolysis reactions [100] (**Figure 6**).

More recently, short chain flavour esters have been efficiently synthesized using a multilayered magnetic biocatalyst with immobilized CaLB. The system was designed by exploring multiple functionalization strategies for magnetic particles to enhance covalent enzyme attachment and by optimizing silane precursor combinations for sol-gel entrapment [101]. In addition, other hydrolases belonging to the cutinase family, such as *Humicola insolens* cutinase (HiC) and *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1), have been immobilized through adsorption onto polypropylene support and applied to the biosynthesis of short-chain esters. Their catalytic efficiency and substrate selectivity across carbon chain lengths from C4 to C12 have been refined through full factorial experimental design [102].

a) Esterification



b) Transesterification



c) Acidolysis

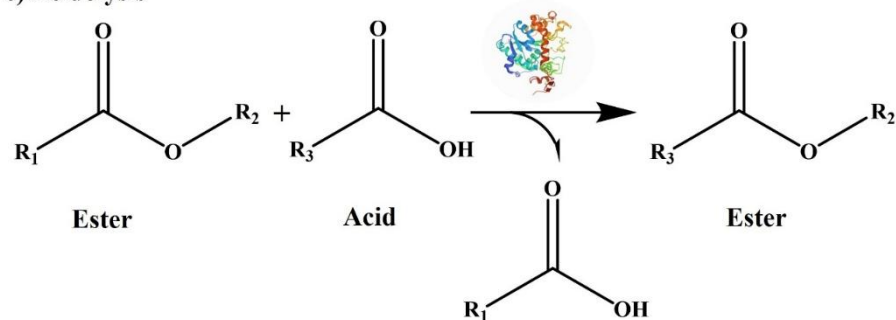
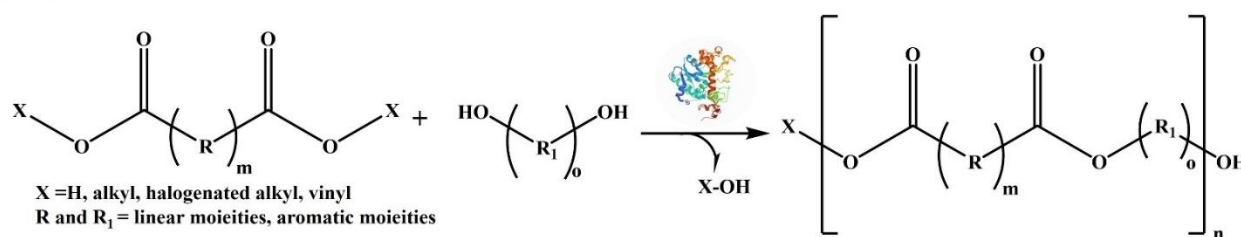


Figure 6: Schematic representation of a) esterification, b) transesterification and c) acidolysis.

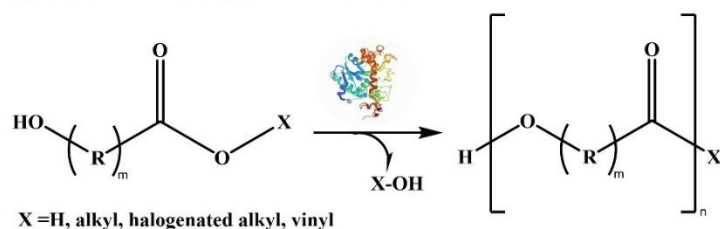
1.6.2 Polyester synthesis

The enzymatic production of polyesters can proceed through two main mechanisms, polycondensation reactions and ring-opening polymerization (ROP). Within this context, lipases have emerged as the most effective catalysts for in vitro polyester formation [103], enabling the synthesis of materials that today find extensive use in packaging, textiles and optical applications. In polycondensation, the most explored transformations involve the reaction of diesters or dicarboxylic acids with polyols (**Figure 7a**), although direct condensation of dicarboxylic acids, oxyacids and their corresponding esters have also been documented (**Figure 7b**). For ROP processes, the enzymatic opening of lactones dominates (**Figure 7c**), reflecting the broad interest in cyclic esters as accessible and versatile monomers [104].

a) Polycondensation of acid or thier esters with alcohols



b) Polycondensation of oxyacids or thier esters



c) Ring-Opening Polycondensation (ROP)

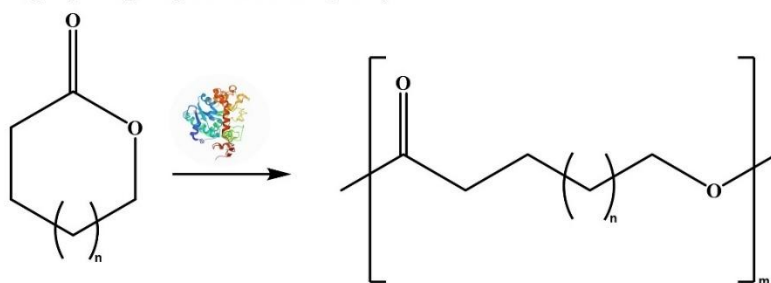


Figure 7: Overview of the main enzymatic routes for polyester formation: a) polycondensation involving either dicarboxylic acids or their esters with alcohols or b) alternatively oxyacids and their derivatives and c) ring-opening polymerization of cyclic monomers.

Early work in this field relied on halogenated diesters and toxic solvents such as pyridine, but research rapidly shifted towards more benign systems, particularly the synthesis of aliphatic polyesters bearing pendant hydroxy or epoxy functionalities that enable further chemical modification or crosslinking [105]. Over the past decade, the enzymatic polymerization has gained renewed attention due to the growing interest to create bio-based polyester and to promote a more sustainable polymer industry built on renewable platform chemicals. This trend aligns with the broader ambition of replacing conventional fossil-based polyesters, such as poly(ethylene terephthalate) (PET), with renewable alternatives like poly(ethylene furanoate) (PEF), which exhibit comparable mechanical and barrier properties but improved biodegradability or depolymerization potential [106].

Given the importance of establishing sustainable catalytic processes, considerable attention has also been devoted to the reusability of enzymes. As discussed in previous section, this has stimulated the development of diverse immobilization strategies designed to enhance catalyst robustness, simplify handling and enable prolonged storage [107]. Among the biocatalysts examined, *Candida antarctica*

lipase B (CaLB) is the most prominent, particularly in its commercially immobilized form Novozym 435 (N435), where the enzyme is physically entrapped within a microporous poly(methyl-methacrylate-co-butyl-methacrylate resin). Nowadays, the advances in nanotechnology have introduced magnetic nanoparticles as innovative supports for biocatalysts immobilization. The covalent attachment between CaLB and Fe_3O_4 prevents desorption and stabilizes the enzyme by constraining its microenvironment. These magnetic supports offer an additional practical advantage: the immobilized enzyme can be rapidly isolated from reaction mixture through the application of an external magnetic field, facilitating its recovery and reuse [108].

The most straightforward class of polyesters accessible through enzymatic polycondensation or ring-opening polymerization (ROP) includes aliphatic polyesters. These materials may be synthesized either as linear copolymers derived from a dicarboxylic acid and a diol, or as homopolymer obtained via ROP of lactones [109]. Polyesters can be produced from hydroxy acids or, more typically, from A-B type monomers in which the A and B functionalities undergo mutual reaction. Alkyl esters of these monomers have likewise served as substrates in lipase-catalyzed polycondensation processes. In A-B type condensations, the formation of the polymer is accompanied by the release of a small molecule that typically must be efficiently removed to achieve high-molecular weights [103]. The earliest studies in this area were conducted by Okamura and colleagues, who demonstrated lipase-mediated formation of oligoesters from diols (AA) and dicarboxylic acid (BB) [85]. Subsequent reports investigated the Novozym 435-catalyzed condensation of adipic acid with 1,4-butanediol [110]. When carried out under solvent-free conditions (4 h at 40°C and additional 10 h at 60°C under vacuum), the reaction led to the generation of narrowly dispersed oligoester ($\bar{D}=1.5$) with a molecular weight of approximately 2200 Da. Performing the analogous reaction in toluene at 60°C resulted in noticeably reduced reactivity, most likely due to the limited solubility of adipic acid in this solvent and produced materials with lower molecular weight ($M_w=1500$ Da) and higher dispersity ($\bar{D}=2.3$). These findings underscore that polymerization efficiency is strongly influenced not only by the chosen enzyme but also by the selected monomers and reaction conditions. Early investigations of enzymatic polycondensation between dicarboxylic acids and polyols reported only low molecular weight products [105]. To achieve higher molecular weights, a continuous removal of the condensation by-product (typically water or alcohol) is required to shift the equilibrium towards polymer formation. Employing diesters instead of the free acids can further shift the equilibrium, as the alcohol released during polycondensation is more volatile than water, facilitating its removal under vacuum and enabling higher conversions and molecular weight [111]. More recently, CaLB has been evaluated both in bulk polymerizations and in organic media (e.g., toluene) for reactions between dimethyl adipate, the dimethyl ester of adipic acid, and linear diols containing 4 to 8 carbon

atoms, such as 1,4-butanediol (BDO) and 1,8-octanediol (ODO), respectively (**Figure 8**).

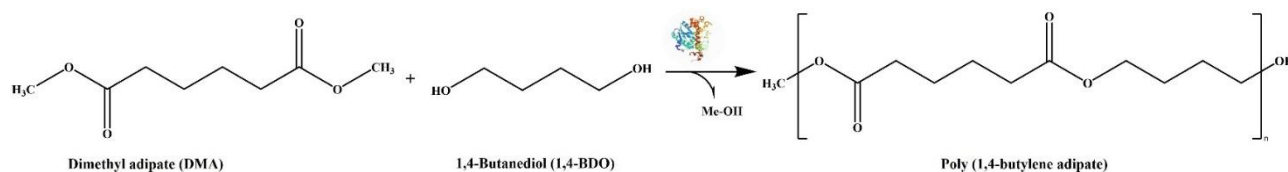


Figure 8: Enzymatic polymerization of dimethyl adipate with 1,4-butanediol catalyzed by CaLB.

Beyond the production of aliphatic polyesters, enzymatic routes to bio-based polymers also target the synthesis of aliphatic-aromatic polyesters via polycondensation. Owing to the growing relevance of furan-derived building blocks, one of the most extensively studied transformations is the polytransesterification between dimethyl furan-2,5-dicarboxylate and linear aliphatic diols containing 2 to 12 carbon atoms (**Figure 9a**). Jiang and co-workers demonstrated that CaLB exhibits increasing catalytic activity toward longer linear aliphatic diols and that elevating the reaction temperature from 80 to 140°C plays a critical role in achieving high molecular weight furan-based polyesters, reaching substrate conversion of 90-95% when C4-C12 diols were employed [112]. An alternative strategy involves the use of 2,5-bis(hydroxymethyl)furan in combination with aliphatic diacid ethyl esters spanning chain length from C4 to C12 to generate aliphatic-aromatic copolyesters, with the furan unit introduced from the diol component (**Figure 9b**) [109]. However, this route has been largely ineffective, yielding products with number-average molecular weights of only 2000 Da. The primary limitation appears to be concurrent etherification during enzymatic polymerization, which inhibits chain growth. Consequently, improved protocols, such as reducing reaction temperature, duration or modification of the organic medium, may be necessary to suppress side reactions [112].

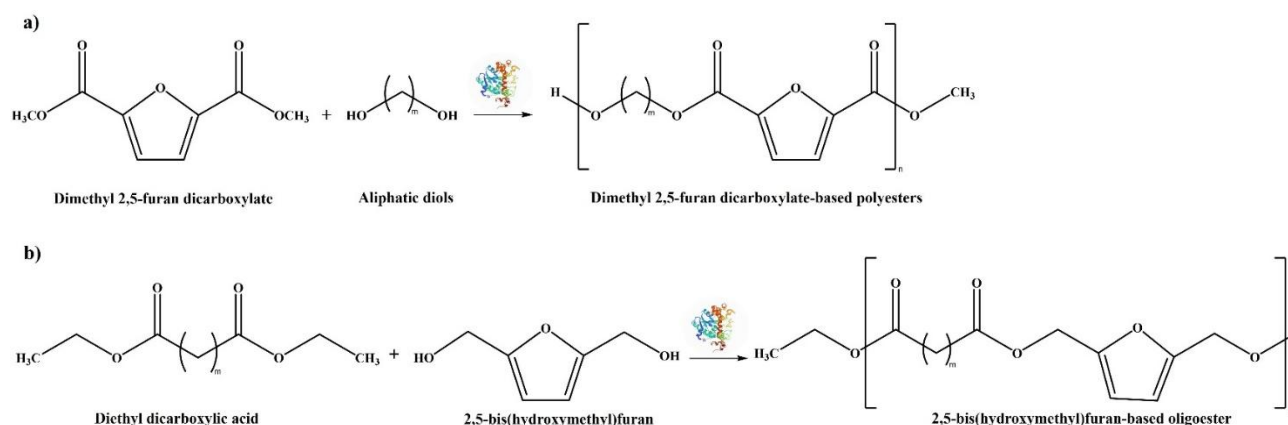


Figure 9: Polycondensation routes to aliphatic-aromatic polyesters using a) dimethyl 2,5-furandicarboxylate and b) 2,5-bis(hydroxymethyl)furan.

In contrast to polycondensation, ring-opening polymerization (ROP) of lactones and carbonates proceeds without the formation of a low molecular weight by product. ROP, frequently cited as one of the most efficient biocatalytic polymerization technique, was first explored by two research groups in 1993 [113]. A well-known example is the enzymatic synthesis of poly(ϵ -caprolactone) (PCL), which has been examined under a wide range of conditions, including both bulk and organic-phase systems [114]. For instance, Gutman and collaborators studied the lipase-catalyzed ROP of ϵ -caprolactone in n-hexane using porcine pancreatic lipase (PPL) as biocatalyst [113]. Concurrently, Kobayashi and co-workers demonstrated that bulk ROP of lactones, catalyzed by *Pseudomonas fluorescens* lipase, yields PCL with 92% of conversion with a M_n of 7700 Da and a dispersity of 2.4 [115]. The late 1990s marked a further advance when Novozym 435 was introduced as a catalyst for the enzymatic ROP of lactones, becoming the predominant biocatalyst for polyester synthesis employing cyclic monomers such as lactones, cyclic diesters and cyclic ketene acetals [116].

1.7 Towards fully circular bioplastic: from renewable bio-based monomers to smart recycling strategies

As discussed in the paragraph 1.6.2, research on enzymatic polymerization has expanded, reflecting worldwide efforts to generate polyesters from renewable monomers and promote a sustainable polymer industry. This transition aligns closely with the broader shift towards circular bioeconomy, which requires materials designed for compatibility with natural carbon cycles and production routes based on selective, recyclable and environmentally responsible catalytic systems. In this context, biocatalysis has emerged as a powerful strategies for efficient synthesis, modification and end-of-life management of bio-derived polymers [117]. The increasing industrial availability of fermentation-based monomers, such as lactic acid, succinic acid, sorbitol and 2,5-furandicarboxylic acid, has expanded the portfolio of renewable polyesters including polylactic acid (PLA), polybutylene succinate PBS and polyethylene furanoate (PEF), while retaining performance levels competitive with conventional fossil-based compounds [118]. This progress consolidates the technological relevance of bioplastics and supports substitution efforts, for example through the introduction of PEF as a potential alternative to PET. Overall, integrating biocatalysis with renewable monomer platform provides a coherent pathway towards high-performance polymers intrinsically compatible with circular material flows [119].

Nowadays, research is increasingly exploring the repurposing of depolymerizing enzymes for synthetic applications, thereby closing the loop between polymer formation and degradation. Therefore, expanding the enzymatic toolbox beyond traditional CaLB is essential to establish more

robust and circular processes. Indeed, as it will be discussed in the Chapter 3, novel thermophilic enzymes belonging to the α/β hydrolase superfamily, traditionally associated with PET depolymerization, were evaluated for the first time as catalysts for polyester synthesis. Demonstrating synthetic activity in these enzymes represents a significant step forward towards enzyme families capable of both polymerization and depolymerization, strengthening the foundation for circular polymer manufacturing and moving towards enzyme-enabled closed loop. Finally, circularity does not depend only on molecular design and biocatalysis but also on engineered functional interfaces capable of improving material performance and simplifying post-consumer management. In this study, as it will be demonstrated in Chapter 4, a Layer by Layer (LbL) surface engineering exemplifies this direction: by functionalizing polyester with magnetically responsive coating, it becomes possible to introduce novel magnetic field assisted sorting strategies that enhance recyclability. This approach offers a catalytic-free but highly impactful method to tackle the challenges of plastic contamination and material separation, two major constraints in current waste management processes. This demonstrates how surface modification can support both performance improvements and more efficient end-of-life separation, thus reinforcing the holistic vision of polymer circularity.

Taken together, the integration of renewable monomers, advanced biocatalysts and smart interface engineering illustrates a converging technological trajectory: designing the next generation of polymers not only for high performance but for full compatibility with circular material ecosystems, from synthesis to sorting. In this perspective, a truly circular approach must also consider the recoverability and reuse of the catalytic systems involved in polymer synthesis. The immobilization of enzymes on magnetic nanoparticles represents an effective and practical solution: it enhances not only enzyme stability and selectivity but also allows rapid, non-invasive catalyst recovery through magnetic separation. This strategy minimizes resource consumption, reduces operational costs and significantly improves the feasibility of biocatalytic processes. When coupled with enzymes and involved in bioplastic functionalization, magnetic nanoparticles contribute to building fully recyclable, high-efficiency catalytic and material system that strengthen the overall circularity of bio-based polymers.

1.8 References

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Appendix

Tutor's request for the insertion of published papers in the Thesis:

In the following page, a document by the Candidate's Supervisors, Professors Alessandro Pellis and Davide Peddis, is attached. The document consists in a declaration confirming the substantial contribution of the Candidate to all the papers constituting part of the present doctoral thesis as Chapter 2 and Chapter 3. The declaration was submitted to the Board of the Doctoral School in Sciences and Technologies of Chemistry and Materials of the University of Genoa that approved it on October 17th, 2025.



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Genova, 02/10/2025

I undersigned Alessandro Pellis, Associate Professor at the Department of Chemistry and Industrial Chemistry of the University of Genoa, supervisor of Francesco Papatola, PhD student of the Doctorate in Sciences and Technologies of Chemistry and Materials (Curriculum in Chemical Sciences and Technologies, XXXVIII cycle), certify, in agreement with the co-supervisor Prof. Davide Peddis, that Francesco Papatola is the co-author of the following articles and/or pre-prints, which belong to the research project of his PhD studies:

- (1) F. Papatola, S. Slimani, F. Fabbri, G.M. Guebitz, D. Peddis and A. Pellis Solventless polyester synthesis using a recyclable biocatalyst magnetic nanoarchitecture. RSC Sustainability, **2025**, 3, 403-412. DOI: 10.1039/d4su00521j
- (2) Papatola, F., Fabbri, F., Kumar, V., Siracusa, Quartinello, F., Ribitsch, D., Varrone, C., Guebitz, G.M., Pellis, A. The quest for the new CaLB: potential of three thermostable polyester hydrolases for esterification reactions. ChemRxiv, 2025, DOI:10.26434/chemrxiv-2025-878j7. This content is a preprint.

Francesco Papatola's contribution can be ascertained from the "Author Contributions" sections of the articles, as reported below:

- (1) F.P. synthesized and characterized the MNPs and carried out the synthesis experiments. F.P. and F.F. immobilized and characterized the enzyme. A.P. and G.M.G. planned the synthesis experiments. F.P. and S.S. wrote the manuscript. S.S. and D.P., planned, carried out and elaborate the magnetic measurements. All authors corrected and reviewed the manuscript. A.P. and D.P. acquired the funding, designed and supervised the work. All authors have read and agreed to the published version of the manuscript.
- (2) F.P., C.S. and V.K. expressed and purified the enzymes. F.P. and F.F. immobilized, characterized the enzymes and planned the experimental design. D.R., C.V. and G.M.G. coordinated the enzyme expression. A.P. planned the synthesis experiments. F.P. carried out the synthesis experiments and the product's characterization. F.P. wrote the manuscript. A.P. acquired the funding and supervised the work. All authors contributed to the final editing and proofreading of the manuscript.

Best regards,

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Chapter 2: Solventless polyester synthesis using a recyclable biocatalyst magnetic nanoarchitecture

Chapter 2 published as paper in the journal “RSC Sustainability”, 3 (2025), 403–412.

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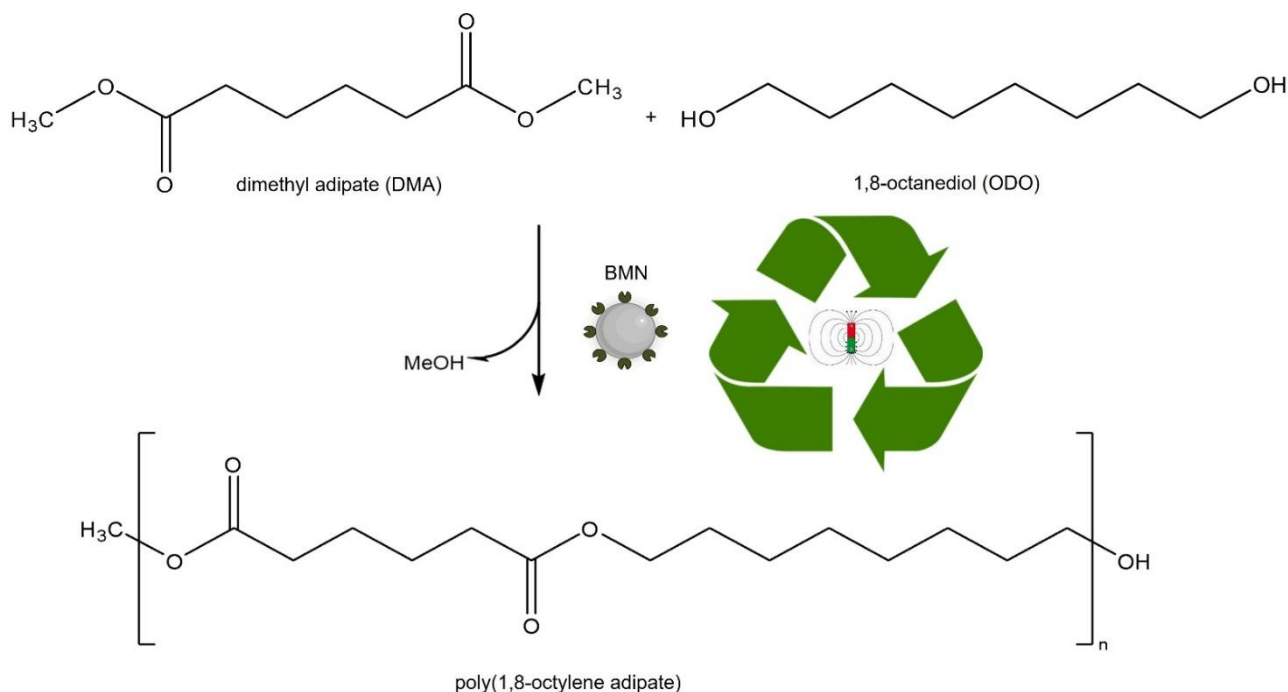
Francesco Papatola, Sawssen Slimani, Filippo Fabbri, Georg M. Guebitz, Davide Peddis,
Alessandro Pellis

2.1 Abstract

Improving enzyme activity and stability as well as preserving selectivity is a must for rendering biocatalysis an economically viable technology. These improvements can be achieved by immobilizing the biocatalyst on the surface of metal oxide magnetic nanoparticles. The aim of this work is to rational design Biocatalyst Magnetic Nanoarchitecture (BMN) consisting of spinel iron oxides nanoparticles having optimized morpho structural (i.e., particles size, shape and crystallinity), textural (i.e., high surface area) and magnetic properties. *Candida antarctica* lipase B (CaLB) was immobilized on the nanoparticles' surface investigating the optimal bioconjugation conditions and performing the biochemical characterizations to quantify protein concentration and to assess enzymatic activity. Once immobilized on the magnetic nanoparticles surface, CaLB was tested for an enzymatic polycondensation reaction to synthesize polyesters starting from renewable monomers such as the dimethyl ester of adipic acid and 1,8-octanediol. Conversion of monomers was >87% over three reaction cycles while the number average molecular weights of the products were between 4200 and 5600 Da with a dispersity <2. Efficient recycling of the enzyme upon magnetic separation was demonstrated for three reaction cycles.

Keywords: biobased polyesters, enzyme immobilization magnetic nanoparticles, solventless reaction systems, biocatalyzed polycondensations

2.1.1 Graphical abstract



The present study proposes enzymatic polymerization reactions to synthesize polyesters employing lipase (CaLB) covalently immobilized onto magnetic nanoparticles (i.e., Biocatalyst Magnetic Nanoarchitecture). This ensures efficient recovery of the biocatalyst in environmentally friendly conditions through the application of an external magnetic field facilitating its re-use over multiple polymerization cycles. This strategy was validated experimentally by carrying out the solvent-free polycondensation of dimethyl adipate and 1,8-octanediol and testing the preparation's recyclability over 3 reaction cycles. A schematic image for overall procedures including CaLB immobilization onto magnetic nanoparticles, polycondensation and enzyme recyclability is reported in ESI (Figure S1).

2.2 Introduction

To satisfy the increasing request for biobased and biodegradable polymers, in line with the “European green deal” [1], the ability of enzymes to transform natural and non-natural compounds into polymers is considered as an environmentally friendly alternative to the traditional chemical synthetic pathways [2]. Biocatalysis is emerging as a fundamental tool within the chemical industry to produce pharmaceuticals and fine chemicals using mild and environmentally-friendly conditions [3]. Around the 1980s a new approach of polymer synthesis employing enzymes as catalysts has been developed, known as enzymatic polymerization [4]. In particular, enzymes from the class of hydrolases (e.g., lipases, cutinases and esterases) are nowadays among the most commonly used biocatalysts for polyester synthesis since they are known for their versatility and thermostability [5]. Among these, the most widely used biocatalyst for the synthesis of polyesters is *Candida antarctica* lipase B (CaLB), due to its commercial availability both as free and immobilized catalyst [6]. However, enzyme applications are in most cases still limited due to the lack of long-term stability, difficult recovery and impossibility of re-use of the costly biocatalyst. These limitations could be overcome by utilizing enzyme immobilization strategies [7] [8]. The immobilization of enzymes presents as the main objective to preserve and eventually enhance some of the enzyme’s properties such as activity, stability and substrate specificity [9]. In addition, the immobilized preparations provide a tool for an easy separation of the biocatalyst from the reaction product facilitating its recovery and re-use. Biocatalysts costs and enzyme recyclability over several cycles of operation are in fact two of the main limiting steps for the cost-effective large scale implementation of enzyme-catalyzed reactions in the chemical industry [10]. In the last two decades, new promising strategies of enzyme immobilization were developed to further improve the separation of the biocatalyst from the reaction product. In particular, the field of nanotechnology has progressed significantly by leading to the fabrication and application of a wide range of nano-supports for the immobilization of enzymes and it is emerging as a general-purpose field having significant impact on catalysis [11]. Among them, magnetic nanoparticles (MNPs) have emerged due to their distinctive physico-chemical and magnetic properties. These MNPs possess several advantageous characteristics, including biodegradability, biocompatibility, low cost and the ability to be tailored with specific surface chemistries [12]. This immobilization strategy not only can provide an active and stable biocatalyst, but also it is a relatively simple and low-cost immobilization procedure that does not require an expensive support [13]. Some critical issues such as optimization of the magnetic properties, mass transfer limitations and low efficacy against insoluble substrates still need to be overcome. This very complex but promising scenario became an exciting playground where biotechnologist and materials physical chemist can

produce novel biocatalyst magnetic nanoarchitecture (BMN) by synergistic rational design of the magnetic and biocatalytic components [14]. The application of BMN represents a promising approach to enable the biocatalyst's immobilization satisfying both the need of preserving the enzyme's activity and the modern's day green chemistry principles that aim to follow the "do not significant harm" principle [15] [16]. In fact, differently from other immobilization strategies based on non-magnetic carriers, their responsiveness to external magnetic field enables a rapid and efficient biocatalyst separation from reaction media simplifying significantly the purification steps in enzymatic reactions mainly by avoiding the use of organic solvents and other filtration systems. High surface to volume ratio, easy surface functionalization and peculiar magnetic properties (i.e., superparamagnetism at room temperature, high saturation magnetization M_s), make MNPs an unique solid support for biocatalyst immobilization [17]. The main advantage of dealing with a superparamagnetic objects (i.e., remanence magnetization and coercivity equal to zero) is that when the external magnetic field is removed, the magnetic object itself is not anymore magnetic. This will prevent clustering phenomena, and the presence of local magnetic fields.

MNPs used as a support for enzyme immobilization have been successfully demonstrated in applications like substrate sensing [18] [19], immunoassays [20] and organic synthesis [21] [22] and several studies have shown that the activity and stability of enzymes immobilized on MNPs are significantly better than unbounded enzymes [23] [24] but without focusing on biocatalyst recycle system. Therefore, the overall intent of this study was to synthesize polyesters employing recyclable biocatalyst magnetic nanoarchitecture. Unlike other immobilization strategies where the recovery process in organic media could determine an irreversible activity reduction of the immobilized enzyme, the idea of the work was to recover biocatalyst without using any kind of solvents, but just a magnetic device, to exploit enzyme recyclability over multiple reaction cycles thanks to the retainment of its activity.

According to the literature [25] [26] [27], for which CaLB exploits excellent selectivity towards long chain diols and acids, enzyme bound nanoparticles were tested for enzymatic polymerization reactions to synthesize polyesters starting from renewable monomers such as dimethyl adipate and 1,8-octanediol studying the preparation's recyclability over 3 cycles of reaction.

2.3 Experimental

2.3.1 Materials and methods

The chemicals $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (>99%), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (>99%), HCl (98%), ammonium hydroxide (30%), ethanol ($\geq 99.8\%$) dimethyl adipate (DMA, >99%), 1,8-octanediol (ODO, >99%), chloroform (99.0-

99.4%), 2-methyl-2-butanol (>99%), 4-nitrophenol (>99%), para-nitrophenyl butyrate (p-NPB), bovine serum albumin (BSA), K_2HPO_4 (>98%) and KH_2PO_4 (>98%), lipase from *Candida sp.*(L3170) and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide-hydrochloride (EDC) were purchased from Sigma-Aldrich.

2.3.2 MNPs synthesis

Spinel iron oxide nanoparticles were synthesized by the co-precipitation method using an aqueous solution of Fe^{2+} and Fe^{3+} with the stoichiometric ratio 1:2, by alkalization with 30% ammonia solution. The synthesis was done according to the following operating procedure: at 60°C, a mixture of $FeCl_3 \cdot 6H_2O$ (>99%, Sigma-Aldrich) and $FeCl_2 \cdot 4H_2O$ (>99%, obtained from Sigma Aldrich) was dissolved in 100 mL 0.01 M HCl solution (obtained from Sigma Aldrich, 98%). The solution was alkalized with 30% ammonium hydroxide. After two hours of magnetic stirring, the resulting precipitate was washed with deionized water for 3 cycles assisted by centrifugation, and adjusting the pH it was possible to obtain stable ferrofluids. The obtained black powders were dried overnight in an oven at 60-70°C before further use [28].

2.3.3 Characterization of MNPs

The crystalline structure was characterized by X-ray diffraction (XRD) measurements, using a TT 3003 diffractometer equipped with a secondary graphite monochromator, employing $CuK\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$). Data were collected in the 10° – 90° 2θ range with a step size of 0.04° and counting 4 sec per step. A vibrating sample magnetometer (VSM Model 10–Microsense) equipped with an electromagnet producing a magnetic field in a range from -2 to $+2$ T was used to investigate the field dependence of magnetization.

Transmission electron microscopy (TEM) analysis was performed by using a Philips CM200 microscope operating at 200 kV and equipped with a LaB6 filament. For TEM observations, the samples, in the form of powder, were prepared by using the following procedure. A small quantity of powder was dispersed in ethanol and subjected to ultrasonic agitation for ~ 1 min. A drop of the suspension was deposited on a commercial TEM grid covered with a thin carbon film. Finally, the grid was kept in air until complete ethanol evaporation.

The Quantum Design PPMS (Physical Property Measurement System) magnetometer was utilized to investigate the magnetic properties of the samples. These samples, in powder form, were secured in polycarbonate capsules with epoxy resin to prevent particle movement during measurements. Field dependence of magnetization measurements were recorded at 5K and 300 K. The thermal dependence

of magnetization was measured using zero field-cooled (ZFC) and field-cooled (FC) protocols. For the ZFC measurements, the sample was first cooled in the absence of a magnetic field, and then the magnetization was measured during warming the sample in an applied field of 2.5 mT. For the FC measurements, the sample was cooled while maintaining the applied field of 2.5 mT, and magnetization was measured during the subsequent warming process keeping the field applied. The field dependence of remanent magnetization was investigated using the IRM (isothermal remanent magnetization) and DCD (direct current demagnetization) protocols. The IRM protocol begins with a demagnetized sample. A small positive magnetic field (H_{rev}) is applied, then removed, and the remanence (M_{IRM}) is measured in zero field. This process is repeated with increasing applied fields until the remanence reaches saturation. In the DCD measurement, the sample has been saturated in a positive direction. Then, a small negative magnetic field opposite to the magnetization direction is applied for a few seconds, then switched off, and the remanence (M_{DCD}) is measured. This process is repeated with increasing magnetic fields until the remanent magnetization reaches saturation. All the magnetic measurements were normalized by the mass of the magnetic phase. Saturation magnetization value (M_s) was determined from the portion at high field of $M=(H)$ curves using as approach to saturation law (LAS) [29].

2.3.4 Immobilization of Lipase on MNPs

After the synthesis of magnetic nanoparticles, *Candida antarctica* lipase B (CaLB) was immobilized onto the surface. The immobilization procedure was based on the formation of covalent bonds between the functional groups present on magnetic nanoparticles and on the enzyme protein upon carbodiimide- EDC activation. Therefore, in a glass vial, 250 mg of magnetic nanoparticles (MNPs) was weighted using an analytical balance. MNPs were added to 2 mL of buffer A (0.003 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6). The reaction mixture was agitated for 10 min with a blood rotator (SB3 model from VWR) after adding 0.5 mL of EDC carbodiimide solution (0.025 g/mL in buffer A). Then, 2.5 mL of CaLB (lipase from *Candida sp.*, obtained from Sigma Aldrich, L3170, 7.1 mg/mL), prepared by mixing 0.7 mL of enzymatic stock with 1.8 mL of Buffer A ($\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6), was added and the reaction mixture was agitated for 24 h at RT with the rotator set at a speed of 40 rpm. Lipase-bound magnetic nanoparticles were recovered from the reaction mixture by placing the glass vial on a permanent magnet. The magnetic particles settled within 30 seconds and the supernatant was used for protein analysis while the precipitates were washed one time with buffer A and then buffer B (0.1M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ pH 8.0). The binding of lipase onto the MNPs was confirmed using FT-IR (Shimadzu IRPrestige-21, equipped with a Specac golden gate single reflection diamond attenuated

total reflection). All samples were analyzed in the 4000 cm^{-1} - 600 cm^{-1} region and Fourier Transform was used as a data-processing technique.

The thermal stability of magnetic nanoparticles before and after enzyme immobilization was determined through a Thermogravimetric (TG) analysis by means of a Mettler Toledo micro and ultra-micro balances with sub-microgram resolution over the whole measurement range. Each sample (80 mg) was measured under air atmosphere and heated from 20 to $800\text{ }^{\circ}\text{C}$ at a heating rate of $20\text{ }^{\circ}\text{C}/\text{min}$.

2.3.5 Esterase Activity Assay

The esterase activity of CaLB activity was determined by monitoring hydrolysis para-nitrophenyl butyrate (p-NPB) (Figure S2). The catalytic activity, which corresponds to the increment of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ϵ 405 nm), was monitored over 5 min in cycles of 30s with a Tecan Plate Reader (Tecan, Grödig, Austria) using 96-well microtiter plates (Greiner 96 Flat Bottom Transparent Polystyrene).

Different enzymes dilutions were prepared using $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 8, 0.1 M. Solution A consisting of 86 μL of p-NPB (p-nitrophenyl butyrate) and 1 mL of 2-methyl-2-butanol was prepared and stored at -20°C until usage. Solution B was freshly prepared each time by mixing 160 μL of solution A and 4 mL of buffer ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer 0.1 M pH 8). 200 μL of the solution B was mixed with 20 μL of the enzyme diluted in buffer. The samples were analyzed in triplicates and a series of blanks (i.e., buffer without enzyme) were included in each activity cycle. The activity was calculated in units (U), where 1 unit is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute.

2.3.6 Protein Concentration Determination

To evaluate the efficiency of the immobilization procedure the protein concentration of the supernatant was determined using the Bio-Rad solution (Coomassie brilliant blue G-250 dye) and different solutions of BSA (Bovine Serum Albumin protein standard, 2 mg/mL, Sigma-Aldrich) as standard protein to obtain the calibration curve (Figure S3). The concentrations used were: 0.025, 0.03125, 0.0625, 0.1, 0.125, 0.2, 0.25, 0.5 and 1 mg/mL. Samples were diluted in 0.1 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer at pH 8 buffer while the dye was diluted 1:5 in mQ water. BioRad assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein (Figure S4). Absorbance was measured at 595 nm (ϵ protein-dye complex) over 5 min in cycles of 18s with a Tecan Plate Reader (Tecan, Grödig, Austria) using a 96-well microtiter plate (Greiner 96 Flat Bottom Transparent Polystyrene) where 10 μL of each sample (in triplicate) and then

200 μ L of BioRad solution was added to each pot. Incubation was performed at 400 rpm for 5 minutes at RT.

2.3.7 Enzymatic synthesis of polyesters

Enzymatic polymerization via polycondensation of dimethyl adipate and 1,8-octanediol was performed in bulk (i.e., solvent-less) condition to be as environmentally friendly as possible using the MNP immobilized lipase as biocatalyst. Equimolar amounts of the two monomers (0.003 mol) and 10% w w⁻¹ of the immobilized enzyme calculated on the total amount of the monomers were left to react at 85 °C for 6 h. After this time, pressure was reduced at 12 mbar for 18 h to remove the by-product, MeOH, that was formed during the reaction. The samples were then analyzed via Proton Nuclear Magnetic Resonance (¹H-NMR) and Gel Permeation Chromatography (GPC) to determine the monomers conversion and the obtained molecular weights.

2.3.8 Characterization of the polyesters: Proton Nuclear Magnetic Resonance (¹H-NMR) and Gel Permeation Chromatography (GPC)

All ¹H-NMR spectra were recorded using a JEOL ECZ400R/S3 at a frequency of 400 MHz using CDCl₃ as the solvent. Gel permeation chromatography was performed at 30°C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17,369 6.0 mm ID × 40 mm L HHR-H, 5 μ m Guard column and a 18,055 7.8 mm ID × 300 mm L GMHHR-N, 5 μ m TSK gel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF as an eluent (at a flow rate of 1 mL min⁻¹). An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the polymers were calculated using polystyrene calibration standards (250–70,000 Da) purchased from Sigma-Aldrich.

2.4 Results and discussion

2.4.1 Biocatalyst magnetic nanoarchitecture

CaLB was immobilized on MNPs using 2% of biocatalyst based on the amount of solid support. For several time-points (after 1,2,4,8 and 24 h), the decrease of the free enzyme activity in the supernatant due to the immobilization onto magnetic nanoparticles is shown in [Figure 1a](#). Results for the protein concentration determination are also presented as residual protein concentration (%) for the same time-points ([Figure 1b](#)). The immobilization results obtained using the lipase B from *Candida antarctica* (CaLB) show that after 24 h of immobilization of CaLB, 59% of the biocatalyst was successfully bound to the magnetic nanoparticles according to the esterase activity assay. This

corresponds very well to 55% bound according to the decrease of the protein concentration in the supernatant (figure 1a and 1b respectively). As visible from the graphs, after 8 h of immobilization of CaLB, <50% of both the residual activity and concentration was detected in the supernatant by showing similar results to those observed after 24 h of immobilization. This could be explained by the fact that magnetic nanoparticle' surface sites were almost completely saturated after approximately 8 h. It is also worth to mention that, in presence of applied magnetic field a significant enhancement in the enzymatic activity can further observed, due to the influence of the magnetic field on the orientation and mobility of the immobilized enzymes. This has been recently proved in a study reported by Wang et al. [30], showing that the application of magnetic field increased the catalytic activity of immobilized CaLB enzymes. This opens interesting perspectives on the role of magnetic nanoparticles and magnetic fields in influencing enzyme activity.

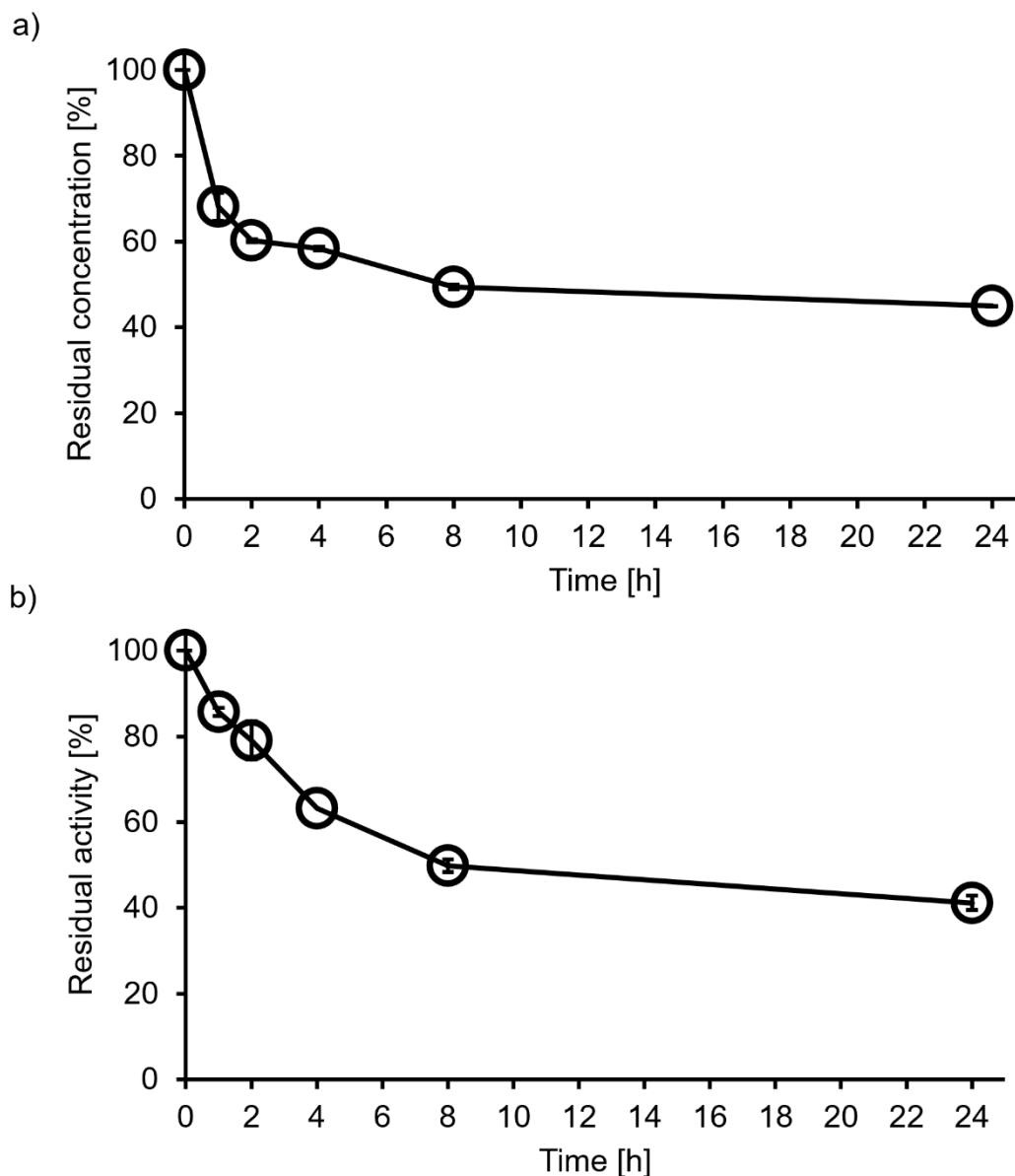


Figure 1: Immobilization of CaLB onto MNPs. Graphical illustration of remaining protein concentration (a) and para-nitrophenyl butyrate activity (b) of the different timepoint supernatants for CaLB concentration of 2%.

Morpho-structural and magnetic properties of the bare (MNPs) and enzyme-functionalized nanoparticles (BMN) were investigated by X-ray diffraction, FT-IR and magnetometry. The X-ray diffraction patterns (Figure 2a) show for both samples reflections typical of iron oxide phase with spinel structure (JCPDS card No.75-449). Any extra phase has been detected and average crystallite size of 9.0 (1) nm was estimated for both samples by the Scherrer equation [31]. It is important to underline that XRD clearly indicated that functionalization process do not induce any relevant structural change in MNPs. To verify the immobilization of CaLB on MNPs surface, FT-IR analysis was performed on bare MNPs, pure CaLB and BMN (Figure 2b). The characteristic absorption band

of CaLB associated with the σ -type C-O (typical of aa residues) is present in CaLB bound MNPs at around 1036 cm^{-1} but is not present in bare MNPs confirming the binding of lipase to the nanoparticles. In addition to the characteristic σ -type C-O absorption band [32], the IR analysis reveals the amide I bond transitions at 1630 cm^{-1} , related to the stretching vibrations of C=O groups and amide II band at 1540 cm^{-1} , due to the N-H bending. In the CaLB spectrum, the amide II transition is obscured by the O-H absorption, and it could be explained by the fact that enzyme was analyzed in water solution. However, in the BMN spectrum, both amide I and amide II transitions are visible. After CaLB immobilization, the absorption peak at 1630 cm^{-1} broadens and shifts, due to the overlapping with O-H bending from MNPs surface (i.e., hydroxyl group). Additionally, the amide II region becomes more evident as the presence of a shoulder in the BMN spectrum absent in bare MNPs. These results support the bonding between the enzyme and the magnetic nanoparticles. Furthermore, as reported in the literature [33] [34], CaLB immobilization could occur through the -NH_2 functional groups of the enzyme onto modified surface of solid support. Nevertheless, since the EDC conjugation chemistry was used, we suppose a reaction between -NH_2 groups, formed from the use of concentrated ammonia solution on MNPs surface (as visible from the jagged behaviour of MNPs from $3500\text{-}2900\text{ cm}^{-1}$), and carboxylic groups of the enzyme. A more detailed discussion on the grafting mode of CaLB on MNPs through the EDC bioconjugation process is reported in ESI (Scheme S1 and Figure S5) as well as TGA analysis of MNPs before and after CaLB immobilization (Figure S6).

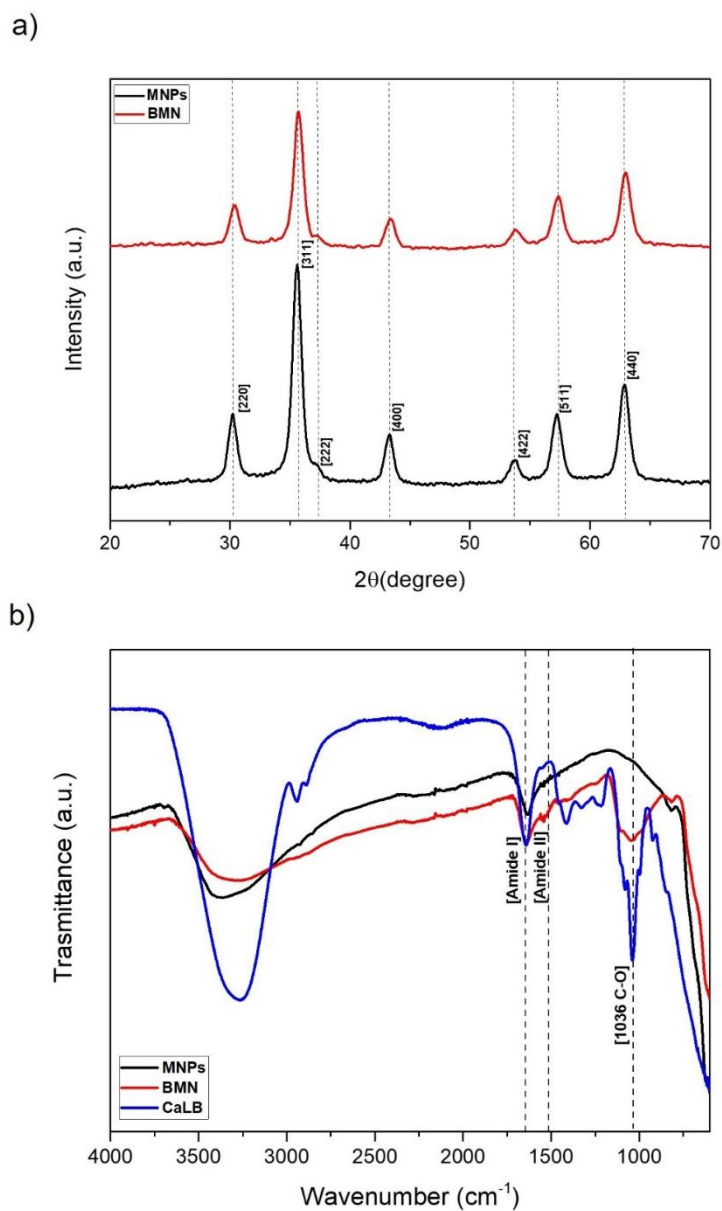


Figure 2: a) XRD pattern of MNPs (black) and BMN (red); b) FT-IR of bare MNPs (black), BMN (red) and pure CaLB (blue).

The typical TEM micrographs (Figure S7) for the bare MNPs show the presence of particles with mean particle size of 10 nm, confirming the XRD results [28].

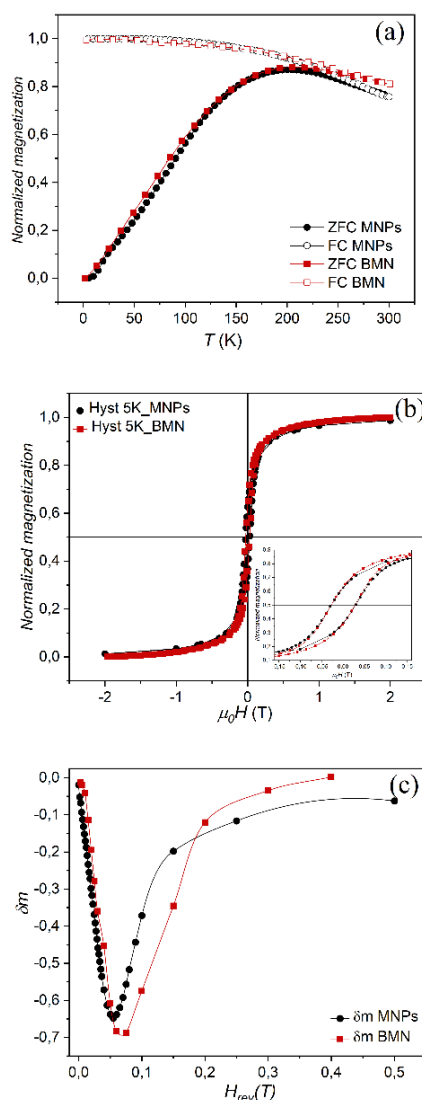


Figure 3: (a) ZFC (full symbols) and FC (empty symbols) curve measured at 2.5 mT, (b) M vs H curves recorded at 5K, inset give details around Zero field, and (c) δm plots for MNPs and BMN samples; in all graph MNPs are indicated by circles and BMN by squares.

To study the effect of CaLB functionalization, a comparative magnetic investigation was carried out on both MNPs and BMN samples. Temperature dependence of magnetization measured by ZFC-FC protocols (Figure 3a) exhibit for both samples typical behaviour of an assembly of interacting single-domain particles [35]. The temperature corresponding to the maximum in the ZFC curve, T_{max} , is directly proportional to the average blocking temperature ($\langle T_b \rangle$) and was found to be the same for both samples within experimental error (Table 1). The blocking temperature (T_b) is defined as the

temperature at which the relaxation time equals the timescale of the experimental technique. T_b is proportional to the anisotropy energy barrier ($E_a = KV$), and due to the distribution in volume of real MNPs systems a distribution of T_b is always present. In this scenario, the mean blocking temperature ($\langle T_b \rangle$) is typically defined as the temperature at which 50% of the particles exhibit superparamagnetic behaviour, identifying the temperature at which 50% of the particles overcome their anisotropy energy barriers [36]. Moreover, there is a noticeable irreversible magnetic behaviour between the ZFC and FC curves. The temperature at which this divergence occurs, T_{irr} , is associated with the blocking temperature of the largest particles [35]. T_{irr} is determined as the point where the difference between M_{FC} and M_{ZFC} drops below 3% [37] [38]. As the temperature decreases, the FC curves display a plateau-like shape, indicating the presence of strong interparticle interactions within both samples. MNPs and BMN samples show small difference in values of T_{max} , T_{irr} and $\langle T_b \rangle$ (Table 1) suggesting that CaLB functionalization does have negligible influence in magnetization dynamics of nanoparticles.

Table 1: Temperature Corresponding to the Maximum in ZFC Curve (T_{max}), Irreversibility Temperature (T_{irr}), mean Blocking Temperature ($\langle T_b \rangle$); Saturation Magnetization (M_s), Reduced Remanent Magnetization (M_r/M_s), Coercive Field ($\mu_0 H_c$)^a

Sample	$T_{max}(K)$	$\langle T_b \rangle (K)$	$T_{irr} (K)$	$M_s (Am^2Kg^{-1})$	M_r/M_s	$\mu_0 H_c(T)$
MNPs	202(6)	100(3)	273(8)	77(3)	0.27(2)	0.028(3)
BMN	214(6)	82(2)	221(6)	69 (3)	0,28(2)	0.027(3)

^a Uncertainties in the last digit are given in parentheses.

Field dependence of magnetization was analyzed at 5 K and 300 K, and when irreversibility is observed the hysteresis parameters, such as saturation magnetization (M_s), coercive field ($\mu_0 H_c$), and reduced remanent magnetization (M_r/M_s), were extracted from the M vs. H curves and are presented in Table 1. At 5 K (Figure 3b), the hysteresis loop shows low values of M_r/M_s and H_c , as expected for spinel iron oxide nanoparticles [39]. Using the LAS equation, an M_s value of 77(3) Am^2kg^{-1} was obtained for bare MNPs, which is close to the bulk value ($\sim 90 \text{ emu g}^{-1}$) [40]. The M_s value for the BMN sample was the same within the experimental error, clearly indicating that the presence of the enzyme does not affect the magnetic features of the MNPs. This result is quite interesting because the presence of a molecular coating and its interactions with the nanoparticle surface can typically induce significant variations in M_s , magnetic anisotropy (i.e., H_c and M_r/M_s) [41] [42].

The effect of interparticle interactions was studied using direct current demagnetization (DCD) and isothermal remanent magnetization (IRM) protocols at 5 K [43] (Supporting Information for details,

Figure S8). For non-interacting single-domain particles with uniaxial anisotropy, where the magnetization reversal occurs by coherent rotation, the IRM and DCD are related through the following equation (1) [44]:

$$m = m_{DCD}(H) - 1 + 2m_{IRM}(H) \quad (1)$$

In this equation, $m_{DCD}(H)$ and $m_{IRM}(H)$ represent the normalized remanence values $M_{DCD}(H)/M_{DCD}(H_{max})$ and $M_{IRM}(H)/M_{IRM}(H_{max})$, respectively, with $M_{DCD}(H_{max})$ and $M_{IRM}(H_{max})$ being the saturation remanence magnetization. Typically, a positive peak in the δm plot indicates the prevalence of magnetizing interactions (i.e., exchange interactions) among nanoparticles; conversely, a negative peak suggests the presence of demagnetizing interactions (i.e., dipole-dipole interactions). As expected, the δm plots of both samples (Figure 3c) shows the dominance of dipole-dipole interactions, with similar interaction strengths observed within experimental error. Being dipolar strength inverse proportional to the interparticle distance, this further supports that the presence of CaLB do not significant influence the morphological properties of our system.

As shown by ZFC-FC, field dependence of magnetization at 300 K (Figure 4) confirm that both samples exhibit superparamagnetic behaviour (i.e., $H_c=0$ and $M_r=0$), with similar M_s values within the experimental error (i.e., M_s (MNPs) $\simeq 61 \text{ A m}^2 \text{ Kg}^{-1}$; M_s (BMN) $\simeq 57 \text{ Am}^2 \text{ Kg}^{-1}$). It is worth to underline that after functionalization values M_s and magnetic susceptibility remain relatively high, ensuring good performance of the materials for magnetic recovery purpose.

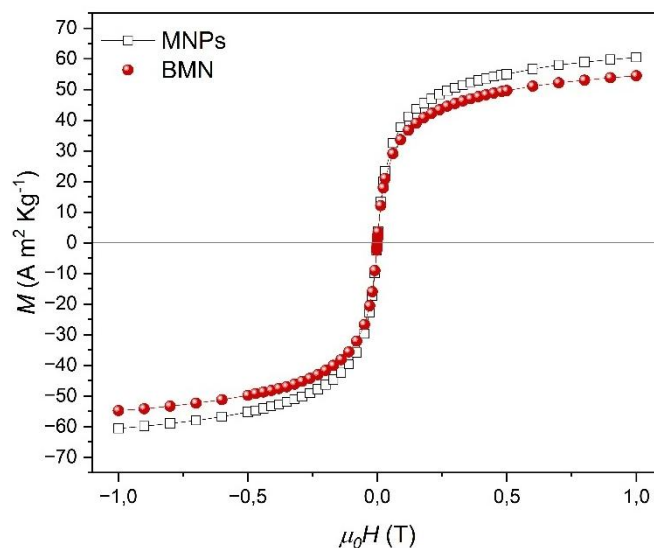


Figure 4: Field dependence of magnetization for MNPs (empty squares) and BMN (full circles) recorded at 300 K.

2.4.2 Polycondensation

The immobilized enzyme preparation was tested for polyesters synthesis reactions. The progression of the reaction was monitored via ^1H -NMR and GPC. By analyzing the ^1H -NMR results (figure 5), it was possible to observe a monomer conversion of 92%, monitoring the intensity reduction of the signal at 3.6 ppm (disappearance of the $-\text{OCH}_3$ group of the diester due to the release of MeOH as by-product) in the ^1H -NMR spectra of the polymerization products together with the intensification of the signal at 4.1 ppm ($-\text{CH}_2-\text{O}-\text{C}=\text{O}$). This does not occur in a control test when the polycondensation reaction is performed using only bare MNPs. As visible in Figure S9, no signal reduction was observed at 3.6 ppm since the $-\text{OCH}_3$ group of the diester does not disappear and $-\text{CH}_2-\text{O}-\text{C}=\text{O}$ is not formed as a demonstration of the unsuccessful synthesis of the final polyester with just 6% of monomer conversion.

Other important properties of the synthesized polyester, such as a number average molecular weight (M_n) of 5600 Da and a degree of polymerization (DP) of ~ 20 were obtained from GPC analysis.

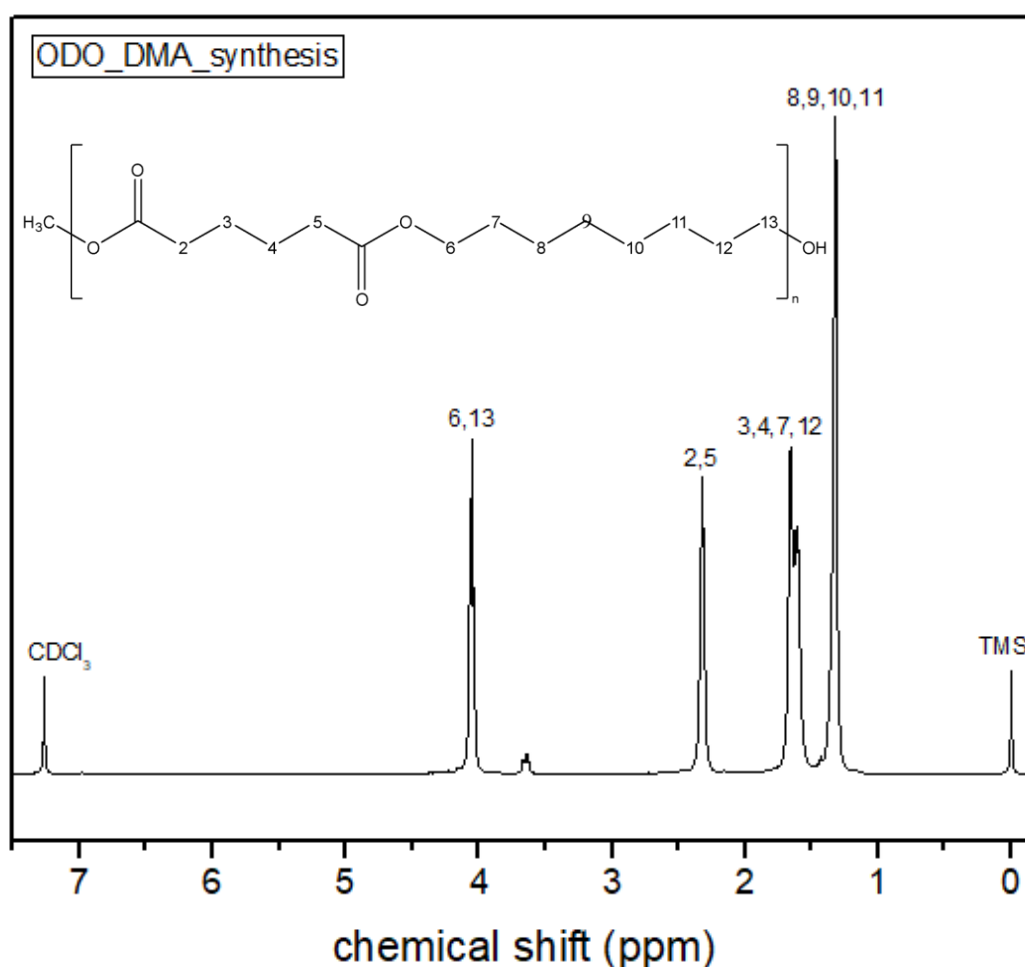


Figure 5: ^1H -NMR spectra of poly(1,8-octylene adipate) synthesized using dimethyl adipate (DMA) and 1,8-octanediol (ODO) as building blocks.

2.4.3 Enzymatic recyclability

In line with the principles of green and sustainable development, a long lifespan and easy recyclability are crucial for the practical application of catalysts in industry. In liquid media reactions, a rapid and efficient separation method is essential to remove the catalyst, allowing for its repeated recycling. Therefore, the chosen separation process should minimize time and energy consumption. In this context, magnetic separation (MS, i.e., to use an external magnet separating magnetic catalysts from the reaction mixture) has recently gained attention as an effective alternative to traditional methods such as filtration and centrifugation. Such a method offers advantages in terms of speed, simplicity, and overall efficiency, making it both economically and technically viable with minimal catalyst loss. Additionally, magnetic separation aligns with green chemistry principles by reducing secondary waste and lowering energy requirements. Accordingly, the efficiency of MS of the BMN under investigation has been evaluated. After the reaction, the biocatalyst was recovered using an external magnetic field, washed, and reused for three cycles (Figure S10 in ESI). Field dependence of magnetization of BMN after each cycle has been measured at 300 K (Figure S11a ESI). A decrease of M_s (Figure 6a, black full circles) has been observed with the increase of cycles indicating an increasing quantity of polyester remain attached to the MNPs. A rough estimation of the percentage of polyester has been determined by equation S1 and reported in figure 6a (red empty squares). After the first cycle a quantity of polyester equal to ~20% of the initial weight stayed attached to BMN allowing a better performance of the catalyst in the subsequence cycles as the polyester chains from the previous cycle might work as nucleation points that help the kickstart of the new reaction cycle. The percentage of polyester slightly increase in the 2nd and 3rd cycles.

Field dependence of magnetization has been investigated also on the polyester after each cycle (see ESI Figure S11b) showing a diamagnetic trend with a susceptibility that, as expected, is decreasing with the increasing of cycles (see ESI Figure S11c). All the magnetization curves have been corrected for the diamagnetic contribution (see ESI Figure S11d) and using the trend of M_s , the percentage of MNPs in polyester has been calculated by equation S1. The M_s decrease with the increase of cycles number indicating a reduced percentage of MNPs, decreasing from 0.009% to 0.006% from 1 to the 3 cycle (Figure 6b).

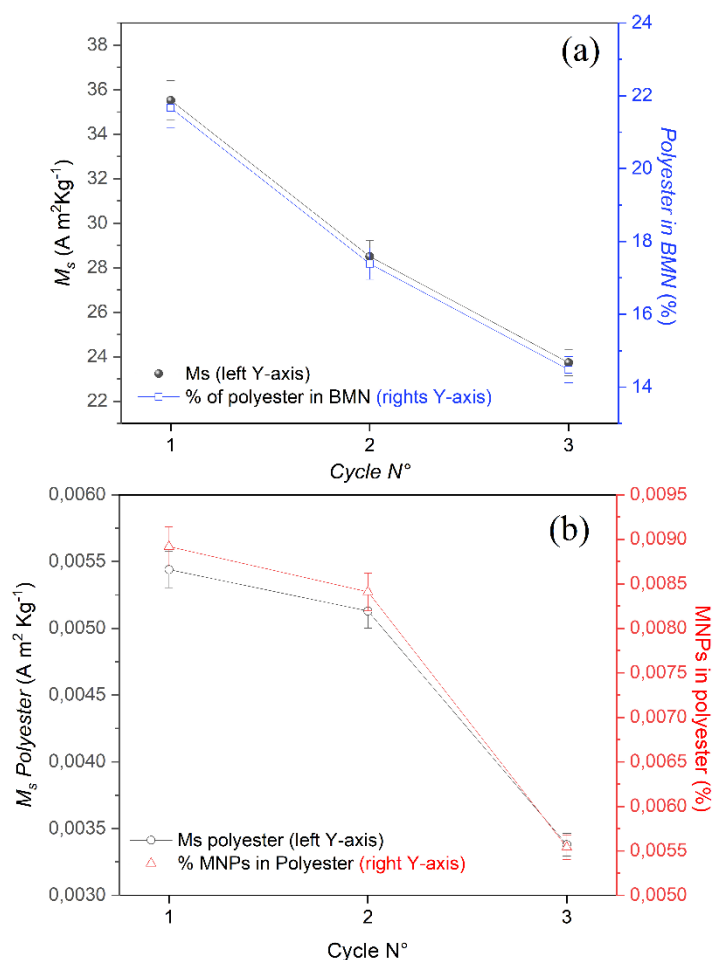


Figure 6: Recovery of the biocatalyst: (a) M_s of BMN and % polyester in BMN after each cycle; (b) M_s of polyester and % BMN in polyester after each cycle.

¹H-NMR and GPC were used to monitor the % of monomer conversion and the molecular weights (M_n & M_w) of the obtained polyesters. As visible in [figure 7](#), through the recovery of the biocatalyst via the application of a permanent magnet, CaLB bound magnetic nanoparticles has retained its activity over several cycles of reaction; the % of monomer conversion was found to be of 89% for the 2nd cycle and 87% for the 3rd cycle, therefore being consistent with the 92% of the 1st reaction cycle. For what concerns GPC analysis, the M_n of 5600 Da obtained for the 1st cycle was higher than the M_n of 4400 Da and 4200 Da for the 2nd and 3rd cycle respectively (~25% M_n reduction) ([table S1](#)). The monitored ¹H-NMR spectra and GPC charts of polycondensation progress over 3 cycles of reaction is reported in ESI ([Figure S12](#)). Hence, as it is reported in the [Figure 7](#), it was verified, in addition to what was previously proven by the literature on different CaLB immobilized preparations [26] [27], that BMN maintain an excellent selectivity towards long-chain diols and dicarboxylic acids

for multiple reaction cycles, exploiting good recyclability due to the retainment of the enzyme's activity able to ensure high monomer conversion rate in polycondensation reactions.

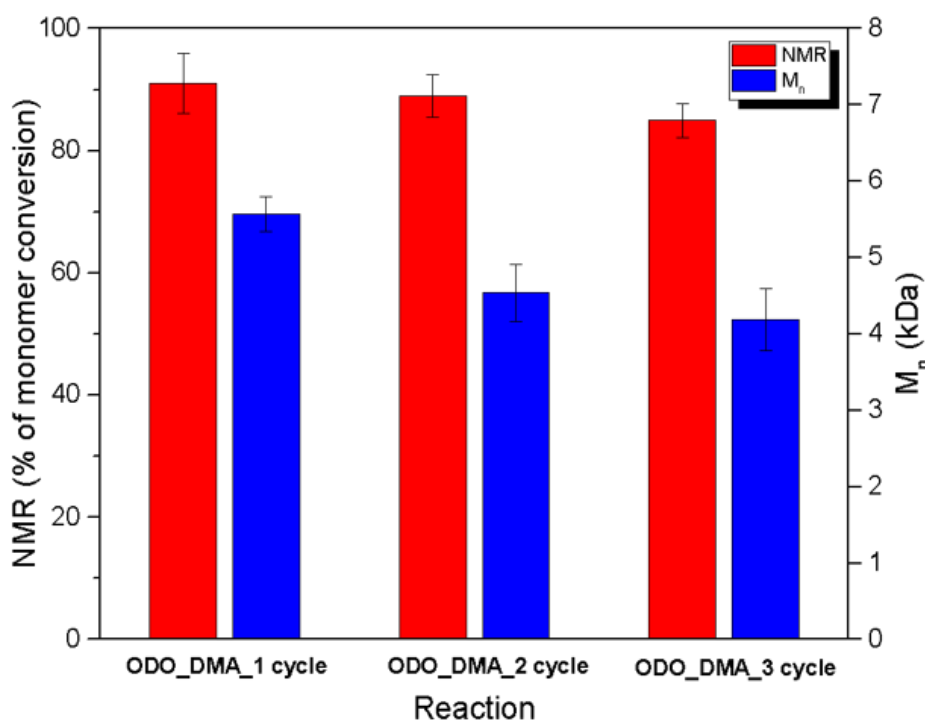


Figure 7: Enzyme recyclability for 3 cycles of polycondensation reactions: progression of reactions was monitored via ^1H -NMR (monomers conversion, red bars) and GPC (obtained number average molecular weight, blue bars).

2.5 Conclusion and future perspective

CaLB was successfully immobilized onto magnetic nanoparticles synthesized by co-precipitation method and enzyme recyclability was tested using dimethyl adipate and 1,8-octanediol as building blocks for biocatalyzed synthesis of polyesters.

Results showed an efficient recyclability over three reaction cycles able to ensure high monomer conversion rate and molecular weights of polyesters; in fact, the tuning of magnetic properties, achieved through the control of primary nanoparticle size, ensures efficiently magnetic recovering of the catalyst (they can be easily separated from the products by an ordinary magnetic device) and their reusability for several reaction cycles. With this paper, the authors want to underline how this not only could enhance the sustainability of enzymatic processes with environmentally friendly processes (since toxic catalysts and organic solvents are avoided) but also contributes to cost-effectiveness and scalability in industrial applications.

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2.7 Electronic Supplementary Information

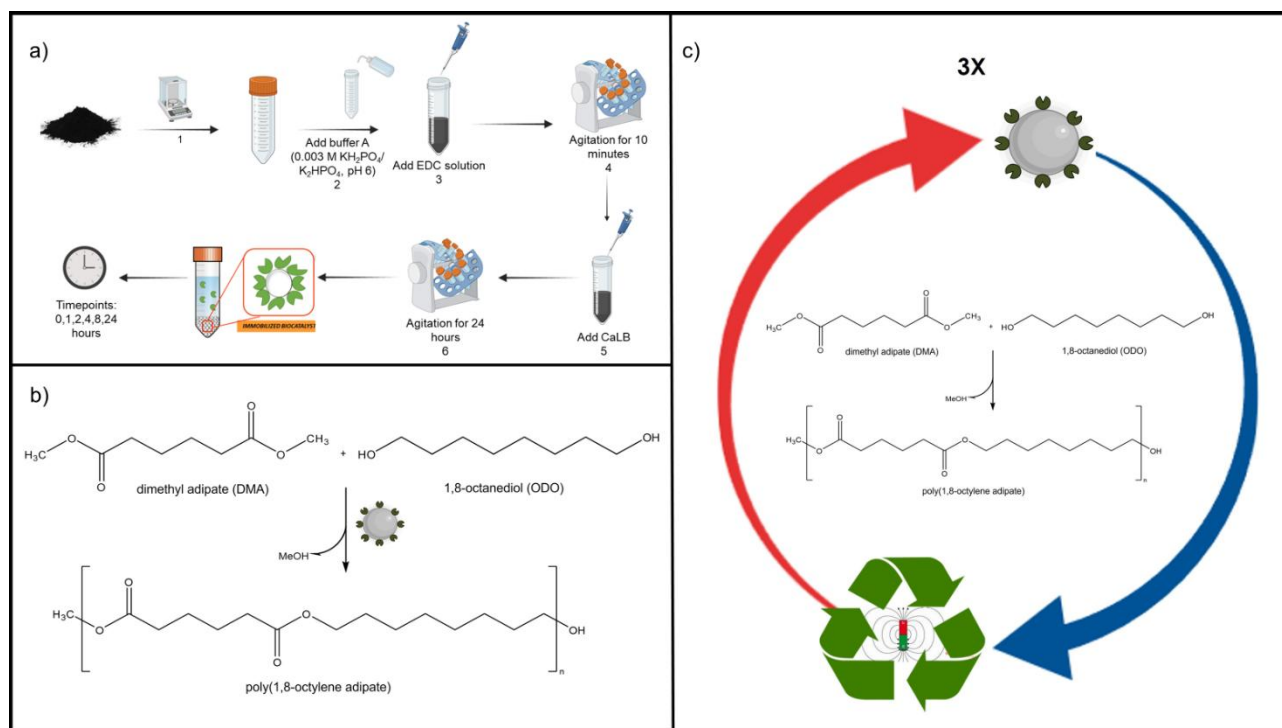


Figure S1: Schematic image for overall procedures including Immobilization of Lipase on MNPs (a), polycondensation (b) and enzyme recyclability (c)

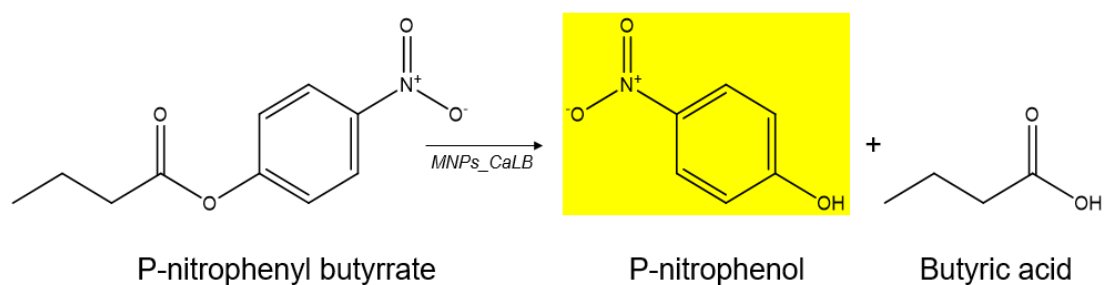


Figure S2: Schematic representation of esterase activity assay.

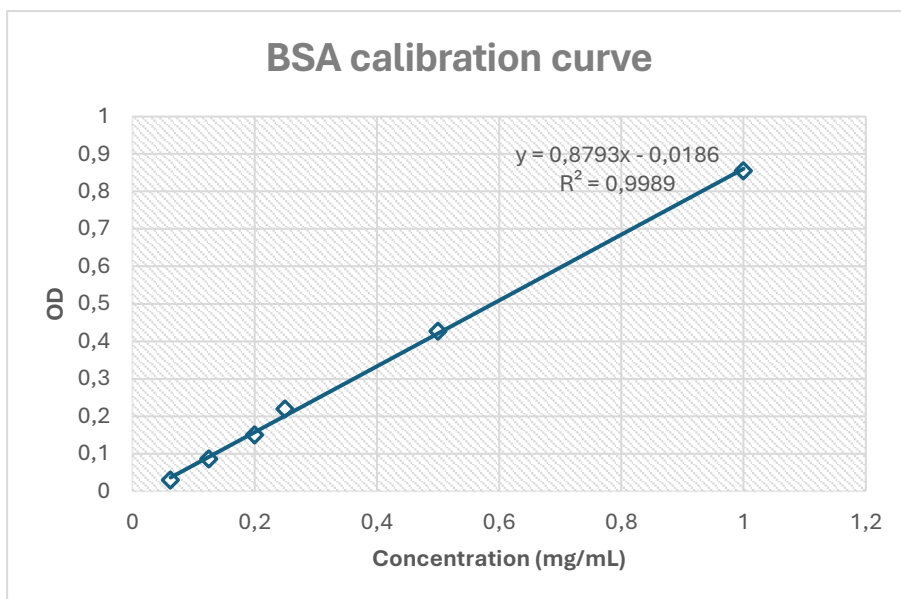


Figure S3: BSA calibration curve.

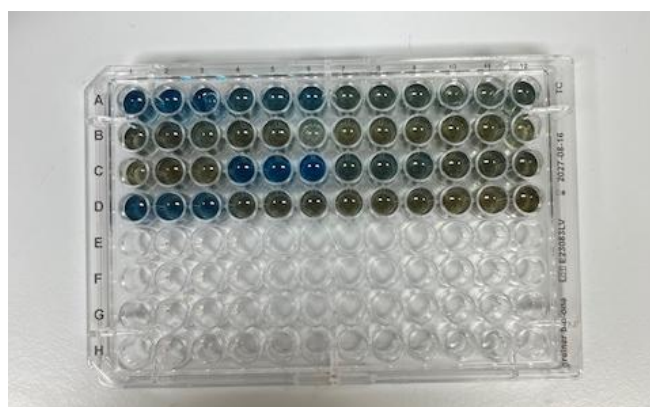
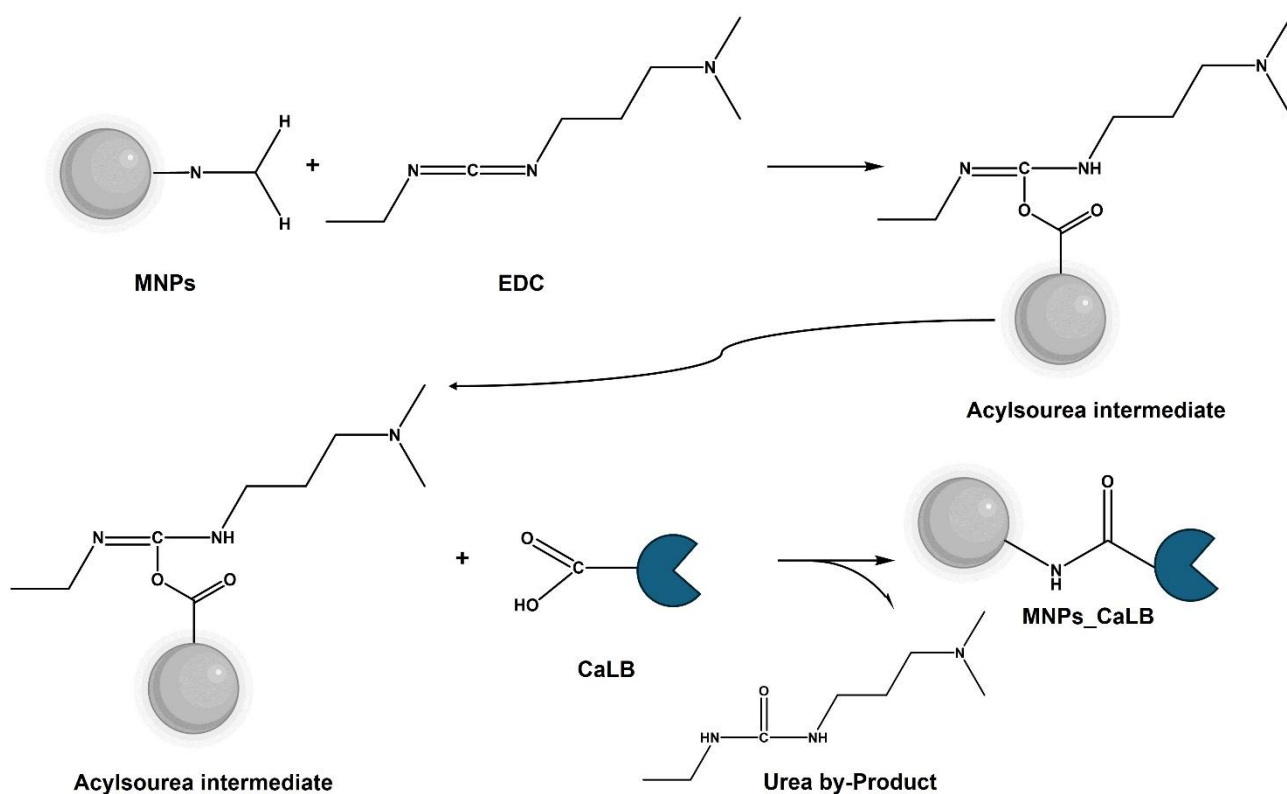


Figure S4: 96-well microtiter plate for protein concentration quantification for the different timepoint (0,1,2,4,8 and 24 h of immobilization).

As visible in the Scheme S1 and confirmed from IR analysis, we suppose that EDC reacts with MNPs to form an active urea derivative intermediate (MNPs_EDC) that is easily displaced by nucleophilic attack from CaLB functional groups in the reaction mixture and the EDC by-product is released as visible from the IR spectra where the characteristic absorption band of MNPs_EDC (C-H at 2970 cm^{-1} ; C-N at 1465 cm^{-1}) disappears in the BMN spectrum and just the absorption band related to the two typical amide bond transitions at 1630 cm^{-1} (amide I region) and 1540 cm^{-1} (amide II region) and the absorption band of CaLB associated with the σ -type C-O (typical of aa residues) at around 1036 cm^{-1} are visible. This absorption band is not present in bare MNPs spectrum confirming the binding of CaLB to MNPs.



Scheme S1: Detailed scheme to describe the chemical mechanism for binding the enzyme to the MNPs.

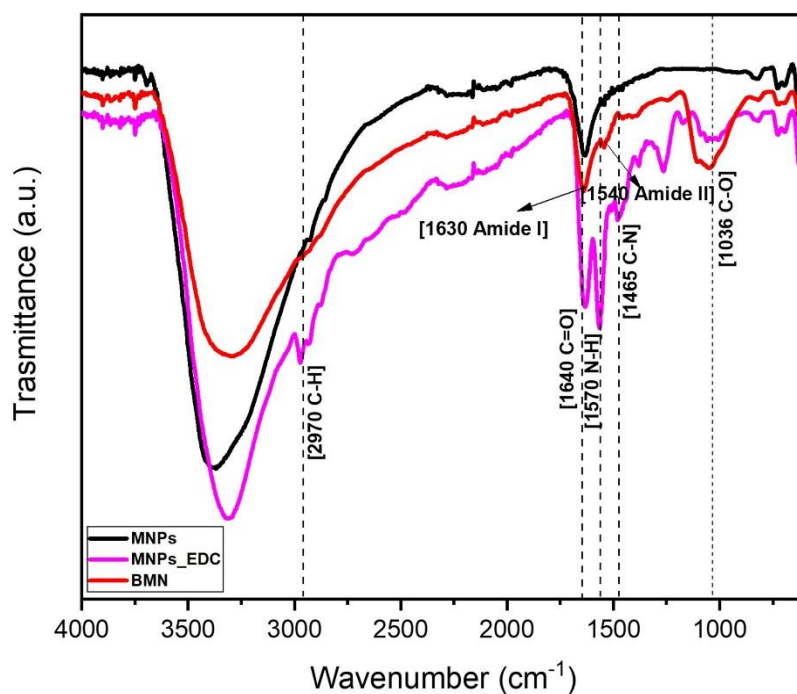


Figure S5: FTIR-ATR spectra in the range 4000 – 600 cm⁻¹ of bare MNPs (black), BMN (red) and MNPs_EDC (purple).

TGA analysis of MNPs before and after Lipase immobilization was performed. Compared to MNPs, BMN presents a weight loss of 10% as a percentage of the enzyme loading. At the initial stage, the mass of BMN was almost 97% when the temperature was raised to 100 °C, indicating a slight mass loss due to the evaporation of water. The second mass loss stage occurred at approximately 200 °C, corresponding to the decomposition of the organic components (C,H, O and N) in the enzyme conformation [1]. The TG curve of BMN is similar to what was previously proven for nano-fructosome encapsulated CaLB enzyme particles by Jang et al [2].

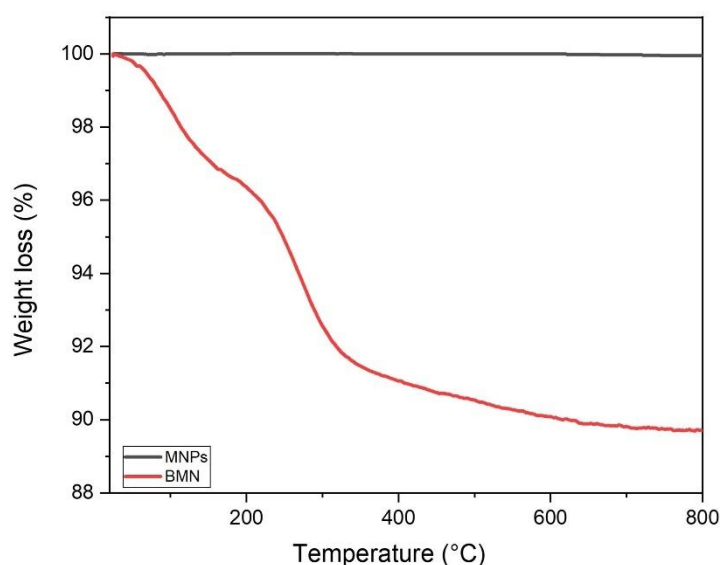


Figure S6: TGA curve of bare MNPs (black) and BMN (red)

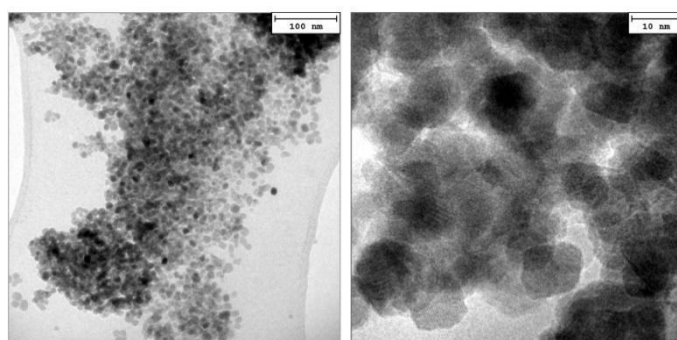


Figure S7: Transmission electron micrographs of magnetic nanoparticles at different magnification

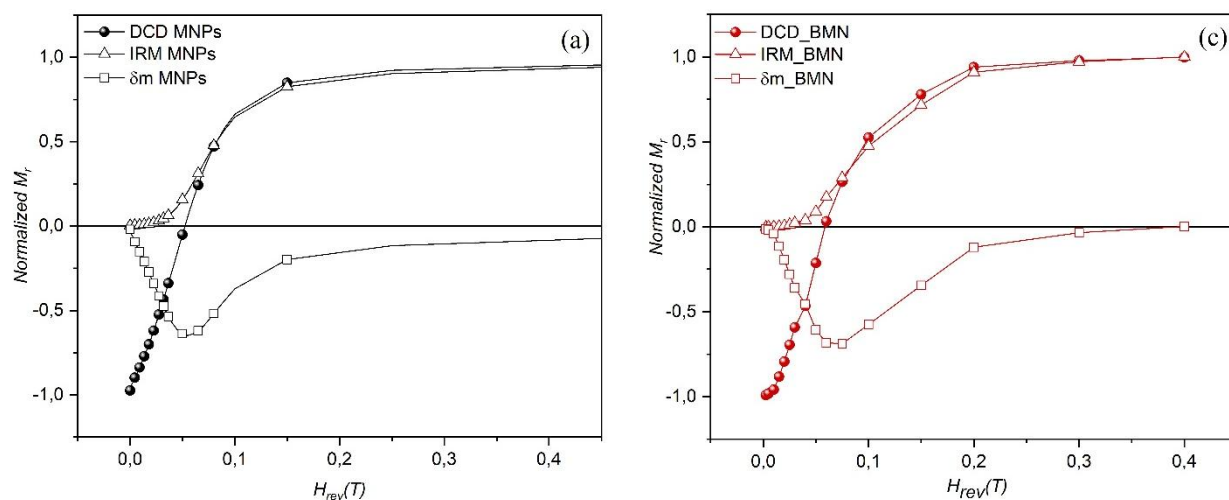


Figure S8: M_{DCD} (empty triangles), M_{IRM} (full 3D circles) and δm plot (empty squares) remanence magnetization for MNPs (a) and BMN (c) samples product

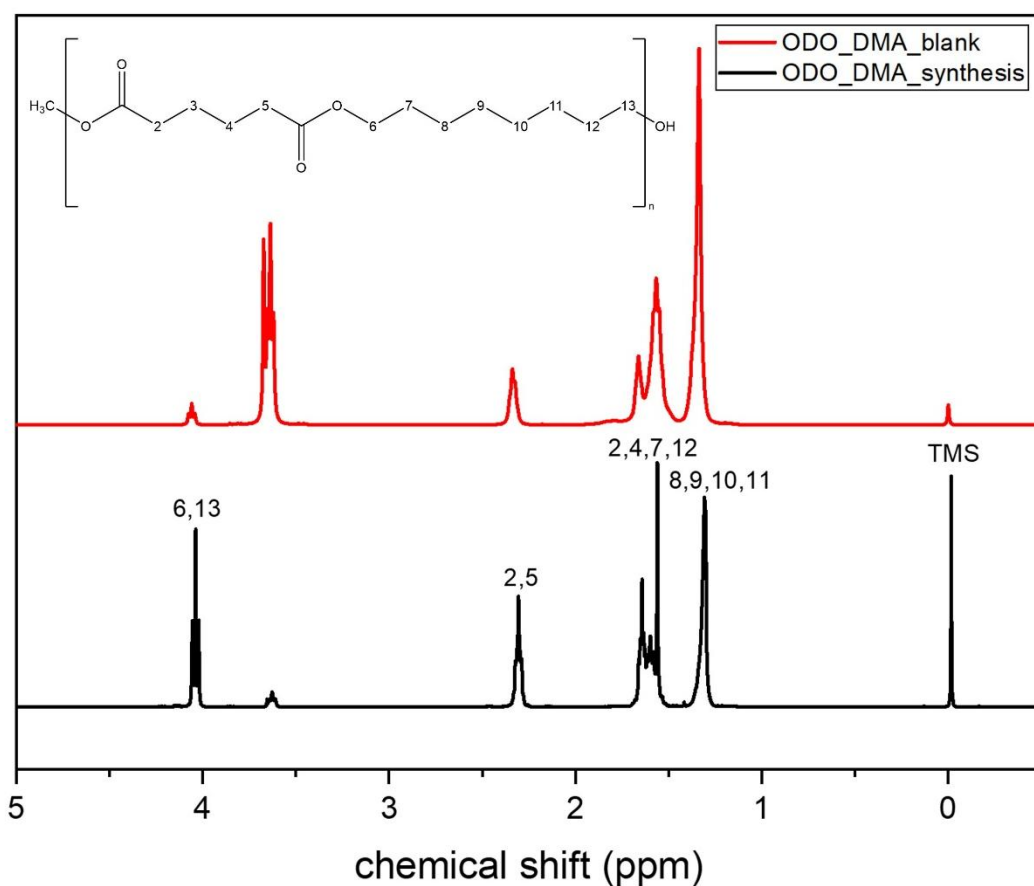


Figure S9: Comparison of ^1H -NMR spectra of poly(1,8-octylene adipate) synthesized using dimethyl adipate (DMA) and 1,8-octanediol (ODO) as building blocks with BMN (ODO_DMA_synthesis) and naked MNPs (ODO_DMA_blank)

Homemade recovery system (Figure S7) has been realized inserting a commercial permanent magnet inside a glass tube; once put the tube inside the 25-mL round bottomed flask, BMN was attracted in few seconds towards the magnet and recovered for a new cycle of reaction. In the following the characteristic of commercial permanent magnet Material/grade: NdFeB / 42MGOe Height(H) = 10 mm , Outer diameter(D) = 10 mm Flux density inside the magnet = 1.28 Tesla max. operation temperature = 180°C



Figure S10: Recovery system to separate biocatalyst from reaction

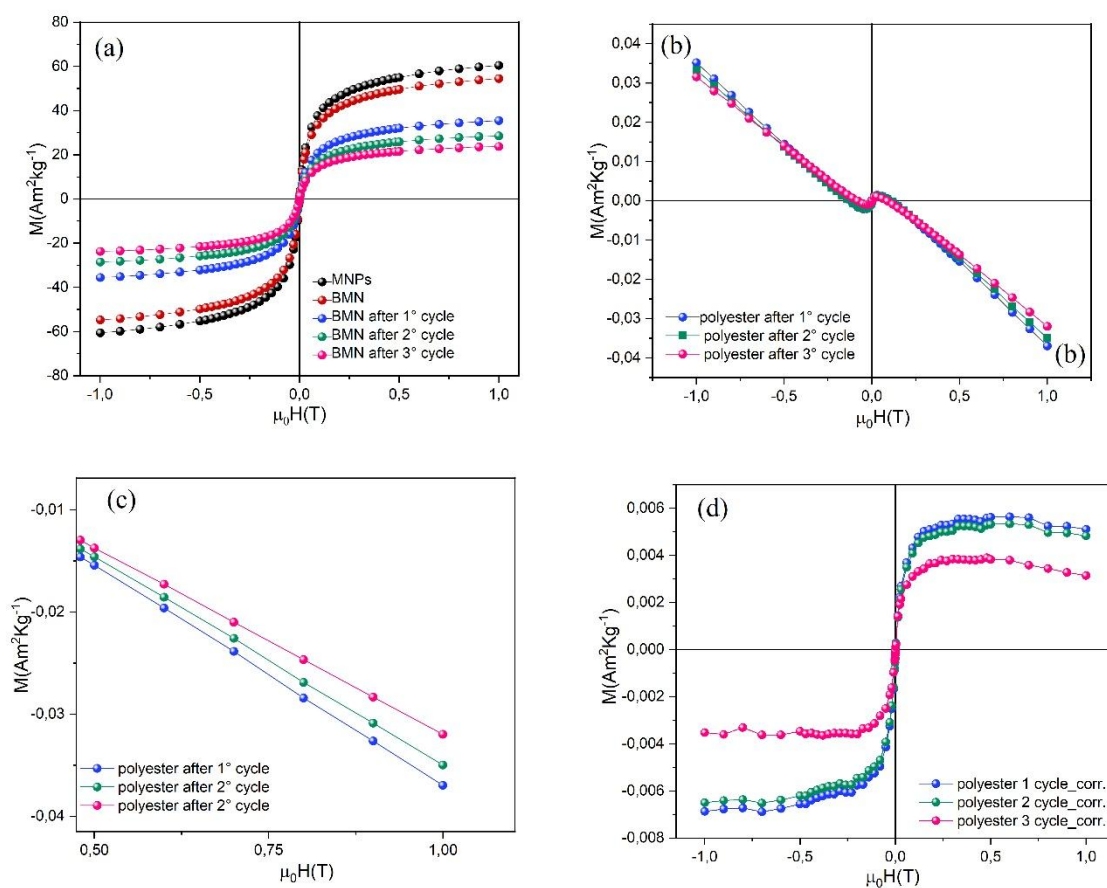


Figure S11: (a) vs M vs H at 300K of MNP, BMN, and MNP_{calc} after the 1st, 2nd, 3rd reaction cycle, (b) M vs H of the polyester after each cycle at 300K; (c) Diamagnetic phase after each cycle at 300K. (d) M vs H of the polyester after each cycle corrected at 300K

The calculation of the percentage of magnetic nanoparticles (MNPs) after each recovery cycle was determined using saturation magnetization data, through the following formula:

$$\% \text{MNPs} = \frac{M_{\text{MNP}}}{M_{\text{rec}}} \times 100 \quad (\text{S1})$$

M_{MNP} initial magnetization of bare particles, M_{rec} is the saturation magnetization after each recovery cycle.

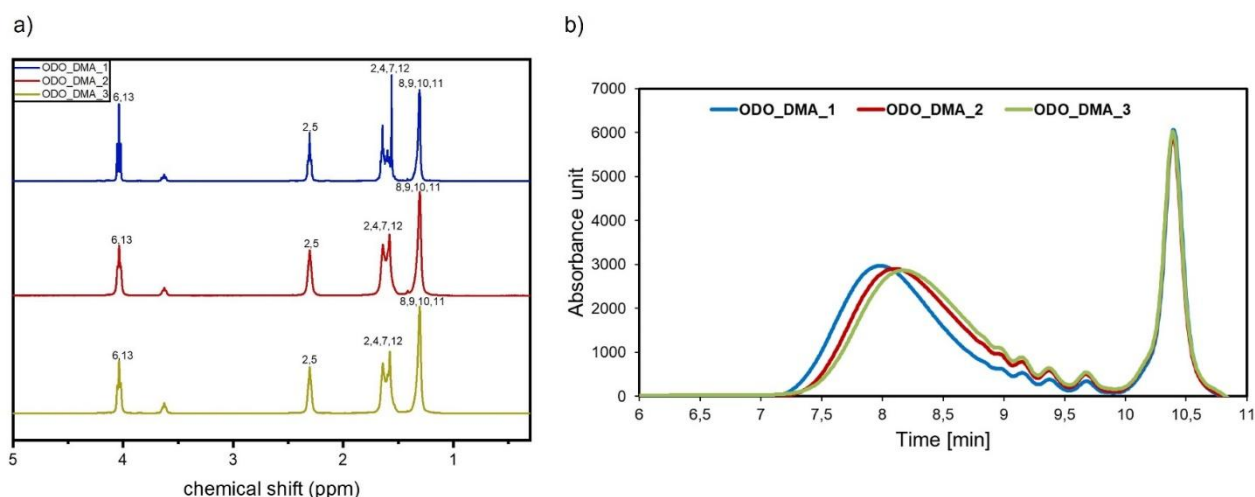


Figure S12: The monitored ¹H-NMR spectra (a) and GPC charts (B) of polycondensation progress over 3 cycles of reaction

Table S1: Comparison between % of monomer conversion and M_n for 3 different cycles of reaction

Reaction cycle	% of monomer conversion	M _n (Da)	M _w (Da)	Dispersity (Đ)
DMA_ODO 1 st cycle	92	5600	11000	1.96
DMA_ODO 2 nd cycle	89	4400	7800	1.77
DMA_ODO 3 rd cycle	87	4200	7000	1.66

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Chapter 3: The quest for the new CaLB: potential of three thermostable polyester hydrolases for esterification reactions

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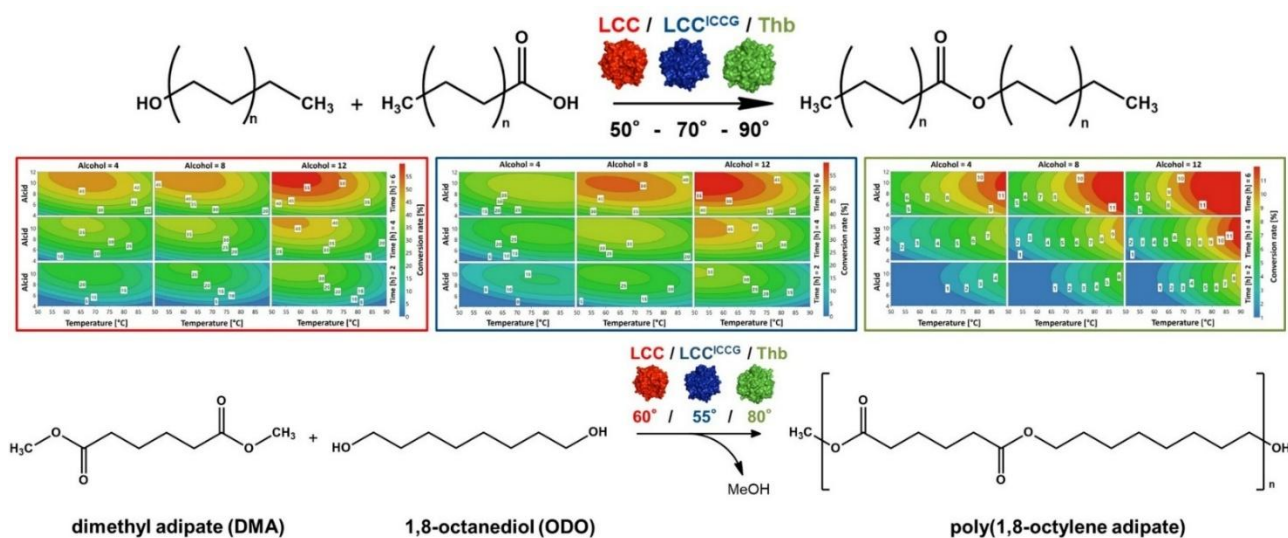
Francesco Papatola, Filippo Fabbri, Virender Kumar, Chiara Siracusa, Felice Quartinello, Doris Ribitsch, Cristiano Varrone, Georg M. Guebitz, Alessandro Pellis

3.1 Abstract

The potential of three thermophilic enzymes from the α,β -hydrolases superfamily, that have recently been described for polyester hydrolysis, was investigated for polyester synthesis, esterification and transesterification reactions. The hydrolases (LCC, leaf-branch compost cutinase; its variant LCC^{ICCG}, Thb from *Thermoanaerobacteriales* bacterium) were recombinantly expressed, purified and immobilized onto polypropylene beads. A design of experiments (DoE) assisted study was performed to investigate their synthetic potential to produce short flavor esters by analyzing their thermostability and selectivity towards alcohols and acids with different chain lengths. The factors considered in the DoE (i.e., temperature, alcohol chain length, acid chain length and reaction time) were optimized using MODDE® software to generate a predictive model defining the optimal synthetic conditions for the three enzymes. In each experiment, the monitored response was the acid conversion rate, quantified with GC-FID analysis. For synthesis, the temperature optima of LCC, LCC^{ICCG} and Thb were 60 °C, 55 °C, and 80 °C, respectively, corresponding to the maximum percentage of monomers conversion for long-chain alcohols and acids as substrates. Polymerization of dimethyl adipate and 1,8-octanediol as building blocks was carried out to confirm the applicability of the obtained model for the synthesis of larger macromolecules via polycondensation reactions. Conversion of monomers, as determined by nuclear magnetic resonance (NMR) analysis, was ~90% for all enzymes while the average molecular weights (M_n) of the polyesters, analyzed by gel permeation chromatography, were between 3600 Da, for LCC and its variant LCC^{ICCG}, and 3800 Da for Thb.

Keywords: α,β -hydrolases, enzyme immobilization, design of experiment (DoE), biocatalyzed synthetic reactions, enzymatic polycondensation

3.1.1 Graphical abstract



A design of experiments (DoE) based approach was employed to investigate the thermostability and substrate selectivity of three enzymes described for polyester hydrolysis, namely LCC, LCC^{ICCG} and Thb in short ester synthesis reactions involving alcohols and acids of varying chain length. The temperature optimum (T_{opt}) of the three enzymes was also tested for enzymatic polymerization of dimethyl adipate and 1,8-octanediol to confirm the applicability of the obtained model for the synthesis of larger macromolecules via polycondensation reactions.

3.2 Introduction

Scientific advances in the field of biocatalysis have led to the development of promising enzymes thus opening extraordinary opportunities not only in the conventional sector of food and detergents but also in green processes having deep impact on health (e.g., pharmaceuticals and cosmetics), environmental protection (e.g., agrochemicals) as well as for the production of fine-chemicals and polymers [1] [2]. Nowadays, enzymes are extensively employed for textile processing and in many other applications like polymer decomposition. For example, certain hydrolases can hydrolyze polyesters such as poly(ethylene terephthalate) (PET) [3] [4] allowing selective recovery of valuable building blocks even from blends and mixed wastes. On the other hand, under specific conditions, enzymes are able to catalyze the reversed reaction to synthesize aliphatic and aromatic-aliphatic polyesters or oligomers with side chains functionalities. In response to the growing demand for bio-based and biodegradable polymers, aligned with the principles of the “European Green Deal” [5], enzymes have emerged as a promising and sustainable alternative to the traditional chemical synthetic pathways offering a viable solution to the ongoing challenge of reconciling mild reaction conditions with high catalytic efficiency and selectivity [6]. Unlike traditional chemical processes, enzymes operate under relatively low temperatures (typically < 100°C) [7] and ensure environmentally friendly processes avoiding the use of toxic metal catalysts such as tin and titanium, which are commonly employed in conventional polycondensation reactions [8]. In this context, biocatalysis is increasingly acknowledged as a powerful strategy in modern chemical manufacturing for the production of bio-based and biodegradable polyesters, offering an environmentally friendly alternative that allow the design of functional polymers which are difficult to achieve via traditional metal-catalyzed reactions [9]. Despite their significant catalytic potential, a broader use of enzymes in this field remains constrained by limitations such as inadequate long-term stability, challenging recovery and the inability to reuse the costly enzymes. These challenges may be addressed by employing enzyme immobilization strategies which facilitate efficient separation of the biocatalyst from the reaction mixture, thereby simplifying its recovery and re-use, while also enhancing key enzymatic properties such as activity, stability and substrate specificity [10] [11] [12].

Nowadays, mainly lipases are used for synthetic reactions, with *Candida antarctica* lipase B (CaLB) being the most commonly employed enzyme for the synthesis of polyesters and flavor esters. This prominence could be attributed to its commercial availability in both free and immobilized form [13], as well as its remarkable properties, including high catalytic efficiency and excellent selectivity. In particular, CaLB, in its immobilized form on acrylic resin (commercially available as Novozym 435, N435), has emerged as the most intensively employed biocatalyst in synthetic applications [14]. Pellis et al. reported the CaLB selectivity pattern in which immobilized enzyme (N435) showed its preference towards long-chain diols

and diesters instead of short-chain compounds in polycondensation reactions obtaining the best combination using dimethyl adipate and 1,8-octanediol at 85°C with high M_n products (7000 Da) [7]. Moreover, other enzymes classified within the α,β -hydrolases superfamily have been extensively investigated as biocatalysts for polyesters synthesis. Among these, the commercial *Humicola insolens* cutinase (HiC) [15] [16] or the more recently reported *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1) [17] [18] have demonstrated promising catalytic performance. In particular, Feder and Gross demonstrated that HiC, when immobilized on Amberzyme oxirane resin, exhibits a high selectivity towards long-chain diols and diacids. Their study revealed an enzyme preference for substrate like dimethyl sebacate and 1,8-octanediol at 70 °C, leading to the formation of polyesters reaching M_{ns} of 6600 Da [19]. Conversely, Thc_Cut1, immobilized on different carriers (such as opal, coral, and amber beads), preferred short chain diol, independent of the diester chain length (C6, C8, or C10), in reactions performed at 50 °C. Differently from reactions employing this kind of supports, where polyesters with a limited molecular weight of 900 Da were produced [18], products with higher M_w (~1900 Da) were achieved when the same enzyme was covalently bound on epoxy resins [6].

Furthermore, in addition to what was previously proven in the literature on different CaLB/HiC/Thc_Cut1 immobilized preparations, Fabbri et al. developed a full-factorial design of experiments to investigate the thermostability and selectivity of these three commercial hydrolytic enzymes (i.e., CaLB, HiC and Thc_Cut1) immobilized onto polypropylene beads confirming the data previously reported in the literature [20].

Esterases, lipases and cutinases primarily split ester bonds in different small and larger molecules, but under low water or organic solvent conditions due to their reversible catalytic mechanism and α/β -hydrolase fold, they can also catalyze esterification and transesterification. This study aims to identify novel thermophilic enzymes suitably for synthesis belonging to the α,β -hydrolases superfamily which have been described to hydrolyze polyesters. For the first time, in this work, three efficient PET hydrolases, namely leaf-branch compost cutinase LCC [21] [22], its mutant form ICCG (LCC^{ICCG}) engineered to improve the thermostability and activity of LCC in depolymerization and recycling activities [23] [24], and Thb (from *Thermoanaerobacteriales* bacterium with high homology to PHL7) [25] [26] [27] were tested in synthesis reactions. The three hydrolases were adsorbed onto polypropylene beads to assess their catalytic efficiency in the synthesis of short-esters while evaluating their thermostability and selectivity towards alcohols and acids of varying chain length. A design of experiments (DoE) assisted study was implemented and the results on flavor esters helped determining the Temperature optimum (T_{opt}) of the three enzymes which were subsequently applied to enzymatic polycondensations using bio-based monomers (i.e., dimethyl adipate and 1,8-octanediol) as building blocks for polyester synthesis, aiming to confirm the applicability of the obtained models also on larger macromolecules.

3.3 Experimental

3.3.1 Materials and methods

Polypropylene beads (Accurell MP1000 surface area of 55,985 m²g⁻¹, particle density of 1.993 cm⁻³, and particle diameter of <1500 mm) were obtained from 3M Deutschland GmbH (Wuppertal, Germany). Dimethyl adipate (DMA, >99%), 1,8-octanediol (ODO, >98%), butyric acid (>99%), 1-butanol (>99%), 1-octanol (>99%), Octanoic acid (>98%), 1-dodecanol (>99%), lauric acid (>99%), 2-methyl 2-butanol (>99%), 4-nitrophenol (>99%), para-nitrophenyl butyrate (p-NPB), K₂HPO₄ (>98%) and KH₂PO₄ (>98%) were purchased from Sigma-Aldrich. Chloroform (CHCl₃ for GC analysis, >99.8%), Chloroform-d (CDCl₃ for ¹H-NMR,) and tetrahydrofuran (THF for GPC, >99%) was purchased from VWR Chemicals (Wien, Austria). The amino acid sequences corresponding to LCC, LCC^{ICCG} and Thb were obtained from the Protein Data Bank (PDB) database and subsequently codon-optimized for the expression in *Escherichia coli* BL21-(DE3). Lysogeny Broth (LB), kanamycin and isopropyl-β-D-thiogalactoside (IPTG) were sourced from Sigma-Aldrich (Germany). *Escherichia coli* BL21-Gold (DE3) electrocompetent cells were obtained from Agilent.

3.3.2 Expression and purification of enzymes

The three enzymes (i.e., Leaf-branch compost cutinase LCC; its mutated form LCC^{ICCG}; and the hydrolase from *Thermoanaerobacteriales* (Thb), were expressed in the electrocompetent *E. coli* strain BL21-(DE3) using pET26b(+) vector. LCC and LCC^{ICCG} were produced and purified as previously described by Kumar et al. [22] [28], while Thb was produced and purified following the method reported by Siracusa et al [27]. Purification efficiency was assessed through an SDS-PAGE analysis. Aliquots from load, flow-through, and eluted fractions were mixed in Laemmli buffer with 2-mercaptoethanol and then loaded onto precast Mini-PROTEAN TGX Stain-Free gels (Bio-Rad). Electrophoresis was left to run at 150 V for 30 min and an already known molecular weight protein marker IV (PepLab, Germany) was used as reference. As visible from figure S1, SDS-PAGE analysis revealed a molecular weight of 30 kDa for both LCC and LCC^{ICCG}, while the molecular weight of Thb was 35 kDa.

3.3.3 p-NPB based Esterase Activity Assay

The esterase activity of the three enzymes was determined based on hydrolysis of *para*-nitrophenyl butyrate (p-NPB). The catalytic activity was calculated by evaluating the increase in absorbance at 405 nm, resulting from the release of *p*-nitrophenol (ε 405 nm). 200 μL of substrate solution were

mixed with 20 μL of the enzyme, previously diluted in 0.1 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer at pH 8 (referred as KPO). Measurements were performed every 18 seconds over a 5-minute period using a Tecan Plate Reader (Tecan, Grödig, Austria) and 96-well microtiter plates (Greiner 96 Flat Bottom Transparent Polystyrene). The samples were analyzed in triplicates and a series of blanks (i.e., buffer without enzyme) were included in each activity cycle. The enzymatic activity of LCC, LCC^{ICCG} and Thb was expressed in units (U), where one U corresponds to the amount of enzyme that catalyzes conversion of 1 μmol per minute, and resulted to be 68.4 ($\sigma=1.2$) U/mg, 57.2 ($\sigma=0.7$) U/mg and 49.2 ($\sigma=0.8$) U/mg, respectively.

3.3.4 Determination of Protein Concentration

Protein concentrations of the three enzymes dissolved in KPO was determined using the Bio-Rad solution (Coomassie brilliant blue G-250 dye) diluted 1:5 in milliQ water. Different solutions of BSA (Bovine Serum Albumin, 2 mg mL^{-1} , Sigma-Aldrich) were used as standard protein to obtain a calibration curve (Figure S2), from which it was possible to determine protein concentration of LCC, LCC^{ICCG} and Thb (0.5 mg/mL, 0.96 mg/mL and 0.72 mg/mL respectively). BSA standard was serially diluted in KPO buffer to get the following concentrations: 0.0625, 0.1, 0.125, 0.2, 0.25, 0.4, 0.5, 1 and 2 mg mL^{-1} . Absorbance was recorded at 595 nm (resulted from the dye's interaction with primarily basic and aromatic aa residue of the protein, ϵ 595 nm) with a Tecan Plate Reader (Tecan, Grödig, Austria) using a 96-well microtiter plate (Greiner 96 Flat Bottom Transparent Polystyrene). 10 μL of each sample (both BSA and enzyme solutions) were put in the wells and 200 μL of 1:5 BioRad solution were added (in triplicate), incubating the mixture for 5 minutes at RT before analysis. A blank was also included using the KPO buffer.

3.3.5 Enzyme Immobilization procedure

In a 50-mL FalconTM tube, 1.0 g of polypropylene beads were accurately weighed and washed three times with 20 mL of acetone at 400 mbar for 5 min to remove the air and facilitate the enzyme loading in the bead's cavities. Subsequently, the beads were first rinsed with 20 mL of deionized H_2O and then twice with 20 mL of the immobilization buffer (KPO buffer pH 8). All washing procedures were performed using a blood rotator (SB3 model from VWR), set at constant speed of 30 rpm. Afterwards, 40 mL of enzyme solutions (0.25 mg mL^{-1} corresponding to 1% w/w enzyme/beads) were added to the beads. The mixture was incubated for 24 h on the blood rotator (RT at 30 rpm). Aliquots were collected over time (0, 1, 2, 4, 8, 24 h) to monitor the progression of immobilization by analyzing the residual enzyme activity (via p-NPB based Esterase Activity Assay) and residual protein

concentration (via Bradford-based Bio-Rad Protein Assay) in the supernatant. After 24 h, the beads were recovered by filtration using a paper filter, washed 3 times with KPO buffer and air-dried for 3 days at RT.

3.3.6 Three-level full factorial design of experiment (DoE)

A 3-level full factorial experimental design was developed using MODDE® Pro 13 Software (Sartorius) to investigate thermostability and substrate selectivity of LCC, LCC^{ICCG} and Thb. The DoE was planned to evaluate all the possible interactions among four selected independent variables studied at 3 levels (i.e., temperature = 50 °C, 70 °C, and 90 °C; alcohol chain length = C4, C8, and C12; acid chain length = C4, C8 and C12; and reaction time = 2, 4, and 6 h) aiming to assess the combined effect of each parameter on the response variable. The experiments were carried out in a randomized order for a total number of 81 reactions for each enzyme (i.e., 4 factors are studied at 3 levels by performing 3⁴ experiments). Additionally, a central point, defined by combination of all independent variables taken at their intermediate levels (specifically: temperature = 70 °C; alcohol = C8; acid = C8; time point = 4 h), was added in the experimental design. The response variable assessed for each condition was the acid conversion rate, determined by Gas Chromatography (GC) analysis.

3.3.7 Enzymatic Esterification Reactions

The reactions for short-esters synthesis were conducted by mixing, in a 50 mL reaction tube, equimolar amounts (6.0 mmol) of dicarboxylic acid and alcohol with an amount of immobilized enzyme (1% w/w enzyme/beads), corresponding to 2.5 % in weight referred to as the total amount of monomers (2.5 % w/w enzyme/total monomers weight). To determine the T_{opt} of LCC, LCC^{ICCG} and Thb for the reactions, enzymatic esterification reactions were performed at three different temperatures (50 °C, 70 °C and 90 °C) using the multipoint reactor Carousel 12 plus reaction station purchased (Radleys, Saffron Walden, UK). Every two hours (2, 4, and 6 h), 10 µL aliquots were collected from each reaction mixture. Each aliquot was subsequently diluted in 10 mL of CHCl₃ and spiked with 10 µL of toluene as an internal standard. In parallel, a blank of each performed reaction was carried out in the absence of the biocatalyst. All the aliquots were then analyzed through gas chromatography (GC) analysis.

3.3.8 Gas Chromatography (GC) analysis

The samples obtained from the enzymatic esterification reaction, and subsequently prepared as described in the section 2.7, were diluted 1:4 by mixing 250 μL of the reaction mixture with 750 μL of CHCl_3 in 1.5-mL HPLC vials. GC analysis was performed over a 45-min run up to 250 $^{\circ}\text{C}$, using CHCl_3 as the needle washing solvent. The GC analysis was conducted using an Agilent Technologies 6890N Network GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a J&W DB-FFAP column (Part No.122-3232; nominal dimension of 30.0 m x 250 μm x 0,25 μm ; 250 $^{\circ}\text{C}$ of maximum operating temperature). The Injector (HO 6890 series) was operated with hydrogen as the carrier gas at a flow rate of 40 mL/min, an airflow rate of 450 mL/min, and nitrogen makeup flow equal to 20 mL/min.

3.3.9 Enzymatic synthesis of polyesters

Enzymatic polycondensation of dimethyl adipate and 1,8-octanediol was performed in solvent-less condition as previously reported by Pellis et al [29]. Equimolar amounts of the two monomers (3 mmol) were mixed in 25-mL round-bottom flask with an amount of immobilized enzyme (1% w/w enzyme/beads), corresponding to 10% in weight referred to as the total amount of monomers (10% w/w enzyme/total monomers weight). Once the enzyme was added, the reaction ran for 6 hours under ambient pressure at the corresponding T_{opt} , previously determined from esterification reactions for all the three enzymes. Afterwards, 25 mL round-bottom flask were connected to a Schlenk line linked to a BÜCHI vacuum pump in order to reduce the pressure at 15 mbar. This reduced pressure was maintained for 18 h (for a total of 24 h of reaction time) both to remove the by-product, MeOH, that was formed during the reaction, and to facilitate the elongation of the polyester.

3.3.10 Polyester characterization: Proton Nuclear Magnetic Resonance (^1H -NMR) and Gel Permeation Chromatography (GPC)

The ^1H -NMR spectra were acquired using a JEOL ECZ-400R/S3 NMR spectrometer equipped with a 5 mm broadband Royal HFX probe. The operating frequency was 400 MHz using deuterated chloroform (CDCl_3) as the solvent. For each measurement, approximately 10 mg of polyester was dissolved in 700 μL of CDCl_3 . Gel permeation chromatography (GPC) analyses were performed using sample solutions prepared at concentration of 2 mg/mL. Analytical-grade THF, stabilized with BHT, was used consistently both as solvent for sample preparation and mobile phase during chromatographic separation. The GPC system operated under the following conditions: an injection volume of 100 μL , a flow rate of 1.00 mL/min and an elution time of 18 min. The column temperature

was maintained at 35 °C with an isocratic Pump pressure of 39 bar. Detection was performed using a refractive index detector set at 35°C and separation was achieved using an Agilent GPC/SEC Plgel 5 µm MIXED-C column (300 x 7.5 mm), equipped with an Agilent Plgel 5 µm Guard Column (50 x 7,5 mm) used as precolumn. The molecular weights of the polyesters were determined from the calibration curve (covering a molecular weight ranging from 532 to 2400000 g/mol), obtained using the Agilent ReadyCal-Kit PSS GPC/SEC Polymer Standards and fitted with a fifth-order polynomial regression curve.

3.4 Results and discussion

3.4.1 Enzyme immobilization on polypropylene beads

LCC, LCC^{ICCG} and Thb were immobilized onto Accurell MP 1000 (polypropylene) beads by employing a biocatalyst loading of 1% w/w relative to the amount of solid support. To assess the efficiency of the immobilization procedure, the protein content and esterase activity were measured in the supernatant collected at defined time-points (0, 1, 2, 4, 8, 24 h) via Bradford Assay and the p-NPB based Esterase Activity Assay, respectively. These measurements allowed to monitor the progressive reduction of enzyme in solutions, thereby confirming its successful adsorption onto the support. As shown in [Figure 1a](#), the enzyme activity declined over time, corresponding to the decrease of free enzyme in the supernatant. Protein quantification results were also expressed as residual protein concentration (%) for the same time-points ([Figure 1b](#)). After 24 h of immobilization, the enzymatic activity in the supernatant was below 2% for LCC and below 1% for LCC^{ICCG} with both enzymes exhibiting residual concentration below 7%, indicating an effective immobilization. Notably, within just 2 h of immobilization, both residual activity and protein concentration for the two enzymes have dropped below 22% pointing out a very similar adsorption pattern onto the support. The comparable adsorption profile of LCC and LCC^{ICCG} was expected since structural integrity was preserved through rational site-directed mutagenesis: the introduced mutation did not alter the catalytic triad of the active site (Ser₁₆₅, Asp₂₁₀ and His₂₄₂) as well as the hydrophobic to hydrophilic ratio on the enzyme's surface ([Figure S3](#)). Briefly, as well documented in the literature [30], in the LCC enzyme structure, the interatomic distances between the C α atoms (4.5 Å) and C β atoms (4.4 Å) of residues Asp₂₃₈ and Ser₂₈₃ were within the range suitable for introducing a disulfide bridge (represented by spheres in [Figure S3](#)). A variant containing the D238C and S283C mutations was created, resulting in a disulfide-bond that enhanced the enzyme's thermal stability, although with reduced activity. To counteract this reduction, the F243I mutation was introduced, yielding the F243I/D238C/S283C (ICC) variant, which showed improved specific activity. Finally, the Y127G mutation was added to further improve thermostability, leading to the development of the quadruple mutant F243I/D238C/S283C/Y127G (ICCG)

[23]. Furthermore, a similar adsorption behaviour was observed also in the case of Thb enzyme, where less than 1% of both residual activity and protein concentration remained in the supernatant after 24 h, indicating that almost all the biocatalyst was effectively adsorbed onto the solid support. In light of these comparable absorption profiles, it was hypothesized that enzyme immobilization efficiency on the hydrophobic polypropylene beads could be influenced by the surface hydrophobicity of the enzyme. To explore this, the composition of surface-exposed amino acids for each enzyme was evaluated by calculating their superficial hydrophobic to hydrophilic ratio (Figure S4). As anticipated above, LCC and its variant LCC^{ICCG} exhibited an identical surface hydrophobicity of 34%; likewise, Thb showed a comparable value of 35%. These findings suggested that the similar immobilization behaviour of the three enzymes could be ascribed to their nearly identical surface hydrophobicity.

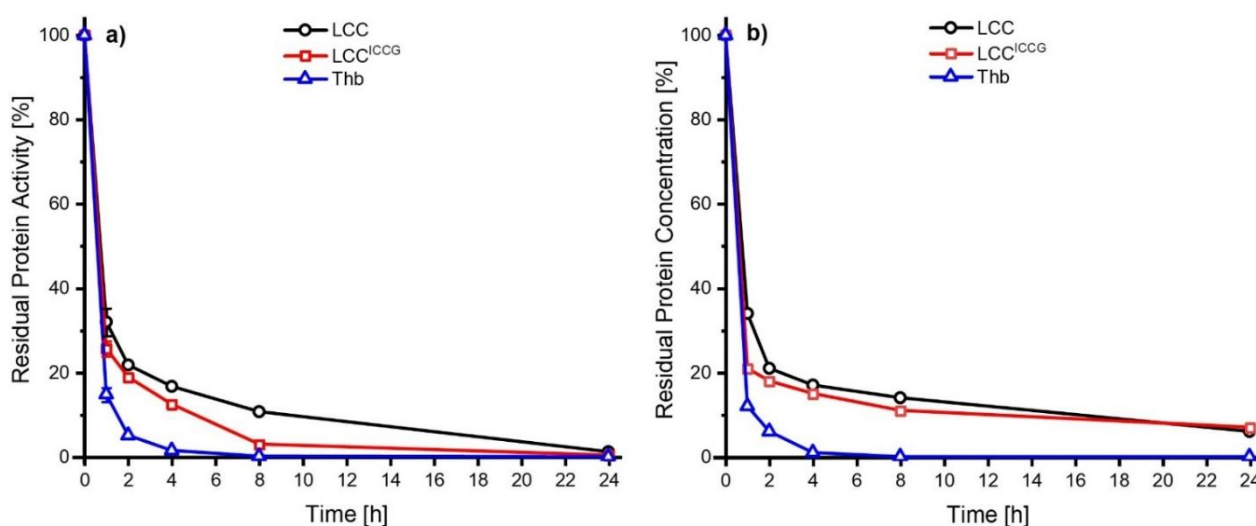


Figure 1: Immobilization of hydrolases LCC, LCC^{ICCG} and Thb on polypropylene beads. Graphical illustration of residual protein activity (a) and residual protein concentration (b) of the different timepoint supernatants for LCC (black circles), LCC^{ICCG} (red squares) and Thb (blue triangles) respectively. All the measurements for the three enzymes were performed in triplicates with standard deviation (σ) < 0,05 in graph a while < 0,4 in graph b with the exception of $t = 1$.

3.4.2 Design of experiment (DoE) assisted study

The potential of LCC, LCC^{ICCG} and Thb in synthetic reactions has not been assessed previously, despite their noted effectiveness in polyester degradation. Therefore, a full factorial experimental design was developed using MODDE® Pro 13 Software to study the thermostability and substrate selectivity of the three enzymes in short-esters synthesis reactions using alcohols and diacids with varying chain length (alcohol chain length = C4, C8, and C12; acid chain length = C4, C8 and C12). As discussed in section 2.7, the DoE was employed to systematically investigate the overall influence of four selected independent variables (temperature, alcohol chain length, acid chain length and reaction

time) on the resulting outcome, with acid conversion rate (measured via GC analysis) serving as response variable for each experimental condition. Moreover, the list of reactions carried out for each enzyme as part of the DoE is reported in Table S1, while figure 2 provides a schematic overview of short-ester synthesis reaction. Upon completion of each DoE reaction, the results of the experiments were graphically evaluated using a 4D contour plot, provided by the software for each enzyme. Model coefficients are plotted along the axes with temperature on the horizontal-axis and acid chain length on the vertical-axis, while the reaction times and the alcohol chain lengths are held constant for each of the nine final profiles that constitute the 4D contour response. The efficiency of the reaction in terms of acid conversion [%] is represented by a color-coded gradient, ranging from blue for lower conversions to red for the highest.

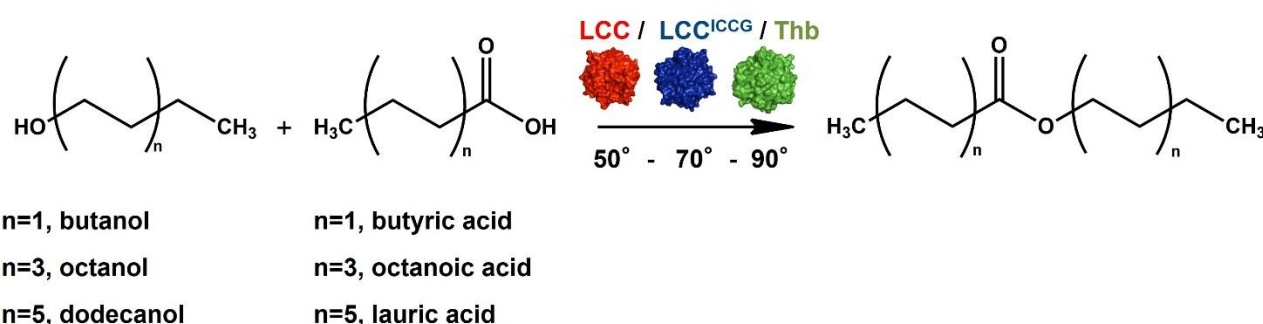


Figure 2: Schematic overview of short-ester synthesis reaction: LCC/ LCC^{ICCG}/Thb-catalyzed synthesis using C4, C8, and C12 alcohol and acid to obtain C8, C12, C16, C20 and C24 esters.

3.4.2.1 LCC-catalyzed esterification reaction: 4D contour response analysis

The LCC 4D Contour Response showed that, as expected, the conversion continued to increase within the time frame explored (6 h), indicating that the enzyme preserved its activity even at high temperatures. As visible from Figure 3, similarly to previously reported trends for CaLB and HiC [20], LCC revealed a clear preference for long-chain alcohols and acids under identical experimental conditions, despite exhibiting comparatively lower conversion rates (~40% efficiency reduction with respect to the commercial hydrolytic enzymes CaLB and HiC where > 90% of monomers conversion were reported [20]. Specifically, when C12 alcohol was used, conversion rates over 55% were achieved after 6 h. On the other hand, aligning with the previously reported CaLB and HiC selectivity pattern [20], when reacting with short-chain alcohols and acids (C4 or C6 chain length), the enzyme showed lower conversion rates (below 30%) even after 6 h of reaction with ~50% efficiency reduction with respect to long-chain alcohol and acids. This behaviour contrasts with that of Thc_Cut1, which exhibited a substrate specificity favouring short-chain alcohols (C4) and medium-chain acids (C8) although showing low overall conversion rates [20], thereby underscoring the different selectivity between these enzymes.

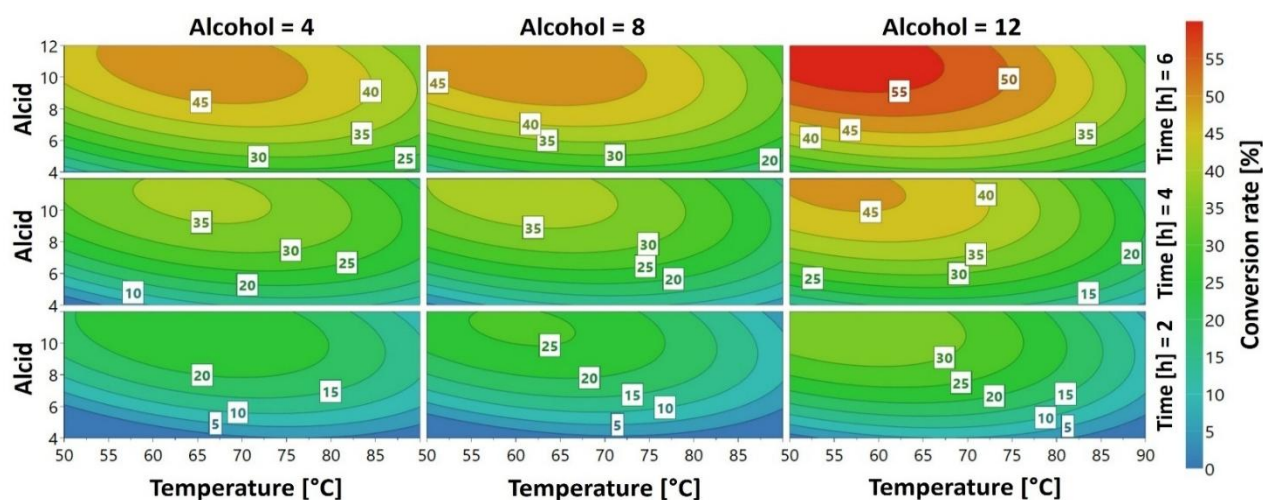


Figure 3: Synthesis of C8, C12, C16, C20 and C24 esters from alcohols and acids of varying chain length: LCC 4D contour response. Every graph illustrates a specific alcohol chain length (with 4, 8 and 12 carbon atoms) at a distinct reaction time (2, 4 and 6 h). The Y-axis corresponds to acid chain length (varying from C4 to C12) while the X-axis represents the Temperature [°C]. Conversion rates (values in the white square) are indicated using different colors, as indicated in the legend on the right.

Moreover, Figure 4 provides additional evidence for LCC's selectivity towards long-chain alcohols and acids demonstrating that the highest efficiency (~69% of conversion rate) was reached using C12 alcohol (1-dodecanol) combined with C12 acid (lauric acid). Furthermore, LCC exhibited its optimal catalytic activity within the 50-60°C range, with a significant reduction in conversion efficiency observed at temperatures above 75°C. To more precisely define LCC optimal temperature (T_{opt}), additional reactions using the most efficient substrate combination (alcohol = 12 with acid = 12) were carried out at selected temperatures in the 50-70°C range (specifically 55°C, 60 °C and 65°C). The highest conversion (~78%) was observed at 60 °C, as reported in Figure 4d, corresponding to the T_{opt} of the enzyme.

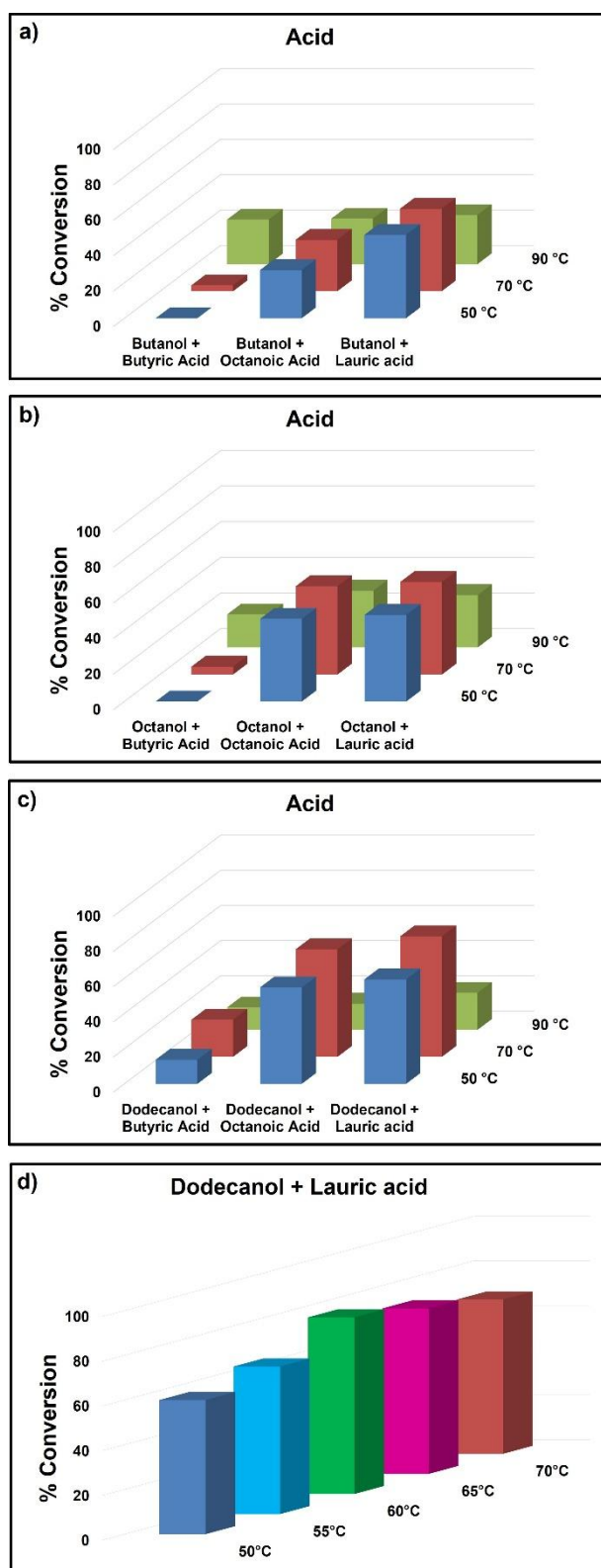


Figure 4: Representation of LCC efficiency, expressed in terms of acid conversion [%] in short-esters synthesis reaction carried out at 50-70-90 °C combining a) C4 alcohol with C4, C8 and C12 acid b) C8 alcohol with C4, C8 and C12 acid c) C12 alcohol with C4, C8 and C12 acid. Reaction time of these graphs = 6 hours; d) Representation of LCC efficiency in the most efficient substrate combination (1-Dodecanol with Lauric acid) carried out in the temperature range between 50-70°C (specifically 55°C, 60 °C and 65°C).

3.4.2.2 LCC^{ICCG}-catalyzed esterification reaction: 4D contour response analysis

The 4D contour response derived from the DoE using LCC^{ICCG} revealed a pattern similar to that observed for LCC. As illustrated in [figure 5](#), LCC^{ICCG} exhibited a clear preference for long-chain alcohols and acids. Notably, a conversion rate exceeding 55% was achieved after 6 h of reaction when using the 12 carbon-chain alcohol. In the case of C8 acid, a ~35% efficiency reduction moving from lower to higher temperature was observed. Conversely, as visible in the upper-left panel of [figure 5](#), LCC^{ICCG} showed limited conversion rates (below 15%) when processing short-chain alcohols and acids (4 and 6 carbon chain length) even after 6h of reaction, with ~75% efficiency reduction than long-chain alcohol and acids.

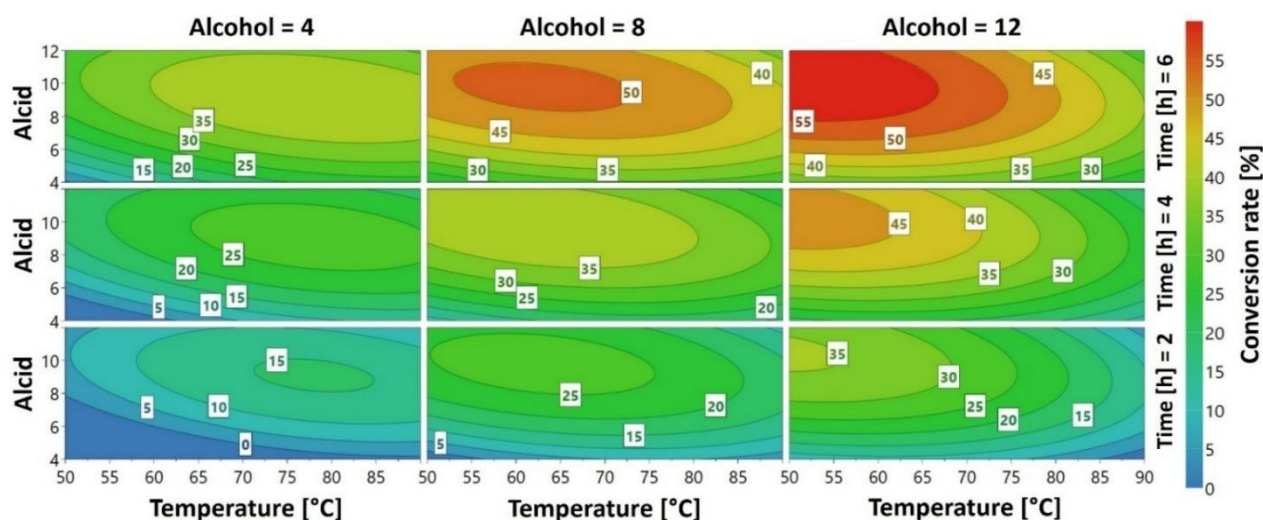


Figure 5: Synthesis of C8, C12, C16, C20 and C24 esters from alcohols and acids of varying chain length: LCC^{ICCG} 4D contour response. Every graph illustrates a specific alcohol chain length (with 4, 8 and 12 carbon atoms) at a distinct reaction time (2, 4 and 6 h). The Y-axis corresponds to acid chain length (varying from C4 to C12) while the X-axis represents the Temperature [°C]. Conversion rates (values in the white square) are indicated using different colors, as indicated in the legend on the right.

Furthermore, [Figure 6](#) demonstrates LCC^{ICCG}'s preference for long-chain alcohols and medium/long-chain acids. Interestingly, although LCC and LCC^{ICCG} exhibited a similar trend with comparable conversion efficiency for this class of substrates, the mutant variant achieved the higher conversion rate (~68%) when using 1-dodecanol (C12 alcohol) and octanoic acid (C8 acid). In terms of thermostability, LCC^{ICCG} showed optimal catalytic activity in the 50-60 °C temperature range. A noticeable reduction in conversion efficiency occurred above 75 °C, in agreement with results obtained for LCC. To accurately determine the T_{opt} of LCC^{ICCG}, a series of reactions were conducted using the most effective substrate combination (alcohol = 12 with acid = 8) focusing on selected temperatures in the 50-70 °C range (specifically 55 °C, 60 °C and 65 °C). As visible from [Figure 6d](#) maximum conversion rate (~74%) was achieved at 55 °C, defining it as LCC^{ICCG} T_{opt}.

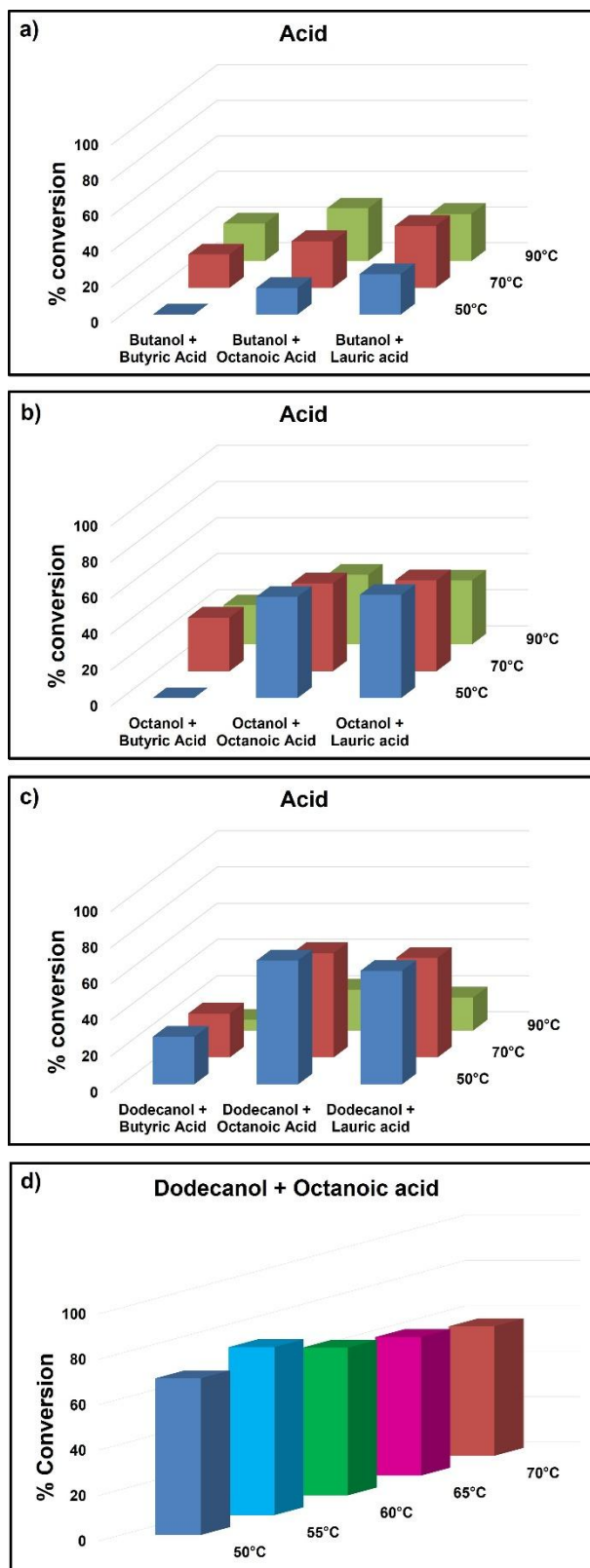


Figure 6: Representation of LCC^{ICCG} efficiency, expressed in terms of acid conversion [%] in short-esters synthesis reaction carried out at 50-70-90 °C combining a) C4 alcohol with C4, C8 and C12 acid b) C8 alcohol with C4, C8 and C12 acid c) C12 alcohol with C4, C8 and C12 acid. Reaction time of these graphs = 6 hours; d) Representation of LCC^{ICCG} efficiency in the most efficient substrate combination (1-Dodecanol with Lauric acid) carried out in the temperature range between 50-70°C (specifically 55°C, 60 °C and 65°C).

Notably, LCC^{ICCG} exhibits enhanced thermostability for PET degradation, functioning efficiently at elevated temperatures [28]. However, during synthetic reactions performed in this study, its optimal activity shifted to lower temperatures. This difference reflects the distinct stability and activity requirements between hydrolytic and synthetic conditions. These results on thermal stability and substrate selectivity suggest that the engineered mutations may contribute to slight alterations in enzymatic behaviour between the LCC and LCC^{ICCG} variants. While the catalytic triad of the active site (Ser₁₆₅, Asp₂₁₀ and His₂₄₂) is conserved, the introduced D238C and S283C mutations, associated with the formation of a disulfide-bond as mentioned above in the section 3.1, as well as F243I mutation are situated in close proximity to the active site (Figure S5). It is hypothesized that these structural alterations are likely to induce conformational rearrangements around the active site, thereby potentially affecting both substrate selectivity and thermostability, not only in depolymerization reactions but also in esterification synthesis.

3.4.2.3 Thb-catalyzed esterification reaction: 4D contour response analysis

Also, in the case of Thb, the conversion rate continued rising across the 6-hour reaction period, indicating sustained enzymatic activity over time at high temperature. However, as visible from Figure 7, the resulting conversions were significantly lower compared to those achieved with LCC and LCC^{ICCG}. The highest conversion was above 11% after 6 hours, although no clear preference towards a specific substrate was observed. Figure S6 further supports the absence of substrate selectivity, as reflected by the consistently similar conversion values across the tested substrate combinations. Specifically, Thb achieved nearly identical conversions when combining C12 alcohol with either C8 or C12 acid (~18%), C8 alcohol with the same acids (~16%), and even C4 alcohol with C8 or C12 acids (~15%). In the case of short-chain acid (C4), conversion varied between 5% and 11% (up to ~12% processing C12 alcohol with C4 acid), with higher efficiency observed at higher temperature. Unlike LCC and LCC^{ICCG}, it was not meaningful to determine precisely at which temperature Thb exhibited its optimal activity, due to the minimal variation in conversion efficiency across all the tested temperatures (just ~6% of variation in the entire 50-90°C range). However, the maximum conversions were reached in the temperature range between 75 and 90 °C. Although Thb showed limited efficiency in short-esters synthesis, this high thermal stability makes it a promising candidate for further synthetic applications, particularly in polycondensation reaction where the T_{opt} will be accurately investigated.

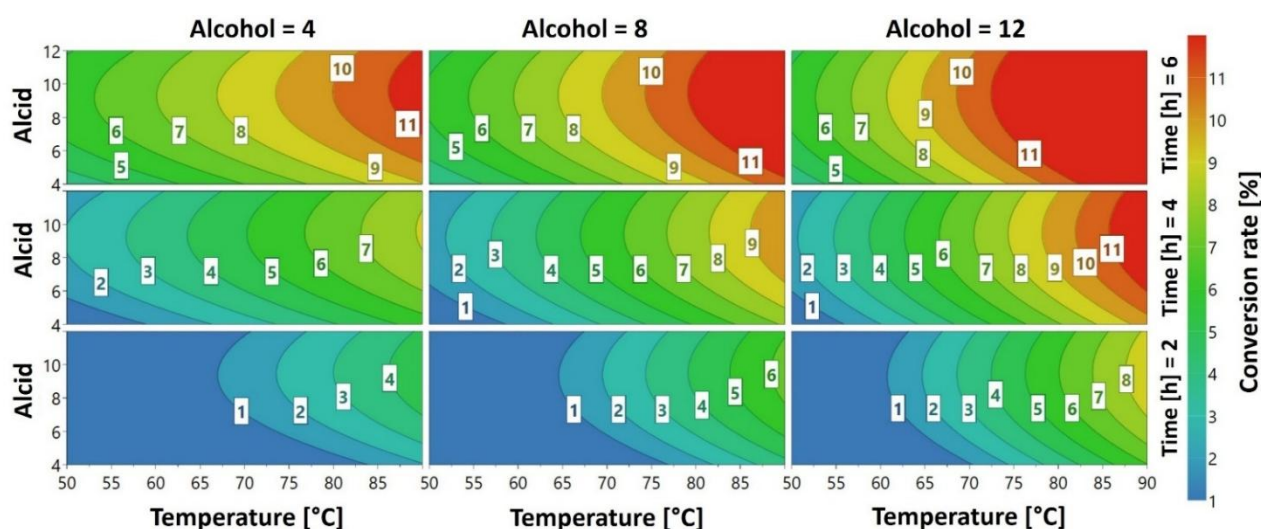


Figure 7: Synthesis of C8, C12, C16, C20 and C24 esters from alcohols and acids of varying chain length: Thb 4D contour response. Every graph illustrates a specific alcohol chain length (with 4, 8 and 12 carbon atoms) at a distinct reaction time (2, 4 and 6 h). The Y-axis corresponds to acid chain length (varying from C4 to C12) while the X-axis represents the Temperature [°C]. Conversion rates (values in the white square) are indicated using different colors, as indicated in the legend on the right.

3.4.3 LCC/LCC^{ICCG}/Thb-catalyzed polycondensation reactions

Short-esters synthesis reactions were conducted to define the optimal synthetic conditions of the three enzymes. To assess the applicability of the developed 4D contour models also in the production of larger macromolecules, the three hydrolases were also compared in polycondensation reactions. In particular, to evaluate the potential of alternative biocatalysts to CaLB in synthetic applications, the new enzymes were analyzed using the monomer combination of dimethyl adipate (DMA) and 1,8-octanediol (ODO), previously identified as the most effective for CaLB-catalyzed polycondensation [7]. Based on this, enzyme catalyzed reactions were conducted at the corresponding T_{opt} of LCC (60 °C) and LCC^{ICCG} (55 °C) using DMA and ODO as biobased precursors for polyester synthesis (figure S7). While the T_{opt} of Thb was precisely determined by performing the same reaction in the 75-90 °C range. The progress of the reaction was monitored by using ¹H-NMR spectroscopy and Gel Permeation Chromatography (GPC). For all the three enzymes, analysis of the ¹H-NMR spectra revealed a similar extent of monomer conversion. As visible from figure 8a, this was evidenced by the intensity reduction in the signal at 3.6 ppm, corresponding to the disappearance of both the -CH₂-CH₂-OH group from the diol and of the -OCH₃ group from the diester, ascribed to the release of MeOH as by-product. Concurrently, an intensity increase in the signal at 4.1 ppm was observed as clear indication of the formation of -CH₂-CH₂-O-C=O ester bond. As expected, comparable monomer conversion rates (~91%), M_n (~3600 Da) and degree of polymerization (~14) were achieved using LCC and LCC^{ICCG} respectively, as shown in Figure 8b and Table S2. These results demonstrated their selectivity towards long-chain diols, confirming the trend seen for the synthesis of short-esters

(Section 3.2 and Section 3.3). When compared to literature reports on enzymatic polymerization of DMA and ODO catalyzed by CaLB and HiC immobilized on polypropylene beads at their corresponding T_{opt} (85°C for CaLB and 70°C for HiC) [20], LCC and LCC^{ICCG} exhibited comparable monomer conversion rates (~6% lower than CaLB and ~5% higher than HiC). Conversely, GPC analysis revealed a M_n of approximately 3600 Da for both LCC and its variant form, which is significantly lower than the 8100 Da achieved using CaLB (~57% M_n reduction), but notably higher than the 1400 Da reported for HiC (~60% M_n increase).

In a next step, enzymatic polycondensation of DMA and ODO was performed using Thb to assess precisely its T_{opt} . The same reaction was carried out focusing on selected temperatures between 75 and 90 °C (specifically 75 °C, 80 °C, 85 °C and 90 °C), predicted as the optimal range from the previously proven model on short-esters synthesis reactions (Section 3.3). As visible from figure S8, the T_{opt} turned out to be at 80 °C, where both monomer conversion rate and M_n were higher than the other temperatures (92% and M_n of 3800 Da) in which Thb exhibited a reduced activity (54%, 62% and 23% of conversion rate while 1250, 1060 and 840 Da at 75°C, 85°C and 90°C respectively). Unexpectedly, ¹H-NMR and GPC analysis (figure 8a and b) revealed that the highest conversion rate of approximately 92% and with a M_n of 3800 Da was achieved at 80 °C, comparable to the values seen for the two LCC variants at a temperature of 60 and 55 °C. Therefore, contrary to the poor efficiency in short-esters synthesis, Thb has been demonstrated to preferentially catalyze reactions involving long-chain diols and diesters in synthetic applications. To the best of our knowledge, such behaviour has not been documented yet and is in contrast with previously published data reporting higher enzymatic efficiency in short-esters synthesis [31].

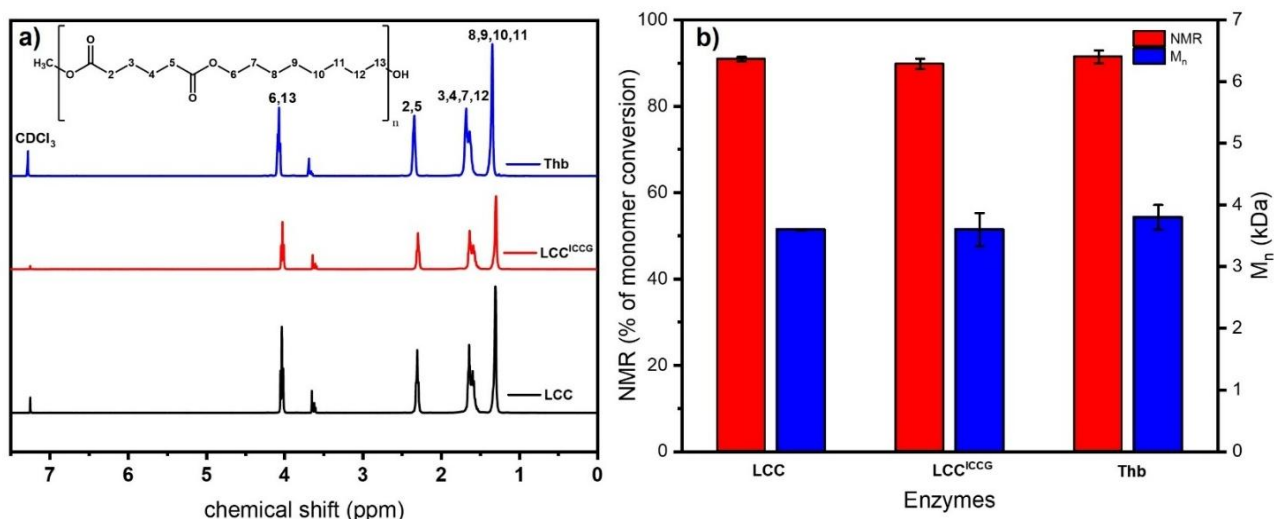


Figure 8: a) ¹H-NMR spectra of poly(1,8-octylene adipate) synthesized using LCC (black), LCC^{ICCG} (red) and Thb (blue) performing the reaction at their corresponding T_{opt} ; b) progression of reactions monitored via ¹H-NMR (% of monomer conversion, red bars) and GPC (number average molecular weight, blue bars) for the three enzymes. All the measurements for the three enzymes were performed in triplicates: the figure shows the mean with standard deviation (σ) < 1,5 % of monomer conversion rate while < 0,3 Da for M_n .

3.5 Conclusions

In summary, LCC, LCC^{ICCG} and Thb were effectively immobilized onto polypropylene beads. A full factorial design of experiments (DoE) was used to determine their optimal synthetic conditions. Both LCC and LCC^{ICCG} demonstrate a clear preference for long-chain alcohols and medium/long chain acids with T_{opt} of 60 °C and 55 °C, respectively. On the other hand, Thb exhibits its highest catalytic activity efficiency at 80 °C, confirming its thermophilic nature, although without showing any selectivity in terms of substrate. To investigate their applicability in the synthesis of higher molecular weight polyesters, the T_{opt} of the three enzymes was tested in polycondensation reactions using dimethyl adipate and 1,8-octanediol as biobased building blocks. The three enzymes catalyzed formation of polyesters with comparable monomer conversion rates and M_n values (91% and 3600 Da for LCC and LCC^{ICCG} vs 92% and 3800 Da for Thb). In conclusion, these results underscore the promising capabilities of these thermophilic enzymes in polyester synthesis, expanding the application of PET hydrolases from depolymerization to synthetic processes. Although exhibiting an approximate 57% reduction in M_n when compared to CaLB, their overall catalytic performance, especially in comparison to HiC and Thc_Cut1, demonstrates their value as viable alternatives for sustainable biocatalyzed synthesis of polyesters.

3.6 References

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3.7 Electronic Supplementary Information

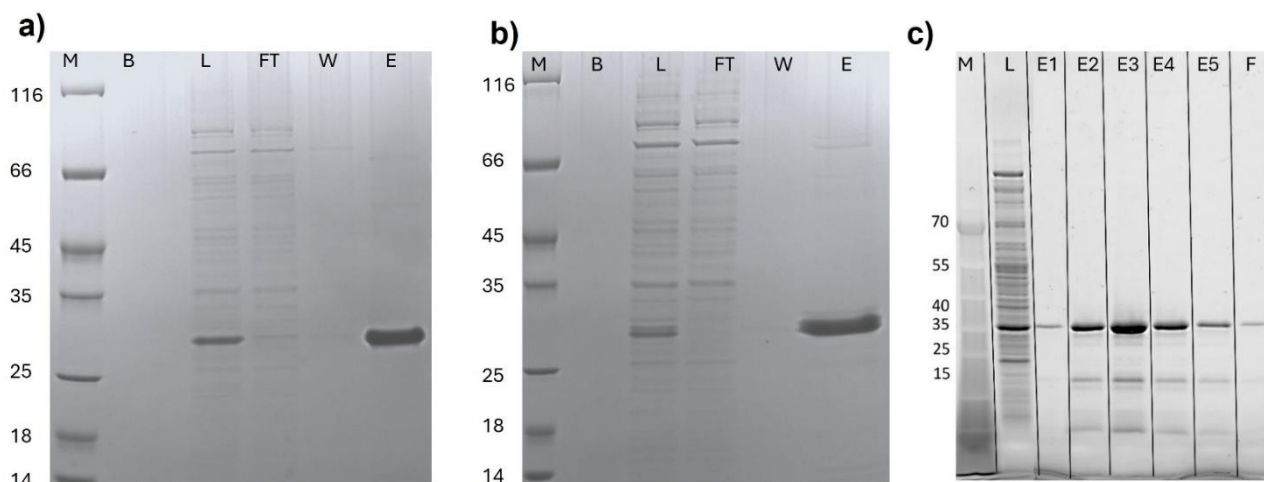


Figure S1: SDS-Page analysis of LCC (a), LCC^{ICCG} (b) and THB (c) to assess the purification of the expressed proteins from *Escherichia coli* supernatant (LCC and LCC^{ICCG}) and THB from the biomass. (M=marker; B=blank; L= Load; FT=flowthrough; W=wash; E, E1, E2, E3, E4, E5 =elution fraction; F= final fraction after collection and buffer exchange).

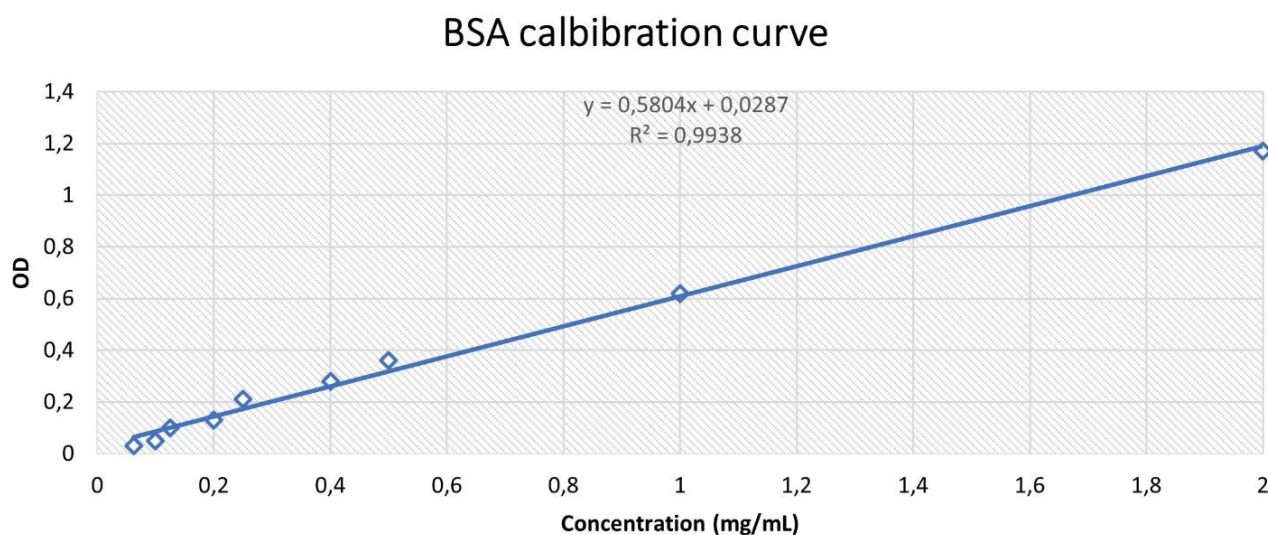


Figure S2: Calibration curve using BSA as standard protein

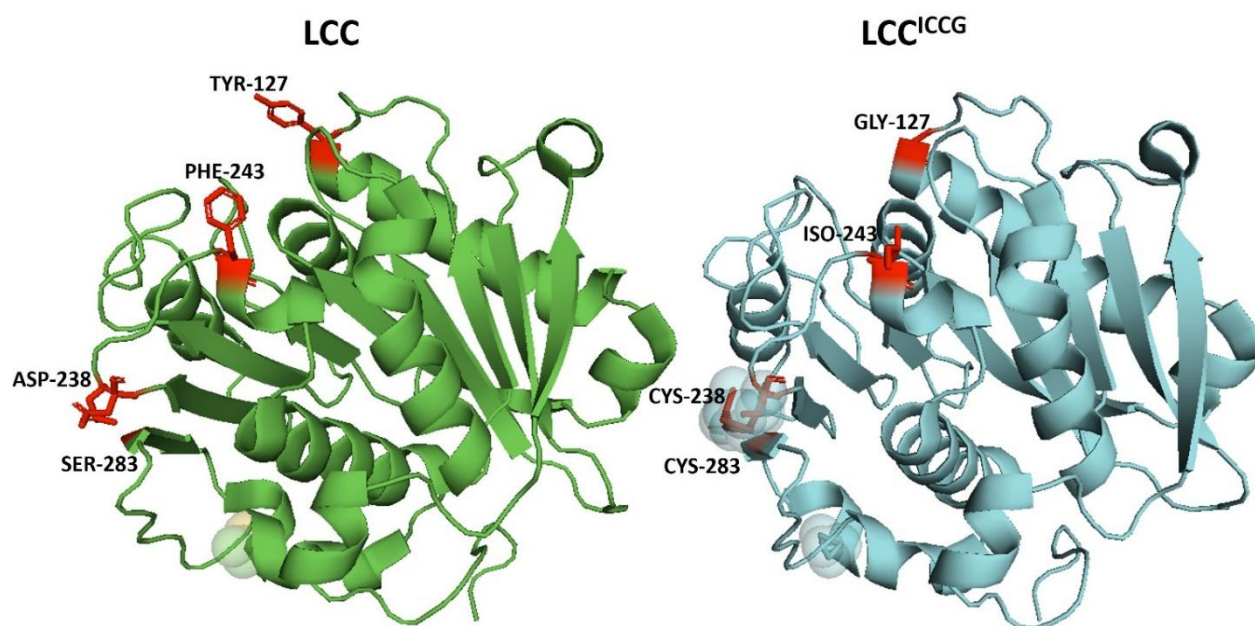


Figure S3: Structural representation of leaf-branch compost cutinase (LCC) and its mutant form (LCC^{ICCG}). In red the four introduced mutation through site-directed mutagenesis

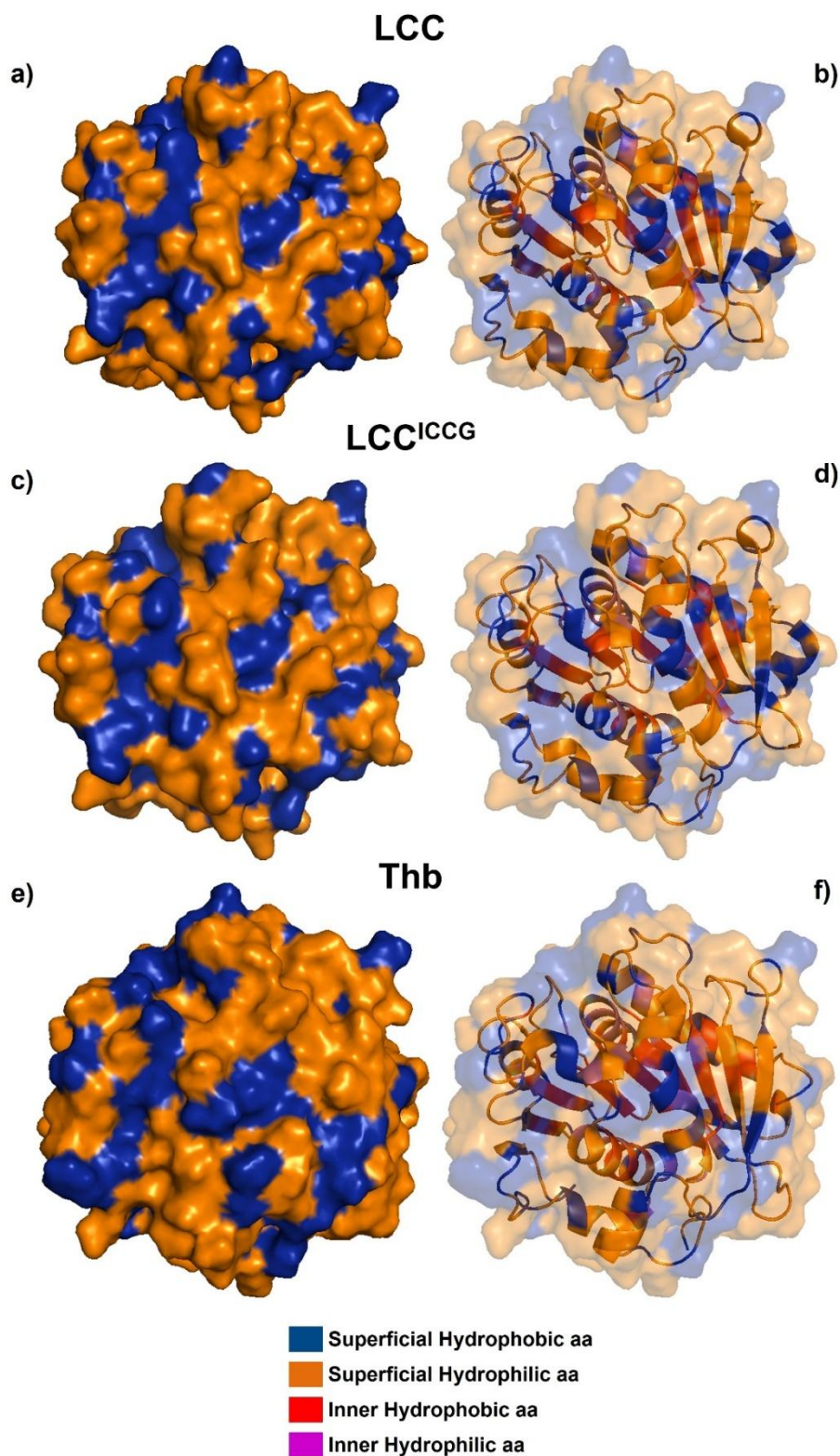


Figure S4: Surface representation of LCC (a), LCC^{ICCG} (c) and Thb (e) with the superficial amino acids components depicted of different colors (blue = hydrophobic; orange = hydrophilic). Inner representation of LCC (b), LCC^{ICCG} (d) and Thb (f) with amino acids residues depicted of different colors (red = hydrophobic; magenta = hydrophilic). PDB code for enzymes: 4eb0 for LCC, 6tbt for LCC^{ICCG} and 7nei_a for Thb.

Table S1: List of the reactions carried out with each enzyme.

	Alcohol	Acid	Temperature (°C)
1	C4	C4	50
2	C4	C4	70
3	C4	C4	90
4	C8	C4	50
5	C8	C4	70
6	C8	C4	90
7	C12	C4	50
8	C12	C4	70
9	C12	C4	90
10	C4	C8	50
11	C4	C8	70
12	C4	C8	90
13	C8	C8	50
14	C8	C8	70
15	C8	C8	90
16	C12	C8	50
17	C12	C8	70
18	C12	C8	90
19	C4	C12	50
20	C4	C12	70
21	C4	C12	90
22	C8	C12	50
23	C8	C12	70
24	C8	C12	90
25	C12	C12	50
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28	C8	C8	70
29	C8	C8	70
30	C8	C8	70

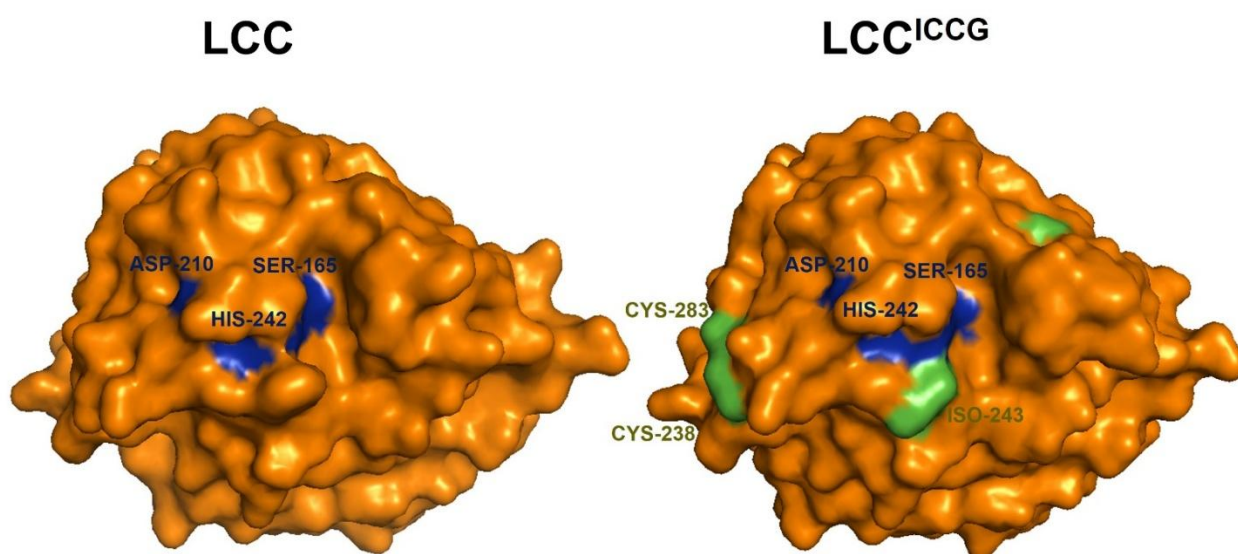


Figure S5: Representation of the D238C, and S283C and F243I mutations (depicted in green) situated in close proximity to the active site Ser₁₆₅, Asp₂₁₀ and His₂₄₂ (depicted in blue).

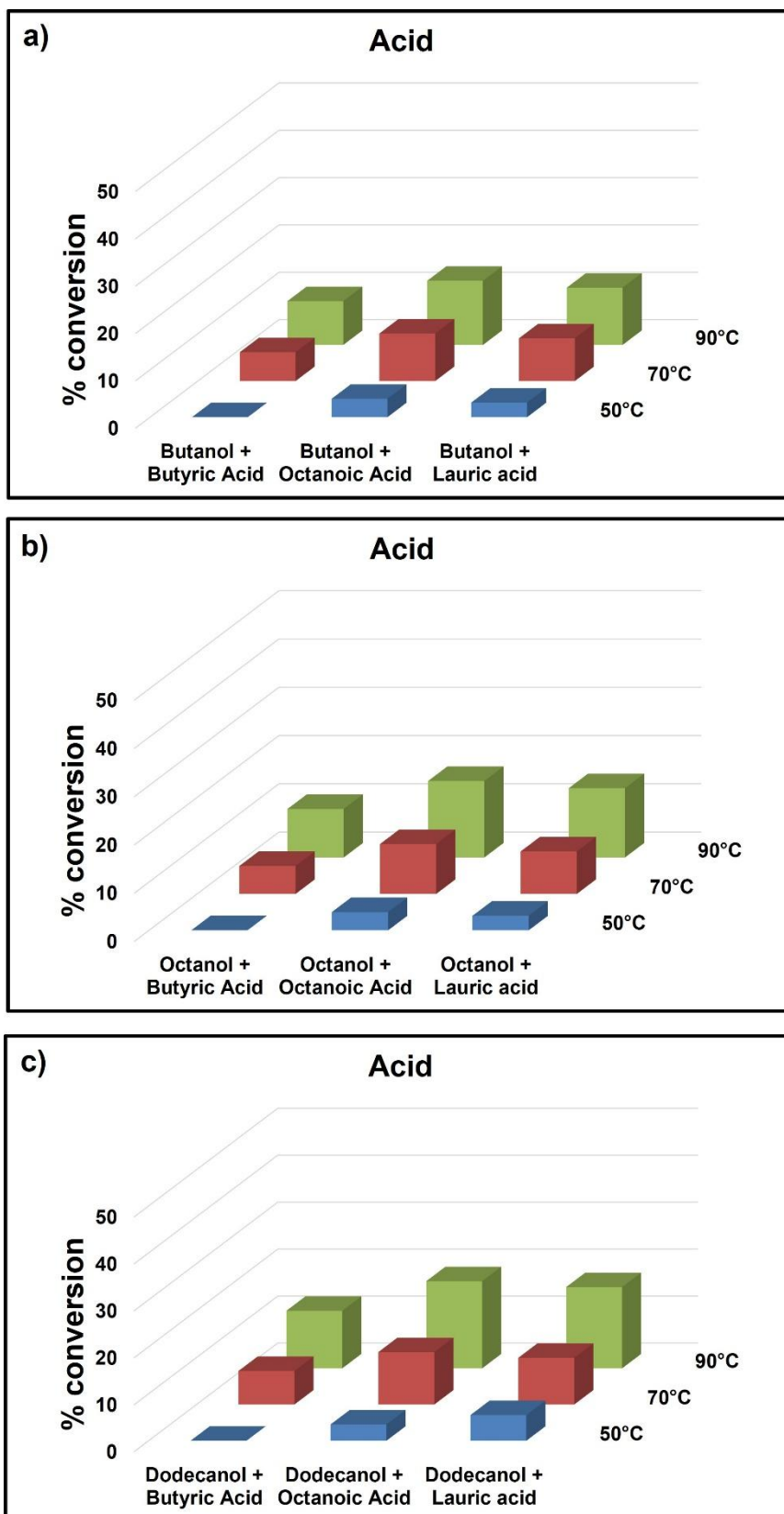


Figure S6: Representation of *Thb* efficiency, expressed in terms of acid conversion [%] in short-esters synthesis reaction carried out at 50-70-90 °c combining a) C4 alcohol with C4, C8 and C12 acid b) C8 alcohol with C4, C8 and C12 acid c) C12 alcohol with C4, C8 and C12 acid. Reaction time of these graphs = 6 hours.

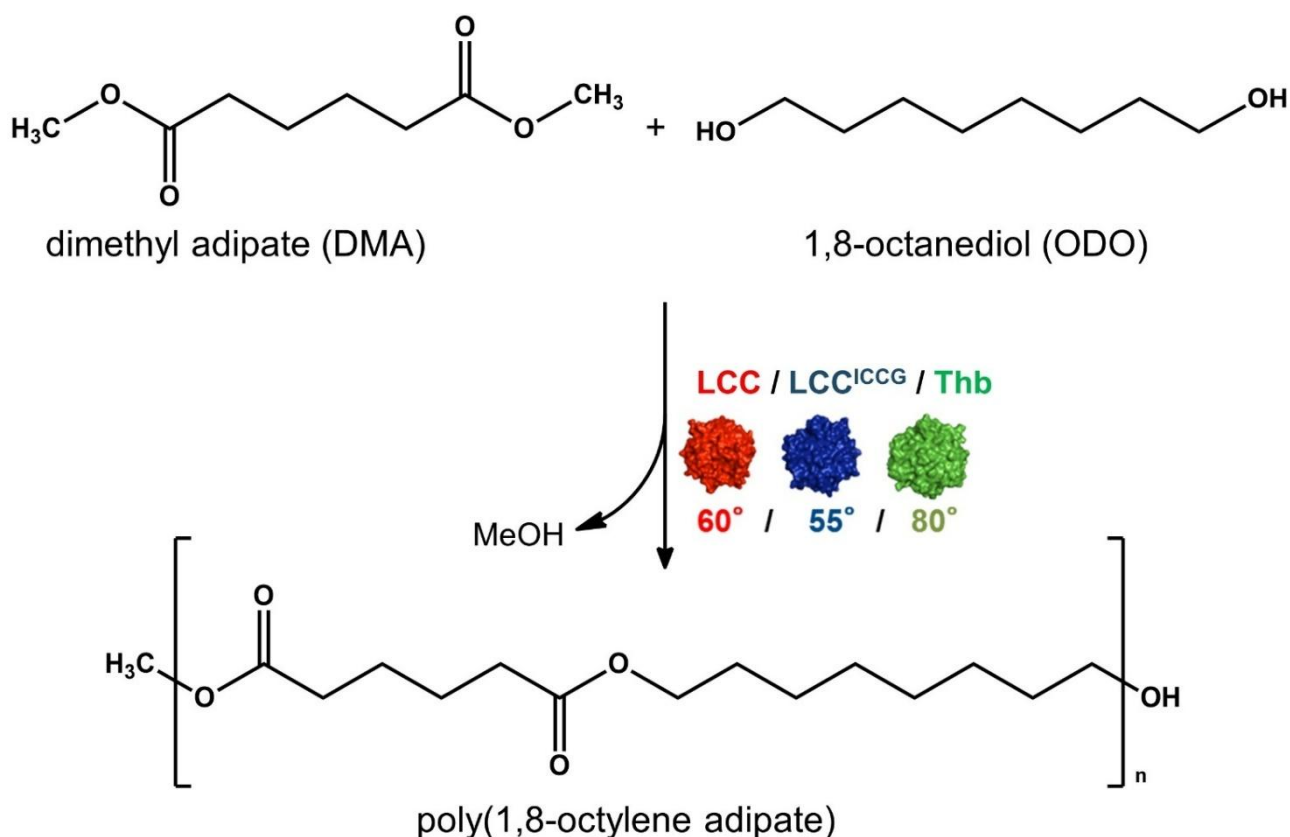


Figure S7: Schematic representation of polycondensation reaction; the corresponding T_{optimum} of each enzymes (specifically 60°C for LCC; 55 °C for LCC^{ICCG}; 80°C for Thb) was tested in the synthesis of aliphatic polyester using dimethyl adipate (DMA) and 1,8-octanediol as building blocks.

Table S2: Comparison between % of monomer conversion and M_n for the three enzymes for reaction (in triplicates)

Enzyme	% of monomer conversion	M_n (KDa)	M_w (KDa)	Dispersity (Đ)
LCC	91.0 (± 0.5)	3.6 (± 0.02)	5.7 (± 0.2)	1.62
LCC ^{ICCG}	90.0 (±1.2)	3.6 (± 0.3)	5.2 (± 0.1)	1.45
Thb	92.0 (± 1.5)	3.8 (± 0.2)	5.9 (± 0.3)	1.55

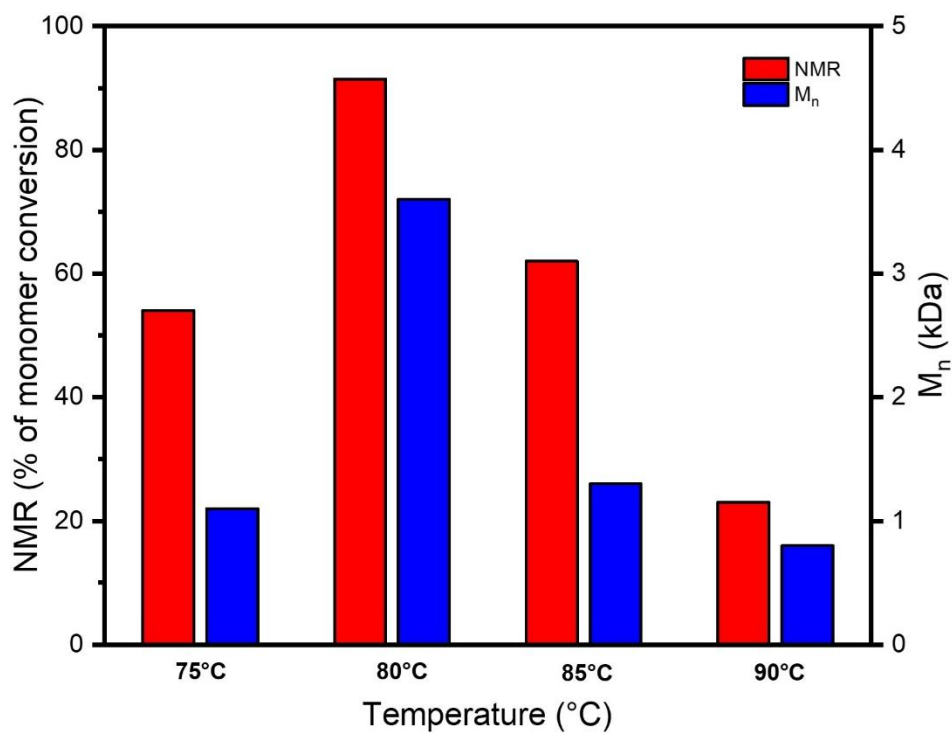


Figure S8: ^1H -NMR and GPC results of enzymatic polycondensation of DMA and ODO at selected temperature (75, 80 and 90°C) to precisely determine the T_{optimum} of Thb.

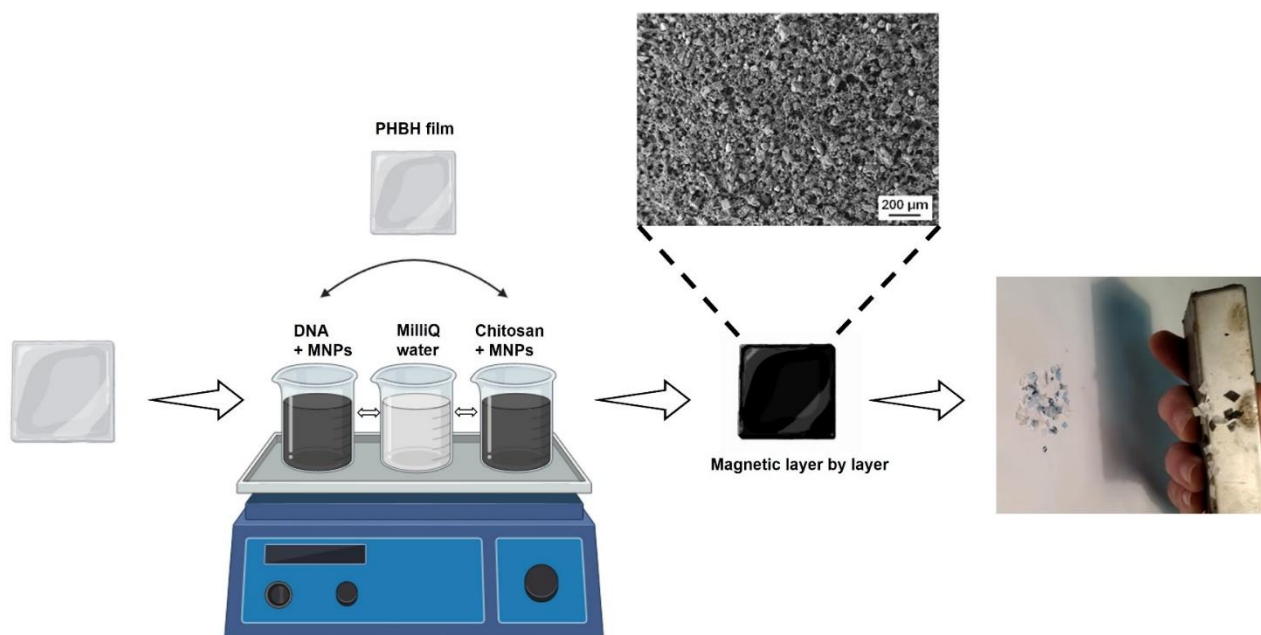
Chapter 4: Easily sortable PHBH films functionalized via a magnetic Layer-by-Layer coating deposition

4.1 Abstract

This work presents an eco-friendly and easily scalable process to modify the surface of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) films, a bioplastic of relevant application interest, making them magnetically active while preserving the intrinsic properties of the polymer. To achieve this, ~10 nm spinel iron oxide magnetic nanoparticles (MNPs), synthesized via coprecipitation method, were assembled using Layer-by-Layer (LbL) deposition with two bio-sourced polyelectrolytes: DNA (polyanion) and chitosan (polycation). An aminolysis reaction was employed to strengthen the interactions between the polymer substrate and the first coating layer. Additionally, the optimal reaction time was determined to maximize surface amine functionalization while minimizing film degradation. The effectiveness of the deposition was demonstrated by both the linear growth of the LbL assembly on a model silicon substrate using FT-IR measurements and by studying the morphology of the coated PHBH films through FE-SEM. These latter measurements showed the formation of a uniform coating after the deposition of 10 bilayers (BL). The 10 BL coated films demonstrated efficient magnetic separation from a mixed polymer waste scraps under a static applied magnetic field. Moreover, these materials undergo enzymatic degradation, with the MNPs that could be easily recovered from the enzymatic solution via magnetic separation, enabling their potential reuse. The proposed approach offers an alternative strategy aimed at tackling the issue of plastic contamination and material sorting during recycling.

Keywords: poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH), magnetic nanoparticles, Layer-by-Layer deposition, DNA, Chitosan, enzymatic depolymerization

4.1.1 Graphical abstract



Preparation of easily sortable functionalized PHBH films via an ecofriendly magnetic layer by layer coating deposition. Environmentally friendly reagents, i.e., DNA (polyanion) and chitosan (polycation), were used in the process after surface functionalization with amino groups and magnetic nanoparticles were incorporated to create magnetic films enabling an efficient separation from other polymer objects during recycling.

4.2 Introduction

The exploitation of biodegradable polymers from renewable sources requires increasing efforts to modify their properties and thus expand their areas of application. Novel strategies that might enable the easy separation of these bio-based materials from other plastics (mostly fossil-based) during recycling are of fundamental importance to ensure an effective recycling of the polymeric materials. Another aspect that must be considered when developing bioplastics-based systems is the need to preserve their unique environmentally positive features such as their biodegradability. This work addresses all the above-mentioned aspects by focusing on a potentially large-scale applicable biopolymer, namely poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH). The PHBH is a copolymer belonging to the polyhydroxyalkanoates (PHAs) family, consisting of two monomeric units: 3HB (3-hydroxybutyrate, short-chain unit) and 3HH (3-hydroxyhexanoate, medium-chain unit). When copolymerized, these monomers impart distinct thermal and mechanical properties to the resulting polymer, improving the overall material performance when compared to other biopolymers. For example, the polyhydroxybutyrate that is obtained from the homo-polymerization of 3-hydroxybutyrate units is biodegradable and has similar properties to polypropylene (PP) and low-density polyethylene (LDPE) [1], but suffers from processability constraints due to its high melting temperature and relatively low degradation temperature. On the other hand, 3HH, derived from 3-hydroxyhexanoic acid, displays a longer side chain than 3HB. This enhances the flexibility and toughness of PHBH by reducing crystallinity and lowering the melting point. Such features allow for a wider processing window with improved thermal stability [2]. In addition, by tailoring the composition of both highly crystalline (3HB) and elastomeric (3HH) units, PHBH copolymers can display tunable mechanical properties [3]. Therefore, thanks to its versatility, PHBH might find be employed in manufacturing processes such as: injection molding [4], extrusion [5] [6], thermoforming [7] [8], foaming [9], non-woven fabrics and fibers manufacturing [10] [11], 3D printing [12], paper and fertilizer coating [13]. Besides the processing features, it is also important to consider some peculiar aspects related to the synthesis/availability of PHBH that make it an extremely promising biopolymer. Indeed, PHBH is synthesized within various microorganisms, depending on factors like microorganism type, environmental conditions, and carbon sources such as amino acids, sugars, and fatty acids. Recently, advances in synthetic biology have led to the development of genetically engineered microorganisms, such as *Escherichia coli*, which can produce PHBH directly from glucose [14] or lauric acid [15], offering faster growth rates and easier purification compared to wild-type microorganisms [16]. Several companies, such as Kaneka Corporation in Japan, which is currently producing commercial PHBH grades, have demonstrated that large scale production and

broad market introduction are possible [1]. It is therefore clear that further improvements in the functional properties of PHBH could expand its use as long as its specific characteristics are not altered, as previously underlined.

A possible strategy implies the use of a surface modification approach such as the layer-by-layer (LbL) technique, which is the method used in this work. The LbL assembly allows for the fabrication of multilayered coatings from the alternate adsorption from the liquid phase of two or more compounds characterized by a specific interaction. The electrostatic attraction occurring between polycations and polyanions in aqueous solutions is the most employed interaction. The simpler LbL assembly alternates two compounds in positively and negatively charged layers depositing, at each cycle, a bilayer (BL). The number of deposition cycles, and therefore BL, defines the thickness of the deposited coating [17]. The LbL assembly has gained considerable interest due to its versatility in coating design and its sustainable features [18], which rely on the use of water as a solvent and mild deposition conditions. In addition, by fine-tuning the deposition parameters, a plethora of components can be assembled to address a wide range of properties e.g. water repellency [19], controlled drug release [20] [21], oxygen barrier [22], sensing [23], antimicrobial [24], and flame retardancy [25].

Beside polyelectrolytes, nanoparticles (NPs) can be easily implemented in a LbL assembly to improve the mechanical [26], thermal [27], and barrier properties [28] of the substrate. In recent years, the LbL approach has been widely applied to biopolymers such as polycaprolactone (PCL) [29] [30] and polylactic acid (PLA) [31] [32]. For the latter, the method was used to improve performance in areas such as drug delivery and food packaging. In particular, considering the use of nanoparticles, Li et al. combined graphite oxide nanoplatelets (GO) with chitosan or branched poly(ethylene imine) (BPEI) in order to improve hydrophilicity, reduce oxygen permeability, and provide antistatic properties [33]. Similarly, Carosio et al. improved the oxygen and water vapor barrier properties of PLA films incorporating a hydrophobic fluorinated polymer (Nafion) in a BPEI/NP assembly [34]. Although the above works demonstrate the feasibility of the LbL method to improve the properties of biopolymers, to the best of our knowledge to date, there is no evidence for the application of the LbL approach as a surface nanostructuring tool to further improve the performance and/or the properties of PHBH. Therefore, the novelty of this work is not uniquely related to the polymer matrix used, but also to the peculiar nature of the LbL assembly employed. The objective of the paper is to functionalize PHBH films to improve recycling by enabling magnetic field sorting procedures. A novel LbL assembly encompassing single-domain magnetic nanoparticles (MNPs) having a high saturation magnetization (M_s) was deposited on the surface of PHBH films using DNA and Chitosan solutions as constituent polyelectrolytes and MNPs dispersing agents. The choice of DNA and Chitosan as bio-sourced building blocks for the multilayer assembly by LbL was determined by their well-known self-

assembly potential as well as by the possibility to target a sustainable coating composition. Therefore, in the proposed approach, the opposite charges of DNA (polyanion) and Chitosan (polycation) are exploited to promote a LbL assembly by ionic interactions, while magnetic nanoparticles are co-dispersed in both solutions to ensure magnetic functionality without interfering with the electrostatic assembly process of polyelectrolytes [35] [36]. The MNPs exhibit superparamagnetic behaviour (i.e., characterized by zero remanent magnetization and coercivity) at room temperature. The main advantage of this behaviour is the absence of residual magnetism once the external magnetic field is removed. This prevents aggregation among the MNPs and avoids unintended magnetic attraction between film fragments and ferrous alloy components after the sorting procedure. In addition, unlike other LbL assembly strategies reported in the literature [37] [38], where MNPs were used as one of the two charged compounds constituting the bilayer, this study presents a LbL strategy that ensures the homogeneous integration of the MNPs therefore maximizing the magnetic functionality within the coating without compromising its LbL electrostatic assembly. The procedure adopted to modify the PHBH-based films consists of a preliminary surface activation of the polymer through a simple and mild aminolysis reaction, which can generate amino groups that can interact with the first DNA layer. Chitosan- and DNA-containing magnetic nanoparticles (DNA_MNPs/CH_MNPs system) were then alternately deposited with intermediate washing steps (**Figure 1**). The produced systems were characterized in detail in terms of their morphological, thermal, and magnetic properties as well as their enzymatic hydrolysis.

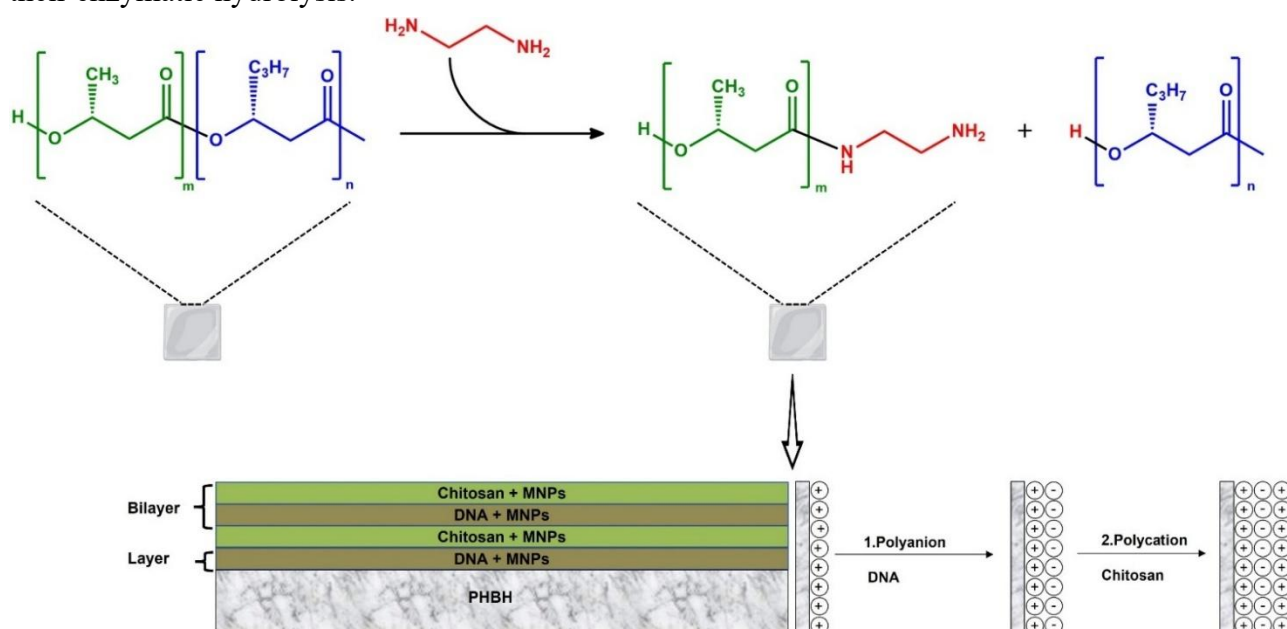


Figure 1: Schematic overview of the LbL deposition process on PHBH based film.

4.3 Experimental

4.3.1 Materials and methods

The chemicals $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (98%), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (97%), HCl (37%), ammonium hydroxide (30%) for MNPs synthesis, chitosan (CH, $M_w \sim 190.000\text{-}310.000$ g/mol, deacetylation degree $\geq 75\%$, viscosity 200-800 cps for $c = 1\%$ in 1% acetic acid), deoxyribonucleic acid (DNA, from herring sperm, crude oligonucleotides, < 50 base pairs, water $\leq 10\%$), acetic acid, ethylenediamine (ED), isopropanol, dichloromethane, and ninhydrin were purchased from Sigma-Aldrich and used as received. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) IamNature B6 A15 C (melt mass flow rate (MFR) at 165°C , 5 kg load = 6 g/10 min) was purchased by Gruppo MAIP (Italy). *Humicola insolens* Cutinase (HiC) (Novozym 51032, product code: 06-3135) was purchased from STREM Chemicals and used as received.

4.3.2 Preparation of PHBH films

PHBH thin films were prepared by casting 10 mL of polymer solution in dichloromethane (20 mg/mL) into a Petri dish (internal diameter = 54 mm). The dishes were left in the oven overnight at 30°C to allow solvent evaporation. The resulting films were subsequently detached and vacuum-dried at 10 mbar and room temperature until constant weight was reached (6 days). After drying, the films (mean thickness = 40 ± 4 μm) were stored at room temperature in a desiccator over activated 4Å molecular sieves.

4.3.3 Aminolysis of PHBH films

To provide the surface of the PHBH films with amino functional groups, 25×25 mm² samples were individually immersed in 25 mL of a 5% ED solution in isopropanol at 30°C for 2 h in a 50-mL Falcon™ tube, using an orbital shaker at 200 rpm. After this treatment, the film samples were removed and washed with 25 mL Milli-Q/isopropanol solution (1:1 v/v) for 5 min, changing the liquid after each period for 1 h. The aminolyzed film samples (coded as PHBH_0) were then dried overnight at room temperature and directly employed for the LbL deposition process.

4.3.4 Ninhydrin Assay

The presence of amino groups on the PHBH film surface was investigated using ninhydrin assay following the method reported by García-García et al. [39]. Specifically, ninhydrin binds to amino

groups present on the films producing a purple pigment with a maximum absorption at a wavelength of 560 nm in the solvent mixture chloroform/isopropanol (1:1), which can be detected and quantified using UV-Visible spectrophotometry. For this test, 3 PHBH film disks (diameter = 16 mm) were immersed in 1 M ninhydrin solution, developed in an oven at 70 °C for 15 min, and dissolved in 1 mL of chloroform/isopropanol (1:1) mixture before analysis. The resulting solution was then ultracentrifuged at 14500 rpm for 10 min, and the clear supernatant analyzed by UV-Vis spectrophotometry.

4.3.5 Spinel Iron oxide nanoparticles

Spinel Iron oxide magnetic nanoparticles were synthesized using the co-precipitation method using an aqueous solution of Fe^{2+} and Fe^{3+} ions in a 1:2 stoichiometric ratio, followed by alkalization with 30% ammonia solution [40]. The synthesis was carried out according to the following procedure: a mixture of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (>99%) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (>99%) was dissolved in 100 mL 0.01 M HCl solution (98%) at 60°C. The solution was then alkalized by adding a 30% ammonium hydroxide solution. After two hours of magnetic stirring, the resulting precipitate was collected by magnetic separation and washed three times with deionized water. The X-ray diffraction pattern (**Figure 2**) shows reflections typical of the iron phase with spinel structure (JCPDS card No.75-449). Any extra phase has been detected, and the average crystallite size of 9.0 (1) nm was estimated by the Scherrer equation [41]. The obtained black powder was dried overnight in an oven at 60°C prior to its use in the LbL deposition process.

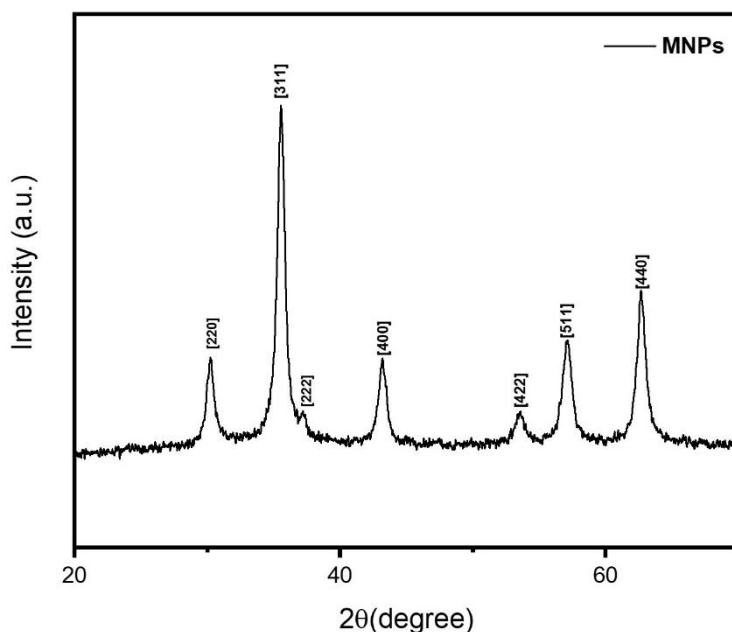


Figure 2: XRD pattern of spinel iron oxide MNPs.

4.3.6 LBL deposition

Bilayers (BL) were deposited by successive dipping of aminolyzed PHBH films (PHBH_0) in 0.5% (w/w) DNA solution and 0.5% (w/w) CH solution to which 0.25% (v/v) of acetic acid was added to dissolve CH, protonating its amino groups. An amount of 1% (w/v) magnetic nanoparticles was added to the DNA and chitosan solutions. Before the LbL deposition, the DNA_MNPs and CH_MNPs solutions were sonicated (5 min; RT; 40 KHz) to promote MNPs dispersion and mixing with the medium. Moreover, the PHBH films were alternately dipped in the DNA (polyanion) and CH (polycation) solutions while being continuously agitated in an orbital shaker (from VWR, KS 3000 I control model). The first DNA layer was achieved by dipping the film in the DNA solution (5 min) and then rinsing with Milli-Q water (1 min) to remove the excess solution. The CH layer was applied using the same procedure to create a BL. The procedure was repeated until the desired number of BL was reached (5, 10, 15, and 20). The samples were coded according to the number of deposited BL (e.g., PHBH_10 indicates 10 BL of DNA and CH deposited onto an aminolyzed PHBH_0 film). Dynamic light scattering (DLS) was employed to measure the zeta potential (ζ) and hydrodynamic size (d_H) of MNPs dispersed in Milli-Q water ($\text{pH} = 7.4 \pm 0.4$), as well as DNA and CH solutions, and the corresponding DNA_MNPs and CH_MNPs mixtures. These measurements allow to investigate the interfacial properties and interaction dynamics between the individual components.

4.3.7 Enzymatic hydrolysis

Enzymatic hydrolysis of films was performed by placing single film specimens (PHBH_10, $5 \times 8 \text{ mm}^2$, mean weight 9.0 mg, $n = 5$) in a 1.5 mL Eppendorf tube, followed by 1 mL HiC solution (5 μM , in 0.1 M KPO buffer, $\text{pH} = 8.0$). The film samples were then incubated at 50 °C in an oven. At the specified time, the film residues were removed manually, washed three times by immersion in 1 mL ultrapure water each time, and vacuum-dried at room temperature for three days until constant weight was reached. The weight loss of specimens was determined gravimetrically on an analytical scale (accuracy: $\pm 0.0001 \text{ g}$).

4.3.8 Characterization techniques

The growth of the LbL assembly up to 10 BL was monitored on a model silicon wafer substrate [(100), single side polished] using a Fourier transform infrared (FT-IR) spectrophotometer (16 scans and 4 cm^{-1} resolution, Frontier, Perkin Elmer) in transmission mode. After each deposition step, the Si wafer was dried, and IR spectra of the deposited layers were collected. Attenuated total reflectance (ATR) FT-IR spectroscopy spectra for the neat, aminolyzed, and coated PHBH films were collected

at room temperature in the $4000\text{--}400\text{ cm}^{-1}$ (16 scans and 4 cm^{-1} resolution) range using an FT-IR spectrophotometer (Frontier, Perkin Elmer, Italy) equipped with Germanium crystal. Differential Scanning Calorimeter (DSC) thermograms for the neat, aminolyzed, and coated PHBH-based films (approx. $5.0 \pm 1.0\text{ mg}$) were measured using a Mettler Toledo “DSC1 STAR^e System[®]” in the temperature range from -100 to 150 °C at a heating/cooling rate of $\pm 10\text{ °C/min}$ under a nitrogen flow of 20 mL/min . To investigate the effect of the number of bilayers on the thermal stability of the resulting films, all the samples up to 20 BL were analyzed by thermogravimetric analysis (TGA) using a Mettler Toledo “TGA/DSC1 STAR^e System[®]” micro and ultra-micro balances with sub-microgram resolution over the whole measurement range.

Each sample (approx. $8.0 \pm 1.0\text{ mg}$), placed into alumina pans, was measured under an 80 mL/min nitrogen flow and heated from 30 to 800 °C at a heating rate of 10 °C/min . SEM analysis of the sample morphology (surface and cross-section) was performed using a Zeiss Supra 40 VP field emission scanning electron microscope (FE-SEM) equipped with a backscattered electron detector. Before analysis, the films were sputter-coated with carbon using a Polaron E5100 sputter coater. Water contact angle (WCA) measurements were performed using an Attension[®] Theta tensiometer using ultrapure distilled water at 22 °C . To determine the contact angle value (θ), film specimens were stuck to microscopy glass slides using bi-adhesive tape, and static contact angles were determined by analyzing a minimum of six drops (volume = $3\text{ }\mu\text{L}$) for each film sample.

The crystalline structure of MNPs was characterized by X-ray diffraction (XRD) measurements, using a TT 3003 diffractometer equipped with a secondary graphite monochromator and $\text{CuK}\alpha$ radiation ($\lambda = 1.5418\text{ Å}$). The data were recorded in the $10^\circ\text{--}90^\circ$ 2θ range with a step size of 0.04° and a counting time of 4 seconds per step.

The field dependence of magnetization was investigated, using a vibrating sample magnetometer (VSM Model 10–Microsense) equipped with an electromagnet generating a magnetic field in the range of $\mp 2\text{ T}$. All the magnetic measurements were normalized by the mass of the magnetic phase, and the saturation magnetization value was determined using the approach to saturation law (LAS) [42] at high fields.

^1H -NMR analysis of the starting PHBH was performed on PHBH solution (CDCl_3 , 30 mg/mL , 25 °C) using a Bruker Avance 500 MHz spectrometer (Bruker, Karlsruhe, Germany). The mean numerical molecular weight and the comonomer composition of PHBH were determined using the equations reported elsewhere [43].

4.4 Results and discussions

The present work focused on the surface modification of films based on poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH), which consists of 89% 3-oxobutyric units and 11% 3-oxohexanoic units, as determined by $^1\text{H-NMR}$ [43], using the LbL method for the first time on this type of polymer. The dense PHBH films were prepared by solvent casting and functionalized by an aminolysis reaction to obtain the proper surface functional groups, namely ionizable amino groups that are positively charged at acidic or neutral pH. These groups were used to ionically interact with the components of the DNA solution (DNA_MNPs), owing to their opposite negative charges, leading to the deposition of a first negatively charged DNA/MNPs surface layer. This initial layer served as a primer for the LbL growth. The subsequent immersion in a CH-based solution containing MNPs (CH_MNPs), with both CH and MNPs having a positive charge, resulted in the deposition of a second, positively charged CH/MNPs layer to form the first BL. The successive alternate deposition of multiple DNA- and CH-based layers led to their LbL assembly (DNA_MNPs/CH_MNPs systems) eventually producing LbL functionalized PHBH-based films. The combination of magnetic nanoparticles with both DNA and CH was selected to ensure their incorporation into both types of layers, allowing for a more homogeneous distribution throughout the multilayer structure. With this approach, the polyelectrolytes not only drive the electrostatic LbL growth, but also act as efficient carriers of the MNPs, enabling their stable integration and uniform distribution within the coating while imparting magnetic functionality to the resulting film.

4.4.1 Study of the surface functionalization of PHBH-based films

The work was preliminary focused on the surface functionalization of PHBH films carried out by aminolysis using a 5% (v/v) solution of ED in isopropanol, a reaction generally applied for the functionalization of polyesters with diamines and involving a nucleophilic attack of the ED amino groups on the PHBH ester bonds, leading to the formation of free amino and hydroxyl groups on the film surface [39] [44]. It is worth underlining that this reaction, which has been widely used for the modification of polyesters such as PLA and PCL [45] [46], has only been described in very few works performed on PHBH [39] [47]. Since aminolysis leads to a progressive decrease in the molecular weight of the surface polymer chains, resulting in material erosion over time [39], the first step was to determine the optimal reaction conditions to achieve adequate surface functionalization without causing significant deterioration of the film. Therefore, the manipulability of the materials was evaluated after different contact times with ED solution (1, 2, 3, and 4 h) by manually bending the films with tweezers (**Figure 3**). After 4 h of aminolysis, a partial embrittlement of the materials was observed, which precluded further use.

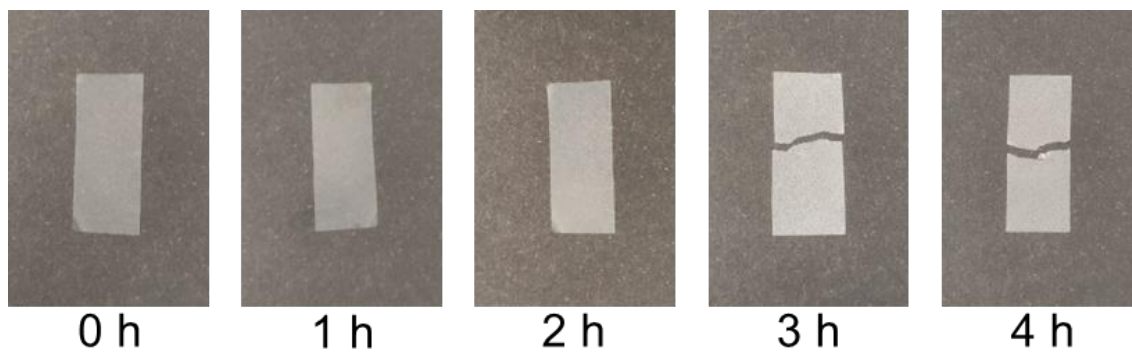


Figure 3: Photographs of PHBH films immersed 5% ED solution in iPrOH for different reaction times, after manual bending of the specimens.

Due to this phenomenon, which can be attributed to polymer degradation triggered by aminolysis [46], it was decided to focus the study on the film functionalized by adopting a reaction time of 2 h (coded as PHBH_0), to limit embrittlement while ensuring the surface functionalization. The validation of the surface functionalization of the films treated with ED for 2 h was investigated using different techniques such as FT-IR, WCA, and ninhydrin assay. No difference was observed when comparing the FT-IR spectra of the neat film with the aminolyzed one, probably due to a low presence of amine and amide groups on the surface of the films, which may be below the instrumental detection limit and cannot be revealed with this technique (**Figure 4**).

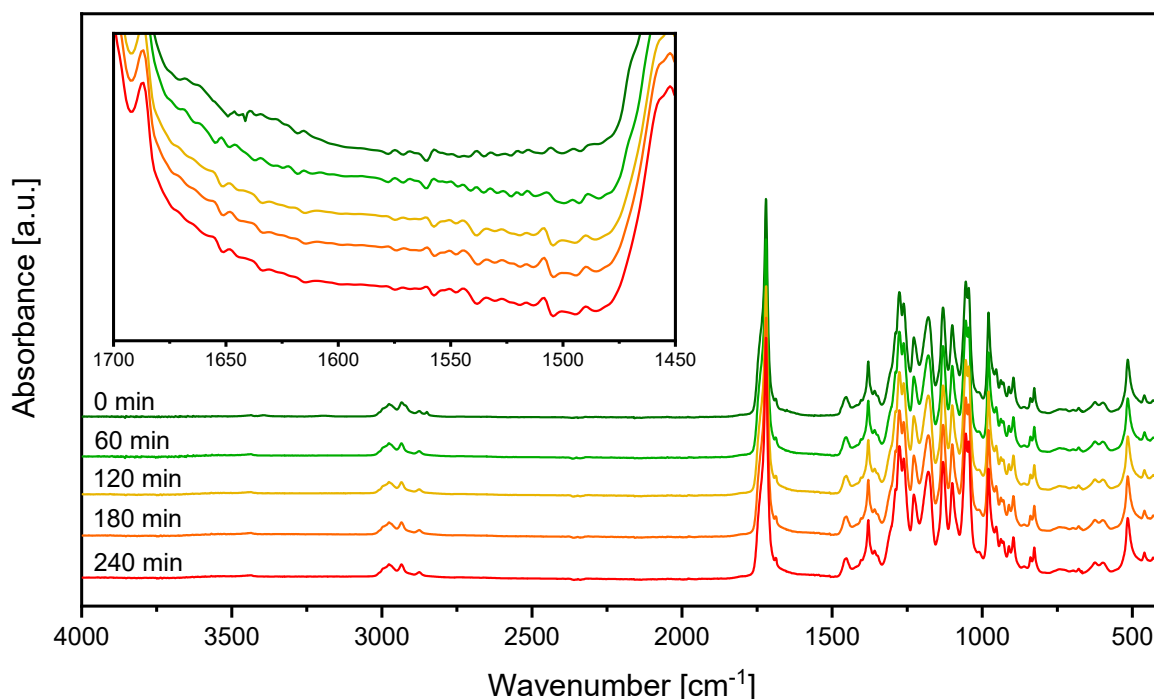


Figure 4: FT-IR spectra of PHBH films before and after contacting with the ED solution for different reaction times.

Nevertheless, WCA measurements of the films before and after the aminolysis treatment (**Figure 5a** and **5b** respectively) revealed a strong decrease in the contact angle value (θ) from about 100° for the pristine material to 65° for PHBH_0, which is consistent with the findings reported by other authors for similar systems [48]. This reduction, indicating an increase in the surface hydrophilicity of the material, indirectly points to the presence of hydrophilic functional groups on the film surface. To confirm the presence and quantify the number of amino functionalities formed on the film surface, the neat system and the ED-treated system were exposed to a ninhydrin solution to develop specific staining, which was subsequently quantified by spectrophotometric analysis. While the neat PHBH film was characterized by negligible content of amino groups, which was below the instrumental detection limit, the analysis of PHBH_0 revealed an -NH_2 concentration of $6.9 \pm 0.3 \cdot 10^{-2} \text{ } \mu\text{mol}/\text{cm}^2$. This clear difference in the amino group content of the two investigated systems (**Figure 5a** and **5b**) supports the effectiveness of the aminolysis reaction. Moreover, it was found that this value corresponds to what is reported in the literature for other PHBH films functionalized by aminolysis reaction with a dendrimeric poly(amidoamine) (PAMAM) [47]. To evaluate the influence of the aminolysis reaction on film morphology and thermal properties, SEM, DSC, and TGA measurements were performed. Regarding the latter analysis, TGA and DTG (**Figure 5e** and **5f**) revealed a slightly different thermal stability of the investigated samples. The onset decomposition temperature (T_{onset}) and the temperature of the maximum decomposition rate (T_{max}) were reduced by 10°C for the film subjected to the ED treatment. These results can be attributed to the reduction in the molecular weight of the surface polymer chains. Nevertheless, it can be assumed that such slight decrease in thermal stability does not affect the subsequent deposition process as well as the material application [49].

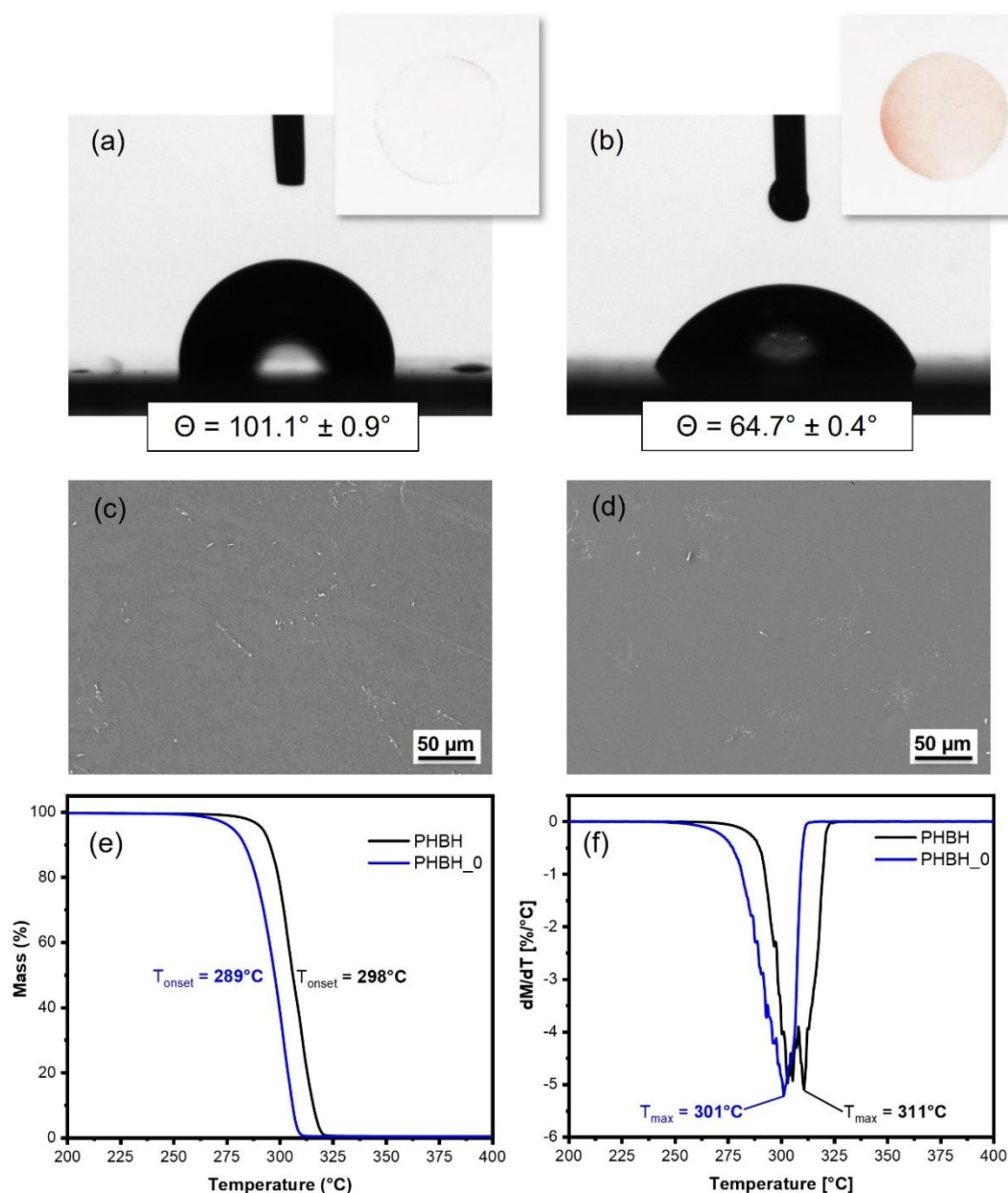


Figure 5: Characterization of PHBH-based films surface functionalization: WCA measurements of PHBH films before (a) and after aminolysis (b) with the corresponding photographs of PHBH films after treatment with ninhydrin solution and development at 70 °C before (left) and after (right) 2 h of aminolysis; SEM micrographs of PHBH (c) and PHBH_0 (d); TGA (e) and DTG (f) analysis of PHBH and PHBH_0.

These results were well supported by the DSC analyses (**Figure 6** and **Table 1**) that displayed an increase in the crystallinity value of the films (χ_c) from 34% for PHBH to 42% for PHBH_0. This variation can be ascribed to the low molecular weight chains on the film surface, which have a greater mobility than the pristine high molecular weight chains and are therefore characterized by a higher tendency for structuring [50].

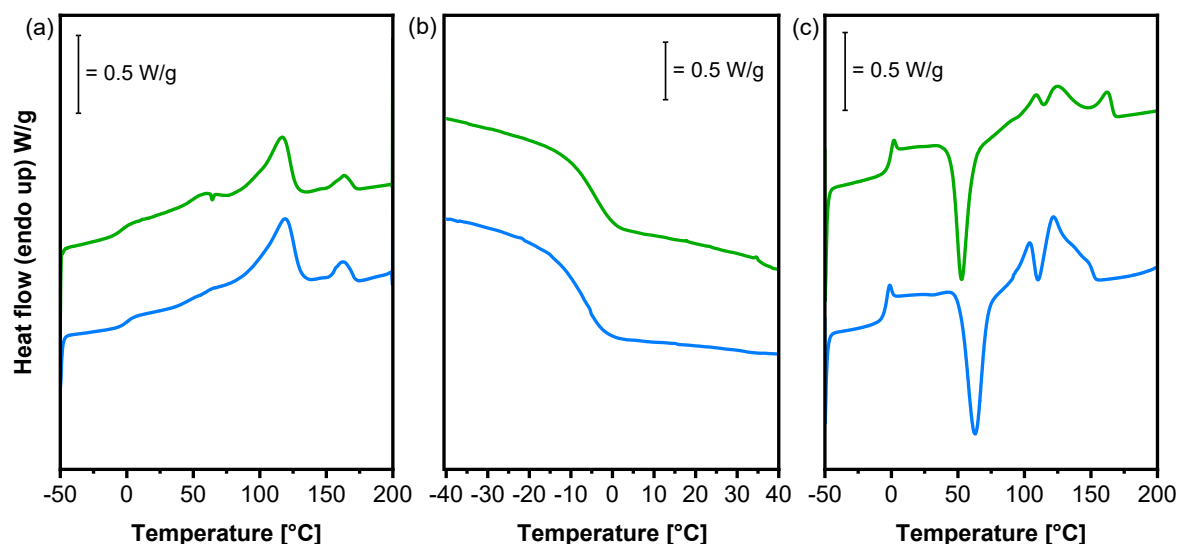


Figure 6: DSC first heating (a), cooling (b) and second heating (c) thermograms of neat PHBH (green), PHBH_0 (blue).

Table 1: DSC characterization of PHBH film before and after aminolysis process.

Sample	First heating				Cooling		Second heating					
	T_g [°C]	T_m [°C]	ΔH_m [J/g]	χ_c [%]	T_c [°C]	ΔH_c [J/g]	T_g [°C]	T_{cc} [°C]	ΔH_{cc} [J/g]	T_m [°C]	ΔH_m [J/g]	χ_c [%]
Neat PHBH	-3	117	50	34	41	2	-2	53	-32	125	34	1
PHBH_0	-2	119	61	42	36	1	-4	63	-39	121	40	1

T_g = glass transition temperature, T_m = melting temperature, ΔH_m = melting enthalpy, T_{cc} = cold crystallization temperature, ΔH_{cc} = cold crystallization enthalpy, T_c = crystallization temperature, ΔH_c = crystallization enthalpy, χ_c is the degree of crystallinity calculated by assuming the ideal enthalpy of fusion for PHBH as 146 J g⁻¹.¹

Despite the slight modification in the thermal properties of the aminolyzed film, SEM analysis showed no significant changes in its surface morphology compared to the starting sample (**Figure 5c** and **5d**). This result, which differs from that of some other treated films based on PHBH and other polyesters that showed significant surface erosion as a result of the aminolysis reaction [39], can be attributed to various aspects related to both the aminolysis conditions (time, temperature, reagent concentration) and the characteristics of the treated materials (molecular weight, crystallinity, film thickness, etc.). Therefore, while a direct comparison with the results reported in the literature is difficult, SEM results highlight that the film functionalization, confirmed by other techniques, affects only a limited thickness of the system surface.

4.4.2 Investigation of MNPs-containing dispersions

Uncoated Iron oxide nanoparticles were selected because of their natural ability to reversibly change their surface charge depending on the pH of the medium and counter-ions [51], making them ideal building blocks for electrostatically driven LbL assemblies. Specifically, the colloidal stability of MNPs in aqueous media is primarily influenced by pH-dependent surface charge, driven by the protonation and deprotonation of surface hydroxyl groups [52]. At low pH, the surface is dominated by positively charged Fe-OH_2^+ groups, which gradually transform into neutral (Fe-OH) and into negatively charged (Fe-O^-) forms in neutral and basic conditions, respectively. As confirmed by DLS measurements (**Figure 7a**), the point of zero charge (PZC) for the system under investigation was identified at $\text{pH} \sim 7.5$, in agreement with previous studies [53], and associated with a clear tendency for nanoparticles' agglomeration. When MNPs are introduced into the DNA solution ($\text{pH} \sim 2.6$), the phosphate backbone retained its negative charge due to the low pK_a values of phosphate groups (< 2) [54], resulting in a strong electrostatic attraction between the negatively charged DNA and the positively charged MNPs (Fe-OH_2^+) under acidic conditions. This interaction was confirmed by zeta potential measurements: the DNA_MNPs complex exhibited a less negative value ($\zeta = -24.5 \text{ mV} \pm \sigma = 5.5 \text{ mV}$) than DNA alone ($\zeta = -35.3 \text{ mV} \pm \sigma = 4.1 \text{ mV}$), indicating a partial charge neutralization upon attraction (**Figure 7b**). Conversely, when MNPs were dispersed into CH solution ($\text{pH} \sim 4.4$), both components carried positive surface charges (Fe-OH_2^+ and NH_3^+ , respectively), which would normally lead to electrostatic repulsion.

However, Chitosan is known to coordinate metal oxide surfaces via its unprotonated amine and hydroxyl functionalities, enabling complex formation even under repulsive electrostatic conditions [55]. Indeed, some works support the formation of such complexes over a wide pH range where chitosan amines are only partially protonated, particularly between pH 4 and 6 [55]. This coordination-driven interaction led to a further increase in surface charge, with the CH_MNPs complex showing a ζ value of $+78.2 \text{ mV} \pm \sigma = 5.8 \text{ mV}$, compared to $+67.3 \text{ mV} \pm \sigma = 4.3 \text{ mV}$ for CH alone (**Figure 7b**). The pronounced charge difference between DNA ($\zeta \sim -35$) and CH ($\zeta \sim +67 \text{ mV}$) supports the feasibility of a LbL approach. This promotes efficient polyelectrolyte adsorption during the alternate deposition cycles, enhancing the coating assembly. Meanwhile, both the polyanionic and polycationic components contribute to successful incorporation of MNPs into the multilayered structure on PHBH films, which occurs through a combination of electrostatic and coordination interaction under carefully controlled pH conditions.

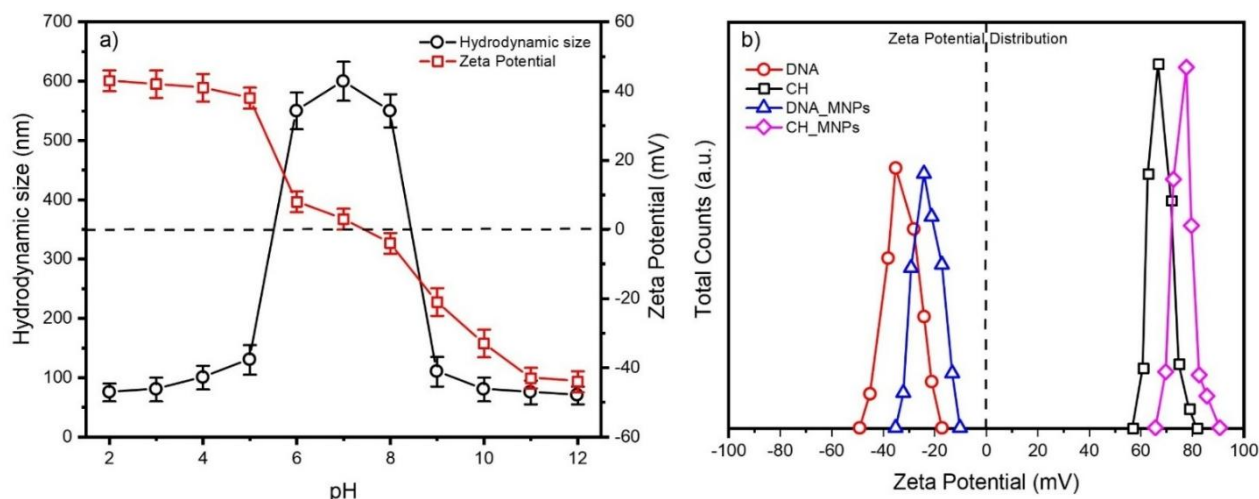


Figure 7: DLS measurements: a) Zeta potential and Hydrodynamic size of magnetite (MNPs) to assess the PZC. Each condition was measured in triplicate, and the error bars represented the standard deviations; b) Zeta potential distribution of DNA (red circles), CH (black square), DNA_MNS (blue triangles) and CH (magenta rhombus).

4.4.3 Study of neat and LbL modified PHBH-based films

To clearly demonstrate the chemical change in the PHBH-based films after the LbL deposition, the IR spectrum of the aminolyzed film (PHBH_0) was compared with those of CH, DNA and a coated film treated with the highest BL number, namely 20 (PHBH_20) (**Figure 8a**).

In the spectrum of PHBH_0, a strong, sharp, and steep band was observed at 1719 cm^{-1} corresponding to the C=O stretching vibration [56]. Other characteristic signals for PHBH were also visible approximately at 1274 , 1180 , and 1054 cm^{-1} , ascribed to ester groups in the polymer structure. Furthermore, PHBH exhibited peaks at 978 , 1227 , and 1262 cm^{-1} , associated with the crystalline phase of the film, while bands at 2871 , 2931 , and 2977 cm^{-1} were attributed to CH stretching, asymmetric stretching of CH_2 , and C- CH_3 , respectively [57]. As far as the PHBH_20 is concerned, the above characteristic peaks disappeared and the signals ascribed to both DNA and CH (**Figure 8b** and **8c** respectively) were distinguished with some wavenumber and intensity variations due to their LbL assembly, as well as the characteristic band at 535 cm^{-1} corresponding to the Fe-O vibrations of MNPs is noticeable. In detail, the signal attributed to the C=O vibration of DNA nucleotide bases (1682 cm^{-1}) was broadened and shifted to a lower wavenumber (1635 cm^{-1}) due to the overlapping with the chitosan asymmetric and symmetric stretching vibrations of protonated amine groups (1640 and 1565 cm^{-1} , respectively). Moreover, PO_4^{2-} stretching vibrations (1210 cm^{-1}) were shifted towards lower wavenumbers (1200 cm^{-1}) due to the electrostatic interaction with NH_3^+ counterions attributed to chitosan (1150 cm^{-1}) in the LbL assembly, while the peak at 1057 cm^{-1} was attributed to the sum

of the C-O-C stretching signals of both chitosan and DNA. These results point out the successful LbL assembly of the two biocomponents and MNPs on the surface of the PHBH-based film.

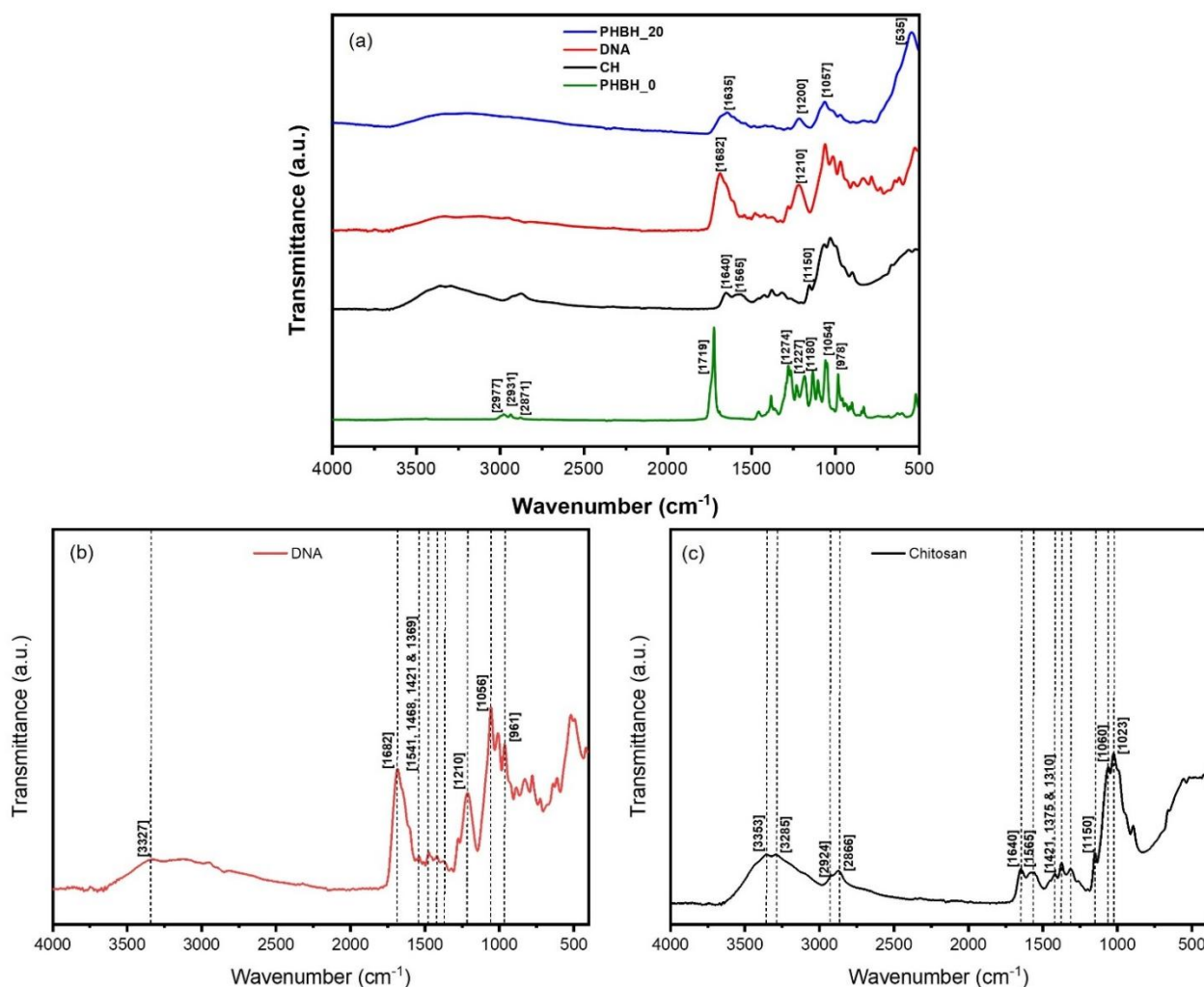


Figure 8: FT-IR spectra of: (a) aminolysis-treated PHBH based films (PHBH_0), DNA, CH and coated film with 20 BL (PHBH_20), (b) DNA, (c) Chitosan

IR signals of DNA (Figure 8b): 3327 cm⁻¹ (O-H stretching); 1682 cm⁻¹ (C=O vibration of Guanine G, Cytosine C and thymine T); 1541, 1468, 1421 & 1369 cm⁻¹ (C=C & C=N stretching); 1210 cm⁻¹ (P=O & PO²⁻ group); 1056 cm⁻¹ (-C-O-C- stretching); 961 cm⁻¹ (-P-O-C- group).

IR signals of CH (Figure 8c): 3353 cm⁻¹ (O-H stretching); 3285 cm⁻¹ (N-H stretching); 2924 (CH₂ stretching); 2866 cm⁻¹ (C-H stretching); 1640 (NH₃⁺ asymmetric stretching vibration); 1565 (NH₃⁺ symmetric stretching vibration; N-H bending and C-N stretching vibration); 1421, 1375 & 1310 (C-H bond); 1150 (NH₃⁺ rocking); 1060 (C-O-C stretching); 1023 (O-H stretching).

The growth of DNA_MNPs/CH_MNPs assembly was monitored as a function of BL deposited on PHBH by recording the IR-ATR spectra of the films coated with 5, 10, 15, and 20 BL (PHBH_5, PHBH_10, PHBH_15, and PHBH_20, respectively) (**Figure 9a** and **9b**).

The evolution of the two more intense peaks at 1564 cm^{-1} and 955 cm^{-1} as a function of the deposited BL was also followed, demonstrating that the coating growth reached a plateau above 10 BL, as the intensity of the two peaks showed no significant changes in the samples with 10,15 and 20 BL (Figure 9c). Moreover, Figure 9d provides additional evidence for the beneficial role of aminolysis in improving the electrostatic affinity between the substrate surface and the initially deposited DNA layer, as the film without any functionalization reaction displayed no evidence of MNPs deposition after a LbL treatment with 10 BL.

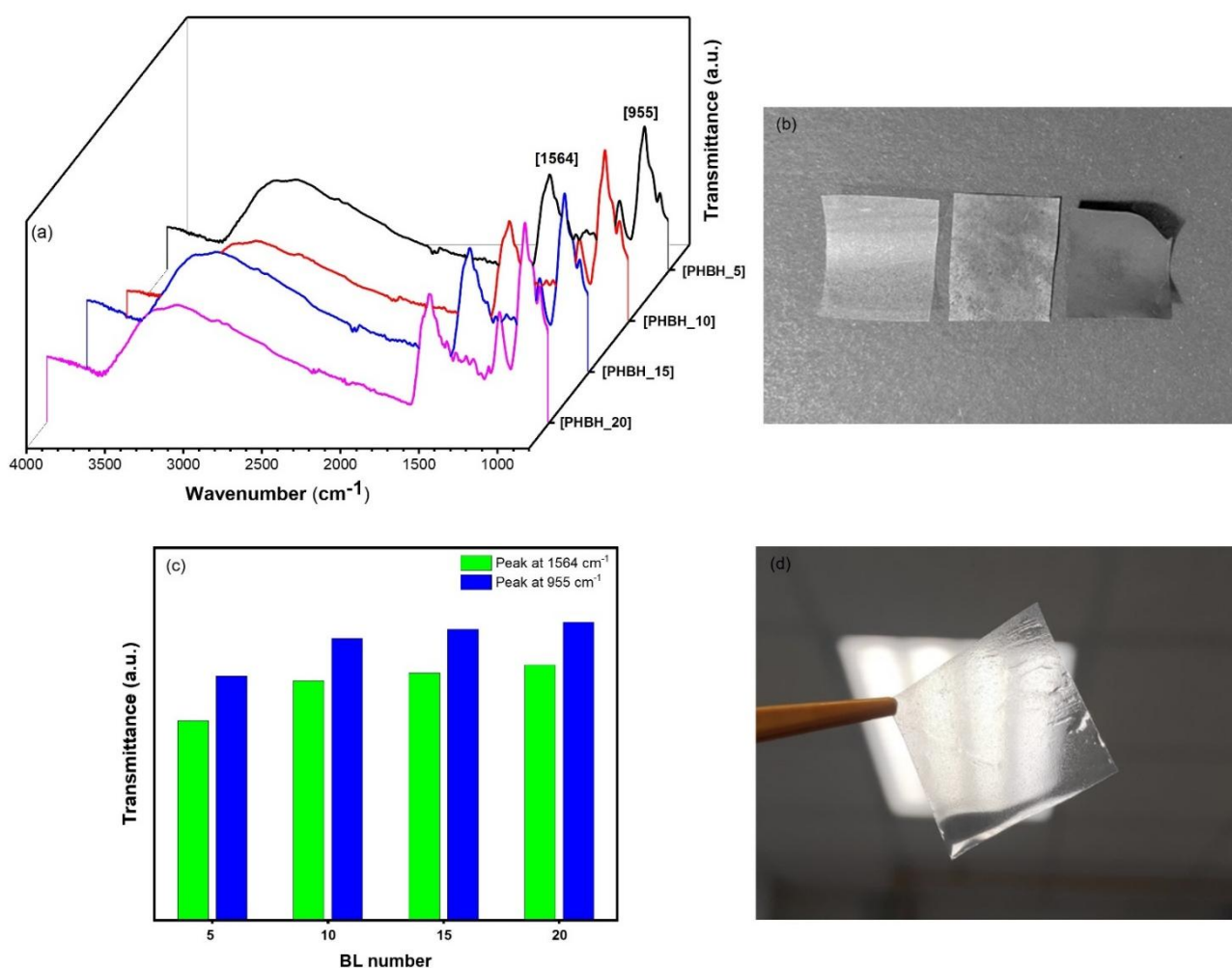


Figure 9: a) IR-ATR spectrum after 5,10, 15 and 20 bilayers (PHBH_5, PHBH_10, PHBH_15 and PHBH_20 respectively) of the DNA_MNPs/CH_MNPs system; (b) Photograph of aminolyzed PHBH films before (left) and after the deposition of 5 BL (center) and 10 BL (right); (c) Evolution of the peak intensity at 1564 cm^{-1} and 955 cm^{-1} as a function of deposited bilayers; (d) Photograph of pristine PHBH film after the deposition of 10 BL.

The thermal stability of the aminolyzed PHBH film and the LbL-treated films was evaluated by thermogravimetric analysis (TGA) in inert atmosphere, comparing the thermograms with those of the

neat biocomponents and the MNPs (**Figure 10b**). As well documented in the literature [58], the decomposition of CH occurred in a single, well-defined step between 250–400°C, releasing H₂O, NH₃, CO, CO₂, and CH₃COOH, producing a carbonaceous residue that is subsequently slowly and continuously converted into polyaromatic structures (final residual mass ~10%). Furthermore, according to reports on DNA [59], an abrupt mass drop was observed near 220°C, due to the chemical decomposition of phosphate groups and the release of phosphoric acid (final residual mass ~40%). As observed in the thermogram of MNPs, magnetite is stable over the entire temperature range, as expected for Fe₃O₄ nanoparticles [60]. In the TGA thermograms (**Figure 10a**), T_{max} showed no significant differences between PHBH_0 and LBL-treated systems with 5, 10, 15 and 20 BL, T_{max} being ~284 °C for PHBH_0 and ~286 °C for all coated films. When the DNA_MNPs/CH_MNPs system was assembled, the resulting films still exhibited a single-step thermal decomposition, which was slightly anticipated with respect to aminolyzed PHBH film, as shown by the T_{onset} (~272°C and 276°C for PHBH_0 and PHBH_5, respectively, vs ~268°C for all the other LBL assemblies). The final residual mass for PHBH_0 was 0%, while for PHBH_5, PHBH_10, PHBH_15, and PHBH_20 was 19, 41, 47, and 48%, respectively, pointing out an increased amount of CH- and DNA-containing magnetic nanoparticles successfully deposited on the surface of the PHBH films. Interestingly, the residual mass increase shows diminishing returns above 10 BL thus pointing out a somewhat plateau in the deposition process.

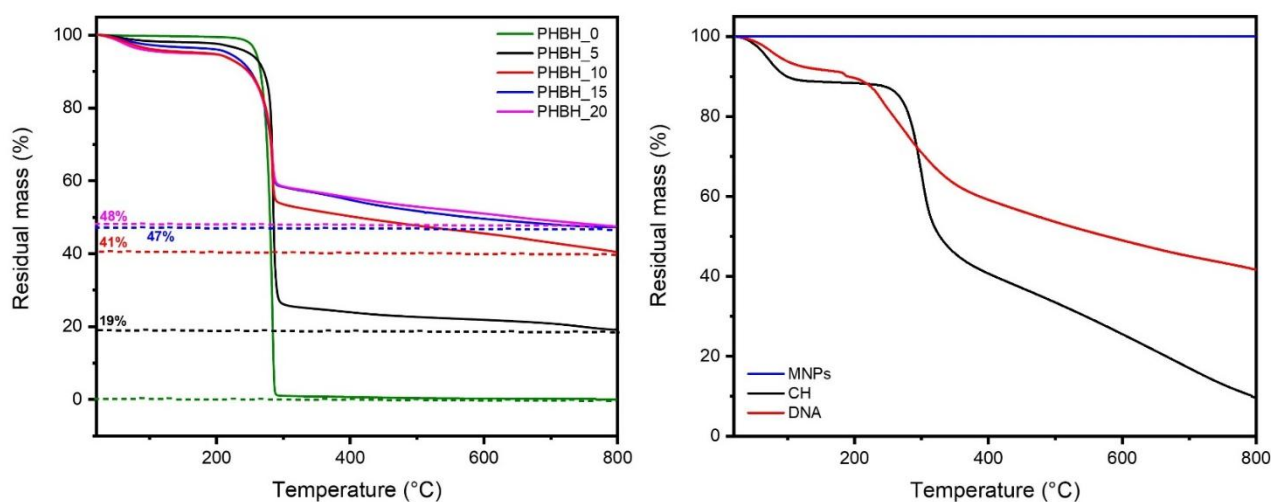


Figure 10: TGA curve of: (a) PHBH_0 and LBL assemblies with 5, 10, 15, and 20 BL (PHBH_5, PHBH_10, PHBH_15, and PHBH_20, respectively) (b) neat DNA (red), CH (black) and MNPs (blue).

The effect of the number of deposited layers on the magnetic properties was investigated by comparing the magnetic behaviour of bare MNPs, the aminolyzed PHBH film, and LbL modified films containing up to 20 DNA_MNPs/CH_MNPs-based BL. The field dependence of magnetization at 300 K (**Figure 11a**) revealed a superparamagnetic behaviour (i.e., coercive field, H_c , and remanent magnetization, M_r , equal to 0), consistent with previously reported results on spinel iron oxide nanoparticles with similar particle size (10 nm) [61] [62], while the aminolyzed PHBH substrate displayed a diamagnetic response. The M_s was found to increase progressively with the number of layers, indicating a corresponding increase in the amount of MNPs deposited onto the PHBH surface as the coating thickness increased. Notably, as illustrated in the inset of **Figure 11a**, the M_s value of the PHBH_20 film ($55 \pm 3 \text{ Am}^2/\text{kg}$) was nearly identical to that of the bare MNPs ($58 \pm 2 \text{ Am}^2/\text{kg}$) within experimental error. This suggests that additional depositions beyond 20 layers would result in minimal enhancement of magnetization. These findings demonstrate that the developed LbL assembly technique effectively imparts magnetic responsiveness to the PHBH films through the surface deposition of DNA_MNPs/CH_MNPs multilayers. The resulting M_s values are sufficiently high to allow for efficient separation of the films from non-magnetic polymer materials under an applied magnetic field. This capability offers promising potential to improve the efficiency and purity of plastic recycling processes by addressing major challenges such as contamination and the separation of mixed plastic waste (**Figures 11b and 11c**).

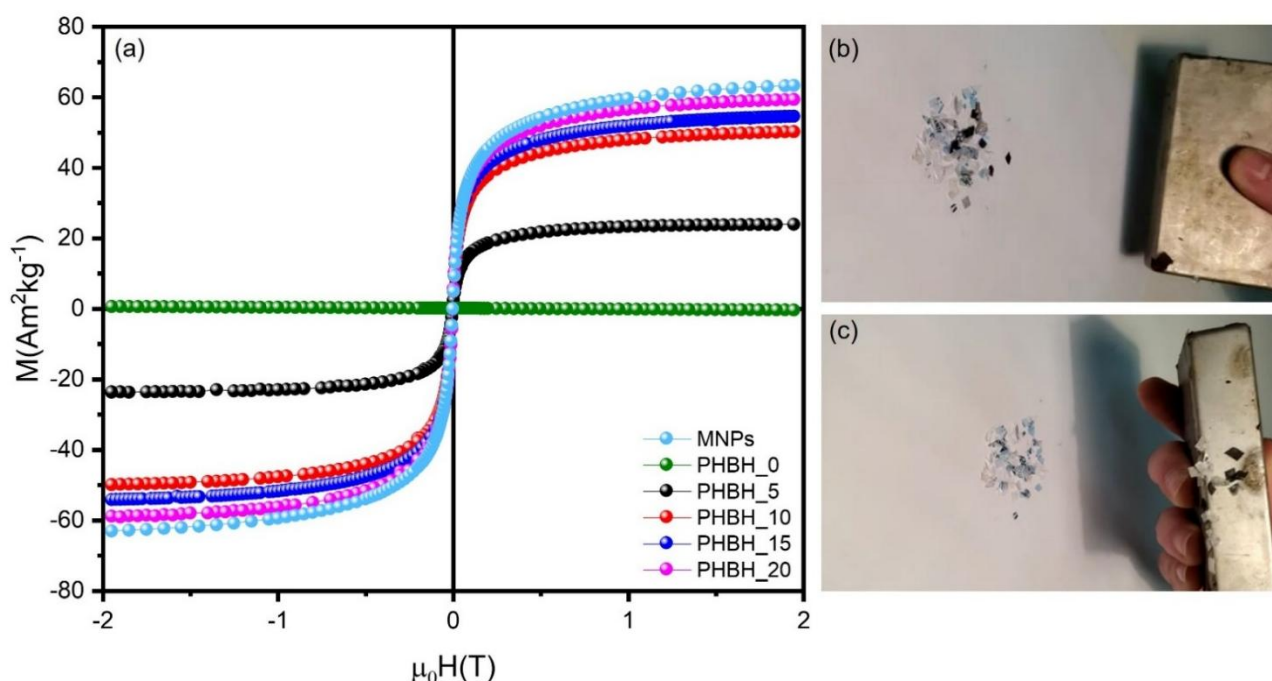


Figure 11: (a) Magnetization curve of bare MNPs, aminolysis-treated PHBH film and PHBH films after 5–20 DNA_MNPs/CH_MNPs depositions; (b and c) Images from magnetic separation tests of PHBH_20 from mixed plastics before and after applying a magnetic field.

Based on the above results, it can be concluded that both TGA and VSM analysis confirmed a limited LbL growth above 10 BL, as shown in **Figure 12**. Therefore, limited differences were observed in the final residual mass and M_s values of PHBH films coated with 10, 15, and 20 BL of DNA_MNPs/CH_MNPs system (PHBH_10, PHBH_15, and PHBH_20, respectively) (**Table 2**). Contrary to what has been reported in the literature for DNA/CH LbL systems [36], where the coating growth displayed a two regimes of linear growth after 10 BL, the presence of MNPs in the present assembly lead to a plateau condition with limit in the growth profile of the coating. This can be ascribed to the formation, during the deposition of 15 and 20 BL, of a highly swollen assembly that limits the coating growth by weakening interactions among the deposited layers that can be either partially or totally removed upon washing.

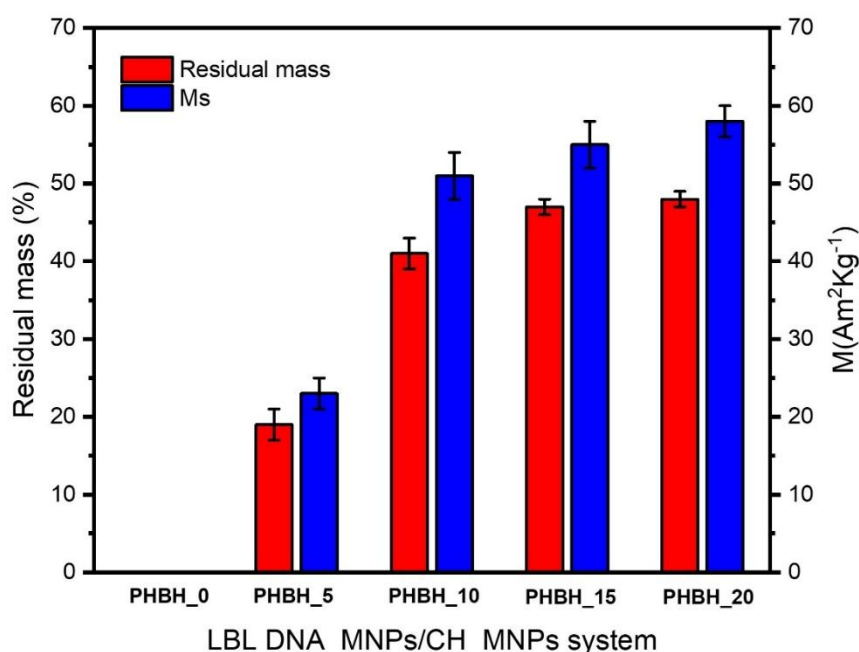


Figure 12: Progression of final residual mass and M_s values of PHBH films after 10,15, and 20 BL (PHBH_10, PHBH_15, and PHBH_20, respectively)

Table 2: Residual mass and Saturation Magnetization (M_s) corresponding to the different PHBH based film after aminolysis treatment and after 5,10,15 and 20 BLs.

Sample	Residual mass (%)	M_s ($\text{Am}^2\text{Kg}^{-1}$)
PHBH_0	0	0
PHBH_5	19 (2)	22 (2)
PHBH_10	41 (2)	51 (3)
PHBH_15	47 (1)	55 (3)
PHBH_20	48 (1)	58 (2)

^a Uncertainties in the last digit are given in parentheses.

FE-SEM analysis was performed on films coated by 5 and 10 BL (referred to as PHBH_5 and PHBH_10) (**Figure 13**). In PHBH_5, areas of exposed substrate alternate with zones where the deposition of the DNA_MNPs/CH_MNPs assembly is clearly visible. In contrast, PHBH_10 exhibited a continuous coating over the entire film surface, with no uncoated regions, suggesting that the deposition of 10 BL was sufficient to achieve complete and homogeneous substrate coverage. Moreover, the cross-section analysis of the samples shown in **Figures 13c** and **13d** confirmed that the thickness and homogeneity of the deposited DNA_MNPs/CH_MNPs assembly increased with the number of BL, which was consistent with the IR-ATR measurements reported in the previous section (**Figure 9a**). Analysis of the SEM micrographs also showed that the deposited coating adhered well to the PHBH substrate, a phenomenon indicative of the good electrostatic interactions between the positively charged amino groups on the film surface and the negatively charged DNA-based solution constituting the first deposited layer.

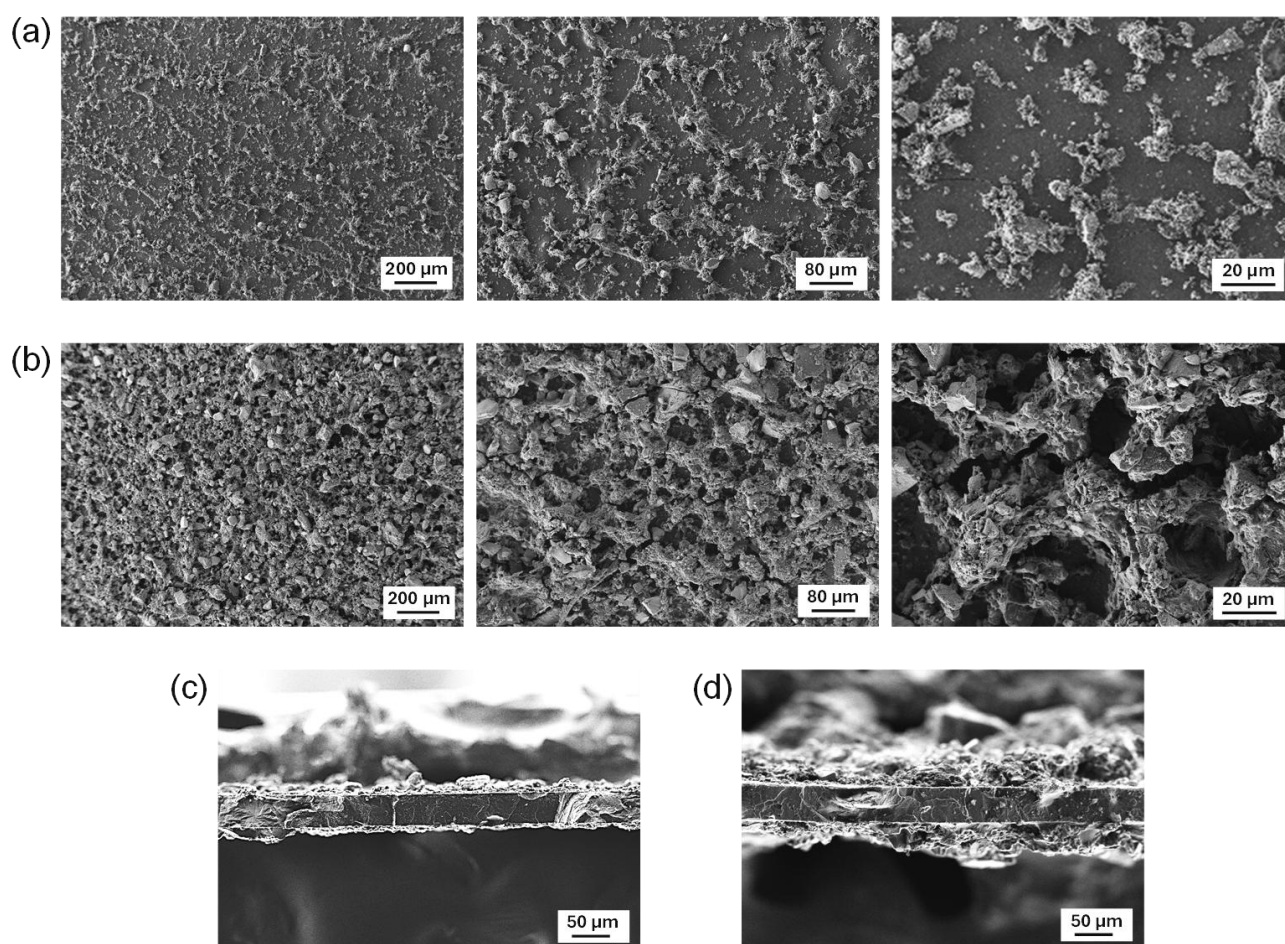


Figure 13: SEM micrographs at different magnifications of PHBH_5 (a) and PHBH_10 (b) and their corresponding cross-sections (c and d).

4.4.4 Study of LbL growth on model silicon substrate

To better investigate the assembly of the DNA_MNPs/CH_MNPs system, the growth of the coating was monitored on a model silicon substrate using FT-IR spectroscopy. The deposition up to 10 BL was investigated, as this is the limit found for the PHBH-based films.

The LbL growth was graphically represented by a 3D graph (**Figure 14**), showing the IR spectra as a function of the BL numbers. Additionally, a magnification of the IR from 1800 to 900 cm^{-1} is given in **Figure 14b**, allowing for a clearer view of this specific region where the main peaks of the assembly can be easily observed. Indeed, the characteristic peaks ascribed to P=O, PO^{2-} and P-O-C groups of the phosphate deoxyribose backbone are visible at 1225 and 969 cm^{-1} , while signals due to C=C and C=N stretching modes (namely, at 1538, 1485, 1421, and 1374 cm^{-1}) confirmed the presence of pyrimidine and purine bases in the DNA_MNPs/CH_MNPs assembly [36]. In all the spectra, including the one collected at low BL, it was possible to observe that the broad peak around 3372 cm^{-1} , associated to the O-H stretching typical of both DNA and CH, overlapped with the stretching of amino groups (3550–3330 cm^{-1}) and the shoulder at 2959 cm^{-1} was assigned to C-H bond of CH [63]. The strong signal at 1696 cm^{-1} was attributed to the C=O vibration of guanine, cytosine, and thymine, while the peak at 1067 cm^{-1} was ascribed to the C-O-C stretching vibrations of both DNA backbone and of chitosan pyranose rings [33] [64], as seen in **Figure 8b e 8c**. The characteristic peak at 546 cm^{-1} , corresponding to the Fe-O vibrations of spinel iron oxide nanoparticles is also visible [65]. To investigate the trend of IR signal evolution as a function of deposited BL, the two more specific peaks at 1067 cm^{-1} and 1696 cm^{-1} were monitored. These specific peaks were found to increase linearly with the number of deposited BL (**Figure 14c**), as previously demonstrated for DNA/CH-based systems [35] [36]. Furthermore, comparing the spectra of DNA_MNPs/CH_MNPs assembly with those of the neat components, it was clear that the signals associated with DNA (i.e., 1696 and 1225 cm^{-1}) were more intense than the CH ones, indicating the formation of a DNA-rich coating (**Figure 14d**).

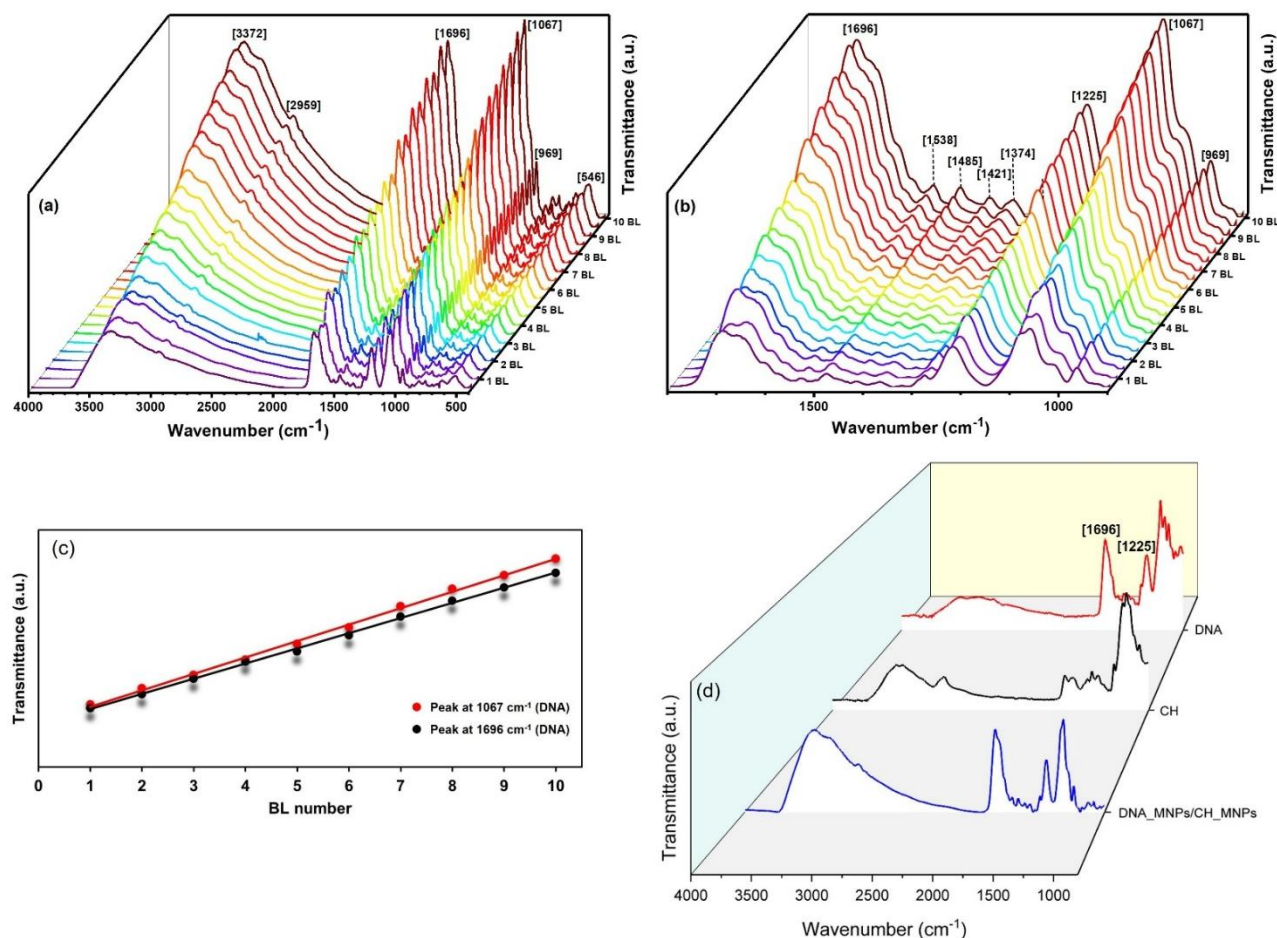


Figure 14: (a) 3D IR spectrum growth on model silicon wafer substrate in the range of 4000–400 cm^{-1} ; (b) Zoom in the range 1800–900 cm^{-1} ; (c) Evolution of the signals at 1067 cm^{-1} as function of BL number; (d) FT-IR spectra of neat DNA, CH and DNA_MNPs/CH_MNPs assembly with 10 BL deposited on silicon wafer in 3D plot in the range 4000–800 cm^{-1} .

4.4.5 Enzymatic hydrolysis of films

To investigate the behaviour of a system coated with the LbL method towards enzymatic hydrolysis, the weight of a film brought into contact with a solution of *Humicola insolens* cutinase, an enzyme that is highly active in the hydrolysis of many polyesters [66] [67], and that has also demonstrated significant hydrolytic activity towards PHAs [43], was monitored over time. The enzymatic hydrolysis of the films coated with 10 BL (PHBH_10) was performed using a 5 μM HiC solution in KPO 0.1 M buffer (pH = 8.0) at 50 °C. This sample was selected because 10 BL ensure a homogeneous coating and approach a plateau in MNPs deposition. As can be seen from the histogram in **Figure 15a**, the mass loss of the films increased dramatically after two days, from 9% to 77% after 24 and 48 h of immersion, which can be related to the complete detachment of the coating from the samples, as also evidenced in **Figure 15b** and **15c**. This phenomenon can be explained by the different behaviour towards enzymatic hydrolysis of the deposited layer and the polymer substrate, as already demonstrated in another work [35]. Interestingly, the detached material based on superparamagnetic

MNPs can be easily separated using a neodymium magnet, avoiding additional time-consuming centrifugation and/or filtration steps. After 40 days of immersion, the residual film mass loss reached 84%, confirming the intrinsic susceptibility of PHBH to HiC-mediated hydrolysis. Overall, these findings demonstrate that these materials can be selectively disassembled into their components, i.e., MNPs and PHBH, under mild conditions, enabling environmentally friendly and efficient recovery (Figure 15d and 15e).

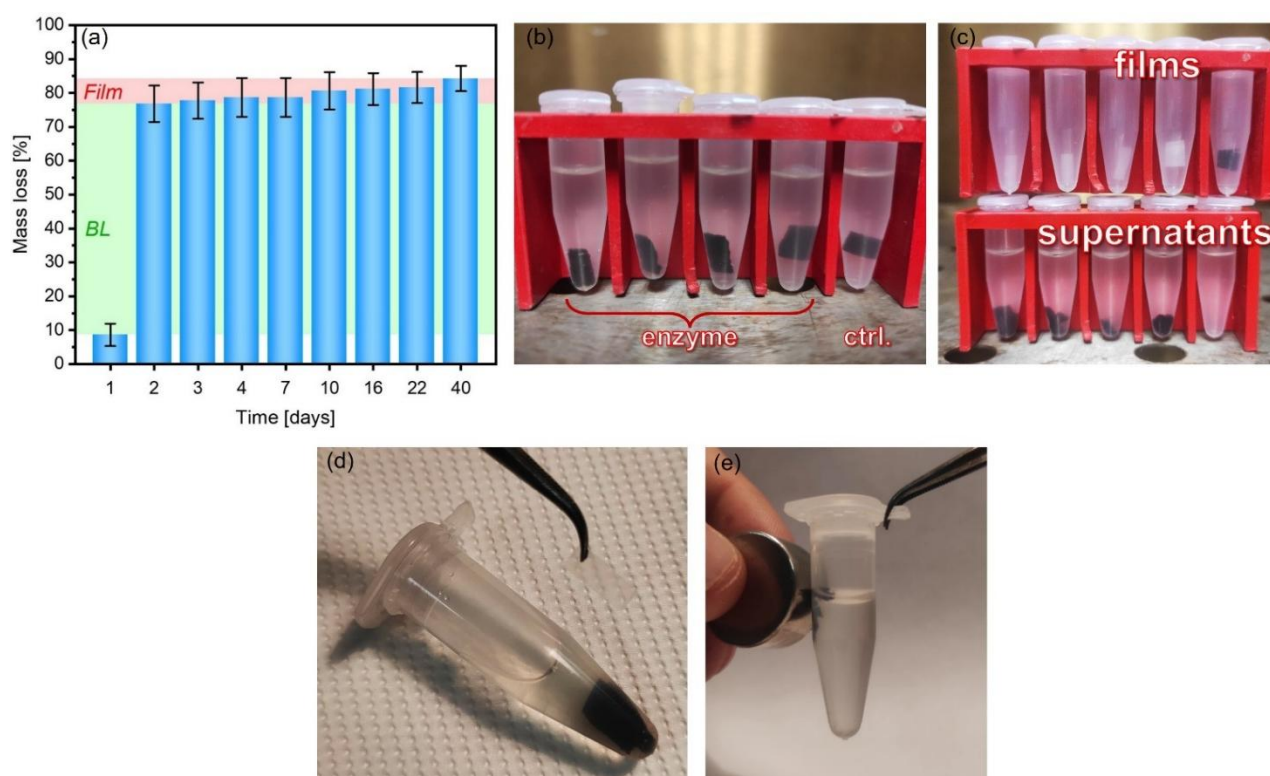


Figure 15: Enzymatic hydrolysis of PHBH-based films using HiC in KPO buffer pH = 8.0 at 50 °C. The pure coating detachment (BL, green) and substrate hydrolysis (Film, red) contributions to the mass loss of the coated specimens are evidenced (a). Photographs of PHBH₁₀ after 1 day of enzymatic hydrolysis in HiC (b). After 2 days of immersion in the enzymatic solution the bilayers were completely detached from the substrates and can be recovered efficiently (c and d). Photographs of PHBH₁₀ after application of a static magnetic field (e).

4.5 Conclusions

In this study, a layer-by-layer (LbL) approach was successfully used to modify poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH)-based films. The developed method consisted in the deposition of alternating layers of DNA and chitosan (CH) embedded with MNPs on the surface of the polymer films, activated by an aminolysis reaction, which enabled an efficient LbL assembly and produced an adherent multilayer coating. The characterization of the multilayer growth and morphology confirmed the progressive and uniform build-up of this DNA_MNPs/CH_MNPs

assembly, with a linear growth up to 10 bilayers (BL) beyond which a plateau was observed, indicating a saturation in the deposition process. The deposition of 10 bilayers (BL) effectively conferred magnetic properties to PHBH films, enabling efficient separation from other polymer materials and addressing the persistent challenge of contamination and material sorting. Moreover, the coating was found to be removable upon exposure to an enzymatic solution, allowing for the biodegradation of the film and the recovery, and potential reuse, of the magnetic nanoparticles (MNPs) via a static magnetic field.

4.6 References

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Chapter 5: Conclusions and future perspective

The field of biocatalysis is undergoing rapid transformation, driven by the growing need for sustainable, efficient and easily recoverable catalytic system for implementing more circular processes. Traditional enzyme catalysis is usually carried out in solution and, while valuable for its selectivity and moderate operating conditions, it is often limited by high-cost, low stability and limitations on the biocatalyst's recyclability. For this reason, immobilization strategies have become fundamental to the advancement of biocatalytic processes. Among the different supports explored to date, this work has explored various enzyme immobilization strategies aimed at improving the sustainability, efficiency and reusability of biocatalysts in the synthesis of bio-based polyesters. The successful immobilization of *Candida antarctica* lipase B (CaLB) onto magnetically recoverable nanoparticles marked a significant step forward in catalyst efficiency and recyclability. By using magnetic nanoparticles as support, the enzyme retained high catalytic activity over multiple reaction cycles, yielding high monomer conversion rates and molecular weights sufficient for the obtained oligomers to be considered viable as additives for plastics manufacturing. The magnetic separation, made possible by tuning the nanoparticle size and properties, represents a significant advancement to facilitate catalyst recovery and process intensification without the use of toxic solvents or aggressive reaction conditions. In parallel, the immobilization of thermophilic enzymes such as LCC, its mutant form LCC^{ICCG} and Thb on classical polymeric supports (PP beads) has revealed promising catalytic behaviour at elevated temperatures, expanding the functional scope of PET hydrolases from depolymerization to new synthetic applications. Although these enzymes have led to the obtainment of polyesters having a lower molecular weight when compared to CaLB, their thermal robustness and selectivity for longer-chain monomers highlight their potential relevance in future biocatalytic platforms as a viable alternative to the commercial (and increasingly expensive) CaLB. The third approach, based on a layer-by-layer (LbL) assembly using DNA and chitosan matrices embedded with magnetic nanoparticles, offered a compelling model for the functionalization of biodegradable film. The resulting PHBH-based films gained magnetic properties and the ability to selectively separate and remove further emphasizing the potential of integrating bio-based materials and smart coatings for circular material lifecycles.

Although all these strategies offer a valuable contribution to the field of green biocatalysis, providing an innovative and comprehensive response to the pressing challenges of sustainable polymer production and their end-of-life management, magnetic supports often have limitations regarding enzyme loading capacity, long-term stability and maintenance of catalytic activity upon repeated use or under process-relevant conditions. In addition, limitations in mass transfer may occur, particularly in systems involving high-molecular weight substrates or poorly soluble reactants. The tuning of the magnetic core itself also requires careful balancing: the increase of particle size can improve magnetic

response but carries the risk of losing superparamagnetic behaviour, which is essential for preventing aggregation and ensuring good dispersibility under reaction conditions. The development of complex magnetic nanoarchitectures (e.g., core-shell systems, hollow nanoparticles or 3D assembled superstructures) offers a path towards optimizing these physical and chemical parameters. Such engineered nanostructures can simultaneously maximize surface area, control the distribution of functional group, stabilize the catalytic layer and maintain magnetic response. Although MNPs represent a significant step forward, the next breakthrough might lie in the adoption of Metal-Organic Frameworks (MOFs) as next-generation supports for enzyme immobilization. At the same time, the exploration of renewable lignocellulosic biomasses, like bamboo powder, as low-cost, sustainable supports for biocatalysts immobilization opens complementary avenues for both batch and continuous flow applications, while contributing to biomass valorization and the broader goals of circular bioprocessing.

Recently awarded the 2025 Nobel Prize in Chemistry for their revolutionary role in the design of crystalline, porous materials with highly tunable chemical functionality, MOFs offer unique advantages: exceptionally large surface area, adjustable pore size, thermal and chemical stability and the ability to be functionalized with specific chemical groups to promote the interactions between enzyme and support. The integration of MOFs into biocatalytic systems offers the opportunity to overcome many of the current limitations. Their structural versatility allows the creation of tailored environments that preserve the conformation and activity of immobilized enzymes. This architecture not only enables higher enzyme loading densities but also improves substrate accessibility to the active sites. Looking forward, the potential synergy between magnetic supports and MOFs could represent a promising new frontier like the development of magnetic MOF-based biocatalyst. These hybrid materials combine the structural adaptability of MOFs with the ease of separability provided by magnetic cores. The result is a class of smart, reusable biocatalyst ideal for automated or continuous flow synthesis systems, where the development of magnetically responsive MOFs further aligns with the goals of catalyst recovery and reuse in sustainable processes. Furthermore, this future direction is strengthened by scientific advances celebrated with the 2024 Nobel Prize in Chemistry, awarded for discoveries in predicting protein structure through artificial intelligence. The ability to accurately model enzyme structures, including surface-exposed residues and active site orientations, now enables rational design of enzyme-support interfaces. This opens new possibilities for moving from empirical immobilization to structure-driven hybrid biocatalyst engineering, where enzyme conformation and activity can be preserved, or even enhanced, through selective interactions with functionalized MOF-scaffolds. Indeed, computational methods are now able to predict which residues

should be targeted for anchoring, how pore geometries should be adjusted to avoid steric hindrance and how the microenvironment can be tuned to increase stability or catalytic turnover.

Together, these two Nobel Prize-recognized discoveries create a unique scientific and technological synergy. The predictive power of protein structure modelling, combined with the modularity and tunability of MOFs, represents a promising frontier in biocatalysis for creating advanced hybrid biocatalytic materials with extraordinary stability, catalytic performance and sustainability. Future research will focus on the synthesis of MOFs tailored for specific enzymes (e.g., CaLB, LCC, LCC^{ICCG}, Thb), the evaluation of their catalytic behaviour under real process conditions and the development of smart, magnetically or enzymatically recoverable systems that close the loop in biocatalytic material lifecycles. This evolution not only advances academic research but also addresses pressing industrial and environmental challenges, potentially positioning these materials in the front line of next-generation green chemical manufacturing.