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Multienzymatic immobilization of laccases on polymeric microspheres: A strategy to expand the maximum catalytic efficiency

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Abstract

Laccase enzymes of were covalently coimmobilized on poly(glycidyl methacrylate) microspheres. The objective of this work was to create a biocatalyst that works efficiently in a wide range of pH. The coimmobilization was performed using two different strategies to compare the most efficient. The results showed that by correctly selecting the enzymes and concentrations involved in the commobilization, it is possible to obtain a biocatalyst that works efficiently at a wide pH range (2.0–7.0). The maximum activity values reached per gram of support for the obtained biocatalyst were 41.90 U (pH 3.0), 40.89 U (pH 4.0), and 39.54 U (pH 6.0). Moreover, the thermal, storage, and mechanical stabilities were improved compared to the free and single-immobilized laccases. It was concluded that enzymatic coimmobilization is an excellent alternative to obtain a robust biocatalyst that works in a wide pH range, with potential environmental and industrial applications.

KEYWORDS

bioengineering, biomaterials, catalysts, morphology, proteins

1 | INTRODUCTION

Laccases, extracellular multicopper enzymes that belong to the group of oxidases were first described by Yoshidain 1883[1]and are one of the oldest known enzyme types. Laccases are found in higher plants[2], fungi[3], bacteria[4], and insects[5] and are excellent biocatalysts with multiple chemical, industrial, and biotechnological applications[6,7] due to their ability to catalyze the oxidation of a wide range of substrates (mainly phenols) using oxygen from the air to produce water as a byproduct.[8]

Laccases act at various temperature and pH ranges, and although their activities are generally higher at low pH values and high temperatures, the optimal conditions of each enzyme vary significantly depending on their source.[9]Additionally, although the greatest effect is on the oxidation of phenols, through the use of mediators, the range of oxidizable compounds can be extended, making it possible to oxidize a greater variety of organic and inorganic compounds.[10]The mediators are a group of low molecular weight compounds with high redox potentials (usually above 900 mV) that have the ability to improve the catalytic activity of the laccases and act as a type of "*electric launcher*." Once oxidized by the enzyme

Abbreviations: ABTS, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); AIBN, α,α′-azoisobisbutyronitrile; ApL, *Aspergillus* sp. laccase; GMA, glycidyl methacrylate; MtL, *Myceliophthora thermophila* laccase; PDI, polydispersity index; PGMA, poly(glycidyl methacrylate); PVP, poly(*N*-vinylpyrrolidone); SEM, scanning electron microscopy; TvL, *Trametes versicolor* laccase.

to generate a very oxidizing intermediate known as comediator or oxidized mediator that diffuses out of the enzyme and are able to oxidize any substrate excluded by size from the enzymes active sites.[11] The ability of laccase enzymes to expand their range of substrates makes them exceptionally versatile and useful in environmental contaminant biodegradation.

Although laccase enzymes have many useful advantages in bioremediation, such as low energy requirements and environmental impacts, as well as high efficiency, their use at an industrial level has been restricted due to some characteristics (low stability, reusability issues, high sensitivity to denaturing agents, and high production costs).[12] Fortunately, many of these limitations can be reduced by enzyme immobilization on suitable supports. The immobilization of the enzyme refers to the confinement of the enzyme in a different phase to the phase in which the substrates and products are found.[13]Due to the interaction with the support, immobilization usually improves the structural stability of the enzymes, avoiding denaturation due to changes in the reaction medium.[14]

Some of the advantages associated with enzyme immobilization are the increased thermal, mechanical, and storage stabilities, the possibility of reuse and the easy extraction of the reaction media, which all enhance enzyme application in industrial processes.[15,16] Enzyme immobilization can be carried out on a wide variety of materials using different methods of immobilization depending on the desired final applications.[17] In the current study, according to the results obtained in previous works,[18–20] nonporous microspheres of poly(glycidyl methacrylate) (PGMA) were chosen as support for multienzyme immobilization. Epoxy-activated supports are almost-ideal ones to perform very easy immobilization of proteins and enzymes at both laboratory and industrial scale. These activated supports are very stable during storage.[21] Hence, they can be easily and long-term handled before and during immobilization procedures. Furthermore, epoxy-activated supports are able to form very stable covalent linkages with different protein groups (amino, thiol, phenolic ones) under very mild experimental conditions.[22–24]

Until now, the need for the use of biocatalysts for industrial and environmental applications within the framework of green chemistry is evident. Additionally, the need for enzyme immobilization is recognized as a necessary technology for the practical and commercial viability of biocatalysts in industry. However, multiple processes remain in which a single immobilized enzyme cannot completely catalyze a reaction and therefore the utilization of multiple cascade enzyme systems are necessary.[25] In nature, this problem is solved by the presence of different enzymes that carry out reactions sequentially, as in the case of the Krebs cycle.[26] In this cycle, a complex of eight highly ordered enzymes reacts sequentially to maintain a high local concentration of the reaction intermediates and reduce losses due to diffusion, which increases catalytic efficiency.[27,28] Due to that, coimmobilization cannot be replaced by the addition of single-immobilized enzymes, since in the single-immobilization a high local concentration of reaction intermediates would not be achieved, which is particularly important when pollutants degradation processes are performed, because there is generation of highly toxic intermediates.[25]

This work focuses on coimmobilization in order to expand the maximum efficiency pH range of the biocatalyst. The obtained biocatalyst could be used in systems where the conditions of the medium change continuously and generate pH changes. Using this biocatalyst, the pH changes of the medium will not generate a loss of the catalytic efficiency, as if it would happen in single-immobilized catalyst systems. However, the idea of this study is to create a precedent that allows seeing coimmobilized enzyme systems as an alternative to develop biocatalytic systems that work efficiently in a wide variety of conditions. These systems include reactions that are carried out under varying pH conditions, degradation of different pollutants in the same sample,[29] or the consecutive degradation of a compound and its degradation products.[25] All of the above, requires the use of coimmobilized enzyme systems since it cannot be carried out with single enzyme immobilized systems.

Until now, various approaches to enzymatic coimmobilization have been proposed to expand the pH range of maximum activity or increase the number of substrates capable of biodegradation.[14,25,29,30] However, the systems reported so far do not reach their maximum possible efficiency in the entire pH range studied possibly because the activities of the individually immobilized enzymes were not taken into account when selecting the amount of enzyme to be coimmobilized. For this reason, a study that relates the amount of enzyme used in coimmobilization and the activity of each enzyme when immobilized individually remains necessary. In this way, a system that works with maximum enzymatic activity for the entire possible pH range according to the enzymes used is possible to obtain.

In an attempt to imitate nature to solve industrial problems and to extend the optimum working pH range for the immobilized enzyme systems, we propose the covalent coimmobilization of three laccase enzymes on the same support and thus take advantage of the diversity of properties that present various types of laccase enzymes to expand the optimum pH range of the resulting polymeric biocatalyst. In this context,

coimmobilization presents several challenges, such as the preservation of enzymatic activity for all immobilized enzymes and allowance of high immobilization percentages. Additionally, because the enzymes are large (>10 kDa) possible steric effects are produced, so the interactions should be reduced to the maximum to maintain enzyme activity. All these aspects can be controlled through optimizing the conditions in which the coimmobilization is carried out as these factors must be suitable for all involved enzymes.

The main aim of this work was to construct a coimmobilized laccase enzyme system to combine and expand the maximum activity range of the three utilized enzymes. To that end, three laccase enzymes were carefully selected (according to the maximum activity pH) and used in the covalent immobilization on previously synthesized PGMA microspheres. The polymeric biocatalyst was characterized by evaluating the enzymatic activity at different values of pH, temperature, reusability, storage stability, and kinetic parameters.

2 | EXPERIMENTAL

2.1 | Reagents and materials

Commercial laccase from *Myceliophthora thermophila* laccase (MtL) was obtained from Novozyme (Bagsværd, Denmark). Commercial laccases from *Trametes versicolor* (TvL) and *Aspergillus sp* (ApL), glycidyl methacrylate (GMA), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Bradford reagent, bovine serum albumin (BSA), poly(*N*-vinylpyrrolidone) (PVP), α,α′-azoisobisbutyronitrile (AIBN), and *N,N*-dimethylformamide were purchased from Sigma-Aldrich (Chile). The reagents for buffers solutions (formic acid, ammonium acetate, and ammonium bicarbonate) were obtained from Sigma-Aldrich (Netherlands). All the chemicals were used as received without any treatment if not otherwise specified.

2.2 | Laccase immobilization on microspheres of PGMA

2.2.1 | Synthesis of PGMA microspheres

Microspheres of PGMA were obtained by dispersion polymerization as reported previously by Vera et al.[19] Briefly, the polymerization of the monomer GMA was carried out in the presence of AIBN and PVP in organic medium (methanol and N,N-dimethylformamide) while stirring for 8 hat 160 rpm under a nitrogen atmosphere at 65 ± 1°C. The obtained microspheres were washed with methanol and characterized by scanning electron microscope (SEM) and Energy-dispersive X-ray spectroscopy (EDX) using a JEOL 6380LV SEM after coating with gold under reduced pressure. The analysis was developed using a Digital Micrograph 3.7.0 image analyzer for GMS 1.2 (Gatan Inc.).

2.2.2 | Single laccase immobilization on polymeric microspheres

All enzymes were previously washed (five times) by ultrafiltration to remove external agents or impurities, following the procedure previously described by Vera et al.[20] Individual laccase immobilization was obtained by separately mixing 10 mg of PGMA microspheres with a 1 ml solution of 0.07 mg/ml of protein of each laccase (TvL, MtL, and ApL). This amount was used based on a previous determination of the optimal protein amount, in which all of the protein involved in the reaction was immobilized (data not shown). The immobilization process was performed in triplicate at pH 5.3 under stirring with a Thermo Scientific Heraeus Heidolph rotatory shaker for 24 hr at 21 ± 1°C. Then, the microspheres were washed three times with consecutive steps of centrifugation and addition of phosphate buffer pH 5.3 to remove the unbound enzyme. After the washing steps, the activity of the free and immobilized enzyme, as well as the protein concentration of the free enzymes was determined. Finally, the immobilized enzymes were characterized and compared with the free enzymes using the methods described later.

2.2.3 | Coimmobilization of multiple laccases

For laccase coimmobilization, two different strategies were performed: (a) coimmobilization of the same amount of protein and (b) coimmobilization of different amounts of proteins based on the corresponding activities of the single immobilized enzymes (see Table 1). In both strategies, the coimmobilization was carried out in triplicate using 10 mg of PGMA in a 1 ml solution and with simultaneous mixing of all the enzymes in the amounts indicated in Table 1. The maximum amount of enzyme added in all cases corresponded to 10% of the carrier. This percentage was determined in previous studies to avoid interference due to blockage of the active site or aggregations between molecules due to immobilization.[20]

The immobilization procedure was the same as performed for the immobilization of the single laccases (described previously). Finally, the coimmobilized

TABLE 1 Enzyme load of single immobilized laccases and protein amounts used for coimmobilizations

Applied laccase	Enzyme load immobilized single laccase (U/g carrier)	Protein amount, Strategy 1 (mg protein/g carrier)	Protein amount, Strategy 2 (mg protein/g carrier)
TvL	1.15 ± 0.05	2.3	5.25
MtL	1.91 ± 0.08	2.3	1.05
ApL	4.63 ± 0.16	2.3	0.70

Abbreviations: ApL, *Aspergillus sp* laccase; MtL, *Myceliophthora thermophila* laccase; TvL, *Trametes versicolor* laccase.

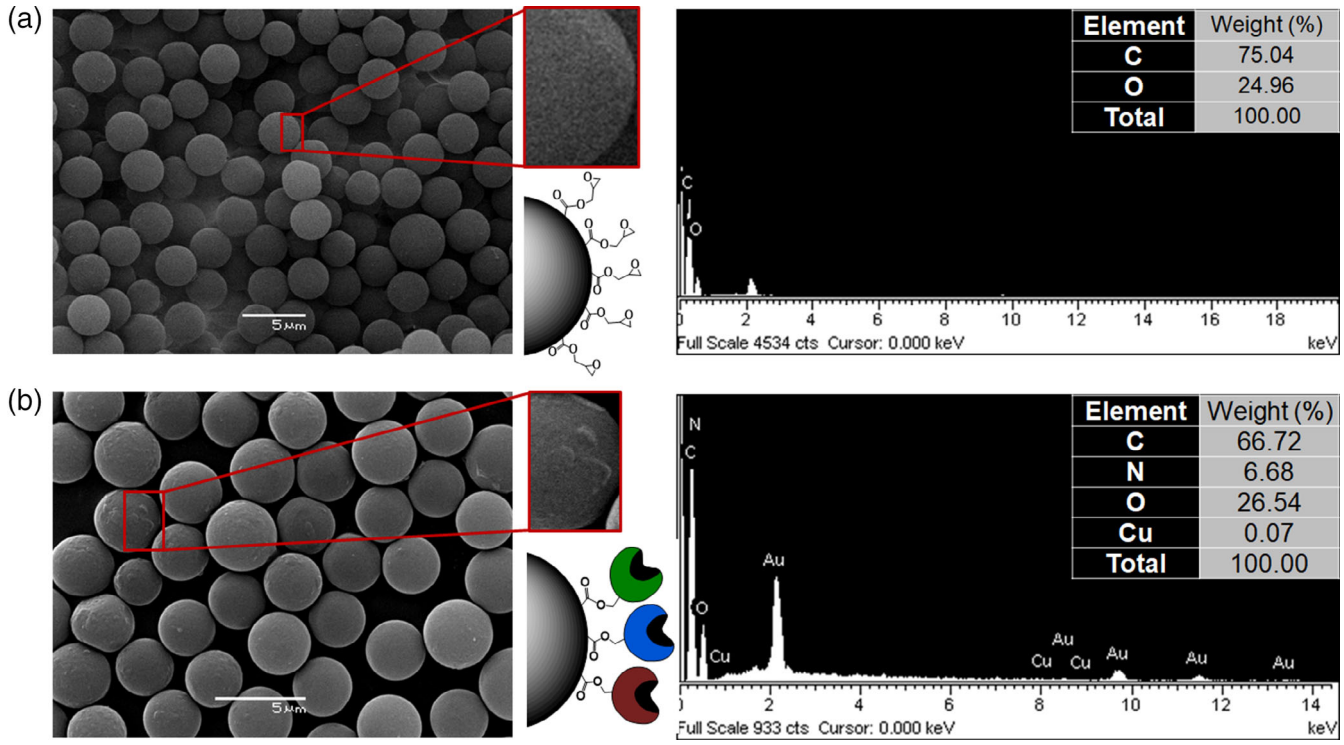

FIGURE 1 Representative SEM images of (a) the obtained neat microspheres of poly(glycidyl methacrylate) (PGMA) with oxirane groups containing surfaces and (b) PGMA after co-immobilization with the TvL, ApL, and MtL enzymes. ApL, *Aspergillus sp* laccase; MtL, *Myceliophthora thermophila* laccase; SEM, scanning electron microscopy; TvL, *Trametes versicolor* laccase [Color figure can be viewed at wileyonlinelibrary.com]

laccases were characterized and compared with the free and single immobilized laccases.

2.3 | Laccase activity assay

The activities of the free, immobilized, and coimmobilized laccases were determined by oxidation of ABTS based on a previously published method.[31] Briefly, the increase in the absorbance was assessed at 420 nm for 5 min using a UV–*vis* spectrophotometer. The reaction mixture consisted of 10 mM ABTS (50 µl) and the buffered sample (170 µl). The enzyme activity was expressed in units (U), which is defined as the amount of enzyme necessary to oxidize 1 µmol of ABTS per minute under the given assay conditions. The measurements were performed in triplicate and the results were reported in U with the corresponding *SD*. The relative activity was calculated as the enzyme activity in each point divided by the laccase activity at the point of the highest laccase activity in the same group of experiments (pH, temperature, reusability, and storage stability).

2.4 | Protein determination

The protein concentration was measured via the Bio-Rad protein assay following a previously established method.[32] Briefly, 200 µl of a diluted Bio-Rad solution (1:5 with MQ-water) were mixed with 10 µl of sample in

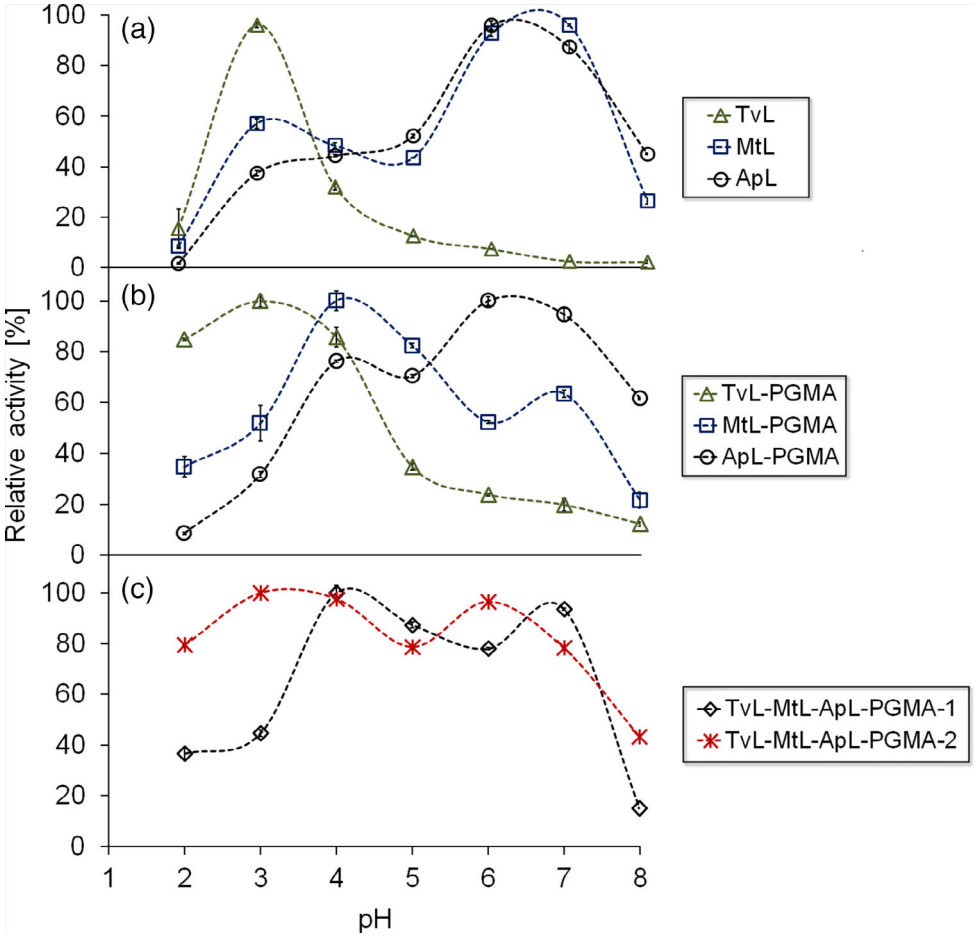

FIGURE 2 The effect of pH on the activity of free (a) immobilized (b) and coimmobilized (c) laccase enzymes at 25°C [Color figure can be viewed at wileyonlinelibrary.com]

a 96-multiwell plate. The plate was shaken at 400 rpm for5 min at 21 ± 1°C. Then, the absorbance was measured at 595 nm using a TECHCOMP UV2310II UV/vis spectrophotometer. BSA was used as a standard for the calibration curve. The measurements were performed in triplicate and reported with the corresponding *SD*.

2.5 | The effect of pH and temperature on laccase activity

The activities of the laccases (immobilized, coimmobilized, and free) under different conditions of pH and temperature were measured following the procedure previously. In this study, the pH values analyzed (2.0–8.0) were maintained using formic acid (pH 2.0), ammonium acetate buffer (0.02 M, pH 3.0–6.0), and ammonium bicarbonate (pH 7.0–8.0).The thermal stability (activity at different temperatures) was measured from 20 to 65 ± 1°C. The measurements were carried out using preheated buffer solutions at the optimum pH of each enzyme (free and immobilized). In the case of the coimmobilized enzymes, the thermal stability was measured at the three pH values with maximum activity (3.0, 4.0, and 6.0). The activities were measured in triplicate and the results were reported with the corresponding *SD*.

2.6 | Kinetics properties

To determine the kinetic parameters including the Michaelis–Menten constant (K_m), the maximum reaction rate (V_{max}), the turnover number (K_{cat}), and the catalytic efficiency (K_{cat}/K_m), the activity of the free and single immobilized enzyme was measured while increasing the ABTS concentration from 0.5 to 20 mmol/L and maintaining the amount of enzyme used in each study. The parameters were determined from the Lineweaver–Burk plot as detailed before.[20]

2.7 | Reusability and storage stability

The reusability of the coimmobilized enzymes was studied following six consecutive reaction cycles. The solution free or immobilized enzymes were mixed with a solution of 5 mmol/L ABTS in buffer solutions at three pH values (3.0, 4.0, and 6.0) and the activity was measured for

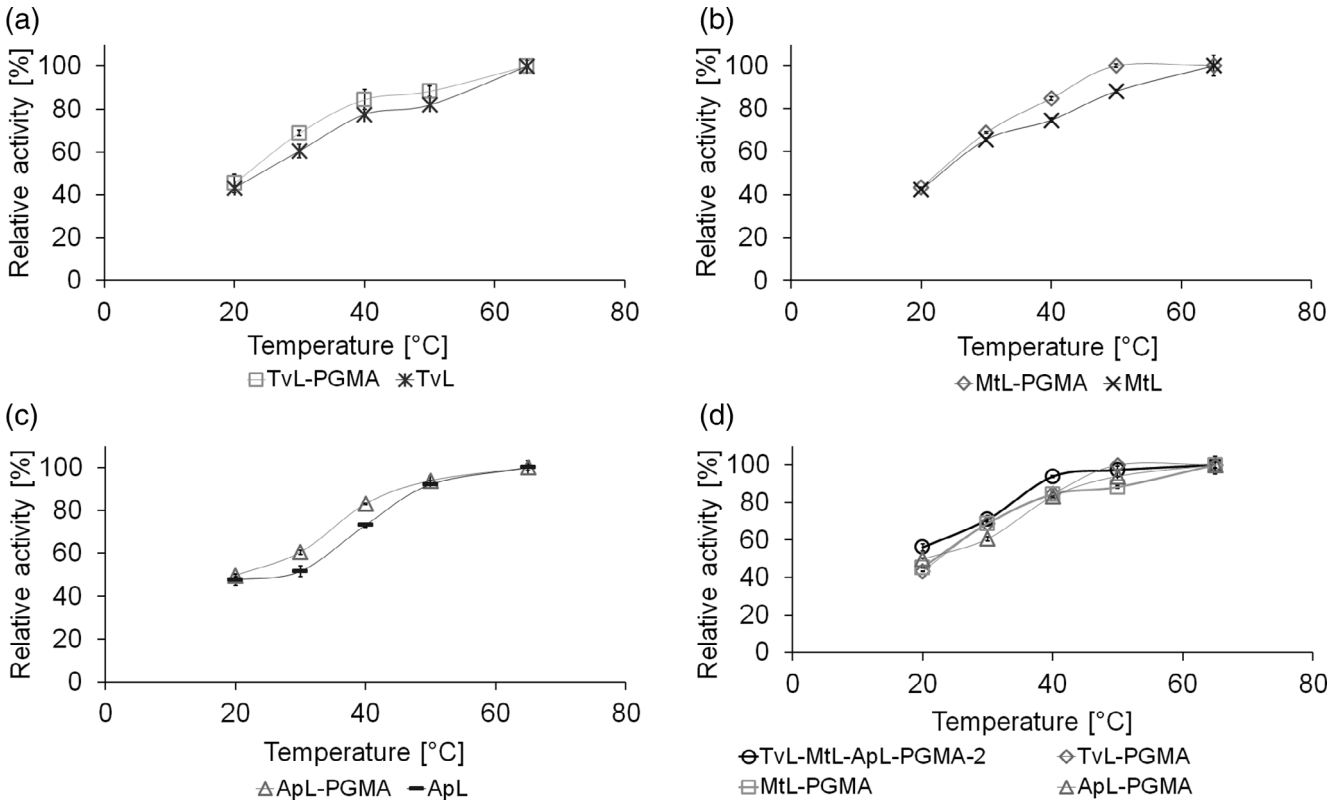

FIGURE 3 The effect of temperature on the activity of free and immobilized enzymes from (a) TvL, (b) MtL, (c) ApL, and (d) coimmobilized and single-immobilized enzymes. ApL, *Aspergillus sp* laccase; MtL, *Myceliophthora thermophila* laccase; TvL, *Trametes versicolor* laccase

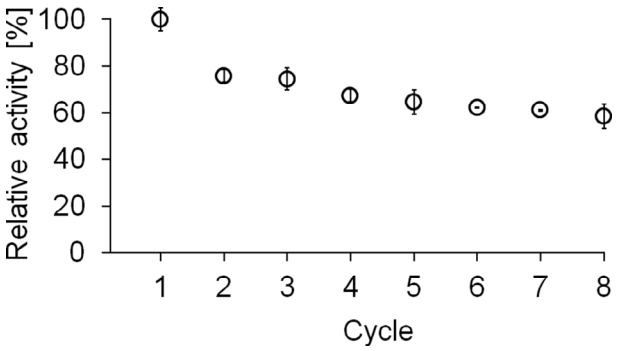

FIGURE 4 Reusability studies on the coimmobilized enzyme system on PGMA microspheres at the three pH values with maximum activity. PGMA, poly(glycidyl methacrylate)

5 min. Subsequently, the immobilized enzymes were filtered and washed with a buffer solution at the pH of the respective study to remove any unreacted ABTS. A new cycle was initiated by reacting the enzymes again with unoxidized ABTS in a fresh buffer solution. For the storage stability study, the laccase enzymes (free, immobilized, and coimmobilized) were stored at two different temperatures (25 ± 1 and 4 ± 1°C) and the activity was measured for 20 days. These experiments were performed in triplicate and were reported with the corresponding *SD*.

2.8 | Statistical analysis

All measurements were performed in triplicate and were expressed with the corresponding *SD* unless indicated otherwise. The significant differences between the means were evaluated using the statistical software SPSS version 25. The data were analyzed by one-way analysis of variance (ANOVA) and the significant differences between the means were analyzed with the Duncan test with a significance of 5%. Additionally, to compare the means between pairs of data, a *t*-test was performed for independent samples with a confidence interval of 95%. The values were considered significant when $p < .05$.

3 | RESULTS AND DISCUSSION

3.1 | Laccase immobilization on PGMA microspheres

The synthesis of the microspheres used for laccase immobilization was carried out by dispersion polymerization. The functional monomer chosen for the polymerization was GMA, which has epoxy groups capable of reacting with the amino groups from the lysine amino acids

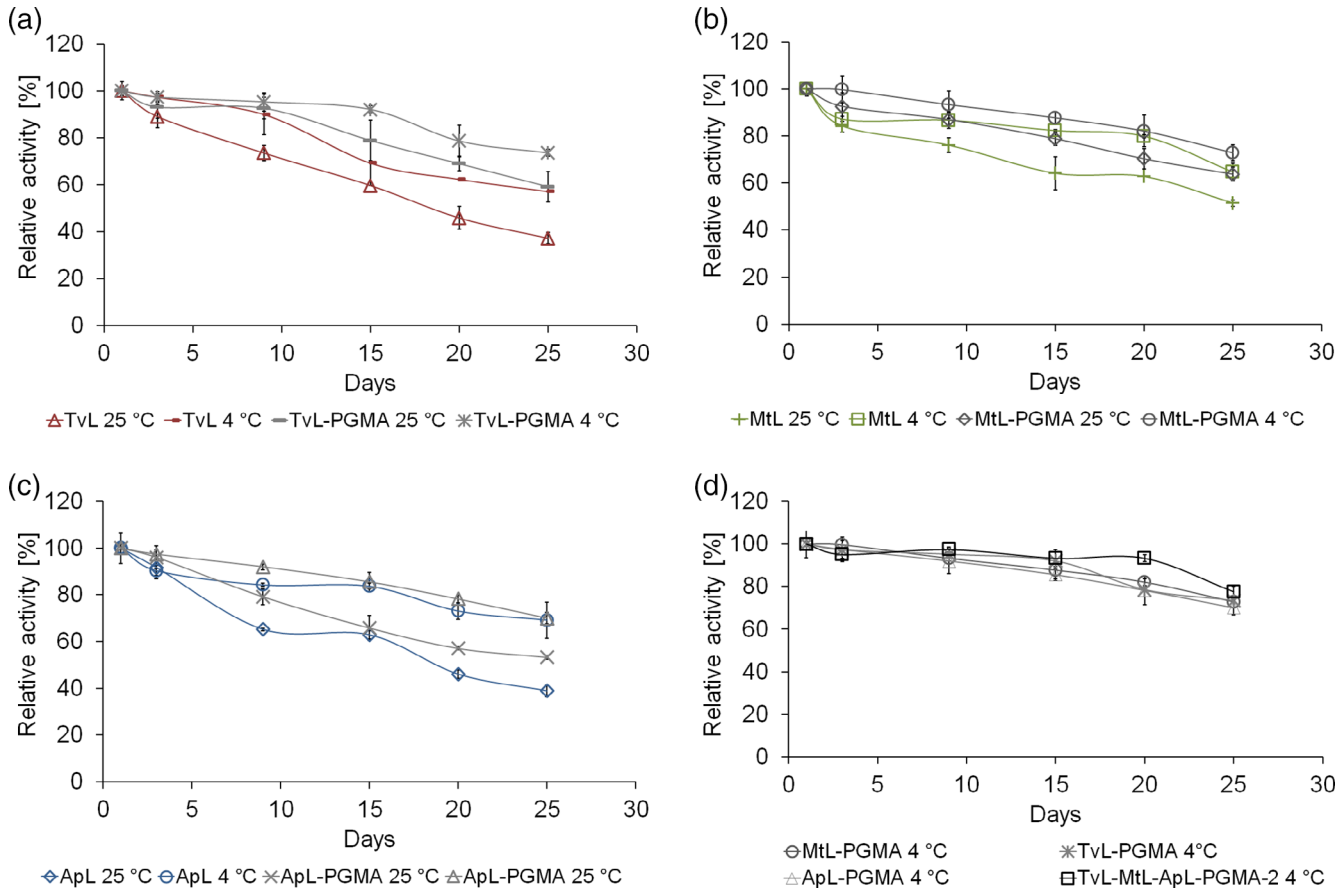

FIGURE 5 Storage stability studies at 25 and 4°C of free and immobilized enzymes from (a) TvL, (b) MtL, (c) ApL, and (d) coimmobilized and single-immobilized enzymes at 4°C. ApL, *Aspergillus* sp. laccase; MtL, *Myceliophthora thermophila* laccase; TvL, *Trametes versicolor* laccase [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 The Michaelis–Menten kinetic parameters (K_M, V_{max}, K_{cat}, and [K_{cat}/K_M]) of free and single-immobilized enzymes

Enzyme	K_M (mM)	V_{max} (μmol/[min mg])	K_{cat} (1/s)	K_{cat}/K_M (1/[s mM])	R^2
MtL free	5.7 ± 0.4	676.4 ± 16.5	1,127.33	197.78	0.991
MtL immobilized	7.3 ± 1.2	395.1 ± 25.6	658.51	90.21	0.971
TvL free	2.3 ± 0.4	355.6 ± 15.9	474.11	204.75	0.881
TvL immobilized	2.5 ± 0.5	110.2 ± 5.3	146.95	58.15	0.876
ApL free	8.3 ± 1.0	614.1 ± 31.2	1,125.7	134.85	0.978
ApL immobilized	5.4 ± 0.8	165.1 ± 9.2	302.64	55.59	0.947

Abbreviations: ApL, *Aspergillus sp* laccase; MtL, *Myceliophthora thermophila* laccase; TvL, *Trametes versicolor* laccase.

present on the enzyme surface to generate covalent bonds.[19,24] The obtained nonporous microspheres (see Figure 1a) were 2.85 ± 0.16 μm with high homogeneity and dispersity of 1.014 ± 0.166.[19,33] These characteristics of size, morphology, and functional group density are suitable for a carrier with multipoint-attachment that reportedly increase the stability of the immobilized enzyme.[34]

Once the carrier was constructed, the laccase enzymes previously selected according to the maximum activity pH (3.0, 6.0, 7.0 for free TvL, ApL, and MtL; and 3.0, 4.0, 6.0 for single-immobilized TvL, MtL, and ApL) were covalently immobilized separately and in combination (see Table 1) to expand the working range of the synthesized polymeric biocatalyst. The immobilization conditions of the laccase on the PGMA microspheres were previously optimized using MtL enzymes.[20] In the studies carried out previously with MtL, the effect of immobilized enzyme concentration was evaluated, in order not to lose enzyme activity due to steric problems.[20] After immobilization, the SEM micrographs revealed a thin and rough layer on the surface of the

microspheres, which can be attributed to the coimmobilized TvL, ApL, and MtL enzymes (see Figure 1b). To confirm that the surface roughness was due to the presence of the enzymes, EDX measurements were performed while monitoring the presence of copper (which is in the enzyme active site). However, because the four copper atoms per enzyme are internal to the active site, the percentage of copper was not significant. During the measurements, the presence of 6.68% N was determined on the rough surfaces (see Figure 1b), which was absent in the microspheres before the immobilization. The measured amount of nitrogen on the surface of the microspheres also confirms the presence of enzymes as a result of the nitrogen from the amino acids such as lysine and alanine in their three-dimensional structure.

3.2 | The effect of pH

The effect of the pH on the activity of free, immobilized, and coimmobilized enzymes was evaluated in the range of pH from 2.0 to 8.0 as depicted in Figure 2. For the free enzymes of TvL, ApL, and MtL (see Figure 2a), the maximum activity values were obtained at pH 3.0, 6.0, and 7.0, respectively. For the individual immobilizations (see Figure 2b), the optimal activity of TvL and ApL was maintained at pH 3.0 and 6.0, respectively. However, the optimum pH of MtL was shifted after immobilization, resulting in a new optimal value of pH 4.0. Furthermore, in all cases the loss of activity at pH values different from the optimal was lower than the loss shown for the free enzymes.

For coimmobilization (see Figure 2c), when Strategy 1 was applied by adding equal parts of protein in the coimmobilization process (TvL-MtL-ApL-PGMA-1), the system showed two activity maximums near pH 4.0 and 7.0. Although, while working pH work range for this system was expanded, the contribution of TvL to the total activity of the polymeric biocatalyst is very low. On the other hand, when immobilization Strategy 2 (TvL-MtL-ApL-PGMA-2) was used, in which the protein addition was based on the specific activity of each single-immobilized enzyme, the resulting new system had three activity maximums near pH 3.0, 4.0, and 6.0. For this strategy, the maximum activity values were 41.90 ± 1.75 U/g of support (pH 3.0), 40.89 ± 1.17 U/g of support (pH 4.0), and 39.54 ± 1.65 U/g of support (pH 6.0).

Additionally, when the pH values differed from the optimal values (except for pH 5.0), the reduction in activity was significantly lower than those presented for the immobilization Strategy 1 ($p < .05$ when comparing the means at pH 2.0, 3.0, 6.0, and 8.0). Therefore, all the coimmobilization processes performed in this study were carried out using coimmobilization Strategy 2 with the amounts of protein indicated in Table 1.

The conservation of a high relative activity at different pH values indicates an excellent synergy of all immobilized enzymes, which apparently show high resistance to denaturation caused by pH changes. This high resistance can be attributed to the multipoint-attachment due to the support used.[34]

Recent publications have reported coimmobilization as an innovative strategy to generate a new system by combining the properties of several enzymes to achieve greater ranges of usability.[14,35,36] Until now, studies such as those carried out by Ammann and coworkers[29] have only considered the coimmobilization of equal amounts of protein, which as demonstrated in coimmobilization Strategy 1 extends and improves the range of functionality of the system. The specific activity that each enzyme contributes to the new polymeric biocatalyst does not optimize the range of action, which results in an incomplete and inefficient system compared to the optimized system presented in this work.

3.3 | Effect of temperature on the activity of enzymes

To assess the effect of immobilization on the thermal stability of the enzymes, the activity was evaluated in the temperature range of 20–65°C as shown in Figure 3. Figure 3a–c compares the activity values obtained for the free and single-immobilized enzymes from TvL, MtL, and ApL, respectively. The enzyme activity increased as the temperature increased, and at 65°C (maximum activity value) the single-immobilized enzymes showed superior activity compared to their free enzyme counterparts, which indicates an increase in resistance to denaturation as a result of immobilization.[37,38] Figure 3d compares the activity of the coimmobilized enzymes with respect to single-immobilized enzymes. The coimmobilized enzyme system achieved greater activity at a lower temperature, which in the case of an industrial process would reduce costs. Additionally, after enzymatic coimmobilization a higher relative activity at 20°C and at 40°C was achieved with a p-value of .003 and .007, respectively. These results demonstrate that the increased stability of the enzyme towards denaturation remain efficient in the coimmobilized system. Because the amount of enzyme added to the system did not exceed 10% with respect to the support, favorable interactions could occur, which increase the stability after immobilization generated mainly by the multipoint attachment between the enzyme (lysine superficial groups) and the support (epoxy groups).

3.4 | Reusability

Reusability is a very important aspect of industrial processes, and was therefore evaluated in the coimmobilized enzyme system at the three maximum activity pH values as shown in Figure 4. After 6 cycles, more than 60% of the initial activity was maintained and the behavior for the three pH values analyzed was similar (in Cycle 6 there no significant differences were found between the three measurements, p-value = .577). These results demonstrate the maintenance of enzyme activity with respect to the others and in general suggest that the system presents good conservation in terms of activity. This aspect highlights the biggest difference between the use of free or immobilized enzymes, because if the free enzymes were simply mixed, the generated system could only be used once. The percentage of activity obtained after Cycle 6 for immobilized enzymes is superior to those previously reported, in which they have found residual activities of around 30% after 7 cycles of reuse.[39–41] There are different explanations for the decrease in activity after the sixth study cycle, such as the leaching of free or noncovalently immobilized enzyme from the support or partial denaturation.[42,43] These results suggest that this biocatalyst can be used at an industrial level, reducing the cost of the process due to the possibility of reuse of the biocatalyst.

3.5 | Storage stability

Storage stability is another key aspect of enzymatic immobilization that generates advantages between the use of solid biocatalyst and the use of free enzymes. The stability was measured at different temperatures (4 and 25°C) for 25 days to evaluate activity preservation at the recommended storage temperature and at room temperature. The results for the free, immobilized and coimmobilized enzymes are shown in Figure 5. In general, when comparing the free and immobilized enzymes, the immobilized enzymes acquired a higher stability at both storage temperatures. As shown in Figure 5a, after 25 days of storage, the increase in stability was 22% (at 25°C) and 16% (at 4°C) for the immobilized TvL compared to the free enzyme. In the case of MtL (see Figure 5b), the improvement in the stability of the immobilized enzyme with respect to the free enzymes was 13% (at 25°C) and 8% (at 4°C). For ApL (see Figure 5c), the difference between the stability of the free and immobilized enzyme was 14% (at 25°C) and 1% (at 4°C). According to the statistical analyses, MtL, TvL, and ApL presented significantly higher values of storage stability at 25 and 4°C. Finally, when comparing the stability of the immobilized enzymes with the coimmobilized enzyme system (at 4°C, Figure 5d), no marked differences were observed at most points (1, 3, 9, and 25 days). No significant differences between the values (p-values equal to 1.00, .964, .439, .101 for 1, 3, 9, and 25 days, respectively) were found, which could indicate that the immobilization and coimmobilization strategies generate a higher storage stability of the immobilized enzymes. The increased stability can be attributed to limitations in the freedom of conformational changes due to the multipoint attachment between the enzyme and the support, which protects the enzyme from denaturation.[18,34,44] In this way, enzymatic immobilization is an excellent alternative to preserve high enzymatic activity for a longer time.[39]

3.6 | Kinetic parameters

The kinetic parameters K_M, V_{max}, K_{cat} and the catalytic efficiency (K_{cat}/K_M) were determined for the free and single-immobilized enzymes and are summarized in Table 2. For the determination of these constants, the experiments were carried out at 25°C and at the previously determined optimum pH for each enzyme. The kinetic parameters were obtained from the Michaelis–Menten and Lineweaver–Burk graphs, which are shown in Figure S1-S3. The K_M value is related to the affinity of the enzyme towards the substrate with a lower value of K_M signifying a higher affinity. In the case of MtL and TvL, the K_M value increased when the enzymes were immobilized, indicating that the affinity of these enzymes towards ABTS decreased after immobilization. In the case of ApL, the value of K_M decreased, which indicates that the affinity of the enzyme towards the substrate increased. The V_{max} value decreased after immobilization for all enzymes, which indicates a decrease in the substrate conversion rate, probably due to the three-dimensional rearrangement of the enzyme caused by the interaction with the support, which could generate diffusional limitations.[45] K_{cat}, which indicates the maximum number of substrate molecules converted into product per unit time per molecule of enzyme, decreased after immobilization in all cases. Finally, the catalytic efficiency (K_{cat}/K_M) also decreased after immobilization in all cases. This tendency is common in immobilization processes due to the enzyme-support interaction.[45,46]

4 | CONCLUSIONS

The study carried out in this work proposes an efficient, unsophisticated, and simple method for the immobilization of three enzymes through a multipoint interaction (TvL,

MtL, and ApL). According to the data shown in this work, a precedent is generated for the correct coimmobilization of enzymes on different supports, which in general must have a high density of active surface functional groups, sufficient mechanical, chemical resistance, and microbial decomposition. The obtained polymeric biocatalyst presented a wide pH range of maximum catalytic activity (3.0–6.0) due to the contribution of each immobilized enzyme on the polymeric support. The obtained system was compared to the free and single-immobilized enzymes in terms of thermal stability. After immobilization, the temperature necessary to reach the maximum relative activity was reduced. Regarding reusability, coimmobilized enzyme system was able to preserve more than 60% of the initial activity after 6 cycles of reuse. In terms of stability, the results showed that both the individually immobilized enzymes and the coimmobilized enzymes were able to retain greater stability at both 25 and 4°C.

The catalytic efficiency decreased for all immobilizations due to the interactions of the enzymes with the support. The results demonstrated that by selecting appropriate enzymes, a system that works efficiently in the desired range of action with higher catalytic activities at different pH values and better mechanical, thermal, and storage stability than many of those previously reported is possible to obtain. Laccase enzymes immobilized on PGMA microspheres can be an excellent biocatalyst with multiple possible applications to industrial, environmental, and laboratory level.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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