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ORIGINAL ARTICLE

Polymeric microspheres as support to co-immobilized *Agaricus bisporus* and *Trametes versicolor* laccases and their application in diazinon degradation

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Abstract The laccase enzymes of *Agaricus bisporus* and *Trametes versicolor* were successfully covalently co-immobilized on poly(glycidyl methacrylate) microspheres. The enzyme load reached after the co-immobilization of both enzymes was 6.75 U g^{-1} carrier. The resulting biocatalyst showed the combined properties of both immobilized enzymes, increasing their optimum pH and temperature ranges. The storage and operational stabilities were also improved after co-immobilization. In presence of mediator (ABTS) the organophosphate pesticide diazinon was 100% biodegraded after 48 h of reaction with 0.2 U/mL of co-immobilized enzymes (at the two maximum activity pH values: 2.0 and 3.0). In the absence of a mediator, the degradation percentages were above 88%. Data showed that, compared with single enzymatic immobilization, the co-immobilization of the two laccases is an easy, efficient, and low cost alternative to expanding the range of work of the biocatalyst, thereby improving the stability and some biochemical properties to generate a powerful alternative for pesticide degradation in a wide range of conditions.

Abbreviations: AbL, *Agaricus bisporus* Laccase; ABTS, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); GMA, glycidyl methacrylate; PDI, Polydispersity Index; PGMA, poly(glycidyl methacrylate); SEM, Scanning Electron Microscopy; TvL, *Trametes versicolor* Laccase

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1. Introduction

Laccases (EC 1.10.3.2) are enzymes belonging to the group of blue oxidases, which are widely distributed in nature including in plants, fungi, insects, and bacteria (Fernández-Fernández et al., 2013). These enzymes are environmentally friendly and efficient biocatalysts with great biotechnological potential, due to their high activity and versatile ability to oxidize a wide variety of substrates (Agarwal et al., 2016; Bayramoglu et al., 2019; Taheran et al., 2017). However, the use of these enzymes in industrial applications could be hampered by some intrinsic properties of free enzymes such as high sensibility to changes of solvent, pH or temperature, low stability, non-reusability, and high production cost, thereby making laccase treatment not viable (Daâssi et al., 2014; Fortes et al., 2016; Gioia et al., 2015; Gonzalez-Coronel et al., 2016; Sun et al., 2015). Laccase immobilization has been proven be the most straightforward way to overcome these problems. Immobilization of laccase on suitable carriers presents several benefits such as longer stability, easy separation from the reaction mixture, reusability, reduction of inhibition by the medium or products, improved pH, thermal stability, and the possibility to be used in any kind of industrial reactors (thus avoiding common solubility problems and being able to arrest the reaction by simple filtering) (Bayramoglu and Arica, 2019; Hijazi et al., 2018; Prasetyo et al., 2016).

Several immobilization techniques on polymer supports and various parameters to be considered have been proposed to obtain the best properties in the immobilized enzyme (Arica et al., 2017a; Lettera et al., 2015; Martínez et al., 2017). Among all the developed strategies, covalent immobilization on nonporous microspheres is a promising alternative that has presented very good results, such as high load of enzymes, increased stability and no diffusion limitation due to the absence of micro and macro pores (Horák et al., 1999; Jiang et al., 2005; Tümtürk et al., 2000; Vera and Rivas, 2017). Among all of the carriers, poly(glycidyl methacrylate) microspheres in the micron range have been shown to be a successful strategy that ensures improved pH, thermal, and storage stability, reusability, and immobilization percentage (Vera and Rivas, 2017). Immobilized laccases have received great attention because of their efficient and low-cost degradation of recalcitrant compounds, including dyes (Pezzella et al., 2014), phenolic contaminants (Ammann et al., 2014), antibiotics (Yang et al., 2017), and pesticides (Vera et al., 2018), among others. Pesticide degradation is important for preventing their negative impacts on the environment and human health, and water contamination (Vidal-Limon et al., 2018).

Organophosphates are among the most widely used pesticides worldwide (Wang and Shih, 2016). Diazinon is an organophosphorus pesticide classified as moderately dangerous (Class II chemical) by the World Health Organization. This herbicide was introduced commercially in 1952, and is widely used in the control of domestic insects and in agriculture due to its efficient ability to inhibit acetylcholinesterase (Zhang et al., 2010). Due to its high efficiency, this pesticide is widely used in different types of crops such as the production of grapes, apricots, blueberries, apples, cabbage, carrots, onions, pears, and various citrus fruits (EPA Office of Pesticide Programs, n.d.; Mirmasoomi et al., 2017). Diazinon is stable at pH 7 and is relatively soluble in water (40 mg/L at 25 °C), has a vapor pressure of 11.97 mPa at 20 °C and a constant of Henry's law of 1.4×10^{-6} atm m^3/mol, which shows that it does not volatilize easily in soil or water and may persist in the environment for at least six months (Mirmasoomi et al., 2017; Shayeghi et al., 2010; The Agriculture & Environment Research Unit (AERU), 2007). This high stability, moderate solubility, and persistence in the soil facilitates the contamination of groundwater (Köck-Schulmeyer et al., 2014), urban fluvial (Bailey et al., 2000), residual (Campo et al., 2013), and drinking water derived from the surface (Zhang et al., 2012), generating as a consequence the presence of diazinon in various agricultural products, thus affecting non-target organisms, representing a great risk to the health of living beings (Tabasideh et al., 2017). In fact, different research studies have shown that diazinon is a toxic, genotoxic, cytotoxic, neurotoxic compound, and a potential chemical mutagen (Hosseini et al., 2015; The Agriculture & Environment Research Unit (AERU), 2007) whose acute oral toxicity is of 300 mg/kg.

For that reason, for decades many methods have been developed for the removal and degradation of diazinon, among which are separation with membranes, adsorption, photocatalysis, irradiation (UV, X-rays and with ionized gamma rays), oxidation (electrochemical or using activated persulfate), ultrasound and photo-Fenton (Basfar et al., 2007; Lazarević-Pašti et al., 2012; Rasoulifard et al., 2014; Real et al., 2007; Wang and Shih, 2016). However, conventional water treatment systems for degradation (chemical and physical methods) are expensive, produce hazardous by products, and have limited degradation ability. In this context, biodegradation using enzymatic methods are emerging as efficient, low-cost, and environmentally friendly alternatives for pesticide degradation (Sharma et al., 2018; Wang and Shih, 2015). Until now, single enzymatic immobilization has proven to be an excellent alternative to improving many laccase properties (Arica et al., 2017a; Bayramoglu et al., 2018). However, the co-immobilization of two enzymes with different properties and ranges of work could have many advantages over single immobilization, such as an expansion of the working range of optimal conditions, an increase in the number of possible target molecules and an increase in the applications that could be included in the system. In this way, it is important to advance in the construction of simple co-immobilized enzyme systems, starting from two enzymes that work at specific pH range. That, in order to have precedents and be able to move forward gradually in the construction of more advanced co-immobilization systems composed of multiple covalently immobilized enzymes.

Thus, as a first step in the construction of a system with multiple co-immobilized enzymes, the aims of this work are to expand the range of the maximum activity in the acid pH region by the co-immobilization of two laccases and to improve the efficiency of the degradation of the pesticide diazinon. Co-immobilization was achieved by a simultaneous reaction of the laccases of *Trametes versicolor* (TvL) and *Agaricus bisporus* (AbL) on the microspheres of poly(glycidyl methacrylate). Finally, the obtained biocatalyst was used in the degradation of diazinon, obtaining excellent results at maximum activity pH values.

2. Materials and methods

2.1. Materials

Commercial laccases from *Trametes versicolor* (TvL, EC 1.10.3.2: specific activity $\geq$ 0.5 U/mg) and *Agaricus bisporus* (AbL, EC 1.10.3.2, specific activity $\geq$ 0.4 U/mg), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate, Bradford reagent, bovine serum albumin (BSA), glycidyl methacrylate (GMA) monomer, radical initiator α,α'-azoisobisbutyronitrile (AIBN), poly(N-vinylpyrrolidone), N,N-dimethylformamide, and diazinon standard were purchased from Sigma-Aldrich (Chile). Acetonitrile (ACN) and methanol of HPLC (high-performance liquid chromatography) grade, citric acid monohydrate, disodium phosphate (Na_2HPO_4), and ethanol were purchased from Merck (Chile). All of the chemicals were analytical grade and used as received without any treatment if not otherwise specified.

2.2. Synthesis and characterization of polymeric microspheres for laccase immobilization

Poly(glycidyl methacrylate), PGMA, microspheres were prepared by dispersion polymerization by using the monomer GMA, as is previously described by Vera et al. (Vera and Rivas, 2017). Briefly, GMA, AIBN, and poly(N-vinylpyrrolidone) were mixed in organic medium under a flow of nitrogen for 8 h at 65 °C. Finally, the obtained microspheres were washed twice with methanol. The sizes and surface morphologies of the PGMA and laccase-PGMA microspheres were investigated by SEM analysis using a JEOL 6380LV scanning electron microscope after coating with gold under reduced pressure.

2.3. Enzyme immobilization by covalent bonding

For the immobilization process, the optimal protein load in which all of the applied laccase was immobilized was determined experimentally by individual immobilization of each laccase on the carrier. After that determination, 10 mg PGMA microspheres were placed in contact with 1 mL solution at different protein concentrations (see Table 1). Control experiments were performed, which immobilized each enzyme separately at the optimal protein load. For co-immobilization, the reaction was developed simultaneously by mixing half of the amount of protein used for individual immobilization experiments (see Table 1). For all cases, the immobilization process was carried out in triplicate at pH 4.2 and stirred for 24 h. Afterwards, the laccase-PGMA microspheres were washed 3 times with buffer solution pH 4.2 (0.1 mol/L citric acid/0.2 mol/L sodium phosphate). Finally, determination of the protein concentration for the free enzymes and activity assays for the free and immobilized enzymes were conducted.

Table 1 Laccases immobilized and co-immobilized on PGMA microspheres.

Applied Laccases	Protein immobilized [mg protein g^{-1} carrier]	Enzyme load [U g^{-1} carrier]
TvL	8.40 ± 0.60	6.82 ± 0.25
AbL	10.25 ± 1.05	7.78 ± 1.02
TvL (4.20 mg protein g^{-1} carrier) and AbL (5.10 mg protein g^{-1} carrier)	9.13 ± 0.17	6.75 ± 0.57

2.4. Determination of the activity for free and immobilized enzymes

The activities of the free and immobilized laccase were determined through the oxidation of ABTS as a substrate since laccases, in general, have high affinity for ABTS, which is oxidized to a stable colored cationic radical ($ABTS^+$) in proportion to enzyme activity. Activity measurements were determine using 0.5 mmol/L of ABTS in a 0.1 mol/L citric acid/0.2 mol/L sodium phosphate buffer solution at the pH of study in each case. The oxidation of ABTS was monitored by an increase in absorbance at 420 nm (ε_{420} = 36,000 $dm^3\ M^{-1}\ cm^{-1}$) for 5 min using a TECHCOMP UV2310II UV-spectrophotometer. One unit of enzyme activity (U) represents the amount of enzyme required to oxidize 1 μmol of ABTS per min under standard assay conditions.

2.5. Protein concentration

For determining the specific laccase activity and the amount of protein immobilized, the Bio-Rad protein assay was used for protein quantification as recently described Pellis et al. (Pellis et al., 2017). Briefly, 10 μL of sample was added in a well of a 96-multiwell plate and was mixed with 200 μL diluted Bio-Rad solution (1:5 in MQ-water). The plate was incubated for 5 min at 25 °C and stirred at 400 rpm. Absorbance was read at 595 nm with a TECAN Infinite M200 Pro spectrophotometer, 0.1 mol/L citric acid/0.2 mol/L sodium phosphate buffer solution was used as blank and protein was estimated using BSA as reference standard. Assays were performed in triplicate, and the results are presented as the mean ± standard deviation.

2.6. Effect of the pH and temperature on activities of free and immobilized laccases

To determine the optimum pH for free and immobilized laccases, their activities under different pH were tested using different 0.1 mol/L citric acid/0.2 mol/L sodium phosphate buffer solutions (pH 2.0–6.0). These experiments were carried out in batch mode, putting the free and immobilized enzymes in contact with 0.5 mM of ABTS at different pH values for 5 min at 25 °C. The effect of the temperature on the activity of free and immobilized laccases was evaluated by incubating the enzyme samples at different temperatures (20 °C to 70 °C) using 0.5 mM of ABTS and a buffer solution pH 3.0. All of the activity measurements were determined in triplicate. The relative activity was calculated as the ratio of laccase activity at one point to the highest laccase activity in the same group of experiments.

2.7. Reusability and storage stability studies

To investigate the reusability of the co-immobilized laccase system, the laccase activity of this system was evaluated through 6 consecutive operational cycles using 0.5 mM ABTS as substrate. At the end of each cycle, the ABTS was removed and the microspheres were washed with 0.1 mol/L citric acid/0.2 mol/L sodium phosphate buffer and deionized water to remove residues of the substrate. In each new cycle, fresh ABTS and buffer solutions were used. For the storage stability assay, individual samples of free and immobilized laccases were stored at 4 °C, and their activities were measured for 20 days at pH3 and 25 °C. The measuring procedure for both cases was carried out in triplicate, and reported whit their corresponding standard errors. The relative activity was calculated as the enzyme activity in each point divided by the enzyme activity in the first cycle in the same group of experiments.

2.8. Statistical analysis

All of the experiments were carried out in triplicate (unless otherwise indicated) and were reported with the corresponding standard deviations. Analysis of variance (ANOVA) was performed using the statistic SPSS Software version 25. In addition, posthoc analysis was performed using Duncan's multiple range test to determine the significant differences between the means. The values were considered significant when $p < 0.05$.

2.9. Diazinon degradation by the immobilized laccase system

The diazinon degradation studies were performed under the optimum pH values for the biocatalyst (pH 2.0 and 3.0) and at 25 °C to have a high activity of the enzyme during degradation. The process was carried out in Eppendorf tubes containing 1.0 mL of 120.0 μg/mL of diazinon and 0.2 U/mL of the co-immobilized enzymes. The degradation was studied at the maximum activity pH values (2.0–3.0) using buffer solutions of 0.1 mol/L citric acid/0.2 mol/L sodium phosphate. In the experiments adding a mediator, 1 mM of ABST was added. The experiments were performed in the dark, incubating at 25 °C and at a fixed agitation speed of 140 rpm. Samples were collected at different time intervals of 0, 1, 6, 12, 24, and 48 h. Each experiment was conducted in duplicate, and controls using microspheres without immobilized enzymes were used. Additionally, experiments using only immobilized enzymes and a mediator were prepared as blank experiments of the degradations with a mediator.

2.10. Analysis of diazinon degradation

After degradation, the immobilized enzyme was precipitated following the Carrez method, which consists of the addition of two solutions (Carrez I and II that contain $K_4[Fe(CN)_6]$ and $ZnSO_4$, respectively), which generates the agglomeration and precipitation of any free or weakly bound enzyme, to prevent it from interfering in chromatographic measurements. After the addition of Carrez I and II solutions, the samples were centrifuged at 12,000 rpm for 10 min, the supernatant was collected and filtered through 0.2 μm syringe filters with a membrane of PTFE (Millipore Simplicity-Syringe Filters). The concentrations of diazinon in the samples were measured using the HPLC-DAD system (YL9100 HPLC System) fitted with a reverse-phase C18 column (RP-18, 150 × 4.6 mm, 5 μm) and using isocratic elution conditions of acetonitrile:water (70:30) at a flow rate of 1.0 mL/min at 30 °C. The injection volume was 20.0 μL. Detection was performed with a diode array detector (YL9160 PDA Detector) at a wavelength of 254 nm. The degree of adsorption and degradation were calculated using the following equations (Arica et al., 2017b; Zeng et al., 2017):

$$C_{ads} = [(C_0 - C_1)V]/m \quad (1)$$

$$C_{deg} = \frac{C_1 - C_2}{C_1} \times 100 \quad (2)$$

where C_{ads} is the concentration of diazinon adsorbed in the microspheres, C_0 is the initial diazinon concentration, C_1 is the concentration after adsorption, V is the volume of the diazinon solution, m is the weight of the microspheres used, C_{deg} is the amount of diazinon degraded and C_2 is the final concentration.

3. Results and discussion

3.1. Characterization of PGMA microspheres

Nonporous PGMA microspheres with an average size of 2.85 μm and a narrow particle size distribution (PDI = 1.014 ± 0.166) were synthesized by dispersion

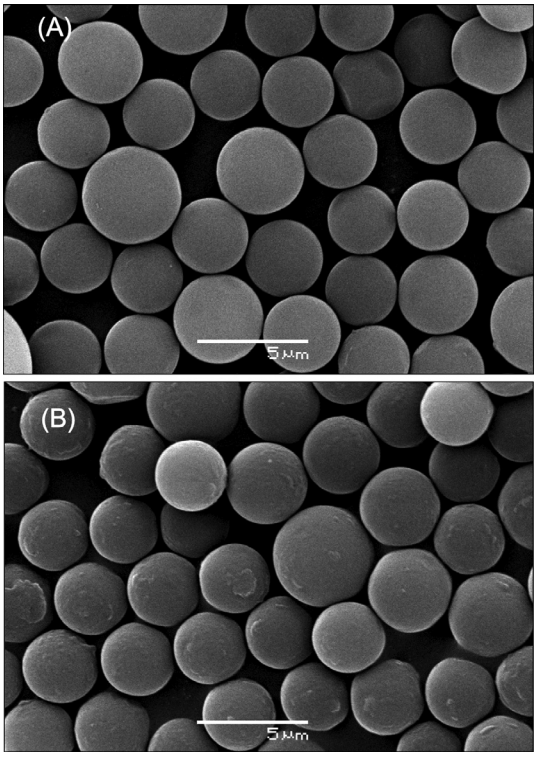

Fig. 1 SEM micrographs of PGMA microspheres (A) before and (B) after laccase co-immobilization.

polymerization (see Fig. 1 (A)). As previously reported by Vera, M. et al. (Vera and Rivas, 2017), this carrier has suitable characteristics for laccase immobilization as follows: the spherical shape of the support, which is related to a high outer surface area available for enzyme immobilization (Horák et al., 1999), high affinity of epoxy group on the carrier for amino groups in the enzyme surface, and high surface density of reactive groups, which allows the multipoint covalent attachment and increases the stability of the immobilized enzyme (Bayramoglu et al., 2018; Mateo et al., 2007).

3.2. Laccases immobilization

Covalent immobilization and co-immobilization of TvL and AbL were carried out during 24 h of reaction at 21 °C. These immobilization conditions were selected according to the previously optimized immobilization of *Trametes versicolor* on PGMA microspheres (Vera and Rivas, 2017). According to the enzymatic activity and the protein concentration analysis before and after immobilization, the total protein immobilized and enzyme load in each case are shown in Table 1. For the immobilization of single laccases, the amount of protein determined in the washing steps was below 11%, which indicated that the reacting enzymes were almost completely immobilized on the microspheres. For the co-immobilized system, the loss of protein with the washing steps was below 5%, which indicated that the two laccases were co-immobilized on the carrier. Therefore, it is evident that the method of immobilization proposed here is a suitable way to achieve co-immobilization of different laccases.

The micrographs of the carrier after immobilization (see Fig. 1 (B)) revealed changes in the surface morphologies of the beads, such as the presence of small layers on the surface of the carrier and the coarseness of laccase-immobilized microspheres surface compared to microspheres without laccases that had a smooth surface. These changes may be due to the attachment of laccase enzymes on the carrier by the formation of covalent bonds (Asgher et al., 2017; Taheran et al., 2017). These results are in agree with those previously reported by Gonzalez-Coronel et al. (2016); Misra et al. (2014), which also showed an increase in the coarseness after covalent laccase immobilization.

3.3. Effect of pH and temperature on the activities of free and immobilized laccases

In this study, the activity values of free, immobilized, and co-immobilized enzymes were tested in a pH range of 2.0 to 6.0, and the results are shown in Fig. 2. For free AbL, free TvL and immobilized TvL, the optimum pH value to obtain the maximum activity of laccase was pH 3.0. However, the loss of activity in pH values different to the optimum pH reduced for immobilized TvL. For immobilized AbL, the optimum pH value in the studied interval was shifted to pH 2.0. This shifting of the optimum pH of work is commonly observed in the formation of covalent bonds between the enzyme and the support and is usually associated with conformational changes and stiffening of the tertiary structure of the enzyme (Mateo et al., 2007). The studies of enzyme properties are necessary after any immobilization procedure, due to the laccase characteristics may be modified as consequence of possible structural changes caused by interactions with the carrier, thus modifying the optimum operating conditions of the enzyme. In this context, the TvL and ApL enzymes were chosen for the co-immobilization study because they are two commercially available enzymes that work at two different pH values when they are immobilized on the PGMA microspheres.

For the co-immobilized system, the enzymes retained the same values of optimum activity as that of the single immobilized systems (pH 2.0–3.0) which allowed for a broader working range. These results show that by using co-immobilization, it is possible to combine the properties of two enzymes in a single system. In this case, the acid optimum range of the co-immobilized system is suitable for achieving the degradation of compounds with high stability at a lower pH, as is the case of many pesticides, such as diazinon and another organophosphates (Gomaa et al., 1969).

With respect to the statistical analysis performed by Duncan's multiple range test, it was observed that at pH 2.0, there was a significant difference between the co-immobilized enzymes and the free enzymes and immobilized TvL. For pH 3.0, a significant difference was established between the co-immobilized enzymes and the free ApL. For the pH values 4.0–5.0, it was found that the relative activity of the co-immobilized enzymes were significantly different from the other enzymes. This shows that co-immobilization generated

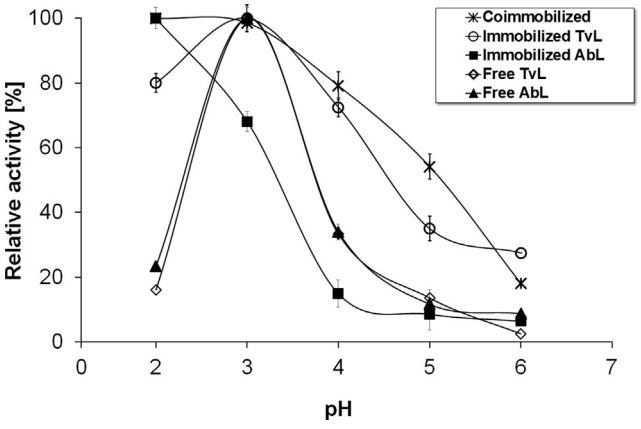

Fig. 2 Effect of pH on the activity of free and immobilized laccases. The points represent the average values, and the error bars show the standard deviations (n = 3).

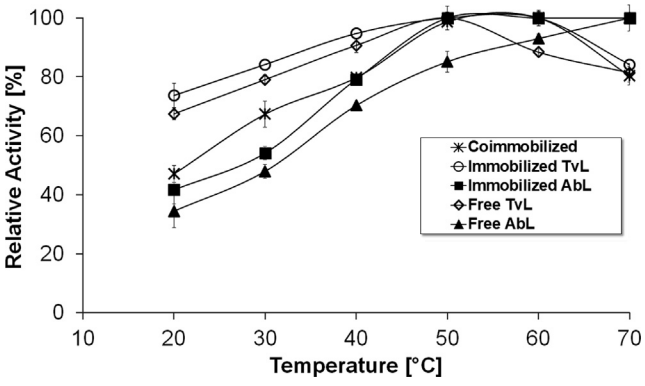

Fig. 3 Effect of temperature on the relative activity of free and immobilized laccases. The points represent the average values, and the error bars show the standard deviations (n = 3).

greater stability at different pH values, thus generating a significant difference ($p < 0.05$) with respect to individual immobilizations and free enzymes. These results indicate the importance of co-immobilization, which allow for mixing the properties of single-immobilized enzymes and creating new characteristics in the synthesized biocatalyst.

The effect of the temperature on the activity of free, immobilized, and co-immobilized laccases was investigated in the range of 20–70 °C as is shown in Fig. 3. In all of the other cases, the activity increased with the increasing temperature until 50 °C. For free TvL, the optimal temperature was 50 °C and, thereafter, decreased by increasing the temperature. In the case of immobilized TvL, the range of optimal temperature was expanded to 60 °C, which indicated an increase in the thermal stability of the enzyme after immobilization, which could be a result of the multipoint attachment on the carrier that protects the enzyme from denaturation at higher temperatures (Chen et al., 2015). For free AbL, the optimal temperature was 70 °C. For immobilized AbL, the range of optimum temperature expanded from 50 °C to 70 °C, thereby increasing the range of work and making it possible to obtain higher activity at lower temperature. Furthermore, compared with the free AbL and with the exception of 70 °C, the activity for immobilized AbL under all of the temperatures were significantly higher ($p < 0.05$) and demonstrated an increase in stability after immobilization. In the case of the co-immobilization of AbL and TvL, the behavior was a mixture between that which was observed for each laccase immobilized separately, and the range of temperature of maximum activity for the co-immobilized system was 50–60 °C. This behavior of the co-immobilized system is evidence of the ability to make a new biocatalyst with the combined properties of properties of two laccases. Besides, this high thermal stability of the enzymes would be extremely useful in systems that work at temperatures above the ambient, as is the case in the case of the textile, pulp and paper and food industries (Fortes et al., 2016).

3.4. Reusability and storage stability studies

Reusability is one of the most important, cost-effective measurements required in industrial applications. Studies of the reusability in the batch were carried out in 6 consecutive cycles,

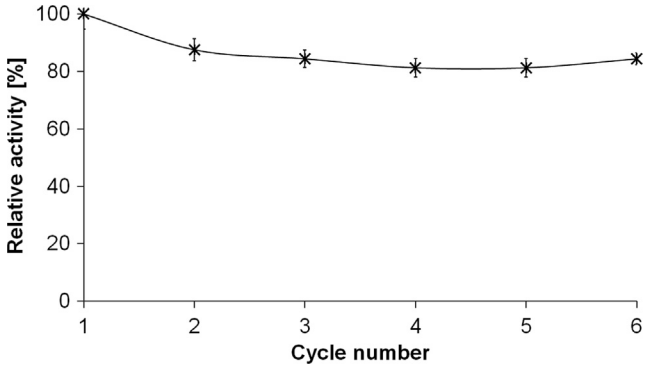

Fig. 4 Reusability of co-immobilized laccases on poly(glycidyl methacrylate microspheres). All of the measurements were performed in triplicate and are shown with the corresponding standard deviation.

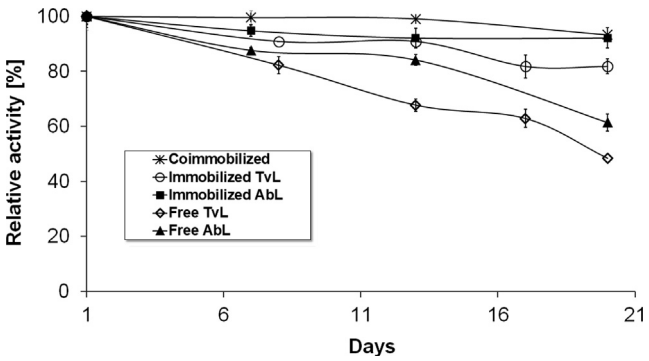

Fig. 5 Storage stability of free and immobilized enzymes at 4 °C. All the measurements were performed in triplicate and are graphed with the corresponding standard deviation.

and the results are shown in Fig. 4. The plot shows that the co-immobilization conserved almost 80% of the initial activity of laccases after 6 cycles of oxidation with ABTS as substrate. According to the ANOVA (with a level of significance of 95%), there were no significant differences between the values obtained for each cycle ($p = 0.07$). These results are in agree with those previously obtained for laccase immobilization by covalent bonds (Asgher et al., 2017; Zhang et al., 2017). The good preservation of the activity for co-immobilized laccases can be attributed to the stability given by the enzymatic immobilization, in this case, by the formation of the covalent bonds with the carrier. This characteristic makes it suitable for industrial applications due to cost reduction in the process that requires repeated batch operations and the ability of stop reactions by simple filtration that are started again without significant losses in activity.

To investigate the storage stability of free and immobilized laccases, all of the enzymes were incubated at 4 °C up to 20 days (see Fig. 5), and the initial laccase activity was set for 100% in each case. The Free TvL and AbL laccases were found to retain 48% and 60%, respectively, of their initial activities after 20 days of storage. At identical storage conditions, the residual activity of the immobilized TvL and AbL increased until 81% and 92%, respectively. In the case of co-immobilized TvL and AbL, the residual activity was 93%. The significant increase ($p < 0.05$) in storage stability after immobilization is evidence of the advantage of immobilization and co-immobilization in preventing laccase from inactivation (Feng et al., 2014). The increase in the storage stability could be attributed mainly to the stabilization of immobilized enzyme by a multipoint covalent attachment on the microspheres, which increased the stability of the enzyme, and thus, reduced drastic conformational changes of laccases under extreme conditions. Similar results of the increase in stability after covalent immobilization have been previously reported by other authors, thus demonstrating that laccase immobilization is a useful way to reduce the cost in industrial applications due to the possibility of retaining higher catalytic efficiency of laccases for a longer duration time (Asgher et al., 2017; Bayramoğlu and Yakup Arica, 2009; Ji et al., 2017).

3.5. Enzymatic degradation of diazinon

The degradation of diazinon, *O*,*O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, by the new

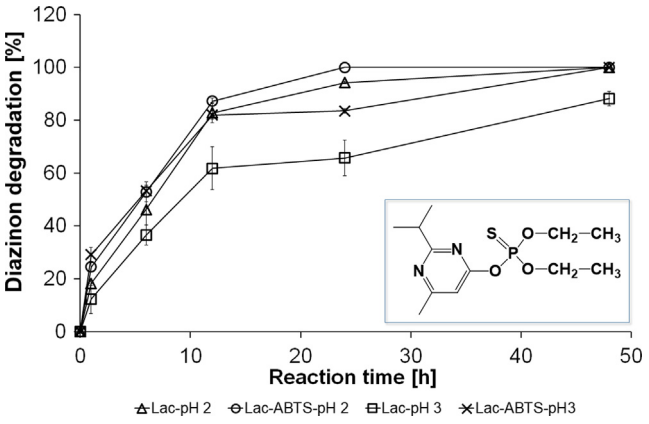

Fig. 6 Diazinon degradation by the biocatalyst with co-immobilized AbL and TvL. All of the experiments were made performed in duplicate and are reported with the standard deviations.

biocatalyst with two co-immobilized enzymes was studied over a period of 48 h in batch mode at pH 2.0 and 3.0. The evolution of the degradation at both pH values was followed by HPLC-DAD following the signal around 4.47 min. The calibration curve was performed in the range of 1–20 μg/mL and a limit of detection of 0.92 μg/mL was reached. In this section, two types of experiments were carried out: the degradation of diazinon without and with mediator (ABTS), as is shown in Fig. 6.

Before carrying out the degradation experiments using the co-immobilized enzymes and diazinon as substrate, the efficiency in the degradation of ABTS as a target molecule was evaluated using both enzymes TvL and AbL (free and individually immobilized). The specific activity of each enzyme serves to estimate its contribution to the degradation of the pesticide diazinon (Sun et al., 2016). For that reason, to have the same contribution of both enzymes, the enzyme load of each enzyme on the carrier in the co-immobilization varied depending on the specific activity of each, as shown in Table 1.

The initial concentration of diazinon added was 120 μg/mL and was selected because although the authorized amount of diazinon in drinking water in different countries is around 0.5–20.0 μg/L (Hamilton et al., 2003), recent studies in which the toxicity of diazinon was investigated for freshwater fish found that diazinon is moderately to highly toxic with LC_{50} values ranging from 90 to 7800 μg/L (Tabasideh et al., 2017). Additionally, it is known that in many countries, farmers add pesticides in an uncontrolled way to improve crop yields, so it is possible to find high concentrations of pesticides in various water sources and that is why the initial amount used was selected (Tabasideh et al., 2017).

The first behavior observed when contacting the microspheres with the pesticide was that the initial concentration measured by HPLC-DAD was lower than that initially added (~30% of the added concentration) which indicates that the obtained biocatalyst initially generates the adsorption of the pesticide, probably due to van der Waals type interactions and hydrogen bonds between the diazinon and the carrier. Hydrogen bonds could be generated by the interactions between hydroxyl groups from the opening of the epoxy groups in the carrier (at the studied pH) and diazinon, as previously reported with other polymers (Motaharian et al., 2016). It results in the reduction of the initial concentration measured by the HPLC-DAD. For this reason, the initial measurements were taken after 5 min of contact in all the samples, in order to avoid confusing the adsorption with diazinon degradation. However, measurements were also made contacting the diazinon with microspheres without immobilized laccases (blank), and it was observed that after 48 h of reaction the adsorption was about 20%. These results indicate that initially the microspheres of PGMA can be used in the removal of low concentration of diazinon by adsorption under the working conditions.

For the experiments using no mediator, approximately 88% of the diazinon was degraded at pH 3.0 after 48 h (Lac-pH 3), while 100% of the initial diazinon was degraded at pH 2.0 (Lac-pH 2) under the same batch conditions. In the experiments using 1 mM ABTS as a mediator, the degradation percentages increased to 100% for both pH values (Lac-ABTS-pH 2 for pH 2.0, and Lac-ABTS-pH 3 for pH 3.0). The effect of the mediator in increasing the rate of diazinon degradation was due to the ABTS being a molecule that acts as an electron shuttle between enzymes and substrate, thereby enhancing the rate of conversion. For that reason, mediators like ABTS are needed to maximize the potential use of laccases as bioremediation agents (Zeng et al., 2017). The increase in the rate of degradation after adding mediator has been published previously in several studies in which laccase enzymes are involved (Bayramoglu et al., 2018; Bayramoglu and Arica, 2019)

According to Fig. 6, the degradations made at pH 2.0 were more effective than those made at pH 3.0 (both in the absence of a mediator), which could be due to a higher specific activity of immobilized AbL, as evidenced in Table 1. However, in multiple studies it has been reported that diazinon degradation processes are favored by decreasing the pH of the solution, which is in agreement with that observed in this study (Dehghani et al., 2019; Tabasideh et al., 2017).

Another important aspect which is shown in Fig. 6, is a decrease in the degradation rate after 12 h of reaction. This effect could be attributed to the possible accumulation of degradation products causing inhibition in the degradation process; another possibility could be the decreasing concentration gradient of diazinon in the medium, as previously reported (Bayramoğlu and Yakup Arica, 2009).

So far the degradation of diazinon has been studied, even proposing degradation mechanisms through oxidation, generating various types of by-products throughout the degradation process (Ayoubi-Feiz et al., 2019; Ménager et al., 2007; Rasoulifard et al., 2014). In this study, the appearance of a peak was observed around 1.27 min (see Fig. S1), probably due to the appearance of 1,2,3-benzotriazin- 4(3*H*)-one as byproduct, as has been observed previously in diazinon degradations under the same conditions of analysis (Ménager et al., 2007). However, due to the overlap with the solvent signal, it was very difficult to quantify it, as well as the quantification of other byproducts that theoretically should appear at very close retention times (Ménager et al., 2007).

Different methods of diazinon degradation have been based on electrochemical oxidation, photocatalysis, ultrasonic treatments, photo-Fenton, and oxidation with activated persulfate has been proposed (Lazarević-Pašti et al., 2012; Rasoulifard et al., 2014; Wang and Shih, 2016). Nevertheless, biodegradation is one of the most rapid, efficient, and eco-friendly

alternatives for pesticide degradation. In this manuscript, has been demonstrated by co-immobilization of the two enzymes that the pH of maximum degradation can be expanded to obtain a new biocatalyst that works efficiently at a wide range of pH values and temperatures by combining the properties of both co-immobilized laccases.

4. Conclusions

Laccase enzymes from *Trametes versicolor* and *Agaricus bisporus* were successfully co-immobilized on poly(glycidyl methacrylate) microspheres. Because of co-immobilization, the maximum activity pH and temperature range was expanded. In addition, the storage and operational stabilities improved after co-immobilization. The biocatalyst containing the co-immobilized laccases was used for diazinon degradation at two different pH values with 1 mM of the mediator ABTS, showing total degradation after 48 h of reaction in both cases. The current research is a contribution in the way to expand the working range of laccase enzymes in the acid region. Nevertheless, future research including enzymes co-immobilization that also work at a wider range of pH is still necessary.

Contributors

Myleidi Vera designed and performed the experiments. Myleidi Vera, Bernabé L. Rivas, Gibson S. Nyanhongo and Georg M. Guebitz analyzed and wrote the paper and approved the final article.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2019.07.003.

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