*Article*

# Nanoencapsulated Laccases Obtained by Double-Emulsion Technique. Effects on Enzyme Activity pH-Dependence and Stability

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**Abstract:** One primary drawback of enzyme catalysis at industrial scale is the short-term service life of the enzymes, they lose their activity due to oxidation or other processes which results in less stability and a shorter lifetime thereby rendering them less efficient. An effective way to increase the stability of the enzymes is to attach them to nanoparticles. In this work, the polymer Eudragit® L 100-55 sensitive to pH was used to prepare laccase polymeric nanoparticles by the double-emulsion solvent evaporation approach. The size and morphology of the nanoparticles obtained were evaluated—as well as the encapsulation efficiency and zeta potential. pH effect on activity and stability was compared between free and immobilized laccase. Their stability was also determined in a sequential assay involving acidic pHs up to alkaline ones. The nanoparticles had a spherical shape with a mean size of 147 nm, zeta potential of −22.7 mV at pH 7.0 and load efficiency of 87%. The optimum pH of both free and immobilized laccases was 3.0, being the nanoparticles more stable at acidic pHs. Thus, this would be the first report of obtaining laccase nanoparticles with potential application in animal feed due to the stability conferred to enzymatic activity at pHs similar to those present in the gastrointestinal tract of rabbits, which would allow their potential use in animal feed.

## 1. Introduction

The global market for industrial enzymes is growing rapidly. These biocatalysts are key to new processes due to their ease of production, substrate specificity and green chemistry. However, industrial applications are often impeded by their lack of long-term operational stability, shelf life and by their recovery and reusability. Enzyme immobilization is one of the strategies to overcome these problems [1]. Several new types of carriers and technologies have been implemented to improve traditional enzyme immobilization in order to enhance enzyme loading, activity and stability and decrease the enzyme biocatalyst cost in industrial biotechnology, among them most recently used is nanoparticle-based immobilization of enzymes [2,3]. The use of immobilized enzymes has several advantages over the

application of free enzymes, they can be recovered and reused, often maintaining activity for large periods of time [3,4]. Enzyme immobilization techniques can be either physical or chemical in nature. Physical approaches involve the creation of nonspecific interactions via hydrogen bonds, ionic and hydrophobic interactions. These methods include entrapment and adsorption that do not require functionalized supports. The entrapment is the inclusion of an enzyme in a polymer network, which is typically an organic or an inorganic polymeric matrix. The entrapment can be performed by gel/fiber entrapping, encapsulation and metal–organic frameworks embedding [1–3]. Among the carriers employed for enzyme immobilization synthetic polymers distinguished by their versatility and easy recovery of the enzymes. Eudragit® is one of such polymers employed. It is an anionic copolymer based on methacrylic acid and methyl methacrylic acid, pharmaceutical grade, designed for controlled release and site-specific drug delivery in the gastrointestinal tract. In particular, the polymer Eudragit® L 100-55 (copolymer based on methacrylic acid and ethyl acrylate) was designed for targeted drug release in the duodenum as it dissolves at pHs higher than 5.5 [5]. Although its pharmaceutical use stands out, it has been only applied up to now in the co-immobilization of laccases and cellulases for use in the textile industry [6]. The co-immobilized enzymes showed higher stability at pH and temperature than the free enzymes [6], highlighting the potential of Eudragit in the stabilization of laccase.

On the other hand, in the livestock industry there is a growing interest in new techniques for improving the digestion of plant-based foods. Recent investigations have focused on enteric particles that do not cause stomach irritation, increase intracellular penetration, retention time and controlled release of the active compound, besides being capable of resisting the gastric acid environment. Enteric micro and nanoparticles (NPs) have been used to improve the bioavailability of protein compounds such as insulin and enzymes [7].

White-rot fungi are known to be potential producers of ligninolytic enzymes, among them laccases [benzenediol oxygen reductases (EC 1.10.3.2)]. Besides being efficient lignin degrading biocatalysts, these enzymes have been applied in pollutant biodegradation, biopulping, biobleaching, phenolics elimination for stabilization and browning of fruit juices, beer/wine, oxidation–reduction reaction in biosensors development, etc. However, laccases have been scarcely tried for possible application in improving the bioavailability of nutrients and digestibility of animal feed, especially for monogastric diets [8]. The beneficial effects of laccases (besides those of cellulases, xylanases and amylases) on rabbit meat have been reported [9]. Nevertheless, as far as we know there is no information on their use in the form of preparation immobilized on polymeric NPs as supplements for animal feed.

Thus, the objective of the present study was to immobilize the laccase produced by *Trametes maxima* CU1, by the double-emulsion solvent evaporation technique, employing the pH-sensitive polymer Eudragit® L 100-55; and evaluate its pH stability in vitro, in order to apply these NPs for pH-dependent site specific release in rabbit's gastrointestinal tract.

## 2. Results and Discussion

### 2.1. Laccase Purification

Purified laccase was obtained from *T. maxima* CU1-submerged cultures with specific activity of 182.3 U mg$^{-1}$ proteins, 28.8-fold purification and a final yield of 53.9%. Comparable results were reported when purified laccases were obtained from other *Trametes* species following similar purification protocols [10]. Although *T. maxima* CU1 laccase purification has been reported before, the strategy applied for its purification resulted in a specific activity of 121 U mg$^{-1}$ and 5-fold purification [11]. Therefore, in order to increase laccase yield in this work cell-free supernatant was subjected to 80% ammonium sulfate salt precipitation and chromatography steps were inverted.

## 2.2. Nanoparticles Characterization

The average particle size of the NPs was 147 nm, with a polydispersity index (PDI) of 0.230 (Figure 1), features of interest for their employment in animal feed. This size could facilitate the in vivo distribution of the NPs and the entry in animal body by ingestion [12]. It has been reported that stomach retains food particles until they are fragmented into pieces smaller than 0.5 mm in diameter [13]. Due to the smaller size of the NPs there should not be any significant delay in their gastric emptying, then they would enter duodenum where its pH > 5.0 would induce deprotonation of the polymer's carboxylic groups and the release of the enzyme would begin as a result of polymer dissolution. Particle size affects the release of the active agent. Smaller particles offer larger surface area. As a result, most of the active agent loaded onto them will be exposed to the particle surface leading to fast release when reaching the site of action [14].
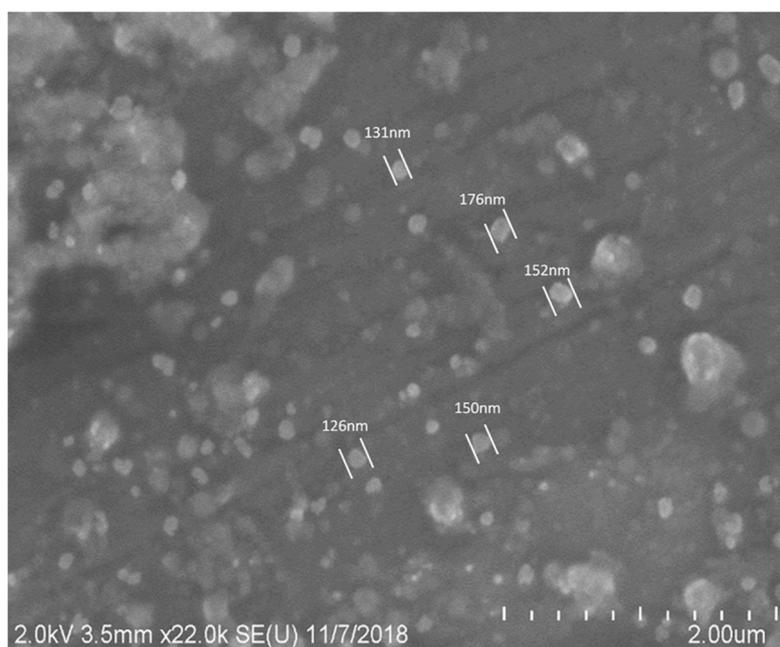


**Figure 1.** SEM image of the nanoparticles (NPs). Their spherical shape is shown.

The surface charge of the NPs was determined by zeta potential measurements. The NPs had a negative charge (zeta potential −0.638 mV at pH 2.0) which was influenced by the pH of the surrounding media decreasing even more at neutral pH (−22.7 mV at pH 7.0) (Table 1). pH 7 may have favored the disintegration of the polymer. The carboxylic groups of anionic particles get protonated if the pH is below the pKa of the carboxylic acid leading to decrease in surface charge of particles. This reduction in surface charge diminishes electrostatic repulsion and increases Van der Waals forces of attraction among the particles facilitating aggregation [13]. In the case of the methacrylic acid (Eudragit® L 100-55) with a pKa of 4.23, at pH lower than this value the surface charge of particles would decrease, thus favoring aggregation [13]. In previous studies applying this polymer for insulin immobilization similar zeta potential values were obtained [15]. In terms of intestinal uptake, apart from their particle size, nanoparticle nature and charge properties seem to influence the uptake by intestinal epithelia [16]. NPs based on hydrophilic polymers, negatively charged, showed a strong increase in bioadhesive properties and were absorbed by both M cells and absorptive enterocytes [16]. Bioadhesion allows the active agent to remain in contact with a particular organ for an extended period of time [17]. Therefore, laccase immobilized on these negatively charged NPs would have longer contact with duodenum and dietary fiber, contributing to a more efficient degradation and improving yield.

**Table 1.** Potential zeta values of laccase immobilized on polymeric nanoparticles (NP + Lac) evaluated at different pHs.

| Treatment | pH | Potential Z Values (mV) |
|---|---|---|
| NP + Lac | 2.0 | −0.638 |
| NP + Lac | 5.0 | −6.2 |
| NP + Lac | 6.1 | −17.00 |
| NP + Lac | 7.0 | −22.7 |

Moreover, the encapsulation efficiency of laccase on Eudragit® L100–55 NPs was found to be 87%. Sharma et al. [13] when using the same polymer to encapsulate the enzyme papain by the double-emulsion W1/O/W2 technique, obtained similar encapsulation efficiency (74.49%). Jelvehgari et al. [15] developed insulin NPs employing Eudragit® L100-55 combined with chitosan and reported an encapsulation efficiency of 30.56 ± 2.76% with particle sizes of 135–199 nm. In this work, higher encapsulation efficiency was attained, and the encapsulation of laccase in NPs by the double-emulsion approach is reported for the first time.

### 2.3. Optimum pH for Enzyme Activity

Fungal laccase are highly nonspecific enzymes with the ability to oxidize a wide variety of aromatic compounds, typically phenols and arylamines. Depending on the chemical nature of the substrate, two types of behaviors (pH-activity profiles) have been reported. (1) With non-phenolic substrates (for example ABTS) most laccases show maximum activity at acid pH, followed by a monotonic decrease at neutral pH). In phenolic substrates (such as 2,6-DMP) biphasic activity curves are presented, with increases in the acid range until reaching the optimum pH close to 4.0, followed by a decrease towards the neutral pH zone [18]. In this work, the effect of pH on free laccase and the immobilized one was tested in the range 2.0–8.0 (Figure 2). Both activities presented the typical bell-shaped curve for phenolic substrates [18]. They exhibited a maximum at pH 3.0 but decreased at pH varying from 3.0 to 6.0.
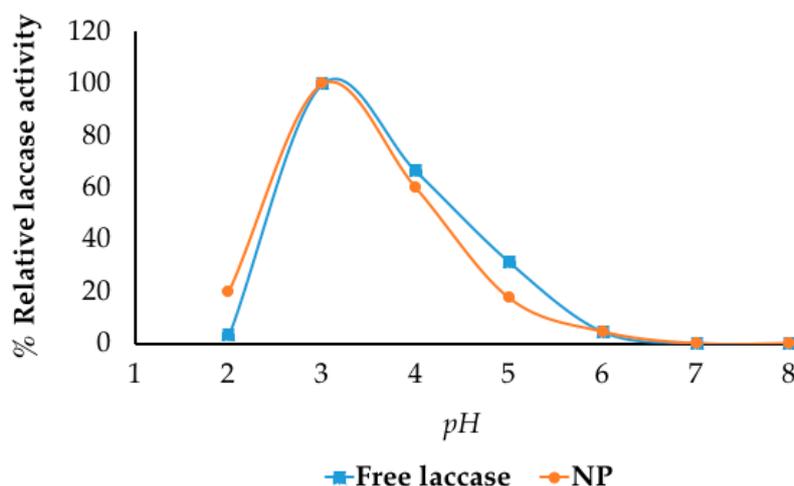


**Figure 2.** Effects of pH on the activity of free laccase and laccase immobilized in Eudragit® L 100-55 nanoparticles (NP).

An increase in pH may have caused a conformational change in laccase structure—especially on its catalytic site—thus inhibiting internal electron transfer and differing reaction product [19]. This suggests that enzyme encapsulation affects neither protein conformation nor enzyme function, keeping this laccase the characteristic behavior of the blue ones [19–21]. The inhibition of T2 Cu site may be explained by the presence of OH⁻ ions, which prevent oxygen reduction to water via the reaction $O_2^- + 2H^+ \rightarrow H_2O$ [18].

### 2.4. Effect of pH on Laccase Stability

Immobilized laccase was statistically more stable ($p \leq 0.05$) than the free enzyme (Figure 3) in the pH range 2.0–5.0. The nanoparticles kept approximately 80% of enzyme activity at pH 2.0 after 24 h. This result may be explained by the reduction in the conformational flexibility and the increase in rigidity of the immobilized enzyme, thus enhancing its resistance against denaturation [22], demonstrating the protective effect of the polymer. Moreover, the enzyme-bound NPs showed Brownian movement when dispersed in aqueous solutions, exhibiting enzymatic activities comparatively better than that of the unbound enzymes.
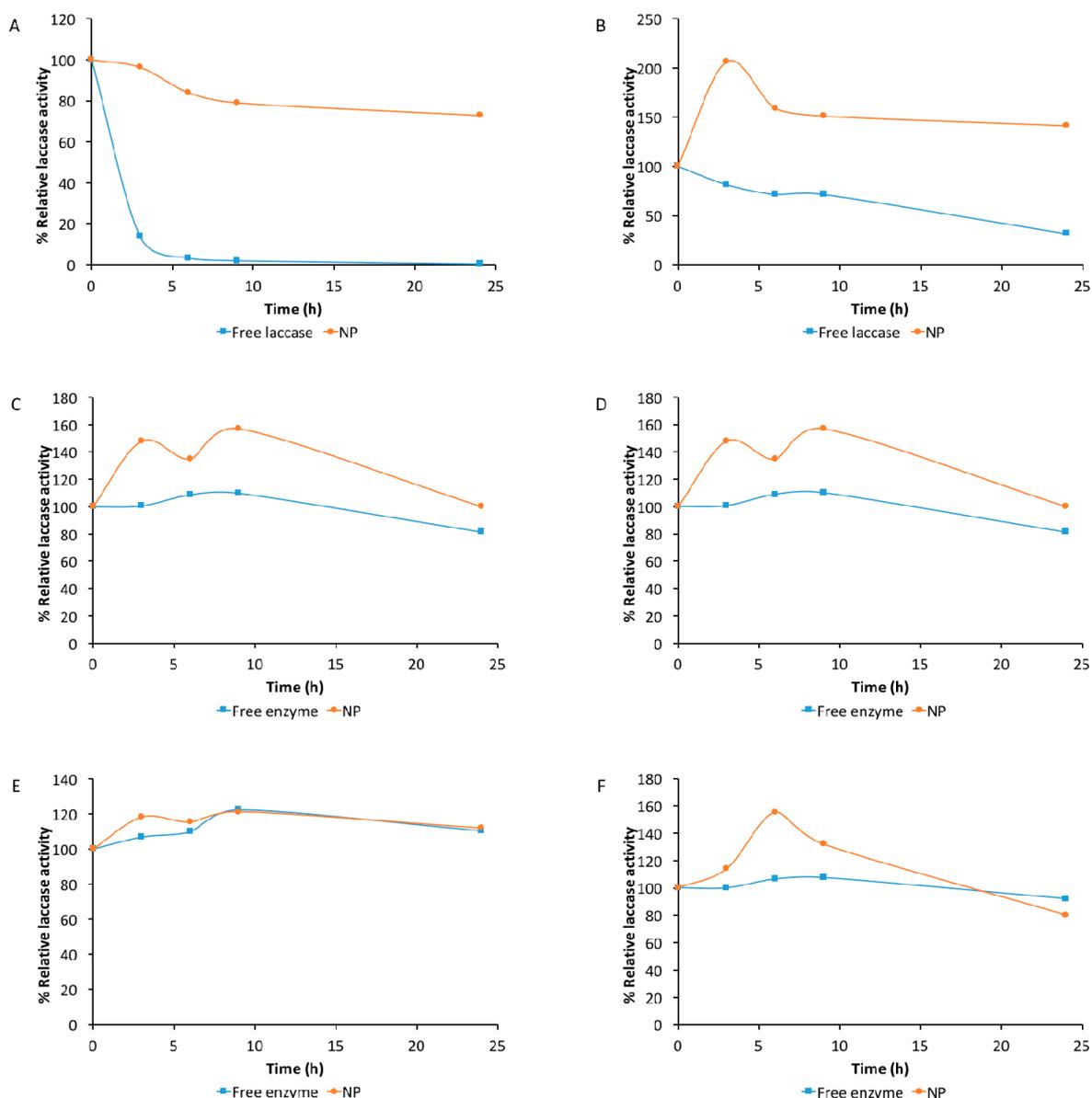


**Figure 3.** pH stability assays carried out by incubating (solid line) free and (dashed line) immobilized laccase on Eudragit® L 100-55 nanoparticles (NP) at 25 °C and different pHs from 2.0 to 7.0 with Britton–Robinson buffer. (**A**) pH 2.0; (**B**) pH 3.0; (**C**) pH 4.0; (**D**) pH 5.0; (**E**) pH 6.0; (**F**) pH 7.0. Samples collected at selected times and residual activities determined.

There was no statistically significant difference between the stability displayed by both free and immobilized laccases at pH 6.0 ($p \leq 0.05$). Eudragit® L 100-55 polymer pharmaceutical grade has been developed for retarded and controlled release of the active agent in the duodenum at pH higher

than 5.5. It offers several benefits, such as improvement in the effectiveness of the active agent, better stability during storage, direct intestinal action and protection against gastric fluids [23]. It is worth mentioning that although most fungal laccases have optimum catalytic activity at acidic pHs, they show maximum stability under neutral/slightly alkaline conditions [24]. On the other hand, in all treatments (excluding pH 2.0), an apparent activity increase was observed in the first minutes of incubation. This could be explained as a result of the substrate/product diffusion through the double layer of the NP. This reactivation effect has been also observed in laccases covalently immobilized in carboxylated polyvinyl alcohol (PVA) due to the interactions between enzymes and matrix [25].

## 2.5. pH Stability Determined in a Sequential Assay Involving Acidic pHs up to Alkaline Ones

Figure 4 depicts the results from stabilization studies performed at different sequential pHs ranging from 2.0 up to 8.0 at 37 °C. The residual activity was quantified at proper intervals. After 2 h at pH 2.0 (simulation of maximum permanence in the stomach), free laccase activity diminished 81.05%, while laccase activity immobilized on the NPs only decreased 13.37%. Residual activity was determined after two extra hours of incubation at 37 °C and pHs 6.0, 7.0 or 8.0. Free laccase exhibited 62, 54 and 58% of its initial activity after the supplementary period of incubation at pHs 6.0, 7.0 and 8.0, respectively. Eudragit® L 100-55 has a pH-dependent solubility and was designed to release the active agent in the gastrointestinal region at pHs 6.0–6.5, i.e., the ileum or upper intestine [26]. Laccase immobilized on Eudragit® L 100-55 NPs kept most of its activity at the acid gastric pH, being active at the more alkaline pHs of the intestinal region where it is released, thus allowing its use as adjuvant in animal feed [26]. The use of ligninolytic enzymes—especially laccases—is an attractive method for detoxification and delignification of feed insoluble fiber fraction [27]. Lignin polymers function as physical barriers that hamper the accessibility of carbohydrates to hydrolytic enzymes and promote their nonspecific adsorption, lowering the number of enzymes available for hydrolyzing carbohydrates and hence diminishing saccharification yields [27]. In this work, the immobilized enzyme exhibited higher stability than free laccase, moreover immobilized laccase activity increased at the end of the sequential assay involving acidic pHs up to alkaline ones. The activity of the immobilized laccase would depend on the diffusion coefficient of the substrate through the polymer matrix to get in contact with the enzyme and react, and then the product is required to diffuse outside the NPs [28]. Thus, the increment in laccase activity observed may be explained by the diffusion of the substrate through Eudragit® polymer that delayed its contact with the enzyme, and when the product was released the activity showed an apparent rise. Previous studies have demonstrated that gastric release of the immobilized active agent depends on the nature of the polymer applied in the process and the pH of the medium [12,23]. The results obtained in the present work are in coincidence with those of Makhlof et al. [29]. When studying the in vitro release profile from PLGA/Eudragit® S100 NPs these authors observed that at pH 1.2 only 20% of the active agent was released while the rest of it diffused slowly and sustained at the pH of polymer dissolution [29]. Adesogan et al. [30] analyzed the effect of pH on 18 commercial fibrolytic enzymes with endoglucanase and xylanase activity, 77 and 61% of them had optimal activities at pHs 4 to 5, respectively, thus limiting the activity at more alkaline pHs and the capacity to degrade forage fiber. On the contrary, although laccase immobilized on Eudragit® L 100-55 NPs exhibited optimum activity at an acid pH (3.0) it demonstrated higher stability in the pH range 6.0–8.0.
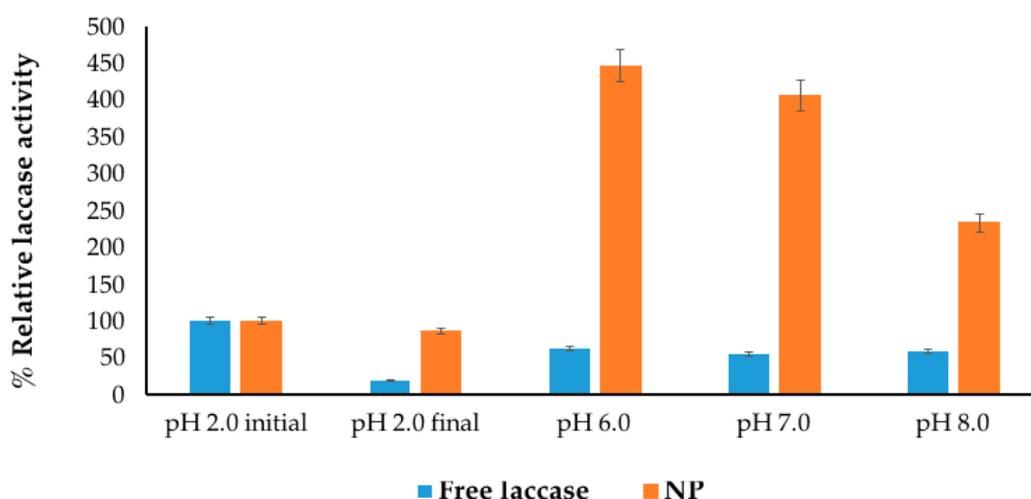
**Figure 4.** Effect of gastrointestinal tract pHs on the stability of free and immobilized laccase on Eudragit® L 100-55 nanoparticles (NP) in an in vitro assay.

Finally, we were able to report for the first time the immobilization of the laccase produced by *T. maxima* CU1, through the double emulsion solvent evaporation technique, using the pH-sensitive polymer Eudragit® L 100-55. In addition, we evaluated the effects on enzyme activity pH-dependence and stability in vitro, in order to apply these NPs for the specific release at the pH-dependent site in the gastrointestinal tract of the rabbit. Future studies will be carried out in model monogastric animals such as rabbits, to evaluate the effects of laccase NPs in the productive parameters of their meat industry.

## 3. Materials and Methods

### 3.1. Culture Media and Reagents

Components of growth media were from Dickenson and Company BD (Le Pont de Claix, France), while the rest of the chemicals were analytical grade obtained from Sigma-Aldrich (St. Louis, MO, USA). Eudragit® L 100-55 polymer was generously donated by HELM México. Solutions and culture media were prepared with double-distilled water from Laboratories Monterrey, S.A (Monterrey, Nuevo Leon, Mexico).

### 3.2. Laccase Purification

*T. maxima* CU1 was used for laccase production. It was provided by the culture collection of the Laboratory of Enzymology, Biology Department from the UANL (Nuevo Leon Autonomous University). The strain was conserved in YMGA (glucose 4 g $L^{-1}$, malt extract 10 g $L^{-1}$, yeast extract 4 g $L^{-1}$ and agar 15 g $L^{-1}$) slants at 4 °C with periodic subcultures every three months. The growth medium consisted of 2% (*w/v*) Kellogg's® Bran Flakes) in 60-mM potassium phosphate buffer pH 6.0 [31]. Four 2-L Erlenmeyer flasks, containing 1 L of growth medium were inoculated with six 0.5-cm-diameter plugs, cut out from the margin of a 5-day-old colony grown on YMGA medium and incubated in a rotatory shaker at 150 rpm and 28 °C for 17 days. To obtain 4 L of supernatants, the culture medium was separated from fungal biomass by filtration through Whatman No 1 filter paper. Culture filtrates were concentrated using 10-kDa ultrafiltration (Millipore prep/scale TFFcartridge, Merck KGaA, Darmstadt, Germany). Proteins were recovered through precipitation with ammonium sulfate at 80%, by agitation overnight at 4 °C. The solution was centrifuged at 5000 rpm 30 min, the supernatant was discharged and the precipitate was suspended in 5 mL final volume of 20-mM potassium phosphate buffer, pH 6.0 and applied in a P-100 Biogel (2.5 cm × 65 cm), equilibrated and eluted with the same buffer. The eluted fractions were assayed for laccase activity with 2,6-dimethoxyphenol [32] and the A280

nm monitored. The fractions with laccase activity were collected and concentrated using Amicon®
ultrafiltration system (Millipore Corp., Merck KGaA, Darmstadt, Germany). Concentrated samples
were applied in a DEAE-Support Macro-Prep® (Bio-Rad, USA) column (2.5 cm × 40 cm) equilibrated
with 20 mM potassium phosphate, pH 6.0. A 20–150-mM potassium phosphate lineal gradient was
applied. SDS-PAGE [33] was performed to verify the purity of the enzyme preparations. The final
fractions of laccase were stored at −20 °C until use.

### 3.3. Laccase Immobilization on Polymeric Nanoparticles

The NPs were obtained by a water-in-organic phase-in-water ($W_1$/O/$W_2$) double-emulsion
solvent-evaporation-modified method. Three hundred microliters of the aqueous enzymatic extract
($W_1$) was added to 8 mL of the organic phase (O), consisting of 300 mg of the polymer Eudragit®
L100-55 and a solvent system of dichloromethane/acetone/isopropyl alcohol in a ratio of 3.46:2.4:2.13 mL.
To prepare the emulsion the mixture was homogenized by sonication (two cycles of 5-min sonication
followed by a 1-min rest period) (Ultrasonic Branson 2510MT, Merck KGaA, Darmstadt, Germany).
Then the first emulsion ($W_1$/O) was added in 12.5 mL of the external aqueous phase containing 6.5 mL
of a solvent system (4.14 mL ethanol: 1.7 mL isopropyl alcohol: 0.66 mL dichloromethane) and 6 mL of
aqueous polyvinyl alcohol (PVA) at 8% p/v. Formed $W_1$/O/$W_2$ phases were agitated at 2000 rpm for 20
min (Eurostar Power-B Ika® Werke, Merck KGaA, Darmstadt, Germany), next 4 mL of 8% PVA were
added at a stirring rate of 1000 rpm for 4 min. Lastly, the organic solvent was evaporated from the
emulsion at reduced pressure (Heidolph Rotatory Evaporator Laborota 4003, Merck KGaA, Darmstadt,
Germany) and the obtained suspension of polymeric particles purified at 60 rpm and 25 °C.

### 3.4. Size and Morphology

Photon correlation spectroscopy (PCS) (Zetasizer Nanoseries; Malvern, Nano-Zs90, Malvern,
Worcestershire, U.K.) was used to study the average particle size of the NPs. The measurements were
made after aqueous dispersion of the NPs (in double distilled water). The size and morphology of the
NPs was evaluated by scanning electron microscope (Hitachi U8000, Hitachi, Kyoto, Japan) at 2 kV.
For these, diluted (1:100) samples were mounted on metal studs and desiccated.

### 3.5. Zeta Potential Analysis

The surface zeta potential of the NPs was measured at different pHs using a laser zeta meter
(Malvern Zeta Seizer 2000, Malvern, Worcestershire, U.K.). Liquid samples of the NPs were diluted
1:100 with distilled water using HCl (pH 2.0) or phosphate buffer 100 mM (pHs 6.0 and 7.0) before
zeta-potential determination. In each case, an average of three separate measurements was reported.

### 3.6. Enzymatic Assays

Laccase activity was determined spectrophotometrically by measuring the oxidation of 2,6
dimethoxyphenyl (DMP) 2 mM in sodium acetate buffer 200 mM, pH 4.5 at 468 nm ($\varepsilon468 = 49{,}600$ $M^{-1}$
$cm^{-1}$) as described by Abadulla et al. [31]. Enzymatic activities were expressed in units (U) defined as
the amount of enzyme required to produce 1 μmol of product. Enzymatic reactions were carried out at
25 °C and evaluated in a UV-vis 1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

### 3.7. Encapsulation Efficiency

In order to measure laccase activity immobilized on the NPs, they were washed with distilled
water and centrifuged at 25,000 rpm/4 °C/3 h. Laccase encapsulated in the NPs and free laccase activity
in the supernatants was estimated as described by Abadulla et al. [31]. The encapsulation efficiency
was estimated by the following formula:

$$\% \text{ EE} = [(\text{LA}_{\text{initial}} - \text{AL}_{\text{supernatants}})/\text{LA}_{\text{initial}}] \times 100$$

where: $LA_{initial}$ corresponds to initial laccase activity and $AL_{supernatants}$ to remnant laccase activity in the supernatant.

### 3.8. Effects of pH on Laccase Activity and Stability

The effect of pH on laccase activity was determined in the range 2.0–8.0 by incubating the enzyme at 25 °C in DMP dissolved in Britton–Robinson buffer (consisting of 1:1:1 mixture of 0.04-M boric, acetic and phosphoric acids, adjusted to the pH required with NaOH 1 M). Data in the graphics appear as relative activity as a function of the pH, considering the average of the maxima obtained as 100%. In the experiments testing the effect of pH on the enzyme stability, the enzyme samples were incubated in Britton–Robinson buffer (pH 2.0–7.0) for different periods at 25 °C. Samples were withdrawn for enzyme-activity determination at standard assay conditions. Residual enzyme activity was calculated by comparison with non-preincubated samples.

### 3.9. pH Stability Determined in a Sequential Assay Involving Acidic pHs up to Alkaline Ones

With the aim of mimicking the pH conditions in the gastrointestinal tract, pH stability was determined in a sequential assay involving acidic pHs up to alkaline ones. Solutions of the NPs and free laccase were incubated at pH 2.0 and 37 °C for 2 h. Afterwards, they were incubated additional 2 h at 37 °C and pHs 6.0, 7.0 or 8.0 (adjusted with phosphate buffer 100 mM). These three experimental conditions resemble the pHs found in different sections of the rabbit's digestive system. Residual enzyme activity was calculated by comparison with non-preincubated samples.

### 3.10. Statistical Analysis

All results were expressed as mean values of three samples ± standard deviation. Statistical significance among samples was evaluated by analysis of variance (ANOVA) followed by Tukey's test using SPSS Statistics software (Version 21, IBM, Burlington, Mass., USA, 2012). A level of probability of $p \leq 0.05$ (5%) was set as statistical significance.

## 4. Conclusions

Laccases have on their catalytic site copper atoms that make their stabilization difficult [3]. Immobilization techniques have expanded the scope of laccase applications, improving enzyme performance. The choice of the most appropriate method for enzyme immobilization must take into account the biocatalyst application [3]. Synthetic polymers have been used for laccase immobilization and applied to degrade nonylphenol and octylphenol [34] and recalcitrant dyes [35,36]. In this work, *T. maxima* CU1 purified laccase was successfully encapsulated in polymeric NPs by a double-emulsion solvent evaporation technique applying the polymer Eudragit® L 100-55 sensitive to pH. The Eudragit® polymer provided to enzyme stability at acidic pHs (2.0–5.0) and conferred it resistance to pH conditions similar to those found in the rabbit gastrointestinal tract, thus allowing its potential use as adjuvant in animal feed.

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