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Publication Date 07 Apr 2020

Article Type Full Research Paper

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Double-emulsion technique applied to laccase immobilization on polymeric nanoparticles

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Abstract

One primary drawback of enzyme catalysis at industrial scale is the short term service life of the enzymes. Enzymes 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, longevity and reusability of the enzymes is to attach them to nanoparticles by applying the double-emulsion technique. 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 was evaluated by Scanning Electron Microscope and particle size distribution was assessed by Photon Correlation Spectroscopy. Encapsulation efficiency and zeta potential were calculated. The effect of pH on laccase activity and stability was compared between free laccase and the immobilized one. 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 289 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 (2.0-4.0). However, this last kept 80% of enzyme activity at pH 2.0 approx., after 24 h. The polymer Eudragit® L 100-55 also conferred them resistance towards the pHs usually found at the gastrointestinal tract. These results suggest the potential use of nanoparticles as adjuvants in animal feed, serving as carriers for oral laccase delivery.

Keywords: animal feed, 2,6-dimethoxyphenol, enzyme immobilization, laccase, *Trametes maxima* CU1

Introduction

The global market for industrial enzymes is growing rapidly. These biocatalyst are key to new processes due to their ease of production, substrate specificity and green chemistry. However, industrial applications are often impede 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 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]. Among the carriers employed for enzyme immobilization synthetic polymers distinguished by their versatility and easy recovery of the enzymes. Eudragit® is one of the 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].

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, 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 [6]. 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. But, have been scarcely tried for possible application in improving the bioavailability of nutrients and digestibility of animal feed, especially for monogastric diets [7]. The beneficial effects of laccases (besides those of cellulases, xylanases and amylases) on productive parameters and carcass yield of rabbits have been reported [8]. Nevertheless, as far as we know there is no information on their use 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.

Results and Discussion

Enzyme production and purification

Purified laccase was obtained from *T. maxima* CU1 submerged cultures with specific activity of 182.3 $U \cdot mg^{-1}$ 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 [9]. 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 [10]. 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.

Nanoparticles characterization

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. It has been reported that stomach retains food particles until they are fragmented into pieces smaller than 0.5 mm in diameter [11]. 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 by polymer dissolution would begin. Likewise, 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. In contrast, the active agent slowly diffuses inside larger particles [12]. In general nanometric-scale favors the in vivo distribution of the NPs, their small size allows them to enter animal´s body by inhalation, ingestion, or through the skin, a crucial factor in order to reach specific tissues [13].

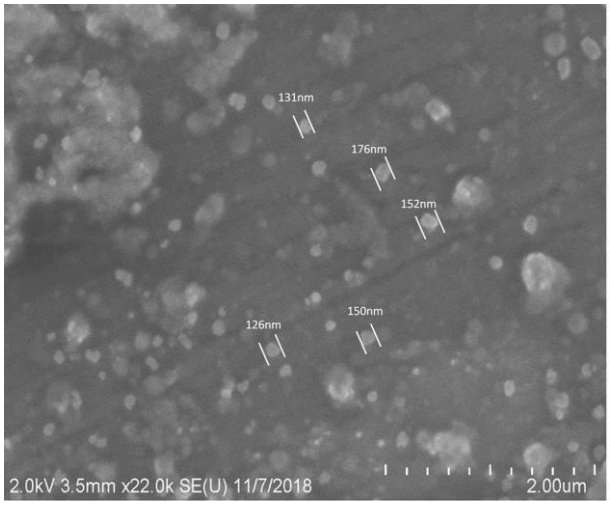

Figure 1. SEM image of the NP. Their spherical shape is shown.

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 pHs (-22.7 mV at pH 7.0) (Table 1). pH 7 might 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 [11]. In the case of the methacrylic acid (Eudragit® L 100-55) with a pKa of 4.23, at pHs lower than this value the surface charge of particles would decrease, thus favoring aggregation [11]. In previous studies applying this polymer for insulin immobilization similar zeta potential values were obtained [14]. In terms of intestinal uptake, apart from their particle size, nanoparticle nature and charge properties seem to influence the uptake by intestinal epithelia [15]. NPs based on hydrophilic polymers, negatively charged, showed a strong increase in bioadhesive properties and were absorbed by both M cells and absorptive enterocytes [18]. Bioadhesion allows the active agent to remain in contact with a particular organ for an extended period of time [16]. 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.

Condition	pH	Potential Z values (mV)
1	2.0	-0.638
2	5.0	-6.2
3	6.1	-17.00
4	7.0	-22.7

Moreover, the encapsulation efficiency of laccase on Eudragit® L100-55 NPs was found to be 87%. Sharma et al. [11] 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. [14] 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 is reported for the first time the encapsulation of laccase in NPs by the double-emulsion approach.

Optimum pH for enzyme activity

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 [17]. They exhibited a maximum at pH 3.0 but decreased on varying the pH from 3.0 to 6.0. Increase in pH might have caused a conformational change in laccase structure and specially on its catalytic site, thus inhibiting internal electron transfer and differing reaction product [18]. This suggests that enzyme encapsulation neither affect protein conformation nor enzyme function, keeping this laccase the characteristic behavior of the blue ones [18-20]. The inhibition of T2 Cu site might be explained by the presence of OH^- ions, which prevent oxygen reduction to water via the reaction $O_2^- + 2H^+ \rightarrow H_2O$ [17].

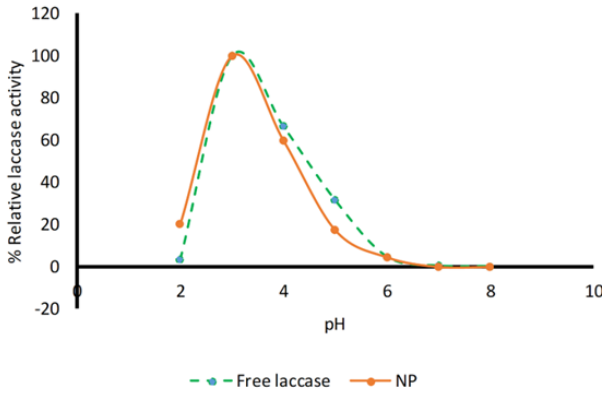

Figure 2. Effects of pH on free laccase activity and immobilized laccase on Eudragit® L 100-55 nanoparticles (NP).

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 nanopatircles kept approx. 80% of enzyme activity at pH 2.0 after 24 h. This result might be explained by the reduction in the conformational flexibility and the increase in rigidity of the immobilized enzyme, thus enhancing its resistance against denaturation [21]. Demonstrating in this way, 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. Furthermore, their stability improved [1].

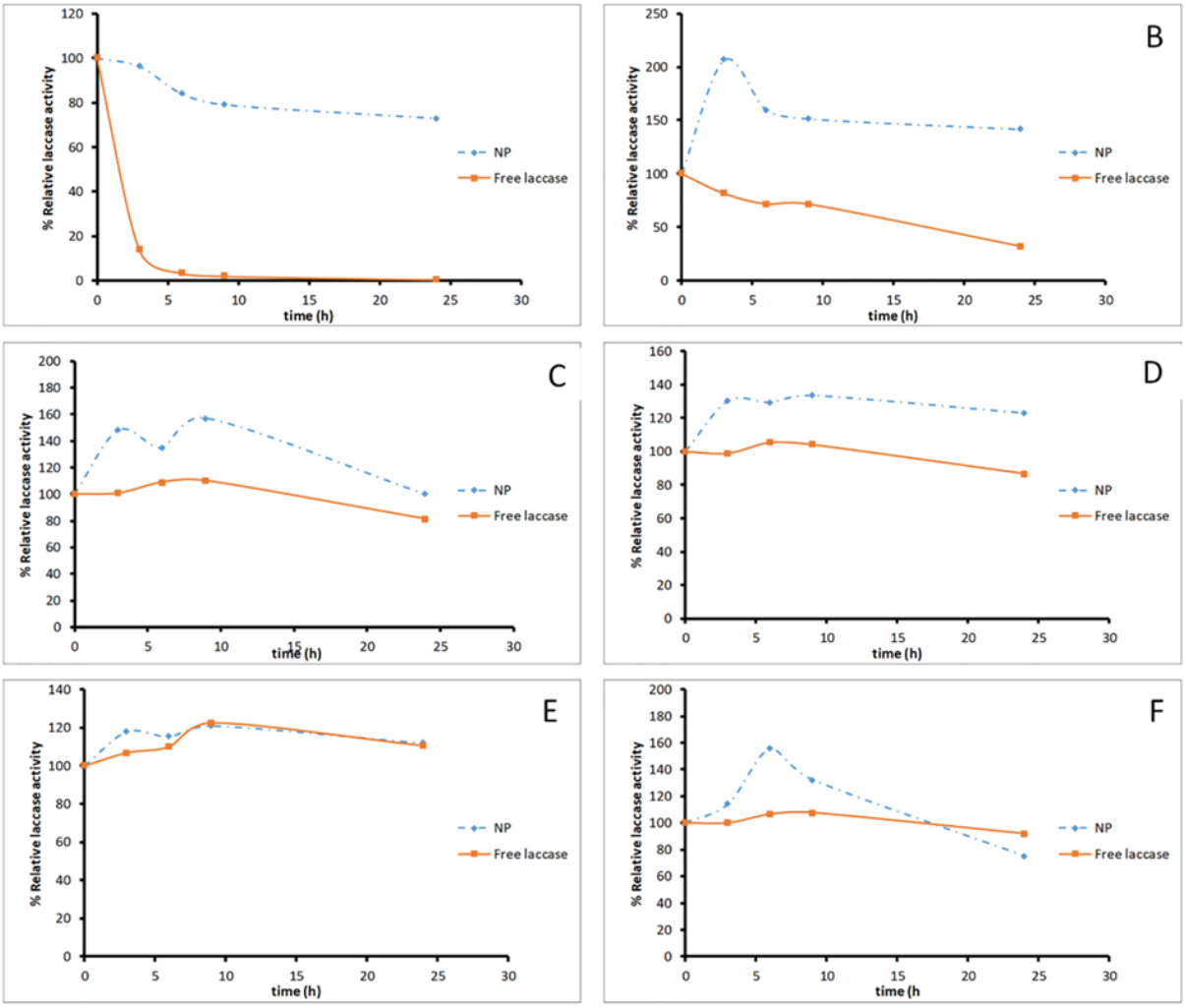

Figure 3. pH stability assays, carried out by incubating free (solid line) and immobilized laccase on Eudragit® L 100-55 nanoparticles (NP) (dashed line) at 25°C and different pHs from 2.0 to 7.0 with Britton-Robinson buffer. A: pH 2.0, B: pH 3.0, C: pH 4.0, D: pH 5.0, E: pH 6.0, F: pH 7.0. Samples were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

There were no statistically significant differences between the stability displayed by both free and immobilized laccases at pHs 6.0-7.0 ($P \leq 0.05$). 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 [22].

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 37ºC. 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 h 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. ileum or upper intestine [23]. 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 [23]. The use of ligninolytic enzymes, especially laccases, is an attractive method for detoxification and delignification of feed´s insoluble fiber fraction [24]. Lignin polymer functions as a physical barrier that hampers the accessibility of carbohydrates to hydrolytic

enzymes and promotes their non-specific adsorption, lowering the number of enzymes available for hydrolyzing carbohydrates and hence diminishing saccharification yields [24]. 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 to the outside [25]. Thus, the increment in laccase activity observed might 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 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 [13, 22]. The results obtained in the present work are in coincidence with those of Makhlof et al. [26]. 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. [27] 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 their activity at more alkaline pHs and their 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. To our knowledge, this would be the first report on laccase immobilized by the double-emulsion

solvent evaporation technique. In addition to providing the evaluation *in vitro* of the potential application in production systems. Therefore, 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.

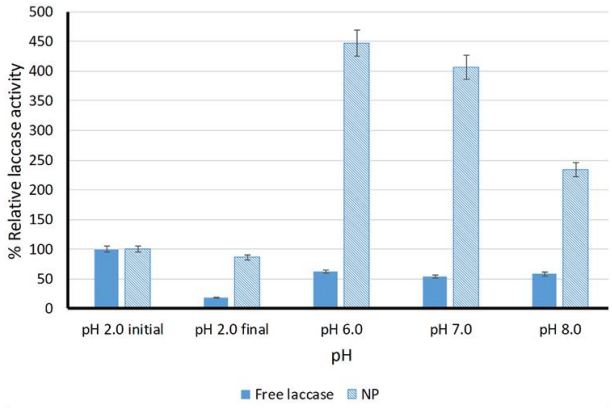

Figure 4. Effect of gastrointestinal tract pHs on the stability of free and immobilized laccase on Eudragit® L 100-55 nanoparticles (NP) in an *in vitro* assay.

Conclusion

T. maxima CU1 purified laccase was successfully encapsulated in polymeric NPs by the double-emulsion solvent evaporation technique. Eudragit® L 100-55 polymer provided the enzyme stability at acid pHs (2.0-5.0), and conferred it resistance to pH conditions similar to those found in rabbit´s gastrointestinal tract. Thus, allowing the release of the enzyme in the duodenum and its action on lignocellulosic components of animal feed increasing their nutritional availability and contributing to improve productive parameters in rabbit meat industry.

Experimental

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.

Laccase production and 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 León Autonomous University). The strain was conserved in YMGA (glucose 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$, yeast extract 4 $g \cdot L^{-1}$1 and agar 15 $g \cdot L^{-1}$) slants at 4 ºC 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 [28]. Erlenmeyer flasks containing 100 ml of growth medium were inoculated with three 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 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). 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 × 65 cm), equilibrated and eluted with the same buffer. The eluted fractions were assayed for laccase activity with 2,6-dimethoxyphenol [29] and the A280 nm monitored. The fractions with laccase activity were collected and concentrated using Amicon® ultrafiltration system (Millipore Corp., USA). Concentrated samples were applied in a DEAE-Support Macro-Prep® (Bio-Rad, USA) column (2.5 × 40 cm) equilibrated with 20 mM potassium phosphate, pH 6.0. A 20-150 mM potassium phosphate lineal gradient was applied. SDS-PAGE [30] was performed to verify the purity of the enzyme preparations. The final fractions of laccase were stored at -20 °C until use.

Laccase immobilization on polymeric nanoparticles

The NPs were obtained by a water-in-oil-in-water (W1/O/W2) double-emulsion solvent evaporation modified method. 300 µl of the aqueous enzymatic extract (W1) were 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 1-min rest period) (Ultrasonic Branson 2510MT). Then the first emulsion (W1/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 W1/O/W2 phases were agitated at 2000 rpm for 20 min (Eurostar Power-B Ika® Werke), next 4 ml of 8 % PVA were added at a stirring rate of 1000 rpm for 4

287 min. Lastly, the organic solvent was evaporated from the emulsion at reduced
288 pressure (Heidolph Rotatory Evaporator Laborota 4003) and the obtained
289 suspension of polymeric particles purified at 60 rpm and 25 °C.

290 Size and morphology

291 Photon Correlation Spectroscopy (PCS) (Zetasizer Nanoseries; Malvern, Nano-
292 Zs90) was used to study the average particle size of the NPs. The measurements
293 were made after aqueous dispersion of the NPs (in double distilled water). The size
294 and morphology of the NPs was evaluated by Scanning Electron Microscope
295 (Hitachi U8000) at 2 kV. For these, diluted (1:100) samples were mounted on metal
296 studs and desiccated.

297 Zeta potential analysis

298 Surface zeta potential of the NPs was measured at different pHs using the Laser
299 Zeta Meter (Malvern Zeta Seizer 2000, Malvern). Liquid samples of the NPs were
300 diluted 1:100 with distilled water using HCl (pH 2.0) or phosphate buffer 100 mM
301 (pHs 6.0 and 7.0) before zeta potential determination. In each case, an average of
302 three separate measurements was reported.

303 Encapsulation efficiency

304 In order to measure laccase activity immobilized on the NPs, they were washed
305 with distilled water and centrifuged at 25000 rpm/4 °C/3 h. Laccase encapsulated
306 in the NPs and free laccase activity in the supernatants was estimated as
307 described by Abadulla et al. [29].

Enzymatic assays

Laccase activity was determined spectrophotometrically by measuring the oxidation of 2,6-dimethoxyphenol (DMP) 2 mM in sodium acetate buffer 200 mM, pH 4.5 at 468 nm (ε_{468}= 49,600 $M^{-1} \cdot cm^{-1}$) [29]. 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, Japon).

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 ºC in DMP dissolved in Britton-Robinson buffer. Data in graphics appear as relative activity as a function of pH considering as 100 % the average of maxima obtained. In the experiments testing the effect of pH on the enzyme stability, the enzyme samples were incubated in Britton-Robinson buffer (pHs 2.0-7.0) for different periods at 25 ºC. Samples were withdrawn for enzyme activity determination at standard assay conditions. Residual enzyme activity was calculated by comparison with non-preincubated samples.

pH stability determined in a sequential assay involving acidic pHs up to alkaline ones

With the aim of mimicking 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.

Statistical analysis

All of the 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 sofware. A level of probability of $P \leq 0.05$ (5%) was set as statistical significance.

Acknowledgments

To the Autonomous University of Nuevo León and the Department of Biological Sciences for the use of their facilities and financial support through the PAICYT project CT724-19

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