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EDTA-Cu (II) chelating magnetic nanoparticles as a support for laccase immobilization

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ABSTRACT

Developments in nanotechnology have led to the discovery of new materials, namely, magnetic nanoparticles (MNPs), that present easy surface functionalization and high surface-to-volume ratios. These properties allow a high mass transfer rate and easy removal from a reaction matrix. Simple separation under an external magnetic field makes them a promising immobilization support for enzymes. In this work, new MNPs were prepared by functionalization with EDTA-TMS and characterized by TEM, FTIR and BET analytical techniques, among others. These MNPs were applied as support for laccase immobilization to create a promising biocatalyst. Despite the known chelating nature of EDTA-TMS, its use for surface modification of MNPs for laccase immobilization is a rather unexplored strategy and is reported here for the first time. At pH 3.5, the immobilization process showed approximately 97% of enzymatic activity recovery. The Michaelis-Menten kinetic properties of immobilized laccase showed a lower V_{max} and a similar K_M compared to free laccase. Regarding operational stability, the immobilized enzyme presented approximately 73% of its initial activity after five sequential reactive cycles. The immobilized enzyme was successfully applied to the biocatalysis of Indigo Carmine dye degradation. These MNPs with immobilized laccase showed important advantages compared to other materials for application in industrial biochemical processes, biocatalysis and biosensors.

1. Introduction

Laccases (EC 1.10.3.2, *p*-diphenol:oxygen oxidoreductase) are multi-copper blue oxidase enzymes that catalyse the oxidation of several compounds coupled to molecular oxygen reduction (Mayer and Staples, 2002). Their redox potential is closely related to enzyme reactivity since it is difficult to oxidize compounds with a high redox potential (Baldrian, 2006). Furthermore, the molecular weight of the substrate can limit laccases' catalytic function since compounds with high molecular weights can have steric properties that impair access to the enzyme's active site (Riva, 2006). To overcome these issues, researchers have explored laccase mediator systems (LMS) to increase the range of laccase substrates (Tavares et al., 2004; Fillat and Blanca Roncero, 2009). LMS are defined as reaction systems wherein small molecules, such as 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole, violuric acid or methyl syringate (MS), are denoted as mediators and are used to enable oxidation of certain compounds by laccase that are usually non-catalysed by laccase. The mediators are low molecular weight molecules that are oxidized by laccase, thus acting as electron carriers between the enzyme and substrate, overcoming the steric barrier between them (Arora and Sharma, 2010). Laccases and LMS are extensively used in several different areas, such as medicine, bioremediation, food, papermaking and textile industries (Kunamneni et al., 2008). Particularly, MS has been described as a good potential mediator for laccases (Rosado et al., 2012). A proposed oxidative cycle for phenolic substrates using MS as mediator is well described in the literature. Its enzymatic catalysis involves the release of a proton and an electron from phenolic substrates when they are oxidized by the mediator. The mediator is then transformed into an intermediate phenoxy radical, which promotes the oxidation of previously formed non-phenolic monomers to either give the corresponding aryl radical or quinone or even to form other phenolic substrates by coupled reactions (the complete proposed oxidative cycle was described by Rosado et al. (2012)).

The textile industry is one of the largest industries in the world and consumes large amounts of synthetic dyes (Ghaly et al., 2014). The

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); APTES, (3-Aminopropyl)triethoxysilane; ATR, Attenuated total reflectance; BET, Brunauer–Emmett–Teller; EDTA-TMS, N-[(3-trimethoxysilyl)propyl]ethylenediamine triacetic acid; FTIR, Fourier transform infrared spectroscopy; IC, Carmine indigo; KM, Michaelis-Menten constant; LMS, Laccase mediator system; MNP, Magnetic nanoparticle; MS, Methyl syringate; NP, Nanoparticle; TEOS, Tetraethyl orthosilicate; TEM, Transmission electron microscopy; VC, Congo red; Vmax, Maximum rate reaction

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production of high volumes of textile wastewater has become a predominant environmental concern since some compounds that are present in these effluents are coloured, toxic and carcinogenic (Ghaly et al., 2014). Currently, besides the classical methods for effluent treatment, enzymatic wastewater decontamination processes are rising as a promising alternative since they are more specific and conform to environmental constraints. Oxidoreductases, such as laccases, are able to oxidize toxic compounds from industrial wastewater (Singh et al., 2015) and thus represent a solution for new technological wastewater plants. Nevertheless, since enzymatic preparations are soluble in aqueous media, for reuse or continuous operation affordability, commercial immobilized enzymes are desirable for profitable operations. Biocatalyst reutilization or continuous operation is a target issue for designing cost-effective industrial processes (Sheldon and van Pelt, 2013).

Currently, several materials are used as matrices for laccase immobilization, such as alginate (Mogharabi et al., 2012), chitosan (Zhang et al., 2009), silica (Tavares et al., 2013), activated carbon (Davis and Burns) and coconut fibres (Cristovao et al., 2012; da Silva et al., 2012; Silva et al., 2014; Tavares et al., 2013), among others. With recent advances in nanotechnology, the development of novel materials, such as nanofibres (Sathishkumar et al., 2014), carbon nanotubes (Silva et al., 2014; Tavares et al., 2015) and titania nanoparticles, (Hou et al., 2014) expands the range of immobilization supports available.

The possibility of immobilizing enzyme on nanoparticles (NPs) presents several advantages, namely, a reduced mass transfer resistance, high effective enzyme load, high surface area and high mechanical resistance (Cipolatti et al., 2014). Nevertheless, the high production cost of some NPs and difficulty of removing them from the reaction media are major drawbacks to the industrial use of such materials (Cipolatti et al., 2014). Magnetic NPs (MNPs) have gained particular importance since they can be simply removed from the reaction medium by applying an external magnetic field (Cipolatti et al., 2014). Iron oxide MNPs are the most commonly used MNPs in biotechnological applications due to the magnetite (Fe_3O_4) NPs exhibiting low toxicity and high biocompatibility (Bucak et al.). The broad range of applications of laccase and advantages of Fe_3O_4 as a magnetic immobilization support material prompt the investigation of new strategies for laccase immobilization to expand industrial applications. Few examples of laccase immobilization on magnetic materials with surface modifications have been described in the literature (Wang et al., 2013, 2008; Bayramoglu et al., 2010). The immobilization of laccase on magnetic NPs via coordination was barely investigated (Wang et al., 2013, 2008), and no research on the optimization of laccase immobilization conditions on MNPs has been reported.

The goal of this study was to prepare and characterize silica-coated magnetite NPs functionalized with (trimethoxysilylpropyl)ethylenediamine triacetic acid (EDTA-TMS) and chelated with copper (II) ions as a metal ligand for laccase immobilization. In this work, the immobilization conditions for laccase on EDTA-Cu (II) chelating magnetic nanoparticles were evaluated for the first time. For this purpose, the optimal pH for enzyme immobilization was determined and the kinetic parameters and reusability of immobilized laccase were also evaluated. Finally, this new application of immobilized laccase was investigated through the LMS reaction, using methyl syringate as a mediator of the degradation of the synthetic dyes Indigo Carmine and Congo Red.

2. Materials and methods

2.1. Materials

Tetraethyl ortosilicate (TEOS, 99.9%), iron (II) sulphate heptahydrate (99%), sodium acetate trihydrate (≥99.0%), sodium (II) phosphate heptahydrate (≥98.0%) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥98.0%) were purchased from Sigma-Aldrich. Glacial acetic acid (99.7%) and citric acid (≥99.5%) were acquired from Panreac. Potassium hydroxide (99.9%) and potassium nitrate (97.2%) were purchased from LabChem. (Trimethoxysilylpropyl)ethylenediamine triacetic acid (EDTA-TMS, 35%) was acquired from Gelest. Ethanol (≥99.9%) was purchased from Carlo Erba. Acetone was purchased from Fluka. Copper (II) sulphate pentahydrate and Congo Red were acquired from Merck. Indigo Carmine was purchased from Acros Organics. Commercial laccase (Novozym ® 51003; physical form: liquid) and methyl syringate (MS) were kindly provided by Novozymes (Denmark).

2.2. Preparation of Cu^{2+}-chelated magnetic nanoparticles

Silica-coated magnetic nanoparticles containing ion chelating moieties at the surface (Fe_3O_4@SiO_2_EDTA-TMS) were prepared as previously reported (Oliveira-Silva et al., 2015). The magnetic core of magnetite nanoparticles (Fe_3O_4) was synthesized by oxidative hydrolysis of $FeSO_4{\cdot}7H_2O$ under alkaline conditions. Then, the magnetic core was encapsulated within amorphous silica shells by hydrolytic condensation of TEOS in an alkaline medium. The resulting nanoparticles were denoted as Fe_3O_4@SiO_2. Finally, the surface of the Fe_3O_4@SiO_2 nanoparticles was modified by grafting the alkoxysilane EDTA-TMS to yield magnetic nanoparticles containing chelating carboxylic acid moieties at the surface, named Fe_3O_4@SiO_2_EDTA-TMS. The reaction was performed under reflux at 70 °C for 24 h.

Chelation of copper (II) ions was performed according to the previously described method (Wang et al., 2008). Briefly, 5 mL of a $CuSO_4{\cdot}5H_2O$ (0.336 mM) aqueous solution was added to 15 mg of Fe_3O_4@SiO_2_EDTA-TMS particles, and the resulting mixture was incubated for 10 h at 30 °C under orbital stirring (150 rpm). The Cu^{2+}-chelated magnetic nanoparticles, hereafter designated as Fe_3O_4@SiO_2_EDTA-TMS_Cu^{2+}, were magnetically recovered, washed with Milli-Q water and dried by solvent evaporation prior to utilization.

2.3. MNPs characterization

Fourier transform infrared (FTIR) spectra of lyophilized particles at different stages of preparation were collected using a Bruker Optics Tensor 27 spectrometer coupled to a horizontal attenuated total reflectance (ATR) cell using 256 scans per spectrum at a resolution of 4 cm^{-1}.

Functionalization of the MNPs was evaluated by elemental analysis of carbon, nitrogen and hydrogen, as performed with Eager 300 equipment.

The morphology of the MNPs was investigated by transmission electron microscopy (TEM) using a Hitachi H-9000 TEM microscope operating at 200 kV. Samples for analysis were prepared through evaporation of dilute suspensions of the particles on a copper grid coated with a film of amorphous carbon.

The specific surface area of the particles was assessed by nitrogen adsorption Brunauer-Emmett Teller (BET) measurements, performed with a Gemini V2.0 Micromeritics instrument. The pore size was calculated from the desorption branch using the Barrett-Joyner-Halenda (BJH) method, and the pore volume was evaluated from the adsorbed amount.

The surface charge of the NPs was assessed by zeta potential measurements performed in aqueous solutions of the particles using Zetasizer Nanoseries equipment from Malvern Instruments.

2.4. Immobilization of laccase

Laccase immobilization was carried out using an enzyme concentration of 0.17 $\mu L_{lac}/mL_{buffer}$. The effect of pH on the immobilization was evaluated through the utilization of buffer solutions with different pH values (0.10 M acetic acid/0.10 M sodium acetate buffer solution with a pH of 3.0; 0.20 M acetic acid/0.20 M sodium acetate buffer solutions with pH values of 3.5, 4.0 and 4.2) in the preparation of the