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### A novel enzyme catalysis reactor based on superparamagnetic nanoparticles for biotechnological applications



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### ABSTRACT

In this research, a new sequential batch reactor (SBR) coupled to an internal magnetic separator was developed. The separator consists of a set of axially magnetized permanent toroidal magnets, distributed along a nonmagnetic steel rod, uniformly spaced with alternate polarity, which provide an external magnetic field of up to 1.2 T. The feasibility of magnetic separation was assessed for the retention of the nanobiocatalyst based on laccase immobilized on silica-coated MNPs. The proof of concept was evaluated for the enzymatic decolorization of the methyl green (MG) dye and the oligomerization of rutin, with virtually complete recovery of the nanobiocatalyst (99%) and high biotransformation efficiency of both compunds. The characterization of the reaction products of MG by laccase was conducted by high performance liquid chromatography (HPLC) with a diode array detector (DAD) coupled to mass spectrometry (MS) using the electrospray ionization source (ESI) (HPLC-DAD-ESI-MS). Moreover, the biotransformation products showed less microtoxicity than the parent compound and increased biodegradability. Beyond laboratory scale experiments, the reactor proposed here was scaled-up to a volume of 100 L and the environmental performance and cost analysis were estimated.

### 1. Introduction

Laccases (benzenediol oxygen oxidoreductases, Enzyme Commission (EC) 1.10.3.2) are biocatalysts with a growing interest for biotechnological and environmental applications due to their high activity, selectivity and specificity. Enzyme immobilization emerges as an alternative to be taken into account to improve enzyme stability and allow easy separation of reaction products, facilitating enzyme retention in continuous operation [1,2]. However, immobilization may also present a number of disadvantages, such as reduced catalytic activity, conformational change of the enzyme or mass transfer limitations, which may prevent the access of the substrate to the active site of the enzyme [3]. Among the different immobilization methods, covalent bonding on various supports, including silica-based supports, epoxyactivated resins and various types of fibers and polymers, has been proven its efficacy for enzyme immobilization [1].

With the recent development of nanotechnology, iron oxide magnetic nanoparticles (MNPs) have gained increasing attention due to their high surface to volume ratio, which makes them good candidates as supports for enzyme immobilization. However, considering that bare iron oxide nanoparticles are likely to lose their magnetism and stability due to their high chemical reactivity and oxidation in air, the dispersion of MNPs may be promoted by providing a suitable surface coating, mainly associated with organic substances such as surfactants, polymers and biomolecules or inorganic substances such as silica and metal oxides [4]. Beyond the stability increase of nanoparticles, the use of a suitable monolayer polymer coating technique allows their conjugation with enzymes. Several research papers have reported higher enzyme stability after immobilization on silica-coated Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles [5], silica-encapsulated nanomagnetic particles [6] or goldcoated magnetic nanoparticles [7]. A recent example of using MNPs for enzyme immobilization has been reported for the co-entrapment of citric acid-magnetic iron oxide (CA-Fe<sub>3</sub>O<sub>4</sub>) nanoparticles and Candida rugosa lipase (CRL) simultaneously with Zn/AMP nanofiber support [8]. The CA-Fe<sub>3</sub>O<sub>4</sub>@Zn/AMP gels could preserve the initial fibrilar structure of Zn/AMP gels and the magnetic nanomaterials grant accessibility for enzymatic immobilization and separation. The CA-Fe<sub>3</sub>O<sub>4</sub>@Zn/AMP gels also exhibited improved stability for pH, T and storage.

One of the main advantages of using MNPs is that they can facilitate the recovery of the biocatalyst from the reaction medium by applying a

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magnetic field. The efficient magnetic separation of the enzyme would imply very low mechanical stress on the nanoparticle compared to centrifugation or filtration [9]. There are recent studies in which magnetic separation has been considered, mainly for algae growth [10], recovery of magnetic components [11], solidification and crystallization processes [12] and photocatalytic reactions [13].

Despite the huge number of papers dealing with MNPs for a wide range of applications: biosensors, electronics, biomedical sciences, chemical industries and other use [14-16], so far, the number of magnetic reactor alternatives is very limited. Crossing the line towards a more effective application of MNPs means to develop a reactor configuration that ensure the separation of the nanoparticles when the reaction medium needs to be withdrawn, while being environmentally friendly and cost effective. Wang et al. [17] applied a magnetically stabilized fluidized bed reactor (MSFB) with laccase immobilized on MNPs for the removal of phenols present in coking wastewater. The MSFB reactor consisted of a glass column and four copper wire coils connected to a power supply, operated with temperature control by means of a water jacket. Ardao et al. [18] designed a stirred tank reactor with a magnet placed in the outlet stream of the reactor to facilitate the separation of the magnetic biocatalyst. A system of valves allowed the flow to be reversed at regular intervals to return the enzyme back to the reactor. One of the challenges of this configuration is to avoid aggregation of the nanoparticles caused by magnetic retention. Another type of reactor corresponds to a dynamic magnetic trap reactor configuration based on two sets of electromagnets [19]. Not only the requirement of energy, but also the remarkable increase in temperature were the major drawbacks associated with its operation.

Taking into account the potentiality of oxidative enzymes as biocatalysts, from biotransformation of pollutants to enzymatic synthesis [20–22], the primary objective of this study was to develop a novel enzyme sequential batch reactor (SBR) based on laccase immobilized onto silica-coated MNPs (Fe<sub>3</sub>O<sub>4</sub>@SiO<sub>2</sub>) for the biotransformation of a dve: methyl green (MG) dve and a flavonoid: rutin. The selection of both types of substrates for enzymatic oxidation is justified by the fact that they represent two different fields of application. On the one hand, MG is a synthetic colorant used to dye wool, leather, silk and can pose acute toxicity to fish [23]. In this context, the enzymatic system may represent an alternative to advanced oxidation treatment. Secondly, a flavonoid was chosen as a model compound, considering that its laccase-mediated biotransformation has been shown to enhance its antioxidant activity [24]. Therefore, the enzymatic system can be considered in this case as a synthesis alternative related to the transformation of the target compound.

The SBR consisted in a tank coupled with an internal magnet module consisting of a non-magnetic rod with aligned permanent magnets in an alternating polarity, covered by a glass sheath. This reactor configuration does not require energy consumption for magnetic separation. The modelling of the magnetic field aims to demonstrate the performance of the magnetic separation system in comparison with the conventional layout of passive magnets. In the pursuit of a real scale development, a pilot-scale reactor (100 L) was designed to compile relevant inventory data for its environmental assessment and cost analysis.

### 2. Materials and methods

### 2.1. Chemicals, enzyme and nanoparticles

3-aminopropyl-triethoxy-silane (APTES) ( $\geq$  98%), 2-2´-azinobis-3ethylbenzothiazoline-6-sulfonate (ABTS) ( $\geq$  98%), glutaraldehyde (25%) commercial laccase from *Trametes versicolor* (activity  $\geq$  0.5 U mg<sup>-1</sup>) were purchased from Sigma-Aldrich. Silica-coated MNPS were prepared and characterized in a previous work [25]. The physico-chemical characteristics of MG and Rutin (purchased from Sigma-Aldrich) along with their molecular structure are summarized in Table 1.

### Table 1

Physico-chemical characteristics and molecular structure of MG and Rutin
--------------------------------------------------------------------------

Compound	Structure	Molecular formula	Molecular weight (g mol <sup>-1</sup> )	рКа
MG	H <sub>3</sub> C. <sup>®</sup> <sub>N</sub> ·CH <sub>3</sub> H <sub>3</sub> C. <sup>N</sup> <sub>N</sub> ·CH <sub>3</sub> H <sub>3</sub> C. <sup>N</sup> <sub>CH3</sub>	C <sub>27</sub> H <sub>35</sub> BrClN <sub>3</sub> ·ZnCl <sub>2</sub>	653.24	1.3
Rutin	HO, O, OH OH QOO, OH OH QOO, OH	$C_{27}H_{30}O_{16}$	610.52	4.3

### 2.2. Enzymatic activity

Laccase activity was determined by monitoring the oxidation rate of 0.267 mM ABTS to its cation radical (ABTS<sup>+</sup>) at 420 nm ( $\epsilon_{420}=36,800$   $M^{-1}\,cm^{-1}$ ) in McIlvaine buffer (80 mM citric acid, 40 mM Na\_2HPO\_4; pH 3) at 30 °C for 7 min (6 s intervals). One unit (U) of activity was defined as the amount of enzyme forming 1  $\mu$ mol min $^{-1}$  of ABTS<sup>+</sup>. All spectrophotometric measurements were carried out on a BioTek PowerWave XS2 microplate spectrophotometer.

### 2.3. Decolorization of MG by laccase immobilized onto silica-coated MNPs

Laccase was immobilized onto silica-coated MNPs by sorption-assisted surface conjugation, based on the aminofunctionalization of the MNPs and glutaraldehyde cross-linking with laccase [25]. Prior to testing in the magnetic reactor, an initial objective was to assess the capacity of the immobilized laccase for the decolorization of the dye. For this purpose, an initial activity of  $1000 \text{ UL}^{-1}$  was tested to decolorize MG ( $20 \text{ mg L}^{-1}$ ) in 10-mL flasks at variable pH between 3 and 7, using McIlvaine buffer (80 mM citric acid, 40 mM Na<sub>2</sub>HPO<sub>4</sub>), sodium acetate and phosphate buffer solutions (100 mM). Samples were withdrawn periodically and analyzed to assess the decrease in absorbance at 630 nm. In parallel, controls lacking laccase but with functionalized silica-coated MNPs were also carried out with the objective of verifying that the decolorization was due exclusively to enzymatic catalysis. In order to determine the effect of pH on the nanobiocatalyst activity and the biotransformation rate of MG, the point of zero charge (pHpzc) of nanobiocatalyst was determined in a Zetasizer Nano ZS from pH 2 to 12.

## 2.4. Development and modelling of the magnetic sequential batch reactor (SBR)

The configuration of the magnetic sequential batch reactor (SBR) included the following elements: peristaltic pumps with adjustable flow rate, a stirred glass reactor of 5 L and a pneumatic cylinder with air for the periodic movement of the magnetic bar inside the reactor. The reactor cover, made of methacrylate, is closed by means of methacrylate flanges that ensure the tightness of the assembly, with ports for the inlet and outlet streams, connection of the stirring rod, hole for the glass tube and a plug/stopper for manual/automatic loading of the magnetic nanoparticles. The operation of the reactor is controlled by its corresponding PLC control with an adjustable timing for sequential operation (Fig. 1). The internal magnetic separator was constructed using commercially available permanent toroidal magnets with axial magnetization (14 NdFeB magnets of  $6 \times 15 \times 6$  mm, Superparamagnet, Germany) inserted in a non-magnetic steel rod, with poles arranged with alternate polarity. A magnet array model was developed to



Fig. 1. Configuration of the sequential batch reactor (SBR) laboratory (5 L) reactor comprising: A) peristaltic feeding pump; B) inlet flow; C) stirring unit; D) internal magnetic separator and pneumatic cylinder; E) peristaltic discharge pump; F) outlet flow and G) programmable logic controller (PLC).

quantify the magnetic field under alternating polarity. The operation of the reactor was carried out in 4 different phases (Fig. SI-1): i) loading of the nanobiocatalyst and feeding of the reaction medium (72 mL min<sup>-1</sup>); ii) reaction stage; iii) separation of the nanobiocatalyst after reaction (2 min); iv) discharge of the effluent (72 mL min<sup>-1</sup>). The retention of the silica-coated MNPs in the reactor was monitored by measuring Fe<sup>2+</sup> concentration in the effluent of the different cycles by flame atomic absorption spectroscopy (FAAS).

The simulation of the magnetic field was performed using COMSOL Multiphysics  $\,^{\circ}$  version 5.3 with AC/DC Module, using the model referred as magnetic fields, no currents (mfnc). The grid was limited to one cylinder with the following dimensions: 100 x 85 mm (diameter x length). As a boundary condition, a zero-magnetic potential for the cylinder surface was considered. Neodymium magnets were simulated using the following physical data: relative magnetic permeability ( $\mu_T$ ) of 1.05 and remnant field (B<sub>T</sub>) of 1.3 T.

## 2.5. Biotransformation of MG and rutin in the magnetic SBR by laccase immobilized onto silica-coated magnetic nanoparticles

The influent containing MG  $(20 \text{ mg L}^{-1} \text{ in } 100 \text{ mM} \text{ phosphate}$ buffer, pH 6) was fed to the magnetic reactor with immobilized laccase added in a single initial pulse of  $200 \text{ U L}^{-1}$ . The reactor was operated in cycles of 6 h and at the beginning of each cycle, laccase activity was measured. In parallel, a control experiment with functionalized magnetic nanoparticles lacking laccase was also performed. The effluent was freeze-dried to evaluate the effect of the different biotransformation products (BP) at concentrations between  $0.5-5 \text{ g L}^{-1}$  on laccase activity after 24 h. The percentage of dye decolorization was calculated from the decrease in the characteristic absorbance of MG (630 nm).

Regarding the oligomerization of rutin, the influent (0.5 g rutin  $L^{-1}$  in 100 mM acetate buffer, pH 5 and 30% ethanol) was transformed in consecutive cycles with 3000 U  $L^{-1}$  of laccase. Rutin concentration was determined by high-performance liquid chromatography (HPLC). Detailed information on the analytical protocol is described in Supplementary Information.

### 2.6. Envisioning the biotransformation of MG present in a textile effluent

To evaluate the influence of the composition of a secondary effluent on enzymatic stability and the biotransformation rate of MG, experiments were carried out with secondary effluent from WWTP (Detailed Composition in Table SI-1). The secondary effluent was spiked with MG (20 mg L<sup>-1</sup>), simulating the composition expected in a real textile effluent. The reactor operated for 6 cycles with immobilized laccase added in a single initial pulse of  $100 U L^{-1}$ . The percentage of dye decolorization was calculated from the decrease in the characteristic absorbance of MG (630 nm). Moreover, the monitoring of the total organic carbon (TOC) concentration using a Shimadzu TOC-L equipment was performed as indicative of dye removal due to enzymatic treatment.

## 2.7. Identification of laccase-catalyzed reaction products from MG decolorization

The identification of MG oxidation products by laccase was conducted in a batch experiment. Each reactor (20 mL) contained 20 mg L<sup>-1</sup> of MG in an ammonium acetate buffer (pH 6) with an initial laccase activity of 1000 U L<sup>-1</sup>. Samples were periodically withdrawn and analysed by high performance liquid chromatography (HPLC) with a diode array detector (DAD) coupled to mass spectrometry (MS) using the electrospray ionization source (ESI) (HPLC-DAD-ESI-MS) to follow the appearance of the different metabolites obtained from the transformation of the parent compound. Detailed information on the analytical protocol is described in Supplementary Information.

### 2.8. Toxicity and biodegradability assays

In order to investigate the potential toxicity of the transformation products obtained after enzyme treatment, a Microtox<sup>\*</sup> test based on the luminescent marine bacterium *Vibrium fischeri* was performed using a Microtox<sup>\*</sup> model 500 Analyzer according to the protocol defined in the Standard Methods [26]. The results were expressed as half of the maximum effective concentration at 15 min (EC<sub>50, 15min</sub>), which



Fig. 2. Modelling of magnetic fields (T) produced by both configurations of magnets array: alternate (a) and series (b) polarity.

corresponds to the concentration of the pollutant that causes a 50% reduction in the light output of *Vibrium fischeri* after 15 min incubation.

Aerobic biodegradability was determined by monitoring oxygen consumption by aerobic sewage sludge in duplicate assays of treated and untreated effluents for 5 days. The concentration of total and volatile solids in aerobic sludge samples was quantified according to Standard Methods [26], at values ranging from 3.1 to  $3.4 \text{ g L}^{-1}$  and 2.1 to  $2.4 \text{ g L}^{-1}$ , respectively. After sampling, the sludge was washed with phosphate buffer (200 mM, pH 7) and stored at 4 °C. Oxygen consumption was measured with an automated OxiTop device (WTW) according to the Standard Methods [26]. The technique was based on the reduction of pressure inside the closed flasks containing the inoculated sample, being proportional to oxygen consumption.

Anaerobic biodegradability was determined by monitoring methane production in triplicate assays of treated and untreated effluent for 17 days in 100 mL bottles. Anaerobic sewage sludge was used as inoculum, and the biomass concentration was fixed at 2 g  $L^{-1}$  of volatile suspended solids (VSS). Biogas production was monitored using a pressure transducer (Centrepoint Electronics), and its composition was analyzed by gas chromatography (HP, 5890 Series II), using helium as carrier gas.

### 2.9. Environmental performance and cost analysis

The design of the reactor was adapted to a scale of 100 L with a daily flow of 400–600 L  $^{-1}$  for a hydraulic retention time of 4–6 h, consisting on the elements described on Section 2.4 coupled with four magnetic bars composed each by 160 toroidal magnets aligned in alternate polarity which permits nanobiocatalyst retention in less than 10 min A short time (4 min) is considered for charge and discharge of the effluent with the pumps selected (D-21 V Dinko). The estimated energy consumption for the reactor was 6.49 kW h m $^{-3}$  of treated water.

### 2.9.1. Environmental performance

The environmental performance of the enzymatic treatment was analyzed according to the Life Cycle Analysis (LCA) methodology and compared with a more widespread advanced oxidation process, such as ozonization. LCA has proven to be a versatile methodology for quantitatively assessing the environmental impacts of products and services [27]. To apply the LCA methodology, inventory data from laboratory and pilot plant experiments was collected. Background data (electricity and chemical production) were obtained from the Ecoinvent<sup>®</sup> database [28–30]. To ensure the validity of the comparison, the functional unit for both processes was defined as the color removal (90%) from an effluent containing  $20 \text{ mg L}^{-1}$  of the dye MG for a treatment volume of 1 m<sup>3</sup>. The environmental assessment was conducted using ReCiPe Midpoint characterization factors [31] and the following impact categories were considered in the analysis: climate change (CC), ozone layer depletion (OD), terrestrial acidification (OT), freshwater eutrophication (EF), marine eutrophication (MEs), human toxicity (HT), photochemical oxidant formation (POF), freshwater ecotoxicity (FET), marine ecotoxicity (MET) and fossil depletion (FD). SimaPro 8.02 [32] was the software used for the computational implementation of life cycle inventory data and the calculation of environmental profiles.

### 2.9.2. Cost analysis

The estimation of capital costs included the reactor vessel, the magnetic separation unit, pumps and PLC control, with a depreciation period of 10 years. The operational costs included the production of the nanoparticles and enzyme, processes that differed significantly in terms of production scheme, chemical and energy requirements, performance and availability. Consequently, different supports (silica-coated magnetic nanoparticles and magnetized silica nanoparticles) and enzymes (Laccase and Manganese Peroxidase) were considered. For the electricity cost, a tariff of  $0.114 \in kWh^{-1}$  was used [33].

### 3. Results and discussion

### 3.1. Characterization of the magnetic reactor and modelling of the magnetic field

Fig. 2 shows a simulation of axial slices of the magnet arrays (limited to 6 for better clarity) of both configurations, alternate and series polarity, showing magnetic fields in the range of 50 m T to 1.2 T. In the case of series arrangement, the magnetic field reaches high values but only at the interspaces between the individual magnets and at the top and bottom of the bar. The magnetic field goes from the north pole of one magnet to the south pole of the adjacent magnet and there is no effect on the outer volume bounded by the glass sheath. For the alternate configuration, when placing face-to-face poles of the same polarity, the magnetic field lines must extend to find the opposite pole, and consequently, the high gradient area (that defines the intensity of the magnetic field) is remarkably superior. It was found that for the alternate one, the magnetic field at 8.5 mm from the axis (corresponding to the outer face of the sheath) has a maximum of about 400 m T, whereas at 11 mm it is below 100 m T. The resulting gradient is approximately 120 T mm<sup>-1</sup>. According to the magnetic force equation:

$$\mathbf{F} = (\mathbf{m} \cdot \nabla) \mathbf{B} \tag{1}$$

where the magnetic force depends on the magnetic moment (m) and magnetic field (B), the mobility of individual particles of approximately 100 nm would not exceed a few mm s<sup>-1</sup>. However, due to agglomeration, the resulting magnetic moment would increase more rapidly than the decrease in mobility, allowing the separation and accumulation in the outside of the sheath [34]. In order to demonstrate the retention capacity of the nanobiocatalyst by the magnetic separation system, the concentration of ferrous ions in the effluent was monitored and it was found that the recovery of the magnetic nanoparticles after 2 min was greater than 99%, which was repeatedly maintained throughout the operation (10 cycles).

Recent research has also used magnetic enzyme reactors, such as Wang et al. [17] who applied a magnetically stabilized fluidized bed reactor for phenol degradation in coking wastewater or Duan et al. [19] who used a reactor with a system of electromagnets and a microcontroller. These reactors require an energy source for the operation of the electromagnet, with the drawbacks of power consumption and temperature increase. In contrast, the use of an internal magnetic separation unit is an alternative to these configurations, with a higher magnetic field on the outside of the sheath, which leads to an efficient recovery of the nanobiocatalyst without associated energy consumption.

## 3.2. Decolorization of MG by laccase immobilized onto silica-coated magnetic nanoparticles

As presented in the description of the operational sequence of the enzymatic magnetic reactor, it is mandatory to establish the reaction time required for the biotransformation of the target compound, in this case, to accomplish extensive decolorization of the dye. Moreover, other basic variables such as pH and enzyme activity must be selected prior to reactor operation and will be application-specific.

The influence of pH on MG decolorization ( $20 \text{ mg L}^{-1}$ ) and enzyme stability was investigated in batch experiments in a range of 3-7 for free and immobilized enzymes (Table 2). An improvement in MG transformation was observed when pH was increased from acidic to neutral conditions. However, MG decolorization was also found in the control with silica-coated MNPs lacking laccase at pH 3 and 7. After 6 h of incubation, 40% and 80% of MG were decolorized at pH 3 and 7, respectively. The coloured cation of triphenylmethyl dyes in basic and acidic medium becomes a carbinol (non-resonant) base [35]. Hassan et al. [36] found that after 4 h of MG incubation at pH 4.5 and 9.6, the concentration decreased by about 10% and 30% respectively. Moreover, a higher MG decolorization was achieved by free laccase for the entire pH range (Table 2). The lower oxidation of the immobilized laccase to phenolic compounds was also observed by Arca-Ramos et al. [37]. These authors reported that this lower oxidation capacity was related to the aggregation of the nanobiocatalyst which rendered to reduced accessibility of the substrate. The stability of the

### Table 2

MG biotransformation and enzyme stability at variable pH by free laccase and laccase immobilized onto MNP for 24 h.

pН	Biotransformation rate ( $\mu M h^{-1}$ )		Control decolorization (%) <sup>a</sup>		Residual activity (%) $^{\rm b}$	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
3	2.27	1.96	42.0	35.0	0.67	66.2
5	5.08	2.83	6.0	4.0	53.9	83.8
6	4.67	4.46	8.0	5.0	78.1	89.5
7	5.89	3.50	93.0	85.0	99.5	99.8

<sup>a</sup> 100% corresponds to no presence of color in the media.

 $^{\rm b}\,$  100% activity corresponds to 1000 U L  $^{-1}.$ 



Fig. 3. Zeta potential curve obtained for laccase immobilized onto  $Fe_3O_4@SiO_2$  suspended in distilled water environment.

nanobiocatalyst and consequently its potential for aggregation is associated with attractive and repulsive forces determined by the zeta potential. Fig. 3 shows the zeta potential profile of the nanobiocatalyst for a pH range between 2 and 12 and depicts the point of zero charge (pHzc) at a pH value around 5.3. Above this pH, the nanobiocatalyst is negatively charged, which is consistent with previous results where higher biotransformation rates were observed at pH 5-7 (Table 2), since the dye is positively charged and there are no repulsive forces between the dye and the nanobiocatalyst. However, the zeta potential values are less than -30 mV (Fig. 3), which indicates that the nanobiocatalyst is not completely stable and partial aggregation may occur, thus reducing the accessibility of the substrate. Enzymatic stability, similar to biotransformation, was significantly improved in a pH range of 3 to 7. However, a slight difference was observed between the free and immobilized enzyme in acidic conditions. After 24 h, the free enzyme retained only 0.7% of its initial activity at pH 3 while the immobilized enzyme maintained 66.2% of its activity at the beginning of the experiment (Table 2). Therefore, in subsequent experiments, pH 6 was selected as the optimal for MG decolorization.

# 3.3. Sequential batch reactor for the biotransformation of methyl green and rutin by laccase immobilized onto magnetic nanoparticles

The designed reactor was used to assess the ability of immobilized laccase to biotransform MG and rutin in repeated 6-h cycles. Immobilized laccase reached a percentage of MG decolorization higher than 95% in the first cycle, decreasing slightly to 87% after the tenth cycle which implies that  $90 \text{ UL}^{-1}$  is sufficient to achieve high decolourization values (Fig. 4). Kunamneni et al. [38] studied the decolorization of MG by laccase immobilized on epoxy-activated carriers, but decolorization was 67% lower. The slight decay on biotransformation after 10 cycles may be due to the accumulation of biotransformation products that influence the oxidation capacity of the nanobiocatalyst. Different concentrations of biotransformation products between  $0.5-5 \text{ g L}^{-1}$  were tested with  $200 \text{ U L}^{-1}$  of immobilized enzyme at acetate buffer pH 5 after incubation for 24 h. The results show that higher values of biotransformation products led to a decline in enzyme activity (Fig. SI-2).

The reactor was also operated for the biotransformation of rutin in an oligomerization process. Immobilized laccase achieved the complete transformation of rutin (0.5 g L<sup>-1</sup>), which was maintained in 5 cycles (Fig. 5). On the other hand, after the first cycle, activity dropped to 65% but remained constant thereafter.

### 3.4. Envisioning the biotransformation of MG present in a textile effluent

The capacity of the nanobiocatalyst to biotransform MG present in a textile effluent was evaluated. The immobilized laccase reached a



Fig. 4. MG decolorization (%, grey bars) and enzyme activity (O) in subsequent cycles of enzymatic treatment with laccase immobilized onto MNP.



Fig. 5. Rutin biotransformation (mg L<sup>-1</sup>, •) and enzyme activity (O) in subsequent cycles of enzymatic treatment with laccase immobilized onto MNP.



Fig. 6. MG decolorization (%, grey bars) and enzyme activity (O) in subsequent cycles of enzymatic treatment with laccase immobilized onto MNP in a secondary effluent from a WWTP.



Fig. 7. Total ion chromatogram of the biotransformation products, recorded at 0 h (a), 4 h (b) and 24 h (c).

Table 3						
Molecular i	ons	peaks	from	HPLC-ESI	mass	spectra.

Peaks	Molecular formula	Retention time (min)	[M + H <sup>+</sup> ]	ESI-MS spectrum ions $[m/z]$	Absorption maximum (nm)
A,MG	C27H37N3O	28.75	418.0	372;269	259.5
В	C <sub>26</sub> H <sub>34</sub> N <sub>3</sub> O	33.91	404.0	358;254.8	254.8
С	C <sub>25</sub> H <sub>32</sub> N <sub>3</sub> O	22.96	390.0	345;254.8	251.4
D	$C_{24}H_{29}N_3O$	18.34	376.0	432	246.8
Е	C23H27N3O	20.38	362.0	351.8;322.8;212.8	242.8
F	$C_{19}H_{25}N_2O$	16.54	297.0	310.8;281.8;267.8	372.4
G	$C_{18}H_{23}N_{2}O$	9.51	283.0	268;253.8;147.8	362.5

percentage of MG decolorization higher than 95% (Fig. SI-3), and was maintained after 6 cycles (Fig. 6). Not only high decolorization of the dye was attained, but also the immobilized laccase retained 95% of its initial activity after consecutive batches of MG decolorization. Although the decolorization of MG was almost complete after 6 h, the removal of

TOC was determined to be 38%. These findings imply that mineralization by enzymatic treatment was not complete, with the generation of biotransformation products from the parent compound.

### 3.5. Evaluation of MG biotransformation products

With the goal of characterizing the reaction products of MG, an additional study was conducted using an HPLC-DAD-ESI-MS. The time course evolution of the initial dye concentration and the transformation products were followed (Fig. 7). The dye and its related intermediates are marked as A–G species in the total ion chromatogram at the beginning of the reaction, after 4 h and after 24 h of enzymatic treatment. Besides, the transformation products were also followed for 28 days. Seven components with a retention time shorter than 35 min were identified (Table 3) and a biotransformation mechanism was proposed (Fig. 8). Other unidentified products with retention times higher than 40 min accumulated over time. The molecular ion peaks appeared in the acidic forms of the intermediates. The results of the HPLC-ESI mass



Fig. 9. Cumulative oxygen consumption during aerobic biotransformation experiments with samples taken after 24 h of laccase-catalyzed transformation of MG (●) and corresponding controls lacking enzyme (○).

spectra are summarized in Table 3. The substrate MG was detected as the carbinol base (A), as confirmed by mass spectral analysis (m/z = 418) and absorption spectra ( $\lambda_{max} = 259.5$  nm) [38,39]. The cationic molecules of the MG dye become the colorless carbinol base,

which is a tertiary alcohol [4-(*N*-ethyl-*N*,*N*-dimethylamino)][4'-(*N'*,*N'*-dimethylamino)][4''-(*N''*,*N''* dimethylamino)]tripehylmethanol, by hydroxide anion. The intensity of A decreased over the reaction time and disappeared after 6 h of enzymatic treatment (data not shown),



Fig. 10. Cumulative methane production during anaerobic biotransformation experiments with samples taken after 24 h of laccase-catalyzed transformation of MG (•) and corresponding controls lacking enzyme (○).



Fig. 11. Comparative environmental profiles (%) for enzymatic treatment (black bars) and ozonization process (grey bar) to each impact category.

similar to what was observed for MG by spectrophotometry in Section 3.2. Compound A was then attacked by the enzyme, producing demethylated intermediates in a stepwise process. Compound B, m/z = 404, resulted from the mono-*N*-demethylation of A, and reached its maximum concentration after 2 h. Other N-demethylated intermediates were observed: C (m/z = 390) after 4 h and D (m/z = 376) and E (m/z = 362) after 24 h (Table SI-2). On the other hand, the attack to the central carbon portion of A produced compound F (m/z = 297), which corresponds to 4-(*N*-ethyl-*N*,*N*-dimethylamino)-4'-(N',N'-dimethylated compound G (m/z = 283) after 28 days of enzymatic transformation (Table SI-2). These intermediates have also been identified during photodegradation of MG by TiO<sub>2</sub> and ZnO [38,39].

### 3.6. Toxicity and biodegradability of the transformation products

Detoxification of dyes by free or immobilized laccases is an important target to consider, since, as previously reported, dye transformation products may lead to more toxic metabolites [40]. The toxicity of the laccase-catalyzed transformation products from MG was evaluated by the Microtox assay. Control and 24-h treated samples showed  $EC_{50\%}$  (15 min) values of 7 and 12%, respectively. The low  $EC_{50\%,15min}$  value of MG reflects the high toxicity of the dye to Microtox<sup>®</sup> test bacteria [33,34]. After laccase treatment, toxicity decreased, suggesting

the generation of biotransformation products less toxic than the parent substrate.

Aerobic biodegradability of the treated effluent was evaluated by measuring the cumulative oxygen consumption over 5 days (Fig. 9). Untreated MG resulted in low oxygen consumption values, confirming the recalcitrant character of this dye. Moreover, laccase treatment increased effluent biodegradability with values of oxygen consumption significantly higher than those found for the control. Anaerobic biodegradability enhanced considerably after enzymatic transformation (Fig. 10). In this sense, after 100 h of anaerobic treatment, the methane accumulated for laccase catalysed transformation products of MG was 87.5% higher than the control.

The superior biodegradability of the enzymatic treatment effluent suggests the generation of more biodegradable products and would therefore favour the applicability of laccase-catalyzed removal system as an environmentally-friendly technology for the removal of synthetic dyes from aquatic environments.

### 3.7. Environmental performance and cost analysis

The magnetic reactor was scaled-up to a volume of 100 L, which can be used as a demonstration example to be applied in different enzymatic processes. Regarding wastewater treatment, this magnetic reaction is not foreseen as an alternative for the treatment of primary

#### Table 4

Estimation cost for wastewater treatment using magnetic nanobiocatalyst within an enzymatic reactor.

	Cost estimation	References
Capital costs PLC		
Investment	7158€	[45]
Capital cost	0.003€m <sup>-3</sup>	
Reactor		
Volume	100 L	
Investment	19225.27€	[45]
Capital cost	0.008€m <sup>-3</sup>	
Separation unit		
Investment	586€	[45]
Capital cost	0.0003€m <sup>-3</sup>	
Total capital costs	0.0113€m <sup>-3</sup>	
Operational costs		
Energy consumption		
Pumps	0.072 kW h (0.4 h)	[46]
-	0.009€m <sup>-3</sup>	[33]
Pneumatic system	0.18 kW h (0.08 h)	[47]
-	0.09€m <sup>-3</sup>	[33]
Nanobiocatalyst		
Enzyme	0.06–1.48 € m <sup>-3</sup>	[42,48,49]
Support material	0.036–2.08 € m <sup>-3</sup>	[43]
Chemicals	0.037€m <sup>-3</sup>	[25,50]
Energy consumption	0.003€m <sup>-3</sup>	Estimated from consumption
		data
Total operational costs Total costs	0.239–3.69€m <sup>-3</sup> 0.25–3.7€m <sup>-3</sup>	

streams, but as an advanced oxidation alternative in concentrated streams to treat reject water from membrane separation systems. Thus, wastewater with high concentrations of recalcitrant contaminants such as dyes, pharmaceuticals or endocrine disrupting chemicals. For this type of compounds, there are previous works that demonstrate the high potential of laccase and other oxidative enzymes for the degradation of these compounds in short periods of time (4–6 h). However, the real application requires systems that enable the recovery of the enzyme and prevent its inactivation.

### 3.7.1. Environmental performance

The development of new processes must meet sustainability criteria. According to the methodology applied in the comparative evaluation of the enzymatic treatment and the ozonisation process, the latter taken as a representative example of an advanced oxidation system implemented at the commercial level, the enzymatic treatment system has less environmental impact in all the categories evaluated (Fig. 11). The enzymatic treatment represents 83% of the ozonation score in FET, almost half of the ozonation in OD and less than 10% of those for ozonation in CC, TA, FE, ME, HT, POF and FD. The ozonisation present a higher environmental impact due to energy consumption necessary in the production of ozone as the activated carbon present for the entrapment of the ozone prior to effluent discharge. In the enzymatic system, the higher impact is also related with the energy consumption by the agitation process but this could be improved changing the agitation system for a common aireation system present in WWTPs. Gabarrell et al. [41] found similar results in the comparison between enzymatic treatment and granular activated carbon (GAC). As in this case, GAC had a greater environmental impact than the enzymatic treatment with values in six categories.

### 3.7.2. Cost analysis

The scale-up of the reactor made it possible to estimate the capital and operation costs associated with a pilot-scale application (Table 4). According to the estimation provided by the equipment manufacturer (ADEPRO Ingeniería, Spain), the total capital investment, including cost of the equipment, instrumentation, freight, taxes, direct and indirect installation costs, should be about 26,969.27 € (in 2018 euros). However, it is necessary to add operating costs to the initial investment, including annual costs related to the use of enzymes (3285–81030€), nanoparticles (1888.9–457189.87€), chemicals (2041.29€) etc. The values of capital and operational costs are referred to the flow of wastewater (in m<sup>3</sup> per year). Considering a useful lifetime of the reactor of 10 years, the capital costs account for a minor contribution:  $0.011 \in m^{-3}$  whereas the operational costs vary depending on the support and enzyme from  $0.239 \in m^{-3}$  to  $3.69 \in m^{-3}$ . The costs of other processes such as ozonation, powder-activated carbon or laccase immobilized on silica nanoparticles were estimated to be 0.078, 0.114 and  $0.130 \in m^{-3}$ , respectively [42]. The estimated costs for the treatment with the magnetic nanobiocatalyst are slightly higher, but the cost could be reduced if more cost-effective production of the biocatalyst was possible. In fact, the main factors with a remarkable cost are the chemicals and energy required. For instance, 70% of total costs of silicacoated MNPs comes from cyclohexane, Igepal CO-520 and IPA  $(0.67 \in m^{-3}, 0.26 \in m^{-3} \text{ and } 0.56 \in m^{-3}, \text{ respectively})$ . Once the costs have been estimated, the scale-up of the novel magnetic enzymatic treatment technology on a pilot scale demonstrated potential technical and economic feasibility.

### 4. Conclusions

In the present work, we have developed an innovative, simple and efficient system for biotransformation processes based on the use of laccase immobilized in magnetic nanoparticles. The reactor configuration described in this paper offers a novel magnetic separation system with proved technical feasibility and low energy consumption. The internal separation unit, composed of a set of toroidal magnets aligned in alternate polarity, provided a magnetic field that guaranteed the almost complete recovery of the nanobiocatalyst (99%). The magnetic SBR was successfully operated for 10 cycles of MG decolorization and 5 cycles of rutin biotransformation. The scale-up of the reactor and the estimation of capital and operational costs demonstrate the potential of this system for other biotransformation reactions, provided that the biocatalyst can be immobilized onto a magnetic support.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jece.2018.09.014.

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### **Further Reading**

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