Design of bioreactor based on immobilized laccase on silica-chitosan support for phenol removal in continuous mode

A.M. Girelli*, L.Quattrocchi and F.R. Scuto

Department of Chemistry, Sapienza University of Rome, P.le A. Moro 5, 00185, Rome, Italy

Abstract

A silica-chitosan support was employed for laccase immobilization. The hybrid support was obtained using calcium ion as linking agent that coordinates silanol and hydroxyl groups of chitosan. The insoluble biocatalyst was then packed in a column and used in a flow system for phenol removal. The immobilized enzyme reactor (IMER) showed a good storage stability (70% of activity in 70 days) and good reusability (90-50% of catalytic activity at the 4th reuse in function of chitosan type). The best performance for the phenol removal was obtained with a low molecular weight chitosan from crab shells at pH 5 and with a flow rate of 0.7 mL/min. The apparent Michaelis–Menten (V_{max}^{app}, K_m^{app}) and the inherent (V_{max}^{inh}, K_m^{inh}) constants were also determined to evaluate the influence of the phenol structure on the performance of the system. The enzymatic oxidation of a phenol mixture (4-methylcatechol, catechol, caffeic acid, syringic acid, vanillic acid, p-coumaric acid, and tyrosol) was followed for 21 h in a continuous mode by HPLC. The phenol mixture removal of 90% was also confirmed by Folin-Ciocalteu assay.

Keywords: immobilized laccase, continuous mode, silica-chitosan support.

* Corresponding author: Prof. Anna Maria Girelli
annamaria.girelli@uniroma1.it
Tel: (+39) 06 4991 747

1.Introduction

The second half of the last century witnessed a rapid deterioration of water environment as a result of the enormous use of many pollutants, which were conventionally discharged into wastewater (Papadaki et al., 2004). Phenolic compounds are among the chemicals of greatest concern as they tend to persist in the environment for a long time, accumulate and exert toxic effects on humans and animals (Anku et al. 2016) even at low concentrations. In addition they can be transformed into other fractions more harmful than the original compounds (Vione et al. 2002). A typical example is the nitration of phenol with nitrite ion on photoinduced oxidation with the formation of 2 - and 4-nitrophenols. Catechols can be oxidized to semiquinone radicals and at a later stage to o-benzoquinones when the reaction is catalyzed by heavy metals (Kulkarni & Kaware, 2013). For this reason, phenolic compounds are included in the list of priority pollulants of the United Environmental Protection Agency (EPA, 2014) and European Union list 74/464.

In particular the phenolic compounds in oil mill wastewater (OMW) are present in concentration range from 0.5 to 24 mg / LOMW (Rahmanian et al. 2013) and if they are released directly into the environment without any treatment they can accumulate on the soils causing problems of flora, fauna and groundwater. Contamination. Typically, phenol concentrations range from 0.5 to 24 mg / LOMW (Rahmanian et al. 2013). Various techniques for phenol removal have been proposed (Rayati & Nejabat, 2017; Soni et al. 2017; Yu et al., 2017), such as conventional methods and advanced methods (AOP). As for conventional treatments there are distillation, adsorption, extraction and chemical oxidation, which show high efficiencies against various phenolic compounds (Dakhil, 2013).

However, conventional processes can have serious shortcomings, such as incomplete removal, formation of dangerous by-products, low efficiency and applicability at limited concentration ranges (Villegas et al., 2016; Villalobos & Buchanan, 2002). Due to their weaknesses, current wastewater treatment technologies are considered ineffective in the complete removal of pollutants, especially organic matter. For this reason, advanced oxidation processes (AOP) have been developed, which use

a combination of oxidizing agents (H_2O_2 or O_3), irradiation (UV or ultrasound) and catalysts (metal ions or photocatalysts) (Kordatou et al., 2018). Obviously, the first indispensable parameter for choosing the best criterion is the level of abatement of pollutants to be obtained, according to the precise terms of the law to be respected, both at national and European level. Once it was established which processes are capable of achieving the objective or not, cost-effectiveness has become the determining parameter if the efficiency is the same as the chosen methods.

However, despite the great efficiency of AOPs, they have numerous disadvantages: relatively high operating costs due to the use of expensive chemical reagents, increased energy consumption and the formation of unknown intermediates which in some cases may be more toxic than the starting compounds (Stasinakis, 2008). Nowadays, the current demand for sustainable methodologies has led to an increase in the use of oxidoreductases in industrial processes, since their use has numerous advantages such as biodegradability and catalytic efficiency (Villegas et al., 2016). However, the harsh industrial conditions cause the enzymes to destabilize, increasing their denaturation. For this reason, the attachment of enzymes to solid particles gives further rigidity and stability to the three-dimensional structure of the protein, which is essential for its activity. Furthermore, immobilization simplifies the management of the biocatalyst and the control of the reaction process, providing easy separation of the enzyme from the product (Mahat & Onojaq, 2016). The combination of both attributes facilitates the reuse of the catalyst in various reaction cycles which increases the economy of industrial biocatalytic processes. Furthermore, the insolubilization of the enzyme confers other advantages, such as improving the stability of the enzyme against temperature, solvents, pH, contaminants and impurities, and improving storage stability (Ji et al., 2017; Liu et al., 2012)

Nevertheless, the enzyme immobilization may denatured the protein. It is caused by distortions, especially if some multi-interactions between the enzyme and matrix occur (Tusek et al., 2013). Nowadays, immobilized enzymes are used for high-fructose corn syrup production, pectin hydrolysis, fruit juices clarification, interesterification of food fats and oils, biodiesel production (Di Cosimo et

al., 2013), and in particular with polyphenol oxidases for detoxification of environmental pollutants, in the decolorization of textile wastewater and in the treatment of pulp and paper (Guzik et al., 2014). As well known, white rot basidiomycetes are the most efficient lignin degraders by means of oxidative reactions catalyzed by phenol oxidases and peroxidases. Between them laccase, a multi-copper enzyme, shows good efficiency in the phenol removal (Tusek et al., 2013; Liu et al., 2012) and in particular *Trametes versicolor* appeared to be one of the best pollutant degrader by reducing phenol by up to 88 % (Rahmanian et al. 2013).

In this scenario, the aim of this study was to point out an insolubilization system of laccase from *Trametes versicolor* by immobilization on a new support, as silica-chitosan, for the removal of various phenols selected among those most present in the OMW from water: phenolic acids (caffeic, p-coumaric, syringic, vanillic), alcoholics (tyrosol) and classic (catechol and 4-methylcatechol). Many studies have previously dealt with immobilized laccase on microparticles, as for examples, of silica beads (Rahmani et al., 2015; Champagne & Ramsay, 2007) and of chitosan beads (Apriceno et al., 2019; Zheng et al., 2016) and on nanoparticles of silica-coated magnetic nanoparticles (Fe_3O_4@SiO_2) (Moldes-Diz et al. , 2018) and of zirconia-silica hybrid support (Jankowska et al. 2019) but it has not been never employed a hybrid support of silica and chitosan. In particular, the linear biopolymer is composed of randomly distributed β-(1-4) linked D-glucosamine (deacetyilated unit) and N-acetyl-D glucosamine (acetylated unit) having several hydroxyls and a number of reactive amine groups in function of deacetylated degree. Therefore, the preparation of the hybrid support was obtained adsorbing Ca (II) ions on chitosan and silica by chelation through OH or NH_2 groups of biopolymer and silanol groups. (Carunchio et al., 1987). In this way it is possible to exploit the good thermal and chemical stability, as well as excellent mechanical resistance and high surface area of silica with the biodegradability, biocompatibility, non-toxicity, inexpensiveness, as well as the good ability to bind the enzymes, of chitosan (Girelli et al., 2020). In addition, with the aim of preserving the catalytic activity of the enzyme, the glycosidic part of the enzyme was involved in the immobilization procedure (Girelli et al., 2020). This peculiarity was obtained treating laccase with

periodate in order to oxidize hydroxyl to aldehydic groups, highly reactive with the available amino groups of chitosan, through the formation of a covalent imine bond type (Apriceno et al., 2018). The whole immobilization process is illustrated in figure 1.

The biocatalyst was then employed in a laboratory-scale reactor in order to be used in a continuous mode. The optimization of key parameters such as type of chitosan and hydrodynamic parameter (flow rate) were investigated. In addition, kinetic parameters, storage and operative stability of bioreactor were performed. The enzymatic oxidation of single phenol and of a model mixture was made in a continuous mode. IMER results were compared of enzymatic with those obtained by the Folin-Ciocalteu spectrophotometric method (Lamuela-Raventos, 2018).

2. Materials and methods

2.1. Chemical and reagents

Laccase from *Trametes versicolor* with a nominal activity of 136 U/mg protein, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonicacid)diammonium salt (ABTS), chitosan form crab shells with low molecular weight (50-190 KDa) (CS1), chitosan from crab shells with medium molecular weight (CS2) (~400 KDa) and chitosan from shrimp shells (CS3) were purchased from Sigma-Aldrich. Potassium meta-periodate was purchased from Carlo Erba (Milan, Italy). Silica LichrosorbSi 60 (5μm)(Merck). Caffeic acid (≥ 98%), catechol (≥ 99%), p-coumaric acid (≥ 98%), 4-methylcatechol (≥ 95%), siringic acid (≥ 95%), tyrosol and vanillic acid (≥ 97%) were purchased from Sigma-Aldrich.

2.2. Chitosan characterization

The chitosan characterization was performed by elemental analysis, using the analyzer EA 1110 CHNS-O. The degree of deacetylation (DD%) was calculated, in accordance to Kaasai,2000, from the carbon/nitrogen (C/N) ratio as follows:

$$DD\% = 100 - ((C/N - 5.145) / (6.861-5.145) * 100)$$

C/N is carbon/nitrogen ratio that varies from 5.145 in completely N-deacetylated chitosan to 6.816 in chitin, the fully N-acetylated polymer.

2.3 Preparation of silica-chitosan support

1 g of silica was placed under stirring in 40 mL of a 0.05 M $Ca(NO_3)_2$ solution at pH 7 for 30 min at room temperature. Then, it was filtered with a 3 D4 gooch and washed with distilled water until the excess of calcium was removed.

Contemporary, in order to dissolve chitosan and obtain a homogenous solution, 0.45 g of chitosan (CS) were placed in 30 mL 1% acetic acid solution under stirring. Successively, pretreated silica was added to 12 mL of CS solution and brought volume to 20 mL with deionized water. After 3 hours, the aqueous phase was removed by evaporation under vacuum. The solid phase was finally washed in turn with a methanolic solution of potassium hydroxide 0.1 M, methanol and distilled water.

2.4 Laccase immobilization procedure

150 mg silica-chitosan support were incubated with 1 mL of oxidized laccase (0.4 U) at pH 7 for 24 h. Then the biocatalyst was washed with deionized water and stored at 4°C. The oxidized laccase was obtained by periodate oxidation, as previously reported, by the authors (Apriceno et al., 2018).

For the flow system, a stainless-steel column (5 cm x 2.1 mm) was dry-packed with ~ 60 mg of biocatalyst and connected between the HPLC pump and the UV-vis detector. The system was equilibrated with 0.1 M citrate-0.2 M phosphate buffer pH 5 for 15 min before use. The IMER was stored when not in use at 4°C after washing treatment with deionized water

2.5 Enzymatic activity assay

<u>Procedure in batch system:</u> 2mL of 0.23 mM ABTS were added to 10 mg/10μL of immobilized/free laccase in a 0.1 M citrate-0.2 M phosphate buffer solution (pH 3.0) until to reach a final volume of 2.7 mL. The laccase activity was detected at 30°C following the enzymatic formation of radical cation

$ABTS^{+\cdot}$ at 420 nm. In this way one unit of enzymatic activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute.

Procedure in the flow system: 20 µL of 2.5 mM ABTS solution were injected onto the HPLC system (composed by the pump, the IMER as stationary phase and the UV-vis detector) and employing a 0.1 M citrate-0.2 M phosphate buffer at pH 5 as eluent. The amount of ABTS radical cation ($ABTS^{+\cdot}$) formed, detected at 420 nm, was pursued by integration of peak areas and by interpolation with a calibration curve previously obtained. Since ABTS cation radical was not available in commerce, it was generated by chemical oxidation of ABTS by sodium persulfate, as reported by Re et al., 1999. Thus, 8 mL of 0.093 mM ABTS and 2 mL of 12.5 mM sodium persulfate was kept in a flask in the dark and at room temperature for 16 hours in order to obtain a stable product. The amount of ABTS radical cation ($ABTS^{+\cdot}$) was detected at 420 nm by a UV-vis spectrophotometer (PG Instrument Limited, Leicester, United Kingdom), considering its molar extinction coefficient (ε = 36000 mol/L*cm). Successively, the solution was diluted several times obtaining a series of standard solutions. Then, 20 µL of each diluted standard solution were directly injected in HPLC system, using silica-chitosan support, without immobilized enzyme, as stationary phase and 0.1 M citrate/0.2 M phosphate buffer pH 5 as eluent. A calibration curve was obtained reporting the $ABTS^{\cdot+}$ peak areas, at 420 nm, in the range 0.0005-0.004 µmol of ABTS radical ($y=1.04\ 10^7x - 1.06\ 10^3$; $r^2 = 0.994$) .

2.6 Immobilization Parameters

The immobilization yield, efficiency and recovery were calculated by the following formulas:

$$\text{Immobilization yield (IY)(\%)} = 100 \times ((U_0-U_f)/ U_0) \quad (1)$$

$$\text{Efficiency (E)(\%)} = 100 \times U_s/ (U_0-U_f) \quad (2)$$

$$\text{Recovery(R)(\%)} = 100 \times U_s/ U_0 \quad (3)$$

where U_0 and $U_f$are laccase activity in the solution before and after the immobilization process, respectively, while U_s is the activity of the immobilized laccase.

2.7 Effect of chitosan types

The effect of 3 chitosan types (CS1, CS2 and CS3) with different molecular weight on the biocatalyst performance was evaluated in "batch" system by comparing the operative stability and the immobilization parameters (immobilization yield, efficiency and recovery) of the biocatalyst.

2.8 Effect of flow rate on IMER performance

A fixed ABTS amount (0.25 mM) was injected onto the HPLC system: the stationary phase was the thermostated IMER at 30°C, and the mobile phase was 0.1 M citrate-0.2 M phosphate buffer pH 5. The injections were made in triplicate at different flow rates (0.5-0.8 mL/min)

2.9 Determination of operational stability

Procedure in batch system: The operational stability of the biocatalysts was assessed in successive batch reaction cycles. After every cycle the system was washed three times with 0.1 M citrate-0.2 M phosphate buffer and was refilled with fresh ABTS substrate in order to detecting the activity of the biocatalyst. The relative enzymatic activity of each cycle was referred to the activity of the first use.

Procedure in flow system: The operative stability of IMER was evaluated detecting the activity in 4 cycles and taking in consideration the ABTS injections in every cycle. The IMER activity was detected injecting 20 µL of a fresh substrate solution (2.5 mM) in 0.1 M citrate-0.2 M phosphate buffer pH 5. Finally, the IMER was regenerated with deionized water as mobile phase in order to remove any traces of substrates and products. Then IMER was reused for a new cycle of injections under the same conditions. The relative enzymatic activity of each cycle was referred to the activity of the initial activity (first cycle).

2.10 Determination of storage stability

The storage stability of IMER and free laccase was assessed by activity evaluation over a 70-day period. The IMER and free laccase were maintained at 4 °C when not in use. The activity assay of both enzymatic forms was carried out as above described.

2.11 Determination of phenol kinetic parameters

In order to verify the laccase action on all the phenols, a UV-visible spectroscopic study was, previously, performed. For this reason, 10 mg of each phenol were dissolved in the minimum amount of ethanol and brought at volume to 10 mL with deionized water. Then a preliminary investigation on each phenol oxidation by laccase was performed in order to individuate the enzymatic reaction and the operative wavelengths. For this reason, 10 µL of free laccase solution (3 U) were added to 2 mL of diluted phenol 1:10 (v/v). The UV- spectra were successively recorded every 10 min for one hour. At the end of the analysis, it appeared that tyrosol and p-coumaric acid were not oxidized by the enzyme while the catechol and 4-methyl-catechol showed a product at 400 nm, the syringic acid at 360 nm, the caffeic acid at 412 nm and the vanillic acid at 390 nm.

For the determination of kinetic parameters, the IMER at 30°C, was firstly equilibrated for 15 min with 0.1 M citrate-0.2 M phosphate buffer pH 5. Then, 20 µL of each phenol solution (caffeic acid, catechol, p-coumaric acid, 4-methylcatechol, syringic acid, tyrosol and vanillic acid), were injected into the HPLC with a flow rate of 0.7 mL/min of 0.1 M citrate-0.2 M phosphate buffer pH 5, as mobile phase. The quinones enzymatically formed were chromatographically detected at the respective maxima wavelengths. The Michaelis-Menten curve was obtained reporting the reaction rates, expressed as the ratio of the products peak areas on the retention times (area/min) as a function of the concentration of injected phenol. The kinetic parameters were determined by the Lineawever-Burk method. The inherent kinetic parameters were, instead, calculated with the Engasser-Horvath graphic method (Engasser-Horvath, 1976).

2.12 Phenolic compounds bioremediation

For the phenol removal investigations, the stainless-still column filled with the immobilized laccase was thermostated at 30°C. 10 mL of a solution containing 75 mg/L of phenol in 0.1 M citrate-0.2 M phosphate buffer pH 5 were pumped and recycled through the IMER at 0.7 mL/min. The degradation was followed both with the spectrophotometric method of Folin-Ciocalteu, for the determination of total phenol content, and with the HPLC method, to evaluate the kinetic of each individual phenol. For both methods, aliquots of sample were taken at set time intervals (8, 14, 21 h) without interrupting the continuous flow system.

With the HPLC the unremoved phenol amount was chromatographically detected at 280 nm. 20 µL of aliquots drawn from reservoir with a flow rate of 0.7 mL/min were injected in a HPLC system at fixed time. The elution was performed employing a gradient between pump A: MeOH: H_2O with 1.3% HCOOH and pump B: deionized H_2O. The optimized gradient was: 0 to 24 min 15% of A, 24 to 35 min 50% A, 35 to 38 min 50% A and 38 to 40 min 20% A. The stationary phase was a prosphere reversed phase C18 column (5 µm) (15 cm x 4.6 mm ID) (Sigma-Alldrich).

For the spectrophotometric Folin-Ciocalteu (FC) method (Lamuela-Raventos, 2018) aliquots of 0.1 mL drawn from recervoir of the phenol mixture was prelevated and put in a 10 mL volume flask. Then 5 mL of deionized water, 0.5 mL of FC reagent, 2 mL of a 20% (w/v) sodium carbonate solution (Na_2CO_3) and deionized water until to reach 10 mL were added. The sample was kept for 30 min at 40 °C and the absorbance was subsequently read at a wavelength of 760 nm against a blank prepared under the same conditions of the sample but replacing the phenols with the citrate phosphate buffer at pH 5. The total phenol concentration, expressed as equivalents (mg/L) of gallic acid, was determined with a calibration curve obtained by appropriately diluting a solution of gallic acid, prepared by dissolving 0.5 g of gallic acid in 10 mL of ethanol and brought to volume in a 100 mL flask with deionized water. The analysis was then performed in the same way as the samples.

3. Results and discussion

The study was initially focalized on the characterization of the hybrid support aimed both for confirming the composite formation and to choose the chitosan with the highest number of amine groups available for immine bonds with the CHO groups of oxidized laccase. Thus, the evaluation was made on the basis of deacetilation degree and the amount of chitosan adsorbed on silica. For the continuous mode it was also fundamental considering the IMER back pressure.

3.1 Chitosan and chitosan-silica support characterization

Generally, the methods used to determine the chitosan deacetylation degree are spectroscopic (IR and NMR), conventional (titration, conductivity, potentiometry) and destructive (elemental analysis and hydrolysis). The elemental analysis has the advantage of being able to be used in the whole range of deacetylation degree in comparison to the other techniques (Kaasai, 2009). The values obtained on the basis of elemental analysis, for the different chitosan samples (table 1), are 78.8%, 74.4% and 70.4 % for CS1, CS2 and CS3, respectively. It appears that chitosan with low molecular weight (CS1) shows the highest degree of deacetylation and the highest percentage of amino groups that can then be involved in the covalent bond with the enzyme. Furthermore, taking into account that % C depends on the molecular weight (PM) of chitosan it is predictable that CS3 has the highest molecular weight having the highest % C.

The elemental analysis of the composites was also carried out in order to verify the chitosan coating in the various composites (table 1). Nitrogen is present exclusively in chitosan and comparing its percentages in the different composites with those in the respective chitosan type, it is predictable a 25%, 17% and 14% of silica covering CS3, CS1, and CS2, respectively.

The investigation was also evaluated by thermogravimetric analysis (TGA). In figure 2 the thermo analytical behavior of silica, pure chitosan and 3 composites of silica-chitosan in the temperatures range between 0 to 700 °C are reported. It appears that chitosan alone shows a weight loss that starts at 270 °C, which is unseen in silica curve. The weight loss indicates that chitosan is starting to decompose at this temperature until to reach a plateau of 30% of its initial weight at approximately

700°C. Similar thermogravimetric curves are achieved with the composites but different percentages of weight loss are obtained. On the basis of the residual weights it is possible to determine the following degree of silica coverage by chitosan: 20% for CS3, 16% for CS1 and 14% for CS2. The reached results are in good accordance with those of elemental analysis.

3.2 Choice of chitosan

To evaluate the type of chitosan (CS1, CS2, CS3) for attaining the best performance of the biocatalysts: operational stability, immobilization parameters (efficiency, recovery and immobilized activity), and the counter-pressures generated by the supports silica-CS in the flow system were investigated.

The operative stability is a very important parameter in the industrial field, since it allows the reuse of the enzyme and the reduction of the process costs. Repetitive measurements of the 3 biocatalysts activity were carried out in “batch” system. The immobilized activity % of each biocatalyst, employing ABTS as substrate, is shown in figure 3. From the histograms it appears that the biocatalyst with silica-CS3 presents the highest operative stability since at the ninth recycle it retains an activity of ~80%, while those with silica-CS1 and silica-CS2 only 50% and 35%, respectively. In addition, since the optimized method must be used in a continuous flow system where back pressures have a fundamental role to allow the analysis, 3 columns were filled with 60 mg of silica-CS1/CS2/CS3 supports and connected to an HPLC pump. The back pressures of the different supports using water as eluent were determined. Results showed that chitosan from shrimp shells (CS3) had the highest counter-pressure value (>250 bar), CS2 27 bar and CS1 the best value (0 bar). This behavior was directly correlated to the chitosan molecular weight ($MW_{CS1=}$ 50-190 KDa, $MW_{CS2=}$ 400 KDa) and viscosity (CS1= 20-300 cP, CS2 >400 cP). CS3, which is presumed to have the greatest molecular weight and viscosity, may increase the resistance to the passage of the eluent phase.

In conclusion, it can be established that "shrimp shells" chitosan (CS3) turns out to be the best for a possible use in” batch” system, since maintains the greatest activity in reuse tests but in a continuous

flow system, the choice of chitosan must be made between CS1 and CS2 which have generated lower back pressure. By the immobilization parameters comparison appears that activity yield (92%), efficiency (32%) and recovery (29%) values are 92%, 32% and 29% for CSI while 86%, 9%, 8% for CS2, respectively. These different values are due to the highest deacetylation degree (78.8%) and chitosan amount adsorbed on silica (17%) for CS1. Finally, considering also the operative stability the choice was focused on chitosan from crab shells with low PM (CS1).

3.3. Optimization of the parameters influencing the phenol removal

The choice of chitosan and the optimization of different parameters like pH, temperature, flow rate for the phenol removal in the continuous flow reactor (IMER) were investigated. In the figure 4 the recycle device used for phenol removal is reported.

3.3.1 Choice of pH

The choice of the most suitable pH was carried out on silica-CS1 system. Considering the data previously reported by the same authors (Girelli et al., 2020) where the results evidenced the highest activity at pH 3 and an high pH tolerance until pH 5.5 towards ABTS and that pH 5 is the value very close to OMW samples (Mulinacci et al., 2001), pH 3 and pH 5 were chosen. In addition, taking into account that biocatalyst application was in continuous mode, the study was made fluxing citrate phosphate buffers on IMER. A back pressure of 110 psi at pH 3 appeared since it may be due to chitosan swelling that increases with pH decreasing. (Rohindra et al., 2004). At pH 5 a low back pressure was instead obtained (<10 psi) indicating a more favorable pH condition for the employment of IMER in continuous mode. This result together with the slight lower laccase activity at pH 5 than pH 3 (Girelli et al. 2020), the value of 5 was chosen as the operating pH.

3.3.2.Choice of hydrodynamic parameter

The flow rate of the mobile phase, which is a key parameter in a continuous reaction was also investigated. This parameter influences the residence time of the reagents in the IMER, the diffusion

rate of the substrate towards the active site and of the product towards the mobile phase. Since the mass transfer limitations arising from immobilization is manifested in an increase in the apparent values of the Michaelis-Menten constants, a study on the individuation of the kinetic parameters at different flow rates was performed. For this reason, different amount of ABTS in the range 0-3.82 mM at pH 5 and 30°C were injected into the chromatographic system in order to determine the rate of ABTS radical cation formation. The apparent values of K_m^{app} and V_{max}^{app} were estimated by the double reciprocal plots of 1/v versus 1/[ABTS]. From the results reported in table 2 it is observed that as the flow rate increases from 0.5 to 0.7 mL/min the K_m^{app} values tend to decrease, while above 0.7 mL/min it tends to slightly increase. This behavior should be due to the fact that the flow rate influences the contact time between the enzyme- substrate (figure S1) and the diffusion of the substrate and product towards the active sites and mobile phase, respectively. For low flow values (< 0.7 mL/min) the predominant effect is the diffusion resistance, while for high flow (> 0.7 mL/min) the contact time becomes short and the efficiency activity tends to decrease.

To eliminate the effect of external diffusion, the inherent kinetic parameters (K_m^{inh},V_{max}^{inh}) were determined by Engasser-Horvath graphical method (Engasser & Horvath, 1976). In this way, assuming that substrate was consumed on the enzyme surface, the laccase activity and the kinetic parameters in the absence of diffusion limitations were evaluated. In order to evaluate it is, however, necessary to consider the surface area present inside the bioreactor (obtained by the product of silica specific surface area (500m^2/g) with the amount of support) and to normalize the amount of formed product respect the retention time and the surface area. The determination of the inherent kinetic parameters is obtained starting from the Michaelis-Menten experimental curve, and tracing to it the tangent at the point of origin, in order to determine the mass transport coefficient “h” as reported by Girelli et al., 2007. Subsequently, it was possible to determine the ABTS amount on the external biocatalyst surface at the different rates in order to obtain a new Michaelis-Menten curve. The K_m^{app},V_{max}^{app} parameters and the mass transport coefficients "h" are shown in table 2 in function of flow rates. It appears that K_m^{inh} values decrease with increasing flow rates until 0.8 mL/min while the

parameter "h" tends to increase until 0.7 mL/min and above this value tends to decrease. Therefore 0.7 mL/min was chosen as optimal flow rate.

3.4. IMER repeatability

In order to assess the reliability of the immobilization process, two equal IMERs, obtained packing two columns (5 cm x 2.1 mm) with silica-chitosan-laccase, were texted. In this way it was possible to compare the activity measured of both IMERs, injecting 20 µL of a 2.5 mM ABTS solution into chromatographic system composed by the pump, IMER and UV detector. The mean peak area values of the radical product enzymatically formed for IMER1 and IMER2 were 46859 and 54535, respectively. The variation coefficient (CV%) of 7.5 % highlighted that the repeatability of the bioreactors preparation was good.

As regards the repeatability of IMER intra-day response the investigation was performed injecting for 5 times 20 µL of a 2.5 mM ABTS solution into the HPLC system. A CV value of 1.7 % showed a good repeatability of the measurements.

3.5. Operational and storage stability of IMER

Operational and storage stability of IMER with laccase immobilized on silica-CS1 support were investigated. since industrial applications require costs reduction. In both cases, the laccase immobilized activity was determined in continuous mode through the analysis of ABTS product peak areas.

For the IMER storage stability at 4°C (when not in use), the measurements after 30, 50 and 70 days were performed. Results, shown in fig 5a, evidence at 70 days a IMER residual activity value of 70% and only 15% for free laccase. This result is very promising since it is possible to store laccase in a more suitable form and ready to use than the soluble form. This finding can be due to the laccase binding on the support that avoid molecules aggregation and alterations in protein conformation.

The reuse of IMER in a continuous mode was assessed according to the number of ABTS injection in the flow system and measuring the activity of the IMER at the starting analysis (cycle 1), after 50

injections (cycle 2), 80 injections (cycle 3) and 105 injections (cycle 4). From figure 5b it is possible to evidence that after 50 injections (cycle 2) the activity decreases of 30% which remains almost constant for 80 injections (cycle 3). In the fourth cycle (after 105 injections) the residual activity tends to decrease slowly until 50% value. However, the decrease in the activity of the immobilized laccase as a result of repeated uses was expected due to the possibility of enzyme denaturation and release during the operation process.

In table 3 the performance and the storage stability of laccase immobilized on various supports, recently published in literature, are compared with those of the proposed biocatalyst. In particular from the table, it appears the biocatalyst obtained in the present study is the only one applied in a continuous mode which is easier and cheaper technology than the batch mode. In addition the system also highlights a storage and operative stability comparable to those of other biocatalysts.

3.6. Kinetic parameters of phenol compounds

Once the phenol affinity for free laccase was verified, the apparent and inherent kinetic parameters of each phenol in the continuous system were determined in order to individuate the affinity of each phenol with laccase. As, well known the activity efficiency of an oxidoreductase as laccase, is correlated to electron transfer from the substrate to the T1 Cu and the difference (ΔEp) between the redox potentials of the substrate and the T1 Cu is the main driving force for the reaction, together with the substrate binding affinity (Xiu et al., 2001). In literature (Gonzalez et al., 2009; Gianfreda et al., 1999) the laccase affinity influence on chemical phenol structure is also reported. Gianfreda et al., 1999, reported the effect of the number and position of hydroxyl groups on the aromatic ring, the nature and molecular mass of the substituents. Gonzalez et al., 2009, evidenced that the substrate affinity towards laccase varies with the substituent position on the aromatic ring (para > meta > ortho). Results obtained in this study show that for the apparent kinetic parameters, reported in table 4, syringic acid has the greatest affinity for active sites of laccase immobilized, followed by caffeic acid, vanillic acid, 4-methylcatechol, and finally catechol while tyrosol and p-coumaric acid presented no

reactivity towards laccase. However, the order obtained in this study does not agree with the electrochemical properties of the compounds, probably due to different external and internal diffusion and partition effects of phenol with the immobilized enzyme (Goldstein, 1976). It appeared, that the biocatalyst performance depends strictly by the presence of the carboxylic acid group in the phenolic structure. In fact, syringic, vanillic, and caffeic acid at pH 5 has a negative charge for the deprotonation of COOH group (pka 3.1-4.2), as shown in table 5, and their diffusion toward active sites of enzyme can be favored for an attractive force of the free protonated amino groups residue of chitosan (pKa 6.5) (Wang et al., 2006) and/or of the protein (pKa 6) (Scheiblbrandner et al., 2017). For simple phenols and tyrosol it appeared that the hydrophobicity, expressed by logP, was the crucial parameter that influenced the apparent kinetic parameters values (table 5). This is confirmed by the fact that catechol and tyrosol with the lowest logP had the lowest enzymatic reaction rate.

So, it is possible to confirm that the kinetic parameters are affected by a number of factors, including redox potential and diffusive and electrostatic phenomena which alter the concentration of substrates at the laccase active sites from bulk solution values.

In order to obtain the kinetic parameters of the individual phenol in absence of the diffusional effects, the inherent constants were determined following the Engasser–Horvath method (Engasser–Horvath, 1976). The results, reported in table 4, evidence that K_m^{inh} values, especially for catechol and 4-methylcathechol, are lower than the correspondent K_m^{app} and that the phenolic affinity order changes. The new order shows that 4-methylcathecol (MC) has the highest efficiency activity towards laccase while caffeic acid the lowest one. This is probably due to the strictly dependence of the chemical features of the phenolic substrate such as the number of-OH groups present, the nature and molecular mass of the substituents and their position on the aromatic rings (table 5). In particular 4-methylcathecol presenting a substituent electron –donating (-CH_3) easier gives up un electron and provides an area of greater density in the aromatic ring from which an electron can easily be removed. This leads to lower oxidation potentials and higher oxidation rate. This is also confirmed by the fact that cathecol respect 4-methycathecol shows always a very lower enzymatic affinity. By contrast,

caffeic acid which has a substituent with electron withdrawing (-COOH) harder gives up un electron and consequently the increased oxidation potential favors a decreased oxidation rate.

However, for the acid phenols it is very difficult to explain the sequence since the activity efficiency depends also on the architecture of the laccase's substrate-binding pockets, related to the substrate's steric hindrance and/or on the partitioning effects related to hydrophobic/hydrophilic interaction (Glazunova et al., 2018; Pardo &Camarero, 2015; Frasconi et al., 2010).

3.7. Phenol compounds removal

The removal of phenol in continuous mode, was performed, at 30°C, by recirculating a model of phenolic mixture solution through IMER and sampling for FC method and for HPLC analysis at prefixed times. The mixture, constituted by catechol, 4-methylcatechol, tyrosol, vanillic acid, syringic acid, caffeic acid and p-coumaric acid, at the same mass amount (mg/L) corresponding at different molar concentration, with the time evidence a change in color from colorless to yellow-pink, indicating a visible proof of the oxidative reaction (Gianfreda et al., 2003).

The effect of phenol concentration on laccase catalytic action was investigated employing three high total concentration of 175, 350 and 525 mg/L in order to assess the ability of the enzyme to transform large amount of phenolic compounds. This study was performed spectrophotometrically by FC assay measuring the total phenolic content, expressed as gallic acid equivalents (mg/L), at fixed times (8 and 14 h). The % degradation, obtained by the %ratio of the residual total phenol amount to the initial one, increased with the increasing of concentrations from 175 to 350 m/ Land then remained approximately constant up to 525 mg/L (table 6). This is probably due to the fact that the system reaches a maximum operating capacity and that a further concentration increase does not lead to any improvement. In addition, in order to establish the operative degradation time, the mixture at 525 mg/L removal was followed up to 21 h and as result a ~90% % degradation value was obtained. Then, in order to confirm that the oxidation action was exclusively due to the laccase and not to a natural degradation process of the phenols, the total phenolic amount was evaluated after a recirculation

process employing a reactor filled with only silica-CS1 support. Spectrophotometric results showed that the absence of any degradation as the amount of 525 mgL^{-1} total phenolic compounds, correspondent to 446 mg/L as gallic acid, remained almost constant (443.6 mg/Lof gallic acid) up to 21 h.

Then, in order to follow the trend of the oxidation kinetics of individual phenolic compounds by IMER, the chromatographic analyses were performed at the optimized conditions: 21h as degradation time and 525 mg/L as amount of total compounds. In figure 6 the chromatograms of phenol removal after their recirculation through IMER at 0, 14 and 21 h are reported. It is possible to observe the peak areas decrease of each compound and the formation of further chromatographic peaks, related to oxidation products.

The results have shown that 4-methylcatecol and caffeic acid are the ones that react faster, while the others start to degrade only after 8 hours (figure 7). It appears that all phenols are almost completely oxidized by IMER at 21 h (90%).

The removal order can be linked to the competitive effects between phenols in the interactions with the enzyme and to their different molar concentrations (Frasconi et al., 2010). In particular p-coumaric acid and tyrosol, which alone have a very low reactivity towards laccase, when incubated with the other phenols, are removed.

A further study is hoped to better clarify the process and improve the removal efficiency of the enzyme.

4. Conclusion

In this work a new immobilization procedure, based on binding of periodate-oxidized laccase onto a silica-chitosan support through the action of calcium ions which coordinate silanol and hydroxyl groups of chitosan, is employed for the bioremediation of 7 phenol compounds in water by a continuous process. The enzymatic activity of the IMER presented good repeatability values and retained 70% value for 70 days and 50% after more than 105 injections. It also appeared that when

all phenols are together, a general increase of the phenol removal was obtained respect than when they were alone. In particular tyrosol and vanillic acid, that have a very low reactivity towards laccase, were removed only in the presence of the other phenols. This result is probably due to a "laccase mediator system" effect ruled by phenols efficiency activity and molar concentrations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to the support from University Sapienza of Rome, Italy.

Supplementary materials

Supplementary data to this article can be found

References

Anku, W.W, Messai, M.A., Govender, P.P. 2016. Phenolic Compounds in Water: Sources, Reactivity, Toxicity and Treatment Methods. Chapter in a book: Natural Sources, Importance and Applications https://doi.org/10.5772/66927

Apriceno, A., Astolfi, M.L., Girelli., A.M., Scuto, F.R., 2019. A new laccase-mediator system facing the biodegradation challenge: insight into the NSAIDs removal. Chemosphere 215, 535-542. https://doi.org/10.1016/j.chemosphere.2018.10.086

Apriceno, A., Bucci, R., Girelli, A.M., 2017. Immobilization of laccase from Trametes versicolor on chitosan macrobeads for anthracene degradation. Anal. Lett. 50, 2308-2322. https://doi.org/10.1080/00032719.2017.1282504

Apriceno, A., Girelli A.M., Scuto, F.R., 2018. Design of a heterogeneous enzymatic catalyst on chitosan: investigation of the role of conjugation chemistry in the catalytic activity of a Laccase from Trametes versicolor. J. Chem. Technol. Biotechnol. 93, 1413-1420. https://doi.org/10.1002/jctb.5509.

Canfora, L., Iamarino, G., Rao, M.A., Gianfreda, L., 2008. Oxidative transformation of natural and synthetic phenolic mixtures by Trametes versicolor laccase. J. Agric. Food Chem. 56, 1398–1407. https://doi.org/10.1021/jf0728350

Carunchio, V., Girelli, A. M., Messina, A., Sinibaldi, M., 1987. Chitosan-coated silica gel as a new support in high-performance liquid chromatography. Chromatographia. 23, 731–735. https://doi.org/10.1007/BF02312665

Champagne, P., Ramsay, J., 2007. Reactive blue 19 decolouration by laccase immobilized on silica beads. Appl. Microbiol. Biotechnol. 77, 819–823. https://doi.org/10.1007/s00253-007-1208-1

Dakhil, I.H., 2013. Removal of phenol from industrial wastewater, using sawdust. Res. Inventy: Int. J. Eng. Sci. 3, 25-31.

Di Cosimo, R., Mc Auliffe, J., Poulose, A. J., Bohlmann,. G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42, 6437. https://doi.org/10.1039/C3CS35506C

DIRECTIVE 76/464/EEC achievements and obstacles in the implementation of council directive. 76/464/EEC on aquatic pollution control of dangerous substances (1976-2002).

Engasser, J.M., Horvath, C.,1976. Diffusion and kinetics with immobilized enzymes, Chapter in book: Immobilized enzyme principles. Appl. Biochem. Bioeng. edited by L.B. Wingard Jr, E. Katchalski-Katzir , L. Goldstein, New York, 1, 127-220.

EPA United States environmental protection agency Priority pollutant list, December 2014.

Frasconi, M., Favero, G., Boer, H., Koivula, A., Mazzei, F., 2010. Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. Biochim. Biophys. Acta. 1804, 899–908. https://doi.org/10.1016/j.bbapap.2009.12.018

Gianfreda, L., Xu, F., Bollag, J.M., 1999. Laccase a useful group of oxidoreductive enzymes. Bioremed. J. 3, 1–25. https://doi.org/10.1080/10889869991219163

Gianfreda, L., Sannino, F., Rao, M.A., Bollag, J.M., 2003. Oxidative transformation of phenols in aqueous mixtures. Water Res. 37, 3205-15. https://doi.org/10.1016/S0043-1354(03)00154-4

Girelli, A.M., Mattei, E., Messina, A., 2007. Immobilized tyrosinase reactor for on-line HPLC application development and characterization . Sensor. Actuat. B 121, 515–521. https://doi.org/10.1016/j.snb.2006.04.076

Girelli, A.M., Quattrocchi, L., Scuto, F.R., 2020. Silica-chitosan hybrid support for laccase immobilization. J. Biotechnol. 318 ,45-50. https://doi.org/10.1016/j.jbiotec.2020.05.004

Glazunova, A.O., Trushkin, N.A., Moiseenko, K.V., Filimonov, I.S., Fedorova, T.V., 2018. catalytic efficiency of basidiomycete laccases: redox potential versus substrate-binding pocket Structure. Catalysts. 8, 152. https://doi.org/10.3390/catal8040152.

Goldstein, L.G., 1976. Kinetic behaviour of immobilized enzyme systems methods. Enzymol. 44, 397-443. https://doi.org/10.1016/S0076-6879(76)44031-4

Gonzalez, M.D., Vidal, T., Tzanov, T., 2009. Electrochemical study of phenolic compounds as enhancers in laccase-catalyzed oxidative reactions. Electroanalysis. 20, 2249–2257. https://doi.org/10.1002/elan.200904678

Guzik, U., Kocurek, K.H., Wojcieszynska, D., 2014. Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. Molecules 19, 8995-9018. https://doi.org. 10.3390/molecules19078995

Jankowska, K., Ciesielczyk, F., Bachosz, K., Zdarta, J., Kaczorek E., and Jesionowski, T., 2019. Laccase Immobilized onto Zirconia–Silica Hybrid Doped with Cu2+ as an Effective Biocatalytic System for Decolorization of Dyes. Materials. 12, 1252. https://doi.org/10.3390/ma12081252

Ji, C., Nguyen, L.N., Hou, J., Hai, F.I., Chen,.V. , 2017. Direct immobilization of laccase on titania nanoparticles from crude enzyme extracts of P.ostreatus culture for micro-pollutant degradation. Sep. Purif. Technol. 178, 215–223. https://doi.org/10.1016/j.seppur.2017.01.043

Kasaai, M.R., 2009. Various methods for determination of the degree of N-acetylation of chitin and chitosan: A Review. J. Agric. Food Chem. 57 (2009) 1667–1676.

Kordatou, I.M., Karaolia, P., Fatta-Kassinos, D., 2017. The role of operating parameters and oxidative damage mechanisms of Advanced Chemical Oxidation Processes in the combat against antibiotic-resistant bacteria and resistance genes present in urban wastewater. Water Res. 129, 208-230 https://doi.org/10.1016/j.watres.2017.10.007

Kulkarni, S.J., Kaware, D.J.P., 2013. Review on research for removal of phenol from waste- water. Int. J. Sci. Res. Public. 3, 1–4.

Lamuela-Raventós, R.M., 2018. Folin–Ciocalteu method for the measurement of total phenolic content and antioxidant capacity, Chapter 6 in Book: Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications, First Edition, Edited by R. Apak, E. Capanoglu Istanbul, F. Shahidi, 107-114. https://doi.org/10.1002/9781119135388.ch6

Liu, Y., Zeng, Z., Zeng, G., Tang, L., Pang, Y., Li, Z., Liu, C., Lei, X., Wu, M., Ren, P. ,Liu, Z. , Chen, M., Xie, G., 2012. Immobilization of laccase on magnetic bimodal mesoporous carbon and the application in the removal of phenolic compounds. Biores. Technol. 115, 21-26. https://doi.org/10.1016/j.biortech.2011.11.015

Mahat, N.A., Onoja, E., 2016. Relevant techniques and mechanisms for enzyme immobilization, Chapter 2 In book: Protocols and methods for developing green immobilized nanobiocatalysts for esterification reactions.Editors: R.A. Wahab, N.A. Mahat, 31-52.

Moldes-Diz, Y., Gamallo, M., Eibes, G., Vargas-Osorio, Z., Vazquez-Vazquez, C., Feijoo, G., Lema, J. M., Moreira, M. T., 2018. Development of a superparamagnetic laccase nanobiocatalyst for the enzymatic biotransformation of xenobiotics. J. Environ. Eng., 144, 04018007.

Mohammadi, M., As'habi, M.A., Salehi, P., Yousefi, M., Nazari, M., Brask, J., 2018. Immobilization of laccase on epoxy-functionalized silica and its application in biodegradation of phenolic compounds. Int. J. Biol. Macromol. 109, 443-447. https://doi.org/10.1016/j.ijbiomac.2017.12.102

Mulinacci, N., Romani, A., Galardi, C., Pinelli, P., Giaccherini, C., Vincieri, F.F., 2001. Polyphenolic content in olive oil waste waters and related olive samples. J. Agric. Food Chem. 49, 3509-3514. https://doi.org/10.1021/jf000972q

Papadaki, M., Emery, R.J., Abu-Hassan, M.A., Dıaz-Bustos, A., Metcalfe, I. S.,. Mantzavinos, D. 2004 Sono catalytic oxidation processes for the removal of contaminants containing aromatic rings from aqueous effluents. Sep. Purif. Technol. 34, 35–42.https://dx.doi.org/10.1016/S1383-475 5866(03)00172-22

Pardo, I., Camarero, S., 2015. Laccase engineering by rational and evolutionary design. Cell. Mol. Life Sci. 72 , 897–910. https://doi.org/10.1007/s00018-014-1824-8

Rahmani, K., Ali, M., Amir, F., Mahvi, H., Gholami, M., Esrafili, A., Forootanfar, H. , Farzadkia, M., 2015. Elimination and detoxification of sulfathiazole and sulfamethoxazole assisted by laccase immobilized on porous silica beads. Int. Biodeterior. Biodegrad., 97, 107-114. https://doi.org/10.1016/j.ibiod.2014.10.018

Rahmanian, N., Jafari, S.M., Galanakis, C.M., 2013. Recovery and removal of phenolic compounds from olive mill wastewater. J. Am. Oil Chem. Soc. 91, 1-18.https://doi.org/10.1007/s11746-013-2350-9.

Rayati, S, Nejabat, F., 2017. Catalytic activity of Fe-porphyrins grafted on multiwalled carbon nanotubes in the heterogeneous oxidation of sulfides and degradation of phenols in water. C R Chim. 20, 967-974. https://doi.org/10.1016/j.crci.2017.05.004.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med. 26, 1231–1237. https://doi.org/10.1016/S0891-5849(98)003153

Rohindra, D.R., Nand, A. V, Khurma, J.R., 2004. Swelling properties of chitosan hydrogels. South Pacific J. Nat. Appl. Sci. 22, 32–35.

Scheiblbrandner, S., Breslmayr, E., Csarman, F., Paukner, R., Führer, J., Herzog, P. L., Shleev, S.V., Osipov, E. M., Tikhonova, T. V., Popov, V.O., Haltrich, D., Ludwig, R., Kittl, R., 2017. Evolving stability and pH-dependent activity of the high redox potential Botrytis aclada laccase for enzymatic fuel cells. Nature 7,13688. https://doi.org/ 10.1038/s41598-017-13734-0

Soni, U., Bajpai, J., Singh, S.K., Bajpai, A.K., 2017. Evaluation of chitosan-carbon based biocomposite for efficient removal of phenols from aqueous solutions. J. Water Process Eng.16,. 56–63. https://doi.org/10.1016/j.jwpe.2016.12.004.

Stasinakis, A.S., 2008. Use of selected advanced oxidation processes (AOPs) for wastewater treatment – A mini review. Global Nest J. 10, 376-385. https://doi.org/10.30955/gnj.000598

Tavares, A. P.M., Rodríguez, O., Fernández-Fernández, M., Domínguez A., Moldes, D., Sanromán, M. A., Macedo, E. A., 2013. Immobilization of laccase on modified silica: Stabilization, thermal inactivation and kinetic behaviour in 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid. Biores.Technol.131, 405-412. https://doi.org/10.1016/j.biortech.2012.12.139

Tušek, A. J., Tišma, M., Bregović, V., Ptičar, A., Kurtanjek, Ž., Zelić, B., 2013. Enhancement of phenolic compounds oxidation using laccase from Trametes versicolor in a microreactor, Biotechnol. Bioprocess. Eng. 18, 686-696. https://doi.org/10.1007/s12257-012-0688-8

Villalobos, D.A., Buchanan, I.D., 2002. Removal of aqueous phenol by arthromyces ramosus peroxidase. J. Environ. Eng. Sci. 1, 65-73 https://doi.org/10.1139/s01-003

Villegas, L.G.C., Mashhadi, N., Chen, M., Mukherjee, D., Taylor, K.E., Biswas, N., 2016. A Short review of techniques for phenol removal from wastewater. Curr. Pollution Rep. 2, 157–167. https://doi.org/10.1007/s40726-016-0035-3.

Vione, D., Maurino, V., Minero, C., Pelizzetti, E., 2002. New processes in the environmental chemistry of nitrite: nitration of phenol upon nitrite photoinduced oxidation. Environ. Sci. Technol. 36, 669-676.https://doi.org/10.1021/es010101c

Wang, Q.Z., Chen, X.G., Liu, N., Wang, S.X., Liu, C.S., Meng, X.H., Liu, C.G., 2006. Protonation constants of chitosan with different molecular weight and degree of deacetylation, Carbohydrate Polymers. 65, 194-201. https://doi.org/10.1016/j.carbpol.2006.01.001

Xu, F., Deussen, H.J., Lopez, B., Lam, L., Li, K., 2001. Enzymatic and electrochemical oxidation of N-hydroxy compounds, redox potential, electron transfer kinetics, and radical stability, Eur. J. Biochem. 268, 4169-4176. https://doi.org/10.1046/j.1432-1327.2001.02328.x.

Yu, Y., Hu, Z., Wang, Y., Gao, H., 2017. Magnetic SN-functionalized diatomite for effective removals of phenols. Int. J. Miner. Process, 162, 1–5. https://doi.org/10.1016/j.minpro.2017.02.001

Zhang, K., Yang, W., Liu, Y., Zhang, K., Chen, Y, Yin, X., 2020. Laccase immobilized on chitosan-coated Fe_3O_4 nanoparticles as reusable biocatalyst for degradation of chlorophenol. J. Mol. Struct.1220, 128769. https://doi.org/10.1016/j.molstruc.2020.128769

Zheng, F., Cui, B.K., Wu, X.J., Meng, G., Si, J., 2016. Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. Int. Biodeterior. Biodegrad. 110, 69-78. https://doi.org/10.1016/j.ibiod.2016.03.004

Table 1: Elemental analysis of different types of chitosan, silica and composites (silica-CS)

	Chitosan (CS1)	**Chitosan (CS2)**	**Chitosan (CS3)**	**silica**	**silica-CS1**	**silica-CS2**	**silica-CS3**
N%	7.30	7.29	7.22	0	1.41	0.99	1.82
C%	40.14	40.63	40.71	0.13	7.02	5.67	10.47
H%	6.92	7.05	7.17	0.31	1.12	1.19	2.21
C/N	5.50	5.57	5.64	-	4.97	5.72	5.75

Table 2: Effect of hydrodynamic parameters (flow rate) on apparent (K_m^{app} and V_{max}^{app}) and inherent (K_m^{inh} and V_{max}^{inh}) kinetic values and mass trasport coefficient (h). Experimental conditions: IMER, pH 5, 30°C, substrate ABTS.

flow rate (mL/min)	K_m^{app} (mM)	V_{max}^{app} (µmol /min)	K_m^{inh} (mM)	V_{max}^{inh} (µmol /sec $^{*}m^2$)	**"h"** (cm/sec)
0.5	23.90	0.147	2.04	$5.5*10^{-6}$	$5.63*10^{-3}$
0.6	1.68	0.018	0.13	$1.1*10^{-5}$	$0.98*10^{-2}$
0.7	0.43	0.010	$0.65*10^{-2}$	$2.8*10^{-5}$	$1.74*10^{-2}$
0.8	0.45	0.009	$0.62*10^{-2}$	$3*10^{-5}$	$1.53*10^{-2}$

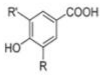
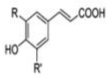
Table 3: Operational and storage stability of laccase immobilized on various supports, recently published in literature, compared with those of the proposed biocatalyst

Support	Application mode	Storage stability (residual activity)	Operational stability (residual activity)	Reference
Chitosan@Fe_3O_4	batch	4 weeks 75.2%	10 cycles 72%	Zang et al., 2020
Zirconia silica hybrid support doped with Cu^{2+}	batch	20 days 80%	7 cycles 40%	Jankowska et al.2019
Epoxi-funtionalized silica	batch	-	5 cycles 61%	Mohamadi et al. 2018
Silica@Fe_3O_4	batch	22 days 80%	8 cycles 70%	Moldes-Diz, 2018
Silica activated with glutaraldheyde	batch	7 days 70%	10 cycles 70%	Tavares et al. 2013
Silica-Ca-chitosan	continuous	70 days 70%	4 cycles and 105 injections 50%	Present study

Table 4: Apparent and inherent kinetic parameters (K_m^{app}, V_{max}^{app}, Km^{inh} , V_{max}^{inh}), transport coefficient (h) of various phenols. Experimental conditions: IMER, pH 5, 30°C, substrate phenols, flow rate 0.7 mL/min.

Phenol	**K_m^{app}** (mM)	**V_{max}^{app}** (peak area/min)	**K_m^{inh}** (mM)	**V_{max}^{inh}** (peak area/ $s^{-}*m^2$)	**h** (10^{-1} cm peak area s^{-1} mmol)
Caffeic acid	1.41	4587	2.59	3.53	2.99
Cathecol	8.05	1718	1.42	1.16	0.157
p-Coumaric acid	-	-	-	-	-
4-Methylcatechol	5.39	1976	1.05	1.62	0.184
Siringic acid	0.74	2074	1.32	2.06	1.27
Tyrosol	-	-	-	-	-
Vanillic acid	1.75	13624	1.11	1.48	0.052

Table 5: Structural and chemical characteristics (pKa, octanol-water partition coefficient (logP), redox potential) of phenols

Class	Structure	Compound	Funtional groups	pKa[a]	LogP[a]	Redox potential*
Simple phenols		Catechol	R= H	9.45	0.88	+0.53 Canfora et al.2008
		4-Methyl catechol	R=CH_3	-	1.37	-
Phenyl alcohols		Tyrosol	R=H R'=H	10.2	0.85	-
Hydroxy-benzoic phenols		Vanillic acid	R = OCH_3 R′ = H	4.16	1.43	+0.71 Canfora et al.2008
		Syringic acid	R =OCH_3 R′= OCH_3	3.93	1.04	+0.68 Gonzalez et al. 2009
Hydroxy-cinnamic phenols		p-Coumaric acid	R = H R′ = H	4	1.74	+0.70 Gonzalez et al. 2009
		Caffeic acid	R = OH R′ = H	3.14	1.53	+0.53 Canfora et al.2008

a: https://chemaxon.com/products/calculators-and-predictors#logp_logd
*oxidation potential versus Ag/AgCl references electrode

Table 6: Effect of total phenol amount on removal % from water. Experimental conditions: IMER, reaction time 8 and 14 h, pH 5, 30°C, continuous mode.

Total phenols (mg/L)	**% Removal (8 h)**	**% Removal (14 h)**
175	15	43
350	38	50
525	33	49

Legends

Figure 1: Scheme of oxidized laccase immobilized procedure

Figure 2: Thermal gravimetric curves pure silica (A), silica-chitosan medium molecular weight (B), silica-chitosan low molecular weight (C), silica-chitosan from shrimp shells (D**)** and pure chitosan (E). Experimental conditions: 1 mg of each samples, oxygen atmosphere, temperature range from 30 to 700°C, and a scanning rate of 10 °C/min.

Figure 3: Reuse comparison between immobilized laccase on silica-CS1 (black histogram); silica-CS2 (dark gray) and silica-CS3 (grey) for 9 consecutive cycles in batch mode. Reaction conditions: 30°C, pH 3 0.1 M citrate-0.2 M phosphate buffer, 0.18 mM 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) as substrate. Values represent the mean ± standard deviations (n=3). The first activity measured was set to 100%.

Figure 4: Recycle device used for dephenolization

Figure 5: Storage stability (A) of free laccase (grey histogram) and IMER (black histogram) and operational stability (B) of IMER. Determination of laccase activity: i) free form: batch mode, 30°C, pH 3 and 0.2 mM 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate); ii) IMER (laccase immobilized on silica-CS1): continuous mode, 30°C, pH 5, injection volume 20 µL 2.5 mM of 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate). IMER when not in use was stored at 4°C.

Figure 6: Chromatographic profiles of the phenol mixture removal from water at three different times initial, 14 h and 21 h. Chromatographic conditions: stationary phase C18, mobile phase gradient elution: Pump A (MeOH: H_2O with 1.3% HCOOH) and pump B (H_2O): 0-24 min 15% pump A, 24 to 35 min 50%, 35 to 38 min 50% and 38 to 40 min 20% A.

Figure 7: Time-course degradation of phenol mixture by laccase immobilized on silica-CS1. Experimental conditions: continuous mode, flow rate 0.7 mL/min, 30°, pH 5, phenol concentration 75 mg/mL

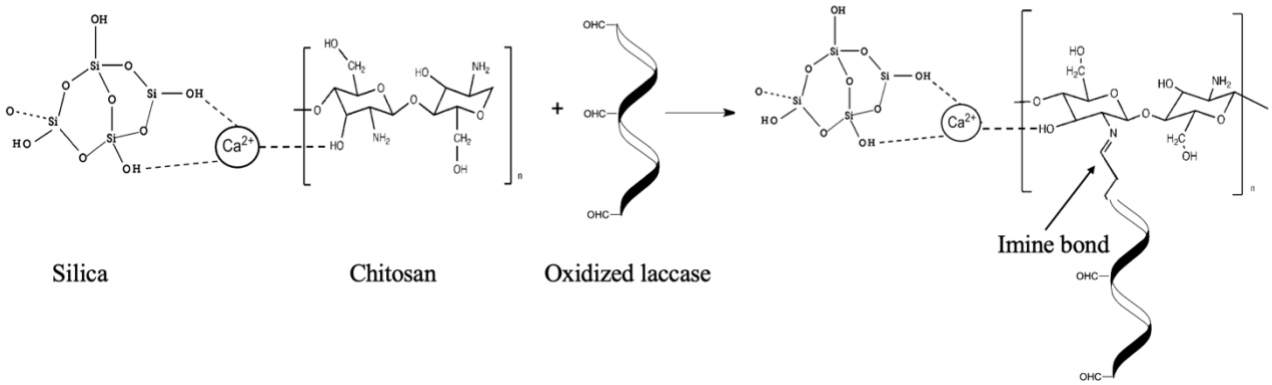


Figure 1

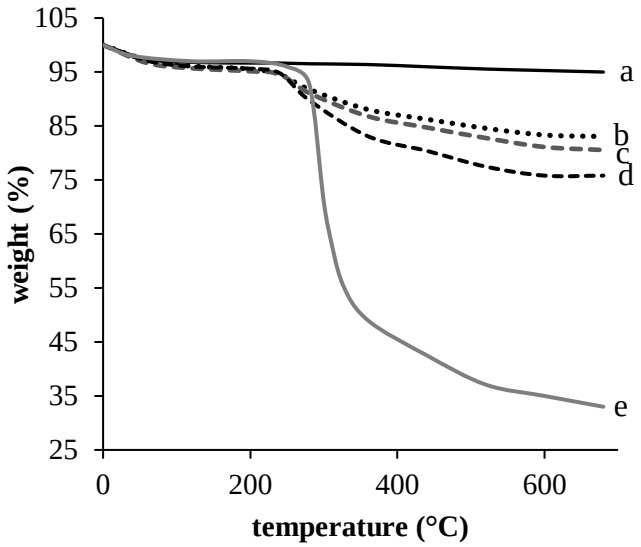

105
95
85
75
65
55
45
35
25
weight (%)
0
200
400
600
temperature (°C)
a
b
c
d
e

Figure 2

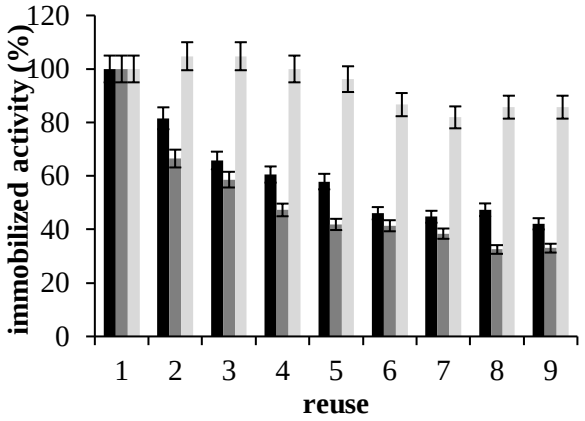


Figure 3

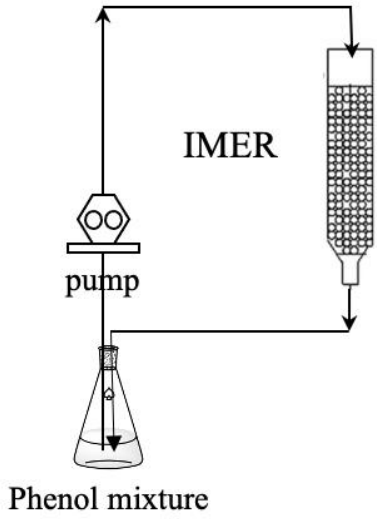


Figure 4

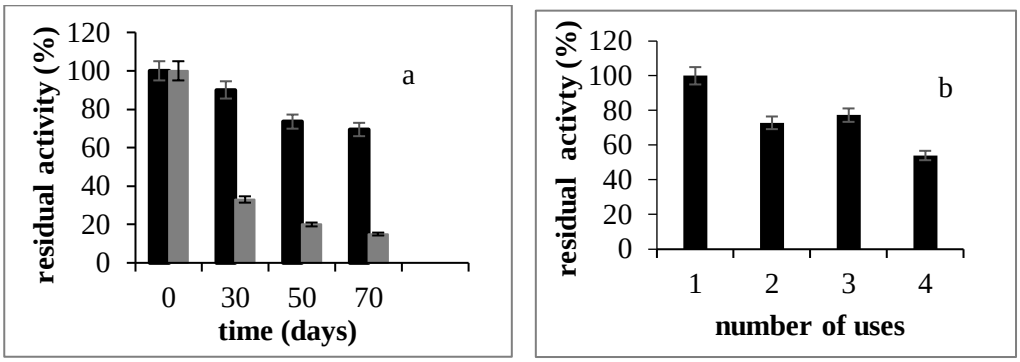

residual activity (%)
120
100
80
60
40
20
0
a
0
30
50
70
time (days)
residual activty (%)
120
100
80
60
40
20
0
b
1
2
3
4
number of uses

Figure 5

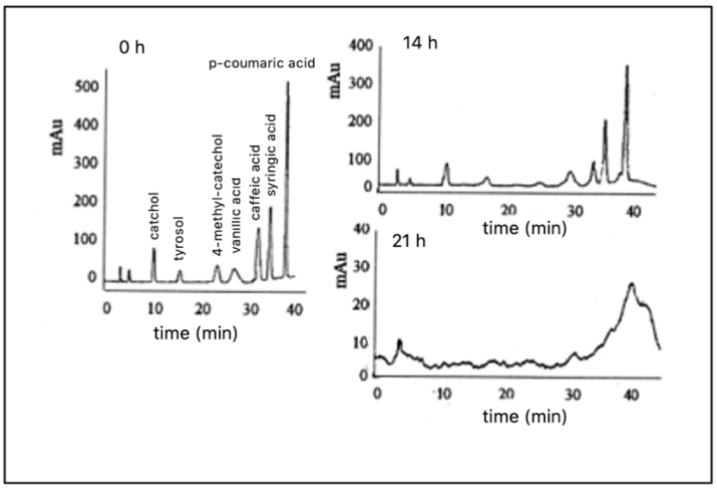

0 h
p-coumaric acid
catchol
tyrosol
4-methyl-catechol
vanillic acid
caffeic acid
syringic acid
mAu
time (min)
14 h
21 h

Figure 6

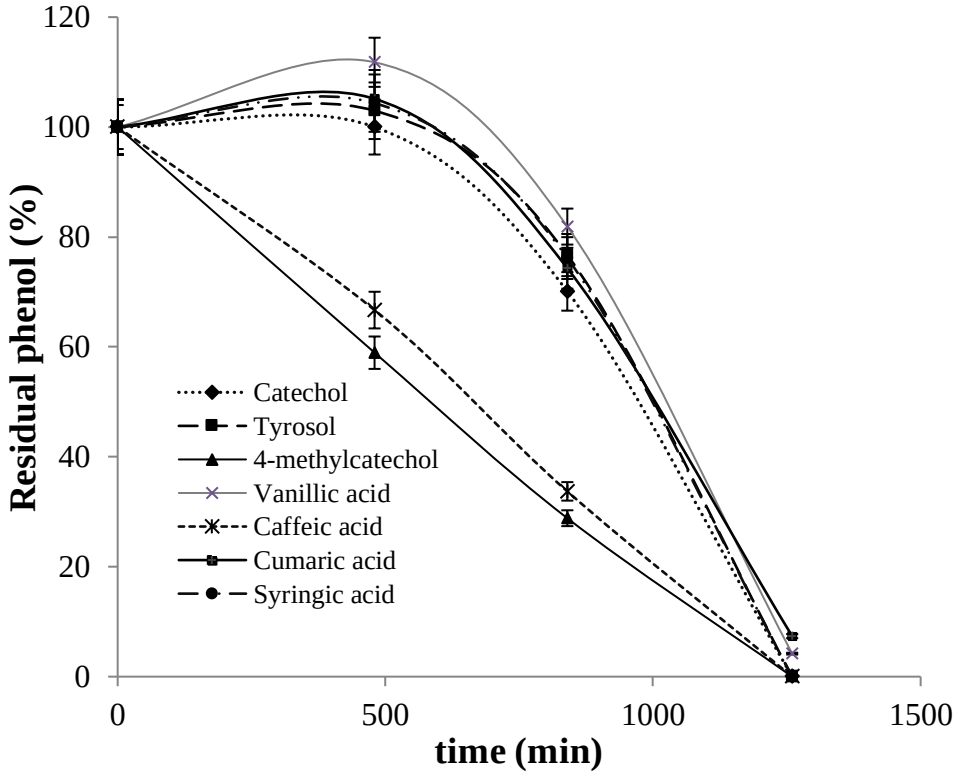

120
100
80
60
40
20
0
Residual phenol (%)
0
500
1000
1500
time (min)
Catechol
Tyrosol
4-methylcatechol
Vanillic acid
Caffeic acid
Cumaric acid
Syringic acid

Figure 7