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Supported biocatalysts for Alizarin and Eriochrome Blue Black R degradation using hydrogen peroxide

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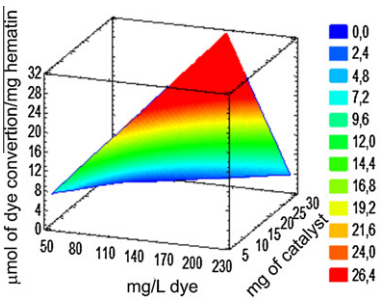
HIGHLIGHTS

- Immobilization of horseradish peroxidase and hematin on different supports.
- Use of supported catalysts in the removal of the dyes AR and EBBR.
- Reuse in target reactions for the best biocatalysts.
- For chitosan–glutaraldehyde–hematin a factorial design was applied for EBBR.

GRAPHICAL ABSTRACT



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ABSTRACT

The goal of this manuscript was to study the immobilization of horseradish peroxidase (HRP) and hematin on different supports at selected conditions to be used in the removal of two synthetic dyes: Alizarin Red (AR) and Eriochrome Blue Black R (EBBR). Reuse in the target reactions was tested for the best biocatalysts. Three supports were selected: magnetite-activated carbon —Fe_3O_4/C—, activated carbon —C— and chitosan —CS—. Several reasons led to discard the use of Fe_3O_4/C and C as supports for hematin and HRP. CS–glutaraldehyde (GA)–HRP not only has low ability to remove EBBR, but also the enzymatic activity of HRP could not be separated from the dye adsorption on CS. However, some adsorptive functions of CS–GA were lost when HRP was supported on it.

In the case of CS–GA–hematin, the most important factors for EBBR removal are dye concentration and mass of catalyst, whereas the temperature has almost no effect on the conversion of dye, considering in all cases the H_2O_2 concentration (0.021 M) constant.

There was not a marked decrease in the removal of AR even after the ninth reuse in the case of CS–GA–hematin. For EBBR removal, with CS–GA–HRP, in the second reuse the catalyst was completely deactivated, whereas with CS–GA–hematin this was found only in the fourth reuse.

1. Introduction

During the last two decades the research regarding the use of enzymes to decontaminate wastewaters containing dyes has seen a sharp increase [1]. The main disadvantage of free enzymes is that they are much less robust than the immobilized ones, making them more susceptible to temperatures higher than 70–80 °C, high substrate concentration and abrupt pH changes. Free enzymes are more likely to be turned off relatively quickly. Another disadvantage is that they cannot be recovered and reused [2].

The immobilization of enzymes is a process that confines or localizes the enzyme in a defined region of space, giving rise to insoluble forms that, ideally, retain their catalytic activity and can be reused repeatedly. Immobilization of enzymes makes them resistant to hostile environments and allows them to be recovered and recycled [3].

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Several methods have been used for the immobilization of enzymes, which include adsorption onto insoluble materials, entrapment in polymeric gels and in membranes, chemical cross-linking using bi-functional or multi-functional reagents and binding to an insoluble "carrier" [4]. The adsorption process is very simple, cheap and effective method of enzyme immobilization, but often reversible [5,6].

A variety of materials as supports for immobilization of many enzymes has been used [7]. These materials differ in particle size and shape, density and porosity. Enzymes can be attached to these supports by adsorption or covalent bonding [8].

Supports activation with a coupling agent such as glutaraldehyde (GA) is one of the most popular techniques for enzyme immobilization. The methodology is quite simple and efficient, and in some cases can even improve the stability of the enzyme by multipoint or multi-subunit immobilization. Furthermore, GA has also been used to introduce intermolecular cross-linking in proteins or to modify proteins adsorbed on aminated supports post-immobilization [9,10].

Peroxidases are versatile catalysts with broad applications in a variety of industrial processes. These enzymes (and in particular horseradish peroxidase or HRP) have been used in the transformation of toxic compounds of industrial origin to preserve the water quality [11–13].

Many materials have been used for the immobilization of HRP including glass, polymers, ion exchange resins and magnetite [14,15]. In recent years, immobilized HRP has also been used in the removal of synthetic dyes [16]. Due to the high cost of HRP, biomimetic alternatives have been explored, being iron porphyrins preferred. In this sense, hematin (hydroxylated hemin) is particularly suitable as a HRP biomimetic.

There are few published reports on the use of hematin in soluble form as a biomimetic of the peroxidase enzyme, and even less on its use in immobilized form [17,18].

Three supports (magnetite-activated carbon (Fe_3O_4/C), activated carbon (C) and chitosan (CS)) were selected in the present work. The Fe_3O_4/C was chosen due to the magnetic properties of magnetite. Because of these properties this iron oxide was used in a previous publication of our group in the adsorption study of the synthetic dyes: Alizarin Red (AR), Eriochrome Blue Black R (EBBR), and Fluorescein (F) [19]. However, the magnetite presents a low specific surface area and a small particle size that make difficult its separation despite of its magnetic properties. For this reason, the magnetite was synthesized on a solid of high surface area such as activated carbon. The fact that activated carbon presents a very high surface area (600–1000 m^2/g) and that a significant fraction (10–30%) of its pore volume is in the range of 300–1000 Å makes this solid very suitable for enzyme immobilization. Daoud et al. [20] carried out various immobilization of enzymes by adsorption on activated carbon and immobilization using GA as crosslinking agent. The magnetic properties of the solid phase allow a rapid separation in a magnetic field applied, so the operating costs are reduced [21–26]. In previous work of our group [27] we performed the comparison of the adsorption capabilities between three iron oxides (goethite, Co-goethite and magnetite) and the CS. Due to the excellent adsorption capacity of CS (especially for AR), we choose CS as a support of the catalysts. CS is a cationic biopolymer, a deacetylated derivative of chitin, the most abundant polysaccharide in nature. In the literature there are numerous reports on chitosan as support for enzyme immobilization through various methods [28,29].

In previous manuscripts [30,31] we presented a comparative analysis of the capabilities of HRP and its biomimetic hematin, free or unsupported, in specific reactions with AR, EBBR and F dyes. These dyes were chosen because their structures are commonly present in dyes of the textile industry.

The former goal of this manuscript was to study the immobilization of HRP and hematin on different supports at selected conditions to be used in the removal of two synthetic dyes: AR and EBBR and to evaluate the enzymatic or biomimetic activity in those dye degradation using experimental design. Besides, the stability of the biocatalysts was tested through reuse assays at selected conditions for both dyes.

2. Experimental

2.1. Materials

All reagents used in this study were of an analytical grade. Horseradish peroxidase was kindly provided by Amano Inc. (EEUU) and was used without further purification. The enzyme has a molecular weight of 41,000 Da, isolectric points of 6.2, 7.2 and 8.8, a stable activity pH range from 6 to 10, with an activity >180 Units/mg for the four aminoantipyrine/phenol reaction in the presence of H_2O_2 (from datasheet available from Amano Inc). Hematin (molecular weight 633.5 g/mol) from Sigma Chemical Co. was employed as provided. AR was provided by The British Drug Houses Ltd., B.D.H. Laboratory Chemicals Group, Poole England. EBBR was supplied by The G. Frederick Smith Chemical Co., Columbus, Ohio.

2.2. Methods

2.2.1. Immobilization of HRP and hematin on chitosan

CS was used as a support for HRP and hematin. We selected covalent binding via GA as the immobilization procedure. The UV/Visible calibration curves were carried out to quantify different organic compounds used in the protocol: GA (at $\lambda_{max.}$ = 293 nm), HRP (at $\lambda_{max.}$ = 403 nm) and hematin (at $\lambda_{max.}$ = 389 nm), all of them in distilled water as the solvent.

The protocol followed for immobilization via activation with GA was the following:

(a) 90 μL of GA 25%, 120 mg of chitosan and distilled water were contacted with magnetic stirring of 450 rpm 1 h at 45 °C. After that, the suspension was centrifuged and GA was measured in the supernatant at 293 nm. The solid was washed several times with distilled and GA was also measured in each supernatant obtained.

(b) The wet solid obtained in (a) was mixed with 5 mg of HRP or hematin and distilled water. The solution was stirred at room temperature for 2 h.

After 2 h the suspension was centrifuged and HRP or hematin was measured in the supernatant. The resulting solid was washed with distilled water and HRP or hematin was also measured in the supernatants obtained by centrifugation. These solids will be identified as CS–GA–HRP and CS–GA–hematin.

2.2.2. Catalytic activity

2.2.2.1. CS–GA–HRP.

2.2.2.1.1. AR. The following experiences were performed to study the catalytic activity of CS–GA–HRP with AR (Table 1):

In all cases aliquots of 20 μL H_2O_2 were added at 5 and 10 min. Enough amount of 0.1 M pH 7 buffer (KH_2PO_4/NaOH) was used. The time of contact was 2 h at room temperature. The complete UV–Visible spectra, initial and after 2 h of reaction, were recorded.

2.2.2.1.2. EBBR. Four experiments were carried out according to Table 2:

In all experiments aliquots of 20 μL H_2O_2 were added at 5, 10, 15 and 20 min. Enough amount of 0.1 M pH 7 buffer (KH_2PO_4/

Table 1
AR removal by CS–GA–HRP at selected conditions.

Exp.	Dye concentration (mg/L)	Solid used	Mass of catalyst (mg)	H_2O_2 9.1% (μL)
1	100	CS–GA–HRP	12.84	40
2	100	CS	1.00	–
3	200	CS	1.00	–
4	200	CS–GA–HRP	1.00	40

Table 2
EBBR removal by CS–GA–HRP at selected conditions.

Exp.	Dye concentration (mg/L)	Solid used	Mass of catalyst (mg)	H_2O_2 9.1% (μL)
1	100	CS	2.00	–
2	100	CS–GA–HRP	2.00	80
3	75	CS	2.00	–
4	75	CS–GA–HRP	2.00	80

NaOH) was used. The time of contact was 2 h at room temperature. The complete UV–Visible spectra, initial and after 2 h of reaction, were recorded.

An attempt was made to perform an experimental design with both CS–GA and CS–GA–HRP using 2 mg of solid in both cases. The removal of EBBR achieved by both solids was very low and clear differences have not been found. Additional experiences only confirmed the previous conclusion, so the factorial design for EBBR with CS–GA–HRP was discarded.

2.2.2.2. CS–GA–hematin. A factorial design to evaluate the catalytic activity of CS–GA–hematin on AR was not explored due to the confounding impact of the AR adsorption on CS, confirmed in a previous work of some of us [27]. So, only an experimental design with EBBR was performed. Previous experiences were conducted to evaluate the optimal conditions for the factorial design (results not shown).

The studied variables were three: temperature, mass of catalyst and dye concentration. The conditions for each experiment were selected as determined by the experimental design, based on previous results using the soluble system [30,31].

2.2.2.2.1. Experimental design chitosan–GA–hematin with EBBR. To perform the experimental design the program STATGRAPHICS® Centurion XVI 2009 from StatPoint Technologies, Inc was used. Since the presence of GA modifies the adsorption properties of CS, CS–GA was used as a blank throughout the experimental design.

In all the experiences 80 μL of 9.1% H_2O_2 were added intermittently leading to volume with 0.1 M pH 7 buffer (KH_2PO_4/NaOH). The contact time was 2 h.

To perform these experiences a calibration curve of EBBR was used, using buffer pH 7 as the solvent (KH_2PO_4/NaOH). The wavelength of maximum absorption of the dye was 543 nm.

The dependent variable was dye conversion (μmol of dye converted/mg of hematin); and the independent variables were dye concentration in mass (mg/L), temperature and mass of catalyst (mg).

2.2.3. Reuse assays

The reuse was performed using 10 mg of supported biocatalyst or biomimetic (CS–GA–HRP and CS–GA–hematin) in all cases.

2.2.3.1. AR. Reuse was performed 50 mg/L of dye. CS–GA–HRP was not tested in the reuse experiences due to the confounding impact of dye adsorption on CS. 80 μL of 9.1% H_2O_2 were added intermittently (20 μL at 5, 10, 15 and 20 min.) when CS–GA–hematin was used. In all cases the final volume was reached with 0.1 M pH 7 buffer (KH_2PO_4/NaOH). The contact time was 2 h at room temperature. The solid used in each step was filtered, washed with 0.1 M pH 7 buffer (KH_2PO_4/NaOH) and recovered for use in the next step. When used the in another day, the catalyst was left as a slurry, in contact with the distilled water and used after filtration in the moment.

The initial experience, 1° and 2° reuse with CS–GA–hematin were done the day 1 of work; 3°, 4° and 5° reuse the day 2; 6°, 7° and 8° reuse the day 3; and 9° reuse the day 4. The initial experience and 1° reuse with CS–GA were performed the day 1 of work; 2°, 3° and 4° reuse the day 2; and 5° reuse the day 3.

2.2.3.2. EBBR. The experiments were performed using 50 mg/L of dye. In all the assays the same procedure described in Section 2.2.3.1 was followed.

2.2.4. Characterization of the supported catalysts

To examine the morphology of the supported catalysts CS–GA–hematin and CS–GA–HRP, the images SEM (Scanning Electronic Microscopy) were recorded with a JEOL 35 CF 1983, Tokyo, Japan. In order to make comparisons the SEM images of CS and CS–GA were also recorded. Also the FTIR spectra of CS, CS–GA, CS–GA–hematin and CS–GA–HRP were recorded using a Thermo Scientific Smart Collector Nicolet 6700.

3. Results and discussion

Several reasons led to discard the use of Fe_3O_4/C and activated carbon as supports for HRP and hematin. One of them is that even using very small mass of supported catalyst the amount of dye initially removed (without the addition of hydrogen peroxide) was close to 100%. The use of higher dye concentrations was unrealistic in some cases, or exceeded the solubility limit in others, as in the case of AR. While magnetite supported on activated carbon caused a decrease of the specific surface area (SSA) from 667 m^2/g (C) to 413 m^2/g (Fe_3O_4/C), the SSA remains high enough to make this solid an excellent dye adsorbent.

In preliminary tests we had observed that magnetite itself had peroxidase like activity in the presence of hydrogen peroxide (results not shown). This is also reported in the literature [32,33] and constitutes an additional difficulty to show the specific activity of HRP or hematin in the dye removal. Taking into account all these considerations Fe_3O_4, Fe_3O_4/C and C were not tested as HRP or hematin support in the present work. Clearly the adsorption abilities of the support should be taken into account when biomimetic or enzymatic dye removal activity is being studied. In this manuscript we take into account these considerations. Unfortunately, in the available literature these concepts and warnings are not very common.

3.1. HRP immobilization

After 1 h of contact as described in Section 2.2.1 (a) 22% of the total GA was adsorbed on CS. In the supernatants after washing GA was not detected, so a great stability of the GA on CS it can be observed. After the conditions of Section 2.2.1 (b) the final percentage of enzyme retained by the support was around 39% of the initial enzyme content in the solution.

3.2. Hematin immobilization

After 1 h of contact as described in Section 2.2.1 (a) 21.4% of the total GA was fixed on CS. The stability of the GA on the support was confirmed by the absence of GA in the supernatants of the respective washes. After 2 h of contact under the conditions of Section 2.2.1 (b) the final percentage of hematin retained by the support was 98%.

3.3. Catalytic activity of CS–GA–HRP

HRP is not desorbed from CS–GA–HRP at pH 7.

3.3.1. AR

When CS–GA–HRP was added to the AR solution, change to colorless was immediate. The recovered solids (CS, CS–GA or CS–GA–HRP) were colored red, showing a significant adsorption of AR on them.

Although high dye removal such as 71% and 89% was found with supported HRP, CS was a so powerful adsorbent that it was impossible to demonstrate the activity of supported HRP to remove/degradate AR. We did not work with higher concentrations of the dye due to its low solubility and, besides, the resulting concentrations would not be representative of those found in a real textile effluent.

3.3.2. EBBR

CS–GA–HRP not only has little capacity to remove EBBR (not more than 27% depending on the experimental conditions), but also the enzymatic activity of HRP could not be distinguished from the dye adsorption on CS. However, it is clear that some adsorptive capacities of CS–GA are lost when HRP is supported on it.

3.4. Catalytic activity of CS–GA–hematin

The results obtained according to the experimental design are shown in Table 3.

The conversion of dye was analyzed as μmol of dye converted per mg of hematin. The comparison considering absolute conversion is not adequate to fit the data.

Dye removal was strongly correlated to the dye concentration and the mass of biocatalyst/biomimetic. The temperature had almost no effect on the dye removal using high H_2O_2 concentration (always 0.021 M).

The equation of the fitted model was:

$$\begin{aligned}&\text{Dye conversion (μmol dye converted/mg of hematin)}\\&= -3.10805 + 0.240333 * \text{dye concentration (mg/L)}\\&\quad - 0.0069717 * \text{mass catalyst (mg)}\\&\quad * \text{dye concentration (mg/L)}\end{aligned}$$

The following three-dimensional (3D) graph shows the impact of the independent variables on the activity per mg of supported hematin (Fig. 1):

3.5. Reuse

At this point it is noteworthy to emphasize that our goal was to highlight this aspect of our supported catalyst. Clearly, this topic is very interesting to be developed in future works.

3.5.1. AR

In the reuse experiments with AR the following results were obtained (Fig. 2):

The percentage of dye removed at a given reuse is expected to be less than the percentage in the previous one. But looking at some of the experiences such as 6 and 7 it can be seen that this did not happen. This was observed when the reuse experiments were not done the same day. In these cases the recovered solid from the last experience was left in the necessary amount of 0.1 M pH 7 buffer (KH_2PO_4/NaOH) to perform the following reuse reaction. In these conditions the desorption of the reaction products from the biocatalysts or the availability of active complexed Fe could be achieved.

Other authors have reported this behavior studying the reuse of supported catalysts. Schultz et al. [34] studied the multiple reuse of immobilized lipase. It is important to note that in this work the immobilized enzyme was only washed once between each cycle and the authors propose that the loss of activity observed was

Table 3
Results obtained following the experimental design. 1 mg supported hematin = 0.04915 mg of hematin.

Exp.	Temperature (°C)	Dye concentration (mg/L)	Catalysts (mg)	% Removed	μmol dye removed/mg supported catalyst	μmol dye removed/mg hematin
1	25	150	10	82.20	0.324	6.596
2	65	150	20	97.21	0.192	3.900
3	65	50	20	98.82	0.064	1.309
4	25	50	10	74.09	0.097	1.964
5	65	150	10	94.53	0.373	7.586
6	45	100	15	98.14	0.167	3.389
7	25	50	20	94.17	0.061	1.248
8	25	150	20	91.74	0.181	3.681
9	65	50	10	92.84	0.121	2.461
10	45	100	15	96.34	0.163	3.327

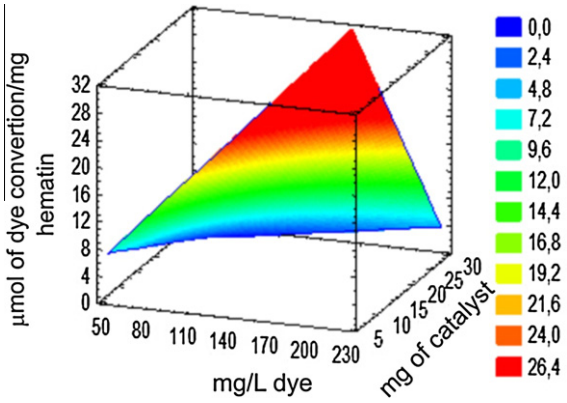

Fig. 1. Tridimensional graph of the dye conversion versus biocatalyst mass and dye mass concentration. Temperature 25–65 °C.

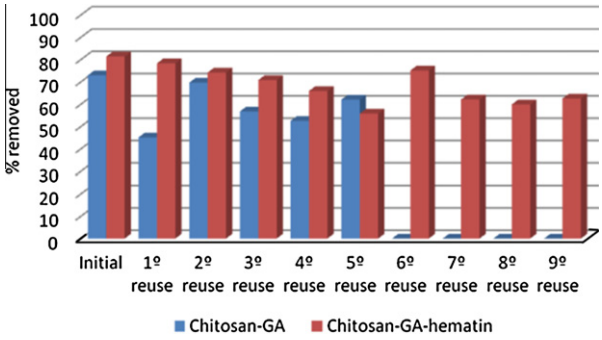

Fig. 2. Reuse experiments with AR 50 mg/L.

Table 4
Reuse for EBBR removal.

Support Use	CS–GA % Dye removal	CS–GA–HRP	CS–GA–hematin
Initial	0	44.47	64.94
1° Reuse	0	8.23	59.41
2° Reuse	–	0	34
3° Reuse	–	0	10
4° Reuse	–	0	0

Table 5
Results of the reuse experiences for EBBR expressed as μmol of dye removed/mg of supported catalyst and μmol of dye removed/mg catalyst.

HRP		Hematin	
μmol dye removed/mg supported catalyst	μmol dye removed/mg HRP	μmol dye removed/mg supported catalyst	μmol dye removed/mg hematin
3.13	160.60	4.56	119.70
0.58	29.73	4.18	109.51
0	0	2.39	62.67
0	0	0.70	18.43
0	0	0	0

caused by lack of washing. The authors confirmed this by a more rigorous washing procedure after the last cycle, thus leading to the successful regeneration of the immobilized enzyme. This is in line with our results with a different enzyme. Furthermore, in our case a marked decrease in enzymatic activity was not observed although the enzyme was not washed between each cycle. Others authors have performed more than a washing step between each cycle and stringent washes leaving the enzyme in a determined buffer and at low temperature to use the enzyme in another cycle of reuse (when the experiences are not performed on the same day) [35–37].

3.5.2. EBBR

The results obtained in the reuse steps were the following (Table 4):

In the case of CS–GA–HRP at the second reuse the catalyst was completely deactivated, while for CS–GA–hematin deactivation just happens in the fourth reuse, with only a slight drop between the initial and the first reuse. Considering that in 10 mg of CS–GA–hematin there is 0.3814 mg of hematin, and in 10 mg of CS–GA–HRP there is 0.1946 mg of HRP, enzymatic activities per mg are reported in Table 5.

3.6. Characterization of the supported catalysts

In Fig. 3 the SEM micrographs of CS, CS–GA, CS–GA–hematin and CS–GA–HRP are shown.

In CS without cross-linking (Fig. 3a) superimposed layers of chitosan are observed and the surface is irregular with a not well defined porosity. However, in CS–GA (Fig. 3b) the chitosan surface changes slightly. Product of the CS crosslinking an irregular porosity and a spongy surface, rough and more fractured with imperfections, with respect to CS without crosslinking are observed (Fig. 3a). These results are consistent with those findings by other authors [38]. In the case of CS–GA–hematin (Fig. 3c) a higher roughness surface is observed. This effect appears to be more marked in the case of CS–GA–HRP (Fig. 3d). There is scarce literature on the analysis of SEM micrographs of supported enzymes on CS–GA.

The FTIR study (not shown) showed no significant changes in the CS bulk bands. A band at 1598 cm^{-1} (assignable to the formation of C=N bond related to the reaction of GA with HRP) suffers an important increase; higher than the increase seen in CS–GA–hematin for that band [39].

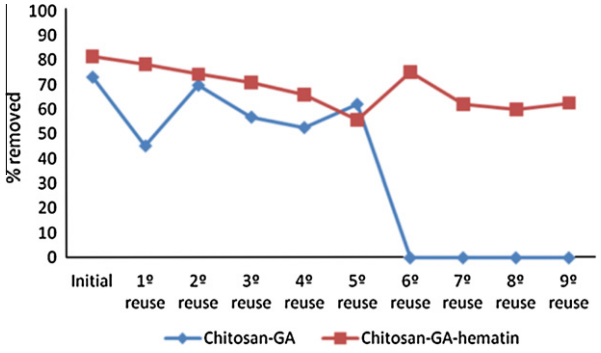

Fig. 3. SEM micrographs of (a) CS, (b) CS–GA, (c) CS–GA–hematin, and (d) CS–GA–HRP. Increase 1000×.

Table 6
Percentage of dye removal using magnetite in absence or presence of H_2O_2 and soluble hematin.

Sample	% Removed		
	Magnetite (adsorption)	Magnetite + H_2O_2	Soluble hematin
AR 100 mg/L	93.80	93.50	93.00
EBBR 25 mg/L	87.60	66.13	62.00

3.7. Comparative study of the efficiencies to remove AR and EBBR using soluble and supported hematin in the presence of hydrogen peroxide and adsorption on magnetite in absence and presence of hydrogen peroxide

Similar conditions (dye concentration and temperature) were selected to compare magnetite in the absence and presence of H_2O_2 and soluble hematin: 100 mg/L of AR and 25 mg/L of EBBR at 25 °C; the mass concentration of magnetite and soluble hematin used were 2 g/L and 25 mg/L respectively [19,30,31].

Two other experiments were carried out using magnetite in the presence of H_2O_2; one with AR and other with EBBR. It should be noted that in the case of using magnetite without hydrogen peroxide only an adsorption process is considered.

Two additional experiments with magnetite in the presence of H_2O_2 were performed to see a possible catalytic effect of magnetite by the presence of H_2O_2. In these assays 2 g/L of magnetite were used but in the presence of 0.021 M H_2O_2 in 0.1 M pH 7 buffer at 25 °C, following the same protocol that with hematin in its soluble form [30,31].

The obtained results are shown in Table 6:

In the case of AR the dye removal is practically the same using adsorption on magnetite, magnetite in presence of H_2O_2 and soluble hematin. However, in the case of EBBR the amount of dye removed by adsorption is higher than the dye removed by magnetite in the presence of H_2O_2 or using soluble hematin. It is important to note that for both dyes, the magnetite in presence of H_2O_2 acts as hematin mimetic, probably generating radicals that absorb in the same UV region of the spectrum than EBBR, giving rise to errors in the dye quantification.

In the literature the results are expressed in almost all cases as percentage of dye removed. Only with the complete information about concentration and absolute mass/mol of dye treated and mass/mol of catalytic material a clear picture of enzymatic activity

Table 7
Mol of dye removed per mol of Fe using magnetite and hematin.

Sample	Magnetite mol of dye removed/mol of Fe	Hematin
AR 100 mg/L	1.04	9.81
EBBR 25 mg/L	0.15	10.51

can be obtained. Regarding the efficiency of a given biocatalyst in dye removal from the solution, the support has also to be tested. In all cases, the results must be expressed per mass or mol of active species of the catalyst or exposed sites of the adsorbent surface. As an example the following Table 7 is shown in order to demonstrate the previously discussed topics.

The density of active sites estimated for the magnetite was 2.7 sites/nm^2 and its specific area 41.7 m^2/g. On the other hand, in the study with soluble hematin always 0.25 mg of hematin (3.95×10^{-7} mol of hematin/Fe) was used. If we compare the efficiency of adsorption of dye on magnetite versus dye removal with hematin expressed as mol of dye removed per mol of Fe exposed at the surface in the case of magnetite, the results shown in Table 7 indicate an efficiency of hematin of about 10 times greater for AR and about 70 times higher for EBBR.

The efficiency of hematin in dye removal was lower as free (58.2%) versus supported (74.1%) in the experiments with 50 mg/L of EBBR at 25 °C. These values however, are closer when reported per mg of hematin 2.35 versus 1.96 μmol of dye removed/mg of hematin. The mass of hematin in the supported catalyst is higher, therefore when the results are expressed as μmol of dye removed/mg of hematin the soluble catalyst is 1.2 times more efficient than that supported. Clearly, part of the Fe in the supported hematin is not available for the catalytic action. Although soluble hematin shows a slightly higher efficiency than the supported one when the results are expressed in this way, it is clear that the use of the supported catalyst offers advantages from the practical and economical point of view, as it can be separated from the solution once the reaction finished and reused again.

4. Conclusions

Three supports were tested as potential part of biocatalysts: Fe_3O_4/C, C and CS. Several reasons led to discard the use of Fe_3O_4/C and C as hematin and HRP supports. CS–GA–HRP not only had low adsorption capacity but also the enzymatic activity of HRP in EBBR removal could not be evident.

In the case of CS–GA–hematin, the most important factors for EBBR removal were the dye concentration and the mass of catalyst, whereas the temperature had almost no effect on the conversion of dye, considering in all cases constant the H_2O_2 concentration (0.021 M). The adsorption of AR on CS is so strong that the impact of this process makes very difficult to evaluate the enzymatic activity of HRP.

There was not a marked decrease in the removal of AR even after the ninth reuse in the case of CS–GA–hematin. For EBBR removal, with CS–GA–HRP, in the second reuse the catalyst was completely deactivated, whereas with CS–GA–hematin this tool place only in the fourth reuse.

Adsorption of dyes on the supports used to immobilize HRP or hematin must be considered as an important confounder when the enzymatic or catalytic activity of HRP or hematin supported is evaluated. In the case of magnetite as part of the support for HRP/hematin immobilization, the impact may be dual: as a dye adsorbent and as a mimetic due to the surface iron reaction with H_2O_2. The appropriate testing of the supports is mandatory to obtain the true enzymatic or biomimetic activity. However, changes in the CS surface adsorption abilities induced by GA or HRP/hematin should also be taking into account.

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