Environmental Engineering and Management Journal

October 2014, Vol.13, No. 10, 2459-2466
http://omicron.ch.tuiasi.ro/EEMJ/

"Gheorghe Asachi" Technical University of Iasi, Romania

REMOVAL OF AROMATIC COMPOUNDS FROM WASTEWATER BY HEMOGLOBIN SOLUBLE AND IMMOBILIZED ON EUPERGIT® CM

María Teresa Pérez-Prior[1], María Isabel González-Sánchez[2], Edelmira Valero[2*]

[1]University Carlos III of Madrid, Department of Materials Science and Engineering and Chemical Engineering, Campus Universitario, E-28911-Leganés, Spain
[2]University of Castilla-La Mancha, Department of Physical Chemistry, Campus Universitario, E-02071-Albacete, Spain

Abstract

The potential application of methemoglobin from bovine blood to remove aromatic compounds in the presence of hydrogen peroxide has been evaluated. The organic compounds herein tested include phenols, amines or hydrocarbons, which are pollutants deriving from different industries and display high persistence and toxicity in waters. The oxidative process has been characterized by soluble hemoglobin and immobilized on Eupergit® CM, using 4-chlorophenol as a model substrate. The obtained data revealed the good effectiveness of the biocatalytic process within the pH 6-8 range, with an optimum pH of 7.5 for the free protein and of 7.0 for the immobilized one. Oxygen evolution in the system was also measured continuously, and indicated that this system significantly contributes to reduce oxygen demand in wastewaters. It was possible to reuse the same hemoglobin-polymer beads as biocatalysts three times. Therefore, the use of hemoglobin in industrial wastewaters treatment may be a good alternative because, despite being somewhat less active, it is able to catalyze the oxidation of different aromatic pollutants, is relatively cheap, and is a food industry waste, which makes the process more ecofriendly.

Key words: aromatic compounds, Eupergit® CM, hemoglobin, peroxidase-like activity, wastewater

Received: May, 2014; Revised final: October, 2014; Accepted: October, 2014

1. Introduction

Thousands of chemicals, including pesticides, biocides, pharmaceuticals, industrial chemicals and chemicals from consumer products are present in wastewaters. The habitual methods to remove these compounds from wastewaters employ microorganisms, adsorption on polymeric resins or activated carbons, extraction with organic solvents, irradiation, chemical oxidation and even ultrasonication (Cailean et al., 2013; Katal et al., 2014). However, none of these methods is free from disadvantages, such as low efficiency or high cost.

Enzymatic bioremediation was proposed some years ago as an alternative approach for the selective removal of pollutants in wastewaters treatment (Klibanov, 1983) in a more environment-friendly way. Laboratory-scale studies have shown that removal of phenols and aromatic amines is possible using horseradish peroxidase (Nicell et al., 1993), polyphenol oxidase (Ikehata and Nicell, 2000) and laccase (D'Annibale et al., 2000). Nevertheless, these enzymes also entail problems when attempting to re-scale such processes to an industrial level, with high cost and low stability being the main drawbacks. Therefore, it is necessary to investigate the catalytic ability of other cheaper biocatalysts that are capable of carrying out the process and improving their stability.

Hemoglobin (Hb) is one of the most important hemeproteins localized in red blood cells in mammals. Besides its crucial function as an oxygen transporter in blood, it is able to catalyze the oxidation of a large number of compounds since in

* Author to whom all correspondence should be addressed: e-mail: Edelmira.Valero@uclm.es

its met state (metHb, Hb-Fe^{III}) and in the presence of H_2O_2, it shows peroxidase-like (González-Sánchez et al., 2009) and catalase-like (González-Sánchez et al., 2011a) activities. The meat industry and slaughterhouses produce large amounts of animal blood, nowadays considered waste. Therefore the use of this protein as an alternative in the removal of organic compounds from contaminated wastewater offers the added benefit of minimizing wastes and rendering the process more ecofriendly.

For a large-scale metHb application to be successful, the protein must be stable and fully functional under the process conditions. Immobilization seems to be the key for optimizing the performance of biocatalysts in industrial processes because it allows operation in the continuous mode, easy protein separation from products, rapid stoppage of reactions, and improved stability. In fact, metHb has been successfully immobilized in different supports (Liu et al., 2012). Eupergit® CM is a methacrylate copolymer with oxirane groups commercially available worldwide, resistant to mechanical and chemical stresses, and adaptable to a variety of configurations and specific processes (Prampano et al., 2010). For all these reasons, this material continues to be used for protein immobilization (Knezevic-Jugovic et al., 2011). However as far as we know, no reports on its use to immobilize metHb have been published.

The purpose of the present paper was to study the potential of bovine metHb, free and immobilized on Eupergit® CM, to be used in the wastewaters treatment for organic compounds removal, under different conditions of pH, H_2O_2, protein and organic compound concentrations. The results obtained indicate that the use of bovine metHb as biocatalyst is a promising alternative.

2. Experimental

2.1. Reagents

Acetaminophen (APAP), 4-aminophenol (4AP), aniline (ANIL), anthracene (ANT), 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 4-*tert*-butylphenol (4TBP), 4-chlorophenol (4CP), 4-cyanophenol (4CNP), hydroquinone (HQ), 4'-hydroxy-acetophenone (4HACP), 4-hydroxybenzaldehyde (4HBZ), 4-hydroxyphenethyl alcohol (4HPA), 4-methoxyphenol (4MP), methyl paraben (MPB), 4-nitrophenol (4NP), phenol (PHE), and Eupergit® CM were obtained from Sigma-Aldrich (Madrid, Spain). H_2O_2 (30%) was purchased from Fluka (Madrid, Spain). All the other reagents were of analytical grade and used without further purification. Solutions were prepared with demineralized water purified in a Milli-Q purification system (18.2 MΩ·cm) (Millipore Corp., Bedford, MA, USA). The following buffers were used: 50 mM sodium citrate (pH 4.0-6.0), 50 mM sodium phosphate (pH 6.0-8.0) and 50 mM sodium pyrophosphate (pH 8.5-9.0).

2.2. MetHb extraction from bovine blood samples

Hb was extracted from the bovine blood samples obtained from a local slaughterhouse. Blood was collected in tubes pre-charged with EDTA (1.5 mg/mL). The tubes were capped off immediately and chilled to about 4ºC.

Hb extraction from bovine blood was carried out as follows: blood samples were centrifuged at 1,100 × *g* for 5 min at 4ºC. As a result of this process, three phases were distinguished (from top to bottom): plasma, a region containing leukocytes and platelets, and erythrocytes. Plasma was eliminated and the resulting pellet was washed with a cold 0.9% (w/v) NaCl solution to eliminate the suspended leukocytes. The mixture was centrifuged at 120 × *g* for 10 min. This process was repeated 3 times. Afterward, erythrocytes were concentrated by centrifugation at 1,100 × *g* for 10 min. To break erythrocytes, a hypotonic dilution buffer (80 mM NaCl, 1.8 mM KCl, 1.3 mM K_2HPO_4 and 5.3 mM KH_2PO_4, pH 7) (Shorr et al., 1993) was used (about 5 erythrocyte volumes). The solution thus obtained was centrifuged at 27,200 × *g* for 45 min and filtered, and the pellet containing the erythrocyte membranes was discarded. The Hb-containing solution was then applied to a Sephacryl S100HR column, MW range 1–100 kDa (Sigma-Aldrich, Madrid, Spain) to eliminate the possible presence of catalase and/or other molecules. Potassium ferricyanide (0.3 mg/mL) was added to the solution to oxidize the protein to the met state. The excess ferricyanide and other low-molecular-weight compounds were eliminated by gel filtration through Sephadex G-25 (Sigma-Aldrich, Madrid, Spain). The stock metHb solutions thus obtained were stored at -20ºC.

2.3. Spectrophotometric measurements

A Perkin-Elmer Lambda 35 ultraviolet/visible spectrophotometer coupled to a Water Peltier System PCB 150 (Perkin-Elmer, Massachusetts, USA) was used in this work. The metHb concentration was calculated in the previously filtered stock solutions (0.22 μm) by using the following molar extinction coefficient: $\varepsilon = 9.48 \times 10^3\ M^{-1}cm^{-1}$ at 500 nm (Zijlstra et al., 1997). The H_2O_2 concentration in the stock solutions was determined at 240 nm using $\varepsilon = 39.5\ M^{-1}\ cm^{-1}$ (Nelson et al., 1972). The concentrations of the aromatic compounds herein tested were calculated by using the ε-values indicated in Table 1.

2.4. HPLC Analysis

An Agilent (Waldbronn, Germany) HPLC 1100 series system equipped with a quaternary pump, a manual sample injection system, a vacuum degasser, a column compartment and a diode array detector was used. Separations were performed on a reversed-phase 5 μm Discovery C_{18} (15 cm × 4.6 mm) from Supelco (Madrid, Spain). Samples were filtered through a 0.45 μm filter prior to injection.

Table 1. The molar extinction coefficients of the aromatic compounds herein studied

Compound	*E (M^{-1} cm^{-1})*	*λ (nm)*	*Reference*
APAP	9.70×10^3	240	González-Sánchez et al., 2009
4AP	2.15×10^3	296	Pérez-Prior et al., 2012
ANIL	1.43×10^3	280	Walton and Reyes, 1983
ANT	9.15×10^4	430	Pavlopoulos, 1992
4TBP	1.82×10^3	275	Pérez-Prior et al., 2012
4CP	1.40×10^3	280	Miland et al., 1996
4CNP	1.90×10^4	247	Ruane et al., 1999
HQ	2.62×10^3	290	Unruh et al., 1998
4HACP	7.71×10^3	294	Nowlan et al., 2006
4HBZ	1.46×10^4	280	Kiljunen and Kanerva, 1996
4HPA	1.60×10^3	275	Pérez-Prior et al., 2012
4MP	7.80×10^3	222	Borra´s et al., 2006
MPB	1.63×10^4	255	Pérez-Prior et al., 2012
4NP	1.70×10^3	405	Lee et al., 2006
PHE	1.40×10^3	272	Al-Kassim et al., 1994

A typical reaction mixture (2-mL final volume) contained metHb (free or immobilized), the organic compound and H_2O_2 in a buffered media at the indicated concentrations. Incubations were performed in an Eppendorf Thermomixer Comfort device (Hamburg, Germany) at 25ºC with mild shaking (300 rpm). The reaction was started by the addition of H_2O_2. Aliquots were withdrawn from the reaction mixture at different times and analyzed by HPLC. Retention times, wavelengths, and the methanol/water ratio used for each substrate were previously reported (Pérez-Prior et al., 2012), except for ANIL (2.2 min, 280 nm, 70:30 methanol:water) and ANT (7.7 min, 251 nm, 80:20 methanol:water). The flow rate of the mobile phase was 1.0 mL/min. The elution conditions were as follows: injection volume, 20 μL and a 20ºC oven temperature (except in the case of ANT, when the oven temperature was 36ºC to reduce the retention time). The solvents used were previously filtered through a 0.22 μm filter and degassed by sonication in a Selecta Ultrasons water bath (JP Selecta, Barcelona, Spain). An Agilent ChemStation B.03.01 revision was used to integrate peak areas.

2.5. Measuremento of oxygen evolution

Oxygen evolution was continuously measured using a Hansatech oxygraph (Hansatech Instruments Ltd., Norfolk, England) equipped with an S1 oxygen electrode based on the Clark oxygen sensor, interfaced online with a PC-compatible computer. The electrode was calibrated using sodium hydrosulfite (Hansatech Instruments Ltd.). The temperature in the reaction chamber was controlled at 25ºC using Selecta water Hetofrig (Barcelona, Spain) circulating bath.

2.6. Immobilization of metHb on Eupergit® CM

Eupergit® CM (1 g) was incubated with different metHb solutions in 50 mM phosphate buffer, pH 7, for 48 h under mild shaking conditions. Afterward, beads were washed 3 times with a solution of 1M NaCl, and 3 other times with 100 mM phosphate buffer, pH 7, to eliminate the unbounded metHb. Finally, they were filtered, dried in a desiccator and stored at -20ºC.

The catalytic activity of the immobilized metHb was measured by monitoring the oxidation of ABTS at 414 nm (ε= 31.1×10^3 $M^{-1}cm^{-1}$) (Childs and Bardsley, 1975) in the presence of H_2O_2 at pH 6 and 25ºC. The experimental conditions were: $[ABTS]_0$ = 1 mM, $[H_2O_2]_0$ = 0.5 mM and 5 mg metHb-Eupergit® CM in a final volume of 5 mL.

2.7. Fitting the data

Data were fitted by using the SigmaPlot Scientific Graphing Software for Windows, version 12.3 (2011, SPSS Inc.).

3. Results and discussion

3.1. Oxidation of aromatic pollutants catalyzed by free metHb

Hemoglobin is not an enzyme. However in its met state, it is able to catalyze the oxidation of a variety of organic compounds to its corresponding free radical in the presence of H_2O_2. This process is accompanied by oxygen evolution (catalase-like activity) in agreement with the catalytic cycle of the protein (González-Sánchez et al., 2009; Pérez-Prior et al., 2012). Subsequently, the corresponding free radical to the substrate polymerizes and can be removed by filtration. Table 2 shows the effectiveness of bovine metHb as a biocatalyst of the oxidation reaction of several organic pollutants with different functional groups in the presence of H_2O_2. All these chemicals are usual in different industries, so they may be found in wastewaters.

3.2. Biocatalytic reaction progress

4CP was chosen as the model substrate to study the optimization of the experimental conditions, mainly due to its solubility in water, its

toxicity and the very large number of industrial applications.

3.2.1. Effect of protein concentration on pollutants removal

To evaluate the influence of the metHb concentration in the soluble form on pollutants removal, a kinetic study of the 4CP oxidation reaction at different initial protein concentrations was performed (Fig. 1). No variation in the 4CP concentration over time was observed in the absence of metHb (●). As the initial protein concentration was increased in the reaction medium, the remaining 4CP concentration at the end of the reaction was progressively decreased until this value was close to zero.

3.2.2. Effect of hydrogen peroxide concentration on pollutants removal

The time courses of the experiments performed at different initial H_2O_2 concentrations are shown in Fig. 2a. In the absence of H_2O_2 (●), the 4CP concentration remained invariable throughout the assay time. In the presence of H_2O_2 however, the 4CP concentration decreased with time to reach a maximum value. The percentage of oxidized 4CP at the end of the reaction increased as the initial $[H_2O_2]_0/[metHb]_0$ ratio increased to reach a maximum value of almost 65% (Fig. 2b). This fact can be attributed to the H_2O_2-induced suicide inactivation phenomenon of metHb (González-Sánchez et al., 2011a); i.e., H_2O_2 behaves in the catalytic cycle of the protein as a suicide substrate because, on the one hand, it is a necessary substrate for the oxidative process, but on the other hand, it irreversibly inactivates the protein.

3.2.3. Effect of the 4CP concentration on oxygen evolution

Dissolved oxygen (DO) determination is one of the most important and useful parameters that indicates the organic strength of a wastewater and determines how satisfactory a biological wastewater treatment operates.

H_2O_2 has been used for many years to reduce both the Biological Oxygen Demand (BOD) and the Chemical Oxygen Demand (COD) of industrial wastewaters (Steiner and Gec, 1992). It can be used alone or with a catalyst. In the present work its use is necessary to switch on the peroxidase-like activity of metHb. As has previously been indicated, besides the progress curve corresponding to the disappearance of the aromatic pollutant, there is a parallel progress curve, which corresponds to oxygen evolution. This means that the metHb-H_2O_2 system significantly contributes to BOD and COD removal in wastewaters. Fig. 3 depicts a selection of progress curves obtained in the oxidation of different initial 4CP concentrations by soluble metHb in the presence of H_2O_2. This Figure clearly illustrates the existing competition between H_2O_2 and 4CP for the protein because the amount of evolved oxygen was reduced as $[4CP]_0$ was increased.

3.3. MetHb immobilization on Eupergit® CM

The immobilization process of the protein on Eupergit® CM occurs by covalent binding between the oxirane groups of the acrylic polymer and the amino functional groups of the polypeptidic chain of the protein at a neutral pH (Katchalski-Katzir and Kraemer, 2000). Nonetheless, it has been previously indicated that this process can also occur through the carboxyl and/or the sulphydril groups at the same pH (Turkova et al., 1978) in a "multi-point-attachment" way. Therefore, N-C and O-C covalent bonds were presumably formed, which are very stable, and which render the immobilization process highly effective.

3.3.1. Optimizing the immobilization process

The immobilization grade in the acrylic polymer depends on the initial protein/Eupergit® CM ratio and the contact time.

Table 2. Optimum pH, initial conditions and percentage of oxidized aromatic compound at the endpoint of the reaction

Compound	*Optimum pH*	*$[H_2O_2]_0/[S]_0$*	*% of oxidized aromatic compound*
APAP	6.0[a]	5	100
4AP	5.0[a]	1	88
ANIL	5.5	2.5	84
ANT	5.0[b]	120	67
4CP	7.5[a]	10	62
4CNP	6.0[a]	5	4
HQ	5.0[a]	5	89
4HPA	6.0[a]	5	68
4HACP	7.0[a]	8	20
4HBZ	6.5[a]	8	17
MPB	7.0[a]	8	16
4MP	5.5[a]	3	76
4NP	6.0[a]	5	12
PHE	6.5[a]	5	49

[a]Pérez-Prior et al., 2012; [b]Laveille et al., 2009 The initial substrate concentrations were: $[APAP]_0$, $[4CP]_0$, $[4CNP]_0$, $[HQ]_0$, $[4HPA]_0$, $[4MP]_0$, $[4NP]_0$, $[PHE]_0$ = 0.5 mM; $[4AP]_0$, $[ANIL]_0$ = 1 mM; $[4HACP]_0$, $[4HBZ]_0$, $[MPB]_0$ = 0.3 mM; $[ANT]_0$ = 8.2 μM. $[MetHb]_0$ = 5 μM, except for ANT, which was 2 μM

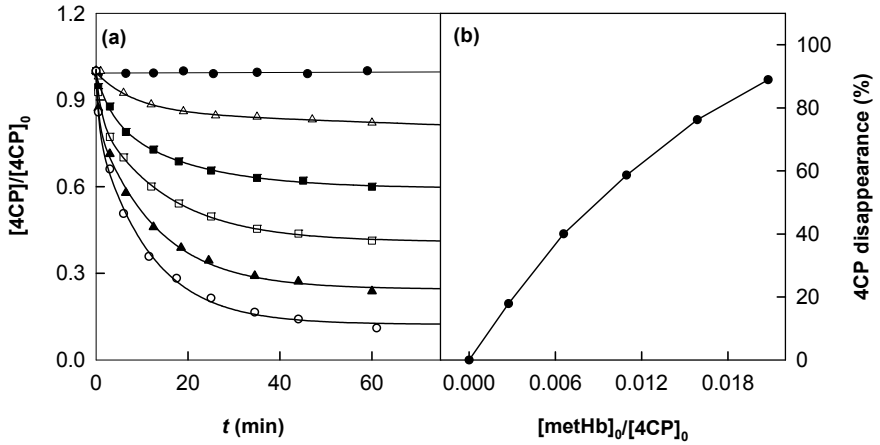

Fig. 1. (a) Time course for 4CP oxidation at the different protein concentrations: $[metHb]_0$ = 0 (●), 1.4 (Δ), 3.3 (■), 5.5 (□), 8.1 (▲) and 10.5 (○) μM. $[H_2O_2]_0$ and $[4CP]_0$ were 2.5 and 0.5 mM, respectively, in 50 mM phosphate buffer, pH 7.5. (b) Percentage of 4CP oxidized at the endpoint of the reaction *versus* the initial $[metHb]_0/[4CP]_0$ ratio used

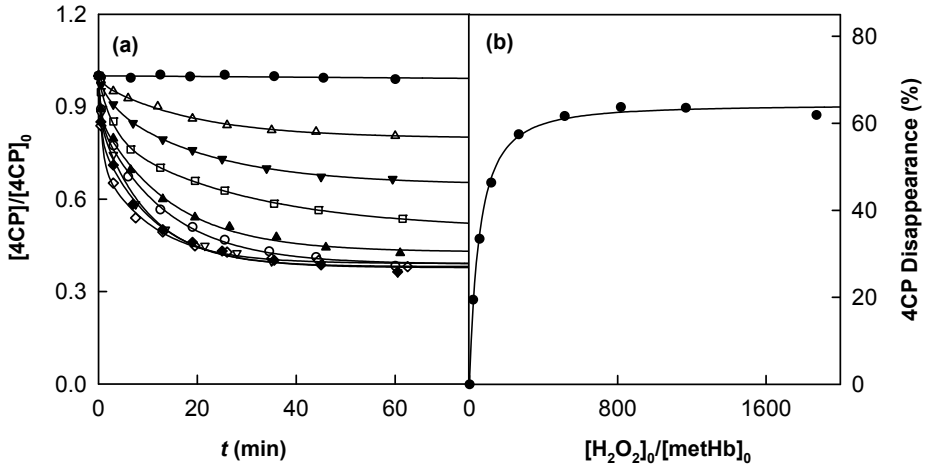

Fig. 2. (a) Time course for 4CP oxidation at the different H_2O_2 initial concentrations: $[H_2O_2]_0$ = 0 (●), 0.1 (Δ), 0.3 (▼), 0.6 (□), 1.5 (▲), 2.8 (○), 4.5 (▽), 6.5 (♦), and 10.3 (◊) mM. $[4CP]_0$ and $[metHb]_0$ were 0.6 mM and 5.8 μM, respectively, in 50 mM phosphate buffer, pH 7.5. (b) Percentage of 4CP oxidized at the endpoint of the reaction *versus* the initial $[H_2O_2]_0/[metHb]_0$ ratio used

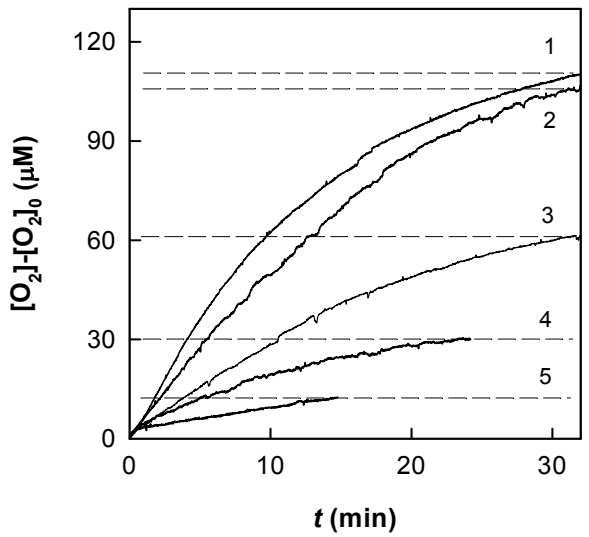

Fig. 3. Time course of O_2 evolution at the different initial 4CP concentrations: $[4CP]_0$ (for curves 1 to 5) = 0, 0.1, 0.3, 0.5 and 1 mM, respectively. $[H_2O_2]_0$ and $[metHb]_0$ were 1.5 mM and 1 μM, respectively, in 50 mM phosphate buffer, pH 6

To attach the maximum amount of protein to the solid support, different experiments were performed at various initial metHb concentrations, with a fixed amount of Eupergit® CM. The free protein concentration was measured at different times from the start of the reaction until a constant value was obtained, which indicates that the system has attained the equilibrium. After 48 h on average, the percentage of immobilized protein was not significantly increased, so this time value was chosen to stop the immobilization process.

The data obtained are shown in Table 3. The maximum specific activity was found using an initial metHb concentration of 129.3 μM, so this concentration was used for further experiments.

3.4. Time course of the oxidative process with the immobilized protein

Fig. 4 offers a selection of the kinetic progress curves obtained during the biocatalytic oxidation of 4CP with the immobilized protein at different initial H_2O_2 (Fig. 4a) and 4CP concentrations (Fig. 4b).

Table 3. Influence of the initial metHb concentration on the immobilization process and specific activity

$[metHb]_0$ (μM)	$[metHb]_{48h}$ soluble (μM)	metHb immobilized (%)	Specific activity (U/mg)
15.5	14.3	7.7	1.10×10^{-4}
18.7	16.0	14.4	1.14×10^{-4}
55.2	44.3	19.7	1.29×10^{-4}
129.3	91.6	29.1	1.81×10^{-4}
144.2	108.1	25.1	1.55×10^{-4}

The experimental conditions were: 50 mg of Eupergit® CM and 5 mL of metHb solutions at different concentrations (pH 7). The rest of experimental conditions as indicated in "Experimental" section. One unit of peroxidase-like activity was defined as the amount of immobilized protein able to oxidize 1 μmol of ABTS per min under these assay conditions

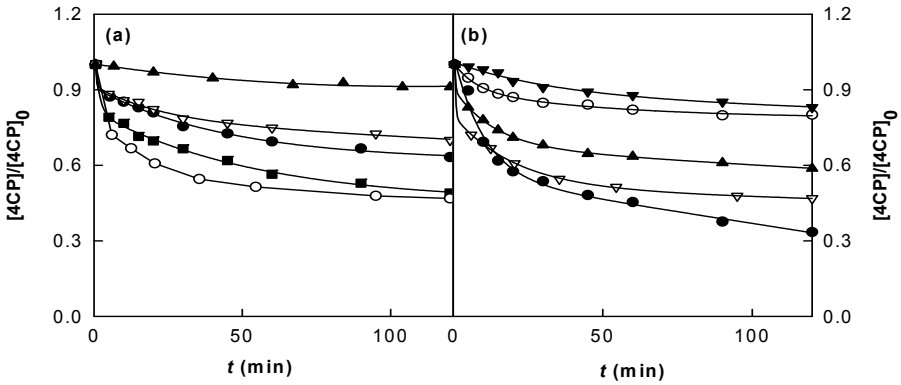

Fig. 4. (a) Time course for 4CP oxidation using metHb-Eupergit® CM at different initial H_2O_2 concentrations: $[H_2O_2]_0$ = 0 (▲), 0.5 (▽), 1 (●), 2.5 (○) and 5 (■) mM. $[4CP]_0$ and [metHb-Eupergit® CM]$_0$ were 0.25 mM and 10 mg/mL, respectively, in 50 mM phosphate buffer, pH 7.0. (b) Time course for 4CP oxidation at different initial 4CP concentrations: $[4CP]_0$ = 2 (▼), 1 (○), 0.5 (▲), 0.25 (▽) and 0.1 (●) mM. $[H_2O_2]_0$ and [metHb-Eupergit® CM]$_0$ were 2.5 mM and 10 mg/mL, respectively, in 50 mM phosphate buffer, pH 7.0

The percentage of oxidized 4CP increased as $[H_2O_2]_0$ was increased at relatively low oxidizing agent concentrations (Fig. 4a). However with higher $[H_2O_2]_0$ values, the amount of substrate oxidized at the end of the reaction lowered, probably due to the H_2O_2-induced suicide inactivation of metHb, which occurs with different peroxidases (Arnao et al., 1990; González-Sánchez et al., 2011b; Valderrama et al., 2002). This means that the amount of H_2O_2 to be added must be optimized, or even that this agent can be gradually added to avoid biocatalyst inactivation. Under the experimental conditions herein used, the optimal H_2O_2 initial concentration was found to be 2.5 mM.

Regarding the initial substrate concentration, the effectiveness of the process improved as the $[4CP]_0$ decreased (Fig. 4b). Finally, the amount of 4CP remaining in the reaction medium was smaller as the amount of metHb-Eupergit® CM was increased (data not shown); e.g., an increment of 20 mg of metHb-Eupergit® CM in the medium led to a 20% increase in 4CP oxidation at the endpoint of the reaction.

3.4.1. pH-dependence of peroxidase-like activity for free and immobilized metHb

The effectiveness of 4CP oxidation catalyzed by metHb is a pH-dependent process. The optimum pH for the free protein was 7.5, whereas it was 7.0 for the metHb immobilized on Eupergit® CM (data not shown). This slight difference could be due to the different environment near the active site of the protein upon immobilization.

3.5. Reusability of immobilized metHb

One of the most important advantages of using immobilized enzymes on an industrial scale is the possibility of reusing them many times. The activity of metHb immobilized on Eupergit® CM was herein assayed in up to three cycles in order to check its reusability with substrate 4CP. In the first use, and under the experimental conditions herein used, the amount of 4CP oxidized at the end of the reaction was almost 65%, whereas it was 18% in the third use.

4. Conclusions

In summary, the results obtained herein reveal that bovine metHb can be used as a biocatalyst in wastewaters treatment because it is able to oxidize a vast variety of pollutants from different types of industry. The immobilization of the protein on Eupergit® CM is an easy process, so the yield is acceptable and allows its reuse. To optimize and ensure the process, more research that focuses on finding new technologies that protect the protein against the unfriendly environments generated mainly by the presence of H_2O_2, necessary for the biocatalytic process is required.

Acknowledgements

This work has been supported by Ministerio de Economía y Competitividad (Spain), Project No. BFU2013-44095-P.

The funding source has not been involved in: the study design; the data collection, analysis and interpretation; writing the report; in the decision to submit this article for publication.

References

Al-Kassim L., Taylor K.E., Nicell J.A., Bewtra J.K., Biswas N., (1994), Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxidase from *Coprinus macrorhizus* in batch reactors, *Journal of Chemical Technology and Biotechnology*, **61**, 179-182.

Arnao M.B., Acosta M., del Río J.A., Varón R., García-Cánovas F., (1990), A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide, *Biochimica et Biophysica Acta,* **1041**, 43-47.

Borra´s C., Berzoy C., Mostany J., Scharifker B.R., (2006), Oxidation of *p*-methoxyphenol on SnO_2-Sb_2O_5 electrodes: Effects of electrode potential and concentration on the mineralization efficiency, *Journal of Applied Electrochemistry*, **36**, 433-439.

Cailean D., Teodosin C., Urgureany F., (2013), Engineering challenges in advanced wastewater treatment, *Environmental Engineering and Management Journal*, **12**, 1541-1551.

Childs R.E., Bardsley W.G., (1975), The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen, *Biochemical Journal,* **145,** 93-103.

D'Annibale A., Stazi S.R., Vinciguerra V., Sermanni G.G., (2000), Oxirane-immobilized *Lentinula edades* laccase: stability and phenolics renoval efficiency in olive mill wastewater, *Journal of Biotechnology,* **77**, 265-273.

González-Sánchez M.I., Manjabacas M.C., García-Carmona F., Valero E., (2009), Mechanism of acetaminophen oxidation by the peroxidase-like activity of methemoglobin, *Chemical Research in Toxicology*, **22**, 1841-1850.

González-Sánchez M.I., García-Carmona F., Maciá H., Valero E., (2011a), Catalase-like activity of human methemoglobin: A kinetic and mechanistic study, *Archives in Biochemistry and Biophysics,* **516**, 10-20.

González-Sánchez M.I., Rubio-Retama J., López-Cabarcos E., Valero E., (2011b), Develpment of an acetaminophen amperometric biosensor based on peroxidase entrapped in polyacrylamide microgels, *Biosensors & Bioelectronics*, **26,** 1883-1889.

Ikehata K., Nicell J.A., (2000), Characterization of tyrosinase for the treatment of aqueous phenols, *Bioresource Technology,* **74**, 191-199.

Katal R., Zare H., Rastegar S.O., Mavaddat P., Darzi G.N., (2014), Removal of dye and chemical oxygen demand reduction from textile industrial wastewater using hybrid bioreactors, *Environmental Engineering and Management Journal*, **13**, 43-50.

Katchalski-Katzir E., Kraemer D.M., (2000), Eupergit® C, a carrier for immobilization of enzymes of industrial potential, *Journal of Molecular Catalysis B: Enzymatic,* **10**, 157-176.

Kiljunen E., Kanerva L.T., (1996), (*R*)- and (*S*)-cyanohydrins using oxynitrilases in whole cells, *Tetrahedron: Asymmetry*, **7**, 1105-1116.

Klibanov A.M., Tu T., Scott K.P., (1983), Peroxidase-catalyzed removal of phenols from coal-conversion wastewaters, *Science,* **221**, 259-261.

Knezevic-Jugovic Z.D., Bezbradica D.I., Mijin D.Z., Antoy M.G., (2011), The immobilization of enzyme on Eupergit supports by covalent attachment, *Methods in Molecular Biology*, **679**, 99-111.

Laveille P., Galarneau A., Drone J., Fajula F., Bailly C., Pulvin S., Thomas D., (2009), Catalase-like activity of bovine met-hemoglobin: Interaction with the pseudo-catalytic peroxidation of anthracene traces in aqueous medium, *Biotechnology Journal*, **4**, 1460-1470.

Lee L.C., Lee Y.L., Leu R.J., Shaw J.F., (2006), Functional role of catalytic triad and oxyanion hole-forming residues on enzyme activity of *Escherichia coli* thioesterase l/protease l/phospholipase L_1, *Biochemical Journal*, **397**, 69-76.

Liu J., Guan J., Lu M., Kan Q., Li Z., (2012), Hemoglobin immobilized with modified "fish-in-net" approach for the catalytic removal of aniline, *Journal of Hazardous Materials,* **217-218,** 156-163.

Miland E., Smyth M.R., Fágáin C., (1996), Phenol removal by modified peroxidases, *Journal of Chemistry Technology & Biotechnology*, **67**, 227-236.

Nelson D.P, Kiesow L.A., (1972), Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV), *Analytical Biochemistry*, **49**, 474-478.

Nicell J.A., Bewtra J.K., Biswas N., Taylor E., (1993), Reactor development for peroxidase catalyzed polymerization and precipitation of phenols from wastewater, *Water Research,* **27,** 1629-1639.

Nowlan C., Li Y., Hermann J.C., Evans T., Carpenter J., Ghanem E., Shoichet B.K., Raushel F.M., (2006), Resolution of chiral phosphate, phosphonate, and phosphinate esters by an enantioselective enzyme library, *Journal of American Chemical Society,* **128,** 15892-15902.

Pavlopoulos T.G., (1992), Triplet extinction coefficients of some organic compounds, *Journal of Applied Physics*, **72**, 845-848.

Pérez-Prior M.T., Gómez-Bombarelli R., González-Sánchez M.I., Valero E., (2012), Biocatalytic oxidation of phenolic compounds by bovine methemoglobin in the presence of H_2O_2. Quantitative structure-activity relationships, *Journal of Hazardous Materials*, **241-242**, 207-215.

Prampano L., Stüber F., Font J., Fortuny A., Fabregat A., Bengoa C., (2010), Immobilisation of horseradish peroxidase on Eupergit C for the enzymatic elimination of phenol, *Journal of Hazardous Materials*, **177**, 990-1000.

Ruane P.H., McClelland R.A., Hegarty A.F., Steenken S., (1999), Photochemical generation of the N-isopropylbenzonitrilium ion and unusually slow trapping by azide ion, *Journal of the Chemical Society, Perkin Transactions*, **2**, 43-48.

Shorr R.G.L., Nho K., Cho M.P., Lee C., Czuba B., Shankar H., (1993), Process for hemoglobin extraction and purification. US Patent, No. *5264555*.

Steiner N., Gec R., (1992), Plant experience using hydrogen peroxide for enhanced fat flotation and bod removal, *Environmental Progress,* **11,** 261-264.

Turkova J., Blaha K., Malaninkova M., Vancurova D., Svec F., Kalal J., (1978), Methacrylate gels with epoxide groups as supports for immobilization of enzymes in pH range 3-12, *Biochimica et Biophysica Acta*, **524**, 162-169.

Unruh L.M., Xu R., Kramer K.J., (1998), Benzoquinone levels as a function of age and gender of the red flour beetle, *Tribolium castaneum*, *Insect Biochemistry and Molecular Biology*, **28,** 969-977.

Valderrama B., Ayala M., Vazquez-Duhalt R., (2002), Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes, *Chemistry & Biology,* **9,** 555-565.

Walton H.F., Reyes J., (1983), *Modern Chemical and Instrumental Analysis* (in Spanish: *Análisis químico e instrumental moderno)*, Reverté, Barcelona, Spain.

Zijlstra W.G., Buursma A., (1997), Spectrophotometry of hemoglobin: absorption spectra of bovine oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, and methemoglobin, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **118**, 743-749.