Environmental Technology & Innovation 30 (2023) 103053

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Biohybrid membranes for organophosphate pesticides degradation: Hyperactivation of immobilized phosphotriesterase by surfactants

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ARTICLE INFO

Article history:
Received 1 December 2022
Received in revised form 3 February 2023
Accepted 5 February 2023
Available online 10 February 2023

Keywords:
Phosphotriesterase
Organophosphates
Pesticides
Biocatalytic membranes
Immobilized enzymes
Regenerated cellulose membrane
Paraoxon

ABSTRACT

The aim of this work is to develop a hyperactivated biocatalytic membrane (BM) for the degradation of organophosphate pesticides (OPs), combining an enzyme-loaded membrane with cationic (cetyl trimethyl ammonium bromide, CTAB) or anionic (sodium dodecyl sulfate, SDS) surfactants. To analyze the effect of surfactants on the catalytic performance of BM, a systematic study of enzyme activity, stability, change in the secondary structure, protein aggregate formation, and kinetic parameters (K_M, K_{cat}, K_{cat}/K_M) was performed. The results showed that the activity of the immobilized phosphotriesterase toward the pesticide paraoxon was greatly enhanced in the presence of SDS and CTAB (90% and 80%, respectively). Circular dichroism, dynamic light scattering, electrophoresis, and kinetics studies made it clear that surfactants affect catalytic performance by either changing the secondary structure and aggregation state of the enzyme (CTAB) or increasing the affinity of the enzyme for the substrate (SDS). Despite the strong improvement in free enzyme activity (2.6 μmol $min^{-1}mg^{-1}$ versus 4.9 and 4.6 μmol $min^{-1}mg^{-1}$ with SDS and CTAB, respectively), a smaller enhancement effect was observed with the immobilized enzyme, which may be attributed to the increased stiffness of the enzyme due to immobilization.

Nevertheless, stable BMs with doubled specific activity were prepared that remained almost constant across various reaction cycles. Moreover, tests in a biocatalytic membrane reactor showed a 96% conversion at one-third of the residence time compared to the literature. The proposed strategy is a real advance in improving the activity/stability of BMs, which is one of the main drawbacks of this technology.

1. Introduction

Contamination of water sources and soils by micropollutants such as pesticides, herbicides, hormones, perfluorinated alkylated substances (PFAS), dyes, human and veterinary drugs, raises serious health concerns due to their toxicity and persistence in the environment (Rasheed et al., 2019). In addition, the potential health and environmental risks resulting from lifetime exposure to the coexistence of these xenobiotics are virtually unknown and difficult to assess, and new technologies to degrade these compounds are urgently needed. Current OP detoxification methods can be divided into physical, chemical, and biobased methods. Physical methods include the use of absorbent compounds (Saleh et al., 2020;

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https://doi.org/10.1016/j.eti.2023.103053

Iwuozor et al., 2022) or surfactant-containing lotions (Magnano et al., 2021; Fentabil et al., 2020). These methods are used to mitigate contamination of aqueous environments and rapidly remove OPs from the skin of contaminated individuals or from other specific sites. However, OPs are not destroyed by these methods, so disposal of used adsorbents or used lotions is a concern. Chemical methods are most commonly used, but the harsh conditions and expensive reagents required limit the use of these methods on a large scale for environmental remediation. In light of this, biotechnological systems that use biomolecules to degrade micropollutants have attracted considerable attention (Štefanac et al., 2021; Hara and Singh, 2021) because they can selectively and more sustainably hydrolyze hazardous compounds (Mazzei et al., 2021; Xu et al., 2021; Goh et al., 2022). In addition, it has recently been reported that the use of a bio-based strategy for bioremediation of pesticides can significantly reduce the cost compared to chemical and physical treatment (about 60%–80% less), which offers obvious advantages in the development of large-scale processes (Sarker et al., 2021).

Phosphotriesterase (EC 3.1.8.1) is an enzyme that catalyzes the hydrolysis of various organophosphate compounds (OPs) (Latip et al., 2019; Adeyinka and Pierre, 2019; Zdarta et al., 2022), and it has been studied to develop either decontamination or detection systems (Prokop et al., 2018; Vitola et al., 2021; Mohd Razib et al., 2021). However, when these enzymes are used in free form, they are unstable at high temperatures and pH variations, show low catalytic activity and stability, and are easily inactivated by solvents and protease (Mazzei et al., 2021).

To overcome these problems, thermophilic phosphotriesterases are more frequently used, which are more stable to environmental conditions (Manco et al., 2018; Xu et al., 2021) and, when immobilized on insoluble supports, allow overcoming the above limitations, such as avoiding contamination, reducting deactivation, and easy recovery and reuse of the biocatalyst (Gebreyohannes et al., 2018; Sharifi et al., 2018). Among the various supports for phosphotriesterase immobilization, synthetic membranes are very promising as they offer both a large surface area and porosity and a wide range of materials (Ranieri et al., 2018; Mazzei et al., 2021). Moreover, they are available in many different conformations, can improve the detection capability of a device by concentrating the analyte (Gentili et al., 2018; Fan et al., 2020), and/or can be integrated into continuous bioremediation systems (Vitola et al., 2019a; Zhou et al., 2018). Although previous work (Vitola et al., 2019a,b, 2021) has demonstrated the specificity of phosphotriesterase for the complete degradation of organophosphate pesticides and the ability of enzyme-loaded membranes to function as continuous biocatalytic membrane reactors, the loss of activity of the immobilized enzyme and the high residence time required for complete conversion of the pesticide remain critical issues to be addressed. Other problems with the above systems are closely related to the problems of enzyme stability during long operating processes, which must be overcome to realize biohybrid membrane systems on a large scale (Malakootian et al., 2020). Emerging strategies are now based on integrating enzymes with solvent engineering or genetic modification (Manco et al., 2018; Holmberg, 2018). Solvent engineering methods are increasingly using surfactants to alter the medium around the enzyme and create a microenvironment that enhances its performance (Duff et al., 2018).

In particular, these molecules contain a polar moiety (ionic or nonionic) and a hydrophobic alkyl chain, and therefore they can modulate the hydrophobic/electrostatic interactions of some enzymes and promote activation and/or stabilization (Liu et al., 2018). Although several literature works show that some free forms of hydrolytic enzymes can be hyperactivated by surfactants (De Oliveira et al., 2018; Holmberg, 2018; Suzumoto et al., 2020), the positive effect is a phenomenon that has not been fully described and, to our knowledge, has never been studied for immobilized enzymes (Bandforuzi and Hadjmohammadi, 2019).

In order to develop a high-performance and more stable biocatalytic membrane for the degradation of organophosphorus pesticides, the effect of two types of surfactants, SDS (negatively charged) and CTAB (positively charged) on a thermophilic phosphotriesterase immobilized on the membrane was investigated. The aim is to produce a more stable and hyperactivated biocatalytic membrane, since the loss of activity during the long-term decontamination process and the low enzyme stability are the main drawbacks of these systems. Moreover, the main effects of surfactants on the enzyme were clarified by evaluating the enzyme activity and stability and determining the kinetic parameters of free and immobilized phosphotriesterase.

2. Materials and methods

2.1. Chemicals

Ultrafiltration membranes of regenerated cellulose (MWCO 100 kDa) were purchased from Merck Millipore (Germany). Phosphotriesterase enzyme was produced from *Sulfolobus solfataricus* (Detoxizymes srl, Italy) (Vitola et al., 2021). Phosphotriesterase concentration was measured with the BCA kit (Thermo Fisher Scientific, USA) using bovine serum albumin (BSA) as a reference standard. HEPES and 2-amino-2-(hydroxymethyl)-1,3-propanediol (trizma), sodium periodate, ethylenediamine, glutaraldehyde, diethyl 4-nitrophenyl phosphate (paraoxon), 4-nitrophenol, SDS, and CTAB were purchased from Sigma-Aldrich.

2.2. Assay of phosphotriesterase activity

Activity assays of free and membrane-bound phosphotriesterase were performed in batch (25 °C) using a UV/vis spectrophotometer (Perkin Elmer Lambda EZ) to measure the absorbance of the product (p-nitrophenol) at 405 nm during

hydrolysis of the substrate (paraoxon). To test the activity of the free enzyme, 10 μL of the phosphotriesterase solution (0.1 mg mL $^{-1}$) were added to1 mL of the assay mixture consisting of paraoxon 1 mM in Tris/HCl 20 mM pH 8.5. To test the activity of immobilized phosphotriesterase, the biohybrid membrane (4.34 cm^2) was added to the assay mixture (10 mL). The reaction rate (mM min^{-1}) was calculated from the slope of the plot p-nitrophenol concentration (mM) versus time (min), while the phosphotriesterase specific activity (μmol min^{-1} $mg_{Enz}{}^{-1}$) was determined from the slope of the plot p-nitrophenol mass (μmol) versus time (min) normalized to the amount of biocatalyst (mg). 15 837 M^{-1} cm^{-1} was the molar absorption coefficient used for quantification of p-nitrophenol. The experiments were repeated at least three times. Results were expressed as the mean (± standard deviation) calculated from the number of different replicates.

2.3. Influence of surfactant concentration on phosphotriesterase activity and kinetic parameters

The specific activity of free and immobilized phosphotriesterase was studied with different surfactant concentrations, ranging from 2 × 10^{-4} to 1 mM for CTAB and from 0.25 to 1.5 mM for SDS. Kinetic parameters were calculated using the Michaelis–Menten kinetic equation (1):

$$V = \frac{V_{max}[S]}{K_M + [S]} \tag{1}$$

where V is the reaction rate (mM s^{-1}), [S] is the substrate concentration (mM), V_{max} is the maximum reaction rate (mM s^{-1}), and K_M is the Michaelis–Menten constant (mM). The catalytic constant or turnover number (K_{cat}) was calculated according to the following Eq. (2):

$$K_{cat} = \frac{V_{max}}{[E]_{Tot}} \tag{2}$$

Here, K_{cat} (s^{-1}) is given by the ratio between V_{max} (mM s^{-1}) and the total enzyme concentration $[E]_{Tot}$ (mM).

2.4. Membrane functionalization procedure

The procedure developed in Militano et al. (2016) was used to functionalize the membrane for enzyme immobilization. A regenerated cellulose membrane (4.34 cm^2) was treated in the dark for 7 h with an aqueous solution of sodium periodate (0.2 wt%) to oxidize hydroxyl groups to aldehyde groups. Then, the oxidized membrane was treated with an aqueous solution of ethylenediamine (5 wt%, used as a spacer) for 15 h. The membrane was then washed with water and treated with an aqueous solution of 5 wt% of glutaraldehyde for 2 h to graft aldehyde groups. All functionalization steps were carried out at 25 °C with gentle stirring.

2.5. Phosphotriesterase immobilization on functionalized membrane

To prepare the phosphotriesterase solution, the protein powder was dissolved in HEPES buffer 20 mM, pH 8.5, in the presence of $CoCl_2$ (0.2 mM). To prepare biocatalytic membranes, covalent immobilization of phosphotriesterase on a functionalized membrane of regenerated cellulose was performed using two different protocols. In the first procedure, the functionalized membrane was soaked in the enzyme solution (3 mL, 0.1 mg mL^{-1}) at 25 °C for 2 h with gentle stirring.

In the second procedure, CTAB (2 mM) was added to the enzyme solution and then incubated with the membrane for 2 h (25 °C) with gentle stirring. Afterwards, the biocatalytic membrane was rinsed with distilled water in both procedures to remove non-covalently bound enzyme.

The amount of immobilized phosphotriesterase was calculated using the mass balance equation (Eq. (3)), which is presented below:

$$m = (C_i \times V_i) - (C_f \times V_f) - \sum (C_{ws} \times V_{ws}) \tag{3}$$

Here, m is the mass of the immobilized protein, while C and V denote the concentration and volume, respectively; the subscripts i, f and ws represents the initial, final, and washing solutions, respectively.

The stability of biocatalytic membranes was investigated using two different approaches (Fig. 1S, Supporting Information). In the first method, the biocatalytic membrane prepared in the presence of CTAB (2 mM) was immersed in a new paraoxon solution (1 mM) for each reaction cycle. In the second approach, the membrane prepared with the first immobilization strategy (without emulsifier) was immersed in an aqueous solution containing CTAB or SDS (2 mM) between the different reaction cycles.

Preliminary experiments with the hyperactivated membranes in a biocatalytic membrane reactor were performed using the system developed by Vitola et al. (2019b). In the mentioned system the feed solution is represented by a mixture of paraoxon (1 mM) and surfactants (2 mM). The applied transmembrane pressure was 0.07 bar, while the flow rate through the hyperactivated biocatalytic membrane was 0.8 ml/min, corresponding to a residence time of 0.3 min.

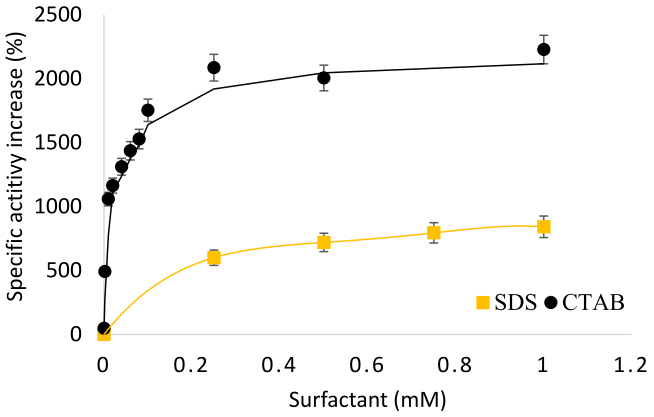

Fig. 1. Increase in phosphotriesterase specific activity (%) as a function of the increase in surfactant concentration. Each data point represents the mean and standard deviation of at least three experiments.

2.6. Characterization of phosphotriesterase in the presence of surfactants

The effect of surfactants on the aggregation and aggregate size of phosphotriesterase in solution was studied by both dynamic light scattering (Zetasizer Nano Series Nano ZS, Malvern instruments) and electrophoresis. Native PAGE electrophoresis was performed in a Mini-Cell system (XCell Sure™ Mini-cell, Invitrogen). Imperial™ Protein Stain (Thermo Scientific) was used to stain the polyacrylamide gel (3%–12%), while NativeMark Unstained Protein Standard (Novex) was used as the gel run standard.

The surface charge of the membrane was measured with the Surpass Electro-kinetic Analyzer (Anton-Paar) at 25 °C and pH from 2 to 9 using a potassium chloride solution (5 mM). The free phosphotriesterase charge in water as a function of surfactant concentration was analyzed using the Zetasizer Nano Series Nano ZS instrument (Malvern Instruments). The zeta potential of phosphotriesterase (without surfactants) was also measured as a function of pH in water and buffers containing 50 mM phosphoric acid, citric acid, potassium dihydrogen phosphate, Tris/HCl, and sodium carbonate in the pH range of 2.5 to 11.0.

The conformational changes of the enzyme were analyzed by circular dichroism (CD) measurements (Jasco J-1500). Spectra were recorded using a spectrometer equipped with a PM-539 detector and a Jasco PTC-510 Cell holder Peltier thermostat. Spectra were recorded at 25 °C using a quartz cuvette (Hellma high precision cell) with a path length of 1 mm from 185 to 260 nm at a scanning speed of 50 nm/min in continuous mode. A data integration time of 2 s, a bandwidth of 1 nm, a sensitivity of 20 mdeg, and a data pitch of 0.2 nm were used. The phosphotriesterase concentration was 0.1 g L^{-1} in 1 mM Tris/HCl buffer pH 8.5. Surfactants were added to the enzymatic solution at the concentration that produced the highest increase in activity. The recorded spectra were the average of three scans subtracted from the buffer spectrum and elaborated using Jasco Spectra Manager (version 2.15.18.1).

3. Results and discussion

3.1. Effect of surfactant concentration on free phosphotriesterase activity

Fig. 1 shows the specific activity of the free enzyme as a function of the increase in surfactant concentration. As can be seen, phosphotriesterase activity increased more than 2000% (i.e., approximately 22-fold) in the presence of the cationic surfactant CTAB (0.25 mM) compared with the enzyme solution without CTAB (specific activity 3.6 $\pm$ 0.3 μmol min^{-1} mg^{-1}). Further increase of CTAB concentration (up to 1 mM) did not increase the enzyme activity.

A much smaller improvement was obtained in the presence of the anionic surfactant SDS. The maximum increase in activity was about 800% (i.e., about 9-fold) and was obtained at a surfactant concentration of 0.75 mM. At concentrations above this value, phosphotriesterase activity remained constant. It is worth noting that the maximum enhancement effect for CTAB was achieved at concentrations slightly below its critical micellar concentration (CMC) (Ruiz-Morales and Romero-Martínez, 2018; Srivastava and Alam, 2020). At concentrations higher than the CMC, micelles can incorporate the substrate into their hydrophobic core, making it unavailable to the active site of the enzyme (Arca-Ramos et al., 2018).

The obtained results clearly show that among the surfactants studied, the cationic surfactant has the strongest effects on the enzyme specific activity. The main difference between CTAB and SDS was the charge of their head group, suggesting that the ionic nature of the surfactant is the most important parameter affecting the hyperactivation of phosphotriesterase. Interactions between enzymes and ionic surfactants can involve both electrostatic and hydrophobic interactions (Wong et al., 2018). In the first case, it is an interaction between the head group and charged amino acids of the enzyme, and in the second case, it is an interaction between the alkyl chain of the surfactant and the hydrophobic residues of the enzyme.

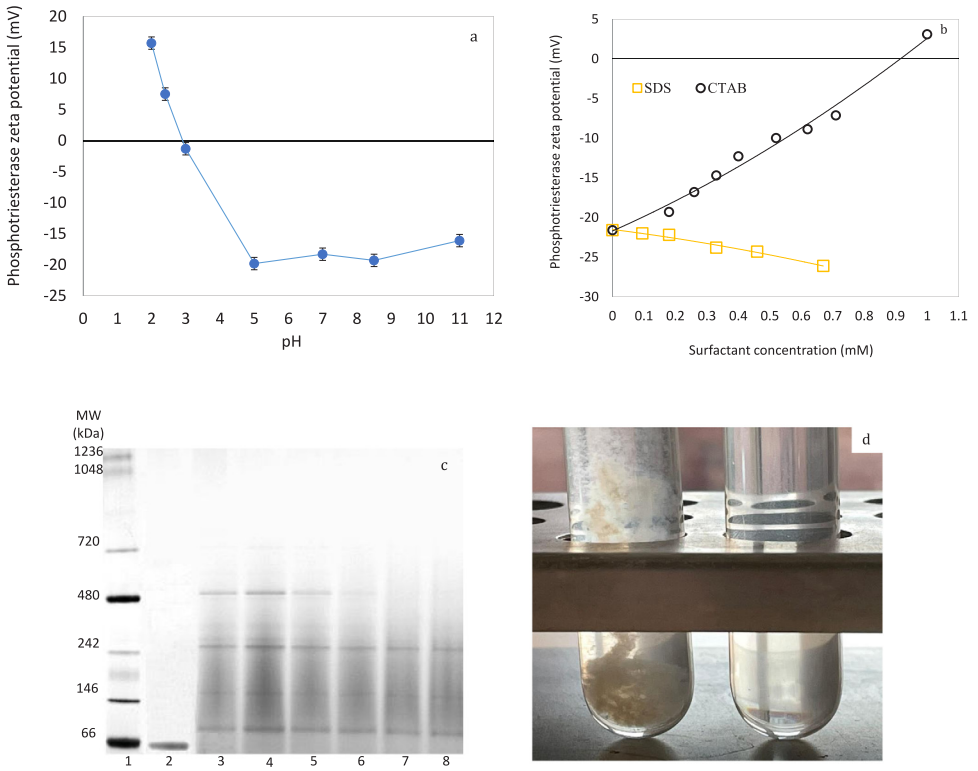

Fig. 2. Phosphotriesterase zeta potential measured at pH 8.5 (2a) and as a function of CTAB or SDS concentration (2b). Native electrophoresis (2c) of phosphotriesterase in the presence of an increasing amount of CTAB. 1: molecular weight (MW) marquer, 2: BSA (0.1 g/L), 3: phosphotriesterase (1 g/L), 4: phosphotriesterase (1 g/L) and CTAB (0.1 mM), 5: phosphotriesterase (1 g/L) and CTAB (0.3 mM), 6: phosphotriesterase (1 g/L) and CTAB (0.5 mM), 7: phosphotriesterase (1 g/L) and CTAB (0.7 mM), 8: phosphotriesterase (1 g/L) and CTAB (1 mM). Enzyme solution (1 g/L) with (left) and without (right) CTAB (1 mM) (Fig. 2d). Each data point represents the mean and standard deviation of at least three experiments.

In particular, the head group of cationic surfactants interacts with the negatively charged side chains of asparagine and glutamic acid, whose alkyl chains then bind to the hydrophobic zone near the electrostatic binding site. On the other hand, the head group of anionic surfactants can interact with the positively charged side chains of amino acids such as arginine, lysine and histidine. The pH of the solution also plays an important role in ionic interactions, as it affects the overall net surface charge of the enzyme. The isoelectric point of the phosphotriesterase used in this work was determined at a pH of about 3 (Fig. 2a). This means that at the working pH (8.5), the protein has a net negative surface charge (-19.3 ± 0.3 mV) and interacts strongly with the positively charged CTAB. Fig. 2b shows the zeta potential of the phosphotriesterase solution as a function of CTAB and SDS concentrations. As expected, a decrease in the zeta potential of the solution was observed with increasing CTAB concentration. The gradual neutralization of the negatively charged groups of phosphotriesterase with increasing surfactant concentration leads to the disappearance of the electrophoresis band near 500 kDa (Fig. 2c), which is caused by the precipitation of heaviest aggregates (Fig. 2d). In contrast, SDS only slightly decreased the zeta potential of the solution, with no obvious changes in protein aggregates, as described below.

The above results demonstrate the strong interaction between CTAB and phosphotriesterase and the concentration-dependent effects on enzyme loading and aggregation/precipitation. To further investigate the effect of surfactants on protein aggregate formation, DLS measurements were performed using enzyme solutions (1 g L^{-1}) with increasing concentration of surfactants. The enzyme with only buffer has a very broad size distribution and is in the form of aggregates (size of monomer 174 ± 14 nm). When CTAB was added to the enzyme solution, larger aggregates are present in the solution (Fig. 3), in good agreement with the native electrophoresis analysis (Fig. 2b), while SDS does not cause any relevant changes in protein aggregates (Fig. 3). Moreover, a precipitate formed in the solution when only CTAB was added (Fig. 2d).

Interactions between surfactants and enzymes can also lead to beneficial changes in enzyme conformation and produce hyperactivated enzymes (Holmberg, 2018). Circular Dichroism measurements were performed to verify the surfactant-induced conformational change of phosphotriesterase.

Far-UV CD measurements were performed in enzymatic solution in the presence of CTAB and SDS at concentrations of 0.25 and 0.75 mM, respectively, as the highest increase in activity was previously observed at these values (Fig. 1). The CD spectra of the enzymatic solution in the absence and presence of surfactants are shown in Supporting Information

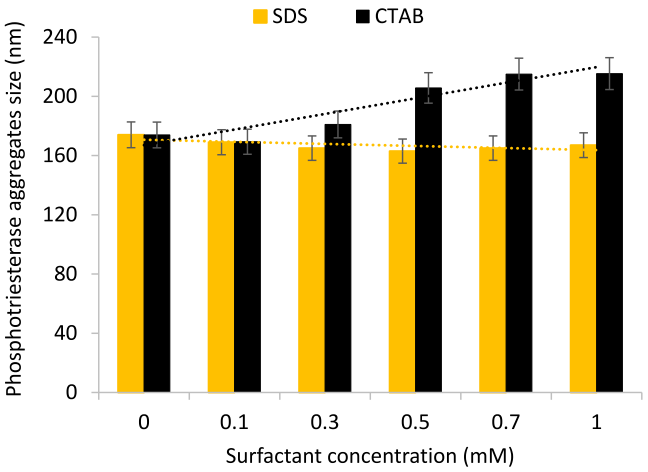

Fig. 3. Size distribution of phosphotriesterase as a function of surfactant concentration. Data represent the mean and standard deviation of at least three experimental series.

Table 1
Content of secondary structures of phosphotriesterase (PTE) in the absence and presence of surfactants.

Enzyme	Surfactant	α helix (%)	β sheet (%)	β turn (%)	Other
PTE	–	36.4	20.9	10.8	32.0
PTE	SDS (0.75 mM)	33.7	25.9	10.5	29.9
PTE	CTAB (0.25 mM)	37.4	6.0	16.8	39.7

(Fig. 2S). Analysis of the pure enzyme reveals a double minimum at 209 and 221 nm, which is characteristic of proteins with high α-helix content (Greenfield, 2006). The spectra (Fig. 2S) are similar to those in the presence of SDS, indicating that this surfactant did not alter the secondary structure of the enzyme, as also shown in Table 1, where the percentage of secondary structures is given. This trend is consistent with the literature, which reported that the effect of SDS on the activation of enzymes may involve a limited conformational change of the enzyme due to the binding of small amounts of the surfactant at a concentration often far below its CMC (Holmberg, 2018; Li et al., 2021). On the contrary, the presence of CTAB caused a decrease in β-sheet content and an increase in β-turn structure and random coil (Table 1). This effect could promote a rearrangement of the enzyme molecular architecture, leading, for example, to a better accessibility of the substrate in the active site.

The presence of surfactants can also increase the availability of the substrate and affect its distribution (Chen et al., 2018). However, they can also decrease the interactions between enzymes and products (Liu et al., 2018) or the binding energy between enzyme and substrate. To better understand the affinity of the enzyme for the substrate and the main constants affecting the reaction kinetics in the presence of surfactants, the parameters K_M, K_{cat}, K_{cat}/K_M of the free enzyme were determined in the absence and presence of surfactants (Table 2). The values of K_M indicate that SDS increases the affinity of phosphotriesterase for paraoxon; indeed, its value is significantly decreased in the presence of SDS compared to that of the enzyme in buffer alone. In contrast, the same K_M value is obtained when CTAB was used. The higher affinity of the enzyme for the substrate in the presence of SDS is probably due to the interaction of the aromatic groups of the organophosphorus pesticides with the hydrophobic chain of SDS. As recently explained in the literature for another enzyme (Bandforuzi and Hadjmohammadi, 2019), an adduct is formed that facilitates the accommodation of the ester groups of the substrate in the hydrophobic pockets of the active site of the enzyme.

On the other hand, the turnover number (K_{cat}) increased significantly (by more than 22-fold) in the presence of CTAB, whereas it increased significantly less (by more than 5-fold) in the presence of SDS. This indicates that the beneficial effect of CTAB is the faster release of the product from the substrate-enzyme activated complex. The higher turnover number is confirmed by the highest enzyme specific activity ($78.7 \pm 1.5 \mu mol\ min^{-1}\ mg^{-1}$) compared to that obtained in the absence ($3.6 \pm 0.3\,\mu mol\ min^{-1}\ mg^{-1}$) and in the presence of the other surfactant (Table 2).

Considering that SDS increases the affinity of the enzyme for the substrate (reduced K_M) and CTAB promotes easy product release (increased K_{cat}), they can be used selectively based on the different devices required. In particular, SDS may be better suited for OPs detection and biosensors development, while CTAB may be better used in biocatalytic devices to increase OPs conversion.

Nevertheless, very high catalytic efficiency (K_{cat}/K_M) was observed in the presence of both surfactants.

Table 2
Kinetic constants determined with the free enzyme in the presence and absence of various surfactants.

Reaction medium	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M (mM^{-1} s^{-1})	Specific activity (μmol min^{-1} mg^{-1})
Tris/HCl pH 8.5	0.48 ± 0.07	2.4 ± 0.1	5.0 ± 0.9	3.6 ± 0.3
Tris/HCl pH 8.5, SDS (0.75 mM)	0.08 ± 0.01	13.3 ± 0.9	166.0 ± 31.5	32.2 ± 1.3
Tris/HCl pH 8.5, CTAB (0.25 mM)	0.42 ± 0.11	53.6 ± 5.4	127.7 ± 31.9	78.7 ± 1.5

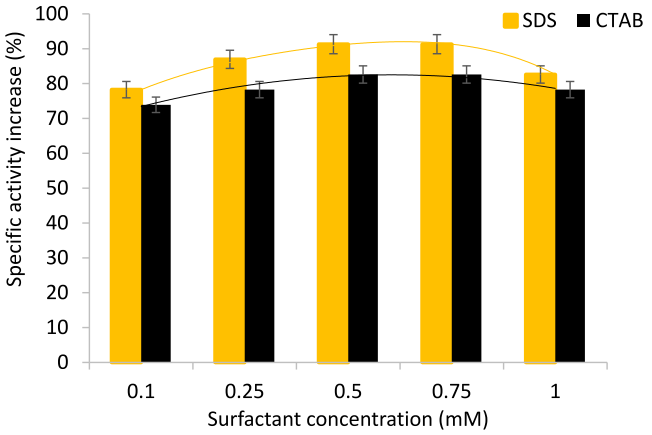

Fig. 4. Specific activity increase (%) of immobilized enzyme as a function of surfactant concentration. Data represent the mean and standard deviation of at least three experimental series.

3.2. Influence of surfactants concentration on biocatalytic membrane activity

The same surfactant concentrations previously tested for free phosphoriesterase, were then used to evaluate their effects on the activity of the immobilized enzyme. The amount of immobilized enzyme was 0.36 mg cm^{-3} and was determined at a contact time of 2 h between the enzyme solution and the functionalized membrane. The contact time was selected based on the highest amount of enzyme immobilized (Fig. 3S, Supporting Information) and the specific activity optimized in our previous work (Vitola et al., 2019b, 2021). Under the above conditions, the biohybrid membrane retained about 72% of its activity compared to the free enzyme.

Fig. 4 shows the percentage improvement in specific activity of the immobilized phosphotriesterase as a function of surfactant concentration. The highest specific activity was obtained at a surfactant concentration of 0.5 mM.

In comparison with the surfactant-free immobilized enzyme (specific activity 2.6 ± 0.2 μmol min^{-1} mg^{-1}), an increase in specific activity of more than 90% and 80% was observed in the presence of SDS and CTAB, respectively. In this case, the effect of CTAB is of the same order of magnitude as that of SDS.

Nevertheless, there is a major difference between surfactant-induced enhancement in specific activity of free and immobilized enzyme. In particular, the enhancement effect of CTAB and SDS on the immobilized enzyme is two and one orders of magnitude lower, respectively, than the enhancement effect obtained with the free enzyme. The lower activation of the immobilized phosphotriesterase is probably a consequence of the stiffening of the enzyme structure due to the formation of covalent bonds, which limit the effect of surfactants. This fact is of particular importance when CTAB is used because, as previously reported (Table 2), it leads to conformational changes of the free enzyme that are now blocked by the covalent immobilization process. Moreover, the concentration of surfactant in contact with the immobilized enzyme may be different from the concentration in the bulk reaction medium. indeed, the positively charged CTAB strongly interacts with the membrane, which is negatively charged (−12.5 mV) at the operating pH of 8.5 (Fig. 4S, Supporting Information). This reduces the interaction with the enzyme.

3.3. Kinetics of immobilized phosphotriesterase in the presence of surfactants

Table 3 lists the values of the kinetic parameters of immobilized phosphotriesterase. The K_M of immobilized phosphotriesterase with and without surfactants are quite similar, confirming that surfactants have no significant effect on the affinity of the immobilized enzyme for the substrate. Compared with K_M of free phosphotriesterase (0.48 ± 0.07 mM), K_M of bound phosphotriesterase was about 5-fold higher, indicating that immobilization has a negative effect on the interaction between substrate and enzyme. This effect was probably due to the reduction of substrate to the enzyme

Table 3
Kinetic parameters of immobilized phosphotriesterase with and without surfactant.

Reaction medium	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M (mM^{-1} s^{-1})	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)
Tris/HCl pH 8.5	3.08 ± 0.16	3.0 ± 0.1	1.0 ± 0.1	2.6 ± 0.2
Tris/HCl pH 8.5, SDS (0.75 mM)	2.99 ± 0.07	5.9 ± 0.5	2.0 ± 0.2	4.9 ± 0.2
Tris/HCl pH 8.5, CTAB (0.25 mM)	2.6 ± 0.18	4.6 ± 0.2	1.8 ± 0.2	4.6 ± 0.2

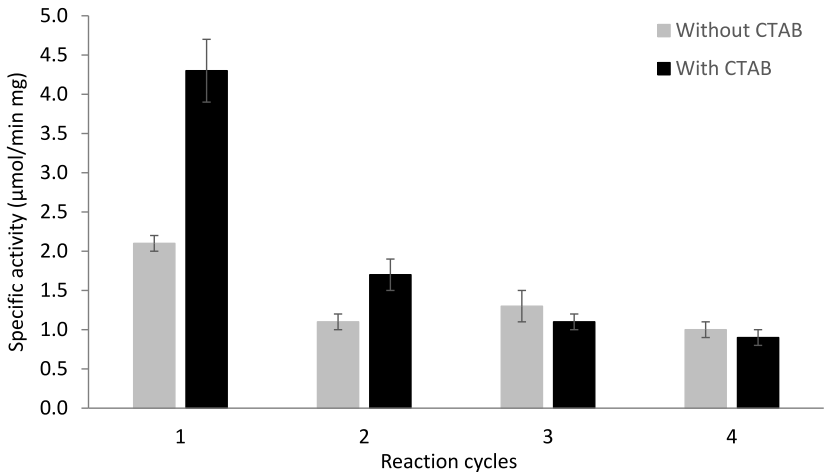

Fig. 5. Specific activity of phosphotriesterase immobilized in the presence of CTAB (0.5 Mm) Each data represents the mean and standard deviation of at least three experiments.

active site as a result of increased stiffening of the biomolecule, increased mass transfer limitations, and conformational changes in the enzyme structure (Grunwald, 2018). The positive effect of surfactants on the immobilized enzyme is in the turnover number and then in the specific activity; in fact, the presence of surfactants increased the K_{cat} values and thus the catalytic efficiency (K_{cat}/K_M). These results indicate that both surfactants can be used to increase the specific activity of immobilized phosphotriesterase in biocatalytic membranes.

The change in the secondary structure of the free enzyme induced by CTAB and the increased K_{cat} indicate that the effect of this surfactant is related to a structural change for better release of the product rather than to a higher affinity of the substrate for the enzyme active site, as previously assumed.

3.4. Stability of the hyperactivated phosphotriesterase membrane

The reusability of the hyperactivated biocatalytic membrane was tested by adding surfactants during the enzyme immobilization process or between the different reaction cycles, as described in the Materials and Methods section.

Fig. 5 shows the specific activity of the biocatalytic membrane upon addition of CTAB during the immobilization process. Application of this strategy resulted in an increase in specific activity compared to the control during the first two reaction cycles. Thereafter, a rapid decrease was observed over time, which was attributed to the continuous desorption of the surfactant from the immobilized enzyme. In contrast, when enzyme was stored in CTAB (or SDS) between cycles (Fig. 6), the specific activity doubled compared with the control without affecting the enzyme stability; in fact, the enzyme half-life time in the absence and presence of SDS and CTAB were 7, 18 and 15 days, respectively. This shows that the surfactants do not irreversibly bind the enzyme and that, in order to positively affect the enzyme activity, they must be "loaded" on the membrane during the different reaction cycles and act like a "co-factor".

Preliminary results of the hyperactivated enzyme-loaded membrane in a biocatalytic membrane reactor showed that compared to other biocatalytic membranes for OPs degradation (Vitola et al., 2019b), an increase in conversion of about 220% was achieved, with a lower residence time (0.30 min compared to 0.96 min).

Given the very high activity and conversion between the different reaction cycles, the membrane thus prepared could be used to develop a high-performance biocatalytic membrane reactor for the continuous degradation of organophosphate pesticides. In addition, this work provides important results and insights for further studies on other enzymatic systems to overcome one of the main drawbacks of enzyme immobilization, which generally increases stability but greatly decreases catalytic activity.

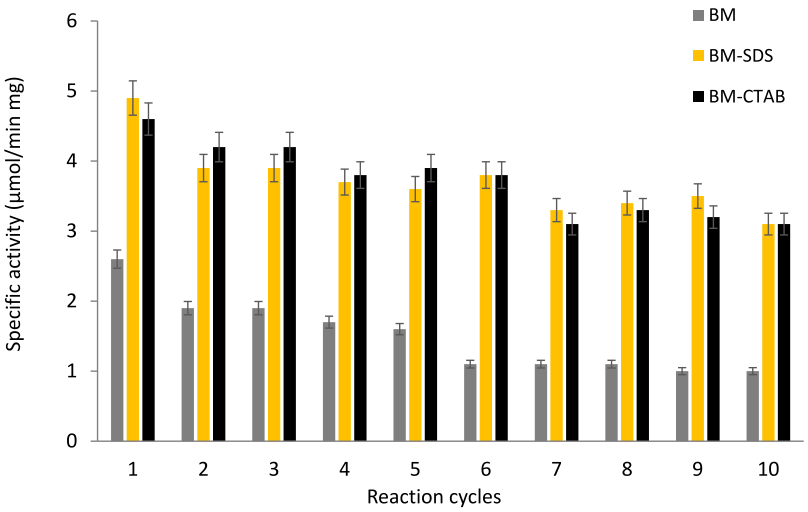

Fig. 6. Specific activity of biocatalytic membranes (BM) stored with and without CTAB (0.5 mM) and SDS (0.5 mM) between the different reaction cycles. Data represent the mean and standard deviation of at least three experimental series.

4. Conclusions

In this work, stable hyperactivated biocatalytic membranes were prepared in which the immobilized thermophilic phosphotriesterase hydrolyzed paraoxon with high efficiency.

The hyperactivated membranes were prepared by studying the enhancement mechanism resulting from the interaction of cationic (CTAB) and anionic (SDS) surfactants with the free and immobilized phosphotriesterase. The results showed that both surfactants significantly increased the activity of free phosphotriesterase, especially when the cationic surfactant was used. CTAB induces conformational changes in the secondary structure and aggregation state of the enzyme, while SDS promotes a higher enzyme affinity for the substrate ($K_{M\ \text{free enzyme with SDS}}$: 0.08 $\pm$ 0.01 mM, vs. $K_{M\ \text{free enzyme}}$: 0.48 $\pm$ 0.10 mM).

When the enzyme was immobilized on the membrane, a similar enhancement effect was obtained with both surfactants. With SDS and CTAB, an increase in specific activity of 90% and 80%, respectively, was obtained compared to the enzyme immobilized without surfactants. Despite the strong improvement in the enzyme activity of the free enzyme, a lower enhancement effect was observed in the immobilized system. This can be attributed to a lower interaction between the surfactant and the enzyme due to a higher "rigidity" of the immobilized enzyme. The lower increase is particularly evident in the case of CTAB, whose effect is triggered by a strong change in the secondary structure of the enzyme (that cannot fully occur due to immobilization).

Considering the increased K_{cat} (from 3.0 $\pm$ 0.1 to 4.6 $\pm$ 0.2 s^{-1}) of the immobilized enzyme in the presence of CTAB and the change in enzyme secondary structure (observed in the free enzyme), a structural change could nevertheless be promoted by CTAB in the immobilized system, allowing better release of the product.

To generate hyperactivated membrane systems for paraoxon degradation, the biocatalytic membranes must be "loaded/saturated" after each cycle with the ionic surfactants that act like cofactors. Under these conditions, the specific activity almost doubled and remained more constant in the different reaction cycles than for the immobilized enzyme in the absence of surfactants. Moreover, the use of the hyperactivated membrane in a biocatalytic membrane reactor allowed a paraoxon conversion of about 96%, requiring only one-third (0.3 min versus 0.9 min) of the residence time reported in the literature. The increase in immobilized enzyme activity and stability promoted by the proposed strategy is a real advance in both the development of a high-performance biocatalytic membrane reactor and in the degradation of OPs, and helps to overcome one of the main obstacles to the large-scale development of this technology.

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.

Acknowledgment

The authors would like to thank POR Calabria FESR-FSE 2014/2020 for the financial support to the SmartMatter project in the ERA.NET Call 2019

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2023.103053.

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