ERYTHROMYCIN DEGRADATION BY AN ESTERASE IN ENZYMATIC MEMBRANE REACTORS

Jose Sanchez Marcano, Institut Européen des Membranes, France
jose.sanchez-marcano@umontpellier.fr
Matthias de Cazes, Institut Européen des Membranes, France
Marie Pierre Belleville, Institut Européen des Membranes, France
Eddy Petit, Institut Européen des Membranes, France
Mathias Salomo, c-LEcta, Germany
Sally Bayer, c-LEcta, Germany
Rico Czaja, c-LEcta, Germany
Jean De Gunzburg, Da Volterra, France

Key Words: Enzymatic membrane reactor, esterase immobilization, erythromycin degradation, ceramic membrane, wastewater treatment.

1 Introduction

Pharmaceuticals products (PPs) and endocrine disrupting chemicals (EDCs) as well as their transformation products have been detected in almost all effluents from sewage facilities, in surface water, in groundwater, adsorbed on sediments and even in drinking water [1,2]. Ecotoxicity studies have demonstrated that pharmaceutical pollutants could affect the growth, reproduction and behavior of birds, fishes, invertebrates, plants and bacteria [3,4]. Some recently published studies report that the presence of low concentrations of antibiotics in the wastewaters may develop antibiotic resistance in the whole environment [5, 6]. As previously reported by Demarche et al. [7], the use of enzymes might be beneficial to enhance or complement conventional wastewater treatments. As far as enzymes are relatively expensive the reuse of the biocatalyst appears to be essential to ensure the economic and industrial viability of the process. Enzymatic membrane reactors appear to be an interesting alternative since they enable to couple reaction and separation [8]. In fact, in such enzymatic reactors, the substrate is continuously brought in contact with the biocatalyst, which is retained by the membrane, either freely circulating with the retentate or fixed on or within the membrane and the reaction products are recovered in the permeate.
This work describes the study of erythromycin degradation by an EreB esterase in free and immobilized forms. It focuses on the comparison between 3 different enzymatic membrane reactors for erythromycin degradation by esterase EreB. In the first configuration the free biocatalyst was confined in the reaction media by a ceramic membrane. In the two other cases, the enzyme was immobilized in the membrane either covalently grafted or adsorbed.

2 Experimental

2.1 Expression and purification of EreB esterase

The gene of EreB [9] which has been provided by Da Volterra has been cloned into c-LEcta's in-house production system under control of a T7 promoter. E. coli W3110 carrying the ereB overexpression plasmid was cultivated in a fed batch fermentation using mineral salt medium at 37°C. After induction of protein production the enzyme was extracted and purified from lysed cells. Total protein content of the purified EreB was 16.2 mg/mL.

2.2 Enzymatic membrane preparation

Two immobilization routes were investigated. In the first method the esterase was covalently grafted on ceramic support following the three steps procedure described by de Cazes et al. [10]. In the second immobilization method the esterase was adsorbed on the ceramic support. The porous supports were put into contact with a 5 $g.L^{-1}$ enzymatic solution prepared in 50 mM phosphate buffer (pH = 7) for 2 hours before being rinsed with the same buffer. Both membrane types were dried at room temperature under vacuum with P_2O_5 and the stored in a dessicator before being used.

2.3 Enzymatic membrane bioreactors

All the degradation experiments were carried out in the same device but when free enzymes were involved the enzymatic membrane was replaced by an ultrafiltration membrane (a 15 kDa three-channel ceramic membrane (membrane length of 25 cm, hydraulic diameter of 0.3 cm) supplied by Tami Industries). Most of the

experiments with free EreB were carried in continuous configuration; the recirculation loop (Fig 1a) was firstly filled with a 100 mg.L^{-1} erythromycin solution prepared in deionised water and mixed with free EreB in order to reach an enzyme concentration of 1 mg.L^{-1}. Then the reactor was continuously feed with a fresh erythromycin solution (100 mg.L^{-1}) to compensate the permeate extraction. Degradation experiments with active membranes were carried out at 25°C in batch configuration (Fig 1b): the permeate valve was closed and the retentate was recirculated at 10 L.h^{-1} in the feeding tank. Irrespective to the operating mode, samples of permeate and retentate were collected periodically and kept frozen until analysis.
The erythromycin content was analyzed by high-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (HPLC-MS/MS).

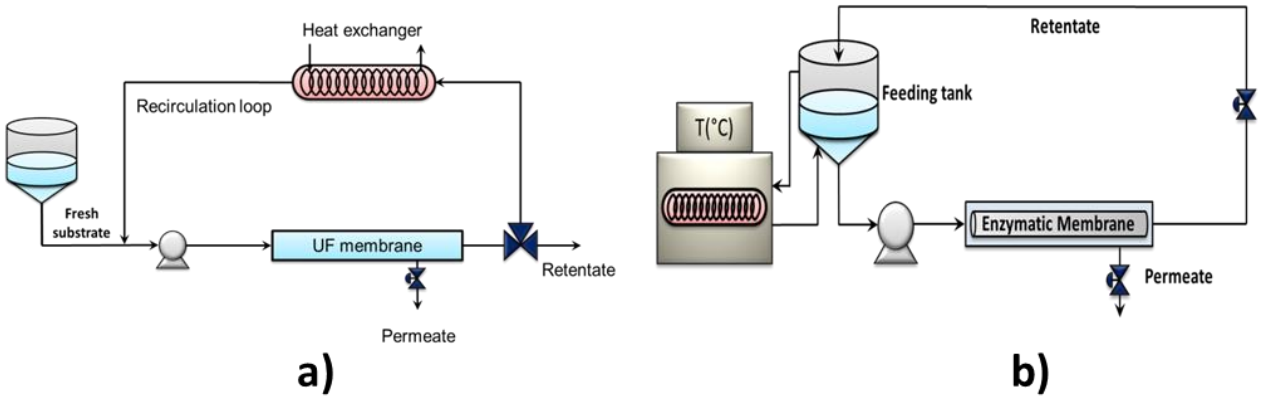

Figure 1. Enzymatic membrane reactors – (a) with free enzyme, (b) with enzymatic membrane

3 Results and discussion

3.1.1 EMR with free enzymes

Preliminary experiments had shown that free EreB showed higher activity than the immobilized derivatives, thus a first set of degradation experiments were carried out in a free enzyme membrane reactor. A 15kDa multichannel membrane was chosen since it was able to retain EreB within the recirculation loop with the reaction medium while the reaction products are continuously removed in the permeate. Firstly the reactor was operated in batch configuration. It was filled with an erythromycin solution (100 mg.L^{-1} in deionised water) which was recirculated at a flow rate of 20 L.h^{-1} while the permeate valve was kept closed. Then a solution of free enzymes was added to the reaction medium in order to reach a concentration of 1 mg.L^{-1}. After five minutes of reaction the conversion reached a constant value (i.e.; higher than 90%) which probably corresponds to the thermodynamic equilibrium. This experiment confirms the very high reactivity of EreB towards erythromycin.
In view to study the EreB stability, a second set of experiments was carried out in continuous configuration (see part 2.3). In this configuration, the reactor was continuously fed at the same level of permeate flow with a fresh substrate solution (100 mg.L^{-1} erythromycin in deionised water) while the retentate recirculation flow was set to 15 L.h^{-1} (i.e. tangential velocity: 0.15 m.s^{-1}). Two feeding flows were tested (3 and 0.3 L.h^{-1}) corresponding to hydraulic retention times of 6 and 60 minutes respectively. The results are presented on Figure 2.
Irrespective to the residence time, at the early first stage of the reaction the concentration of erythromycin is very low (< 10%). This result is in good agreement with experiments in batch conditions (not presented here) which showed that the reaction occurs as soon as enzymes are added. When the hydraulic retention time is low (Figure 2a), the concentration of antibiotic in the permeate remains negligible during 5 minutes. Afterward the concentration increases rapidly up to 80-90% of the initial concentration. The substrate accumulation in the system showed that the feed rate was higher than the degradation rate. This result is certainly due to the fact that the residence time was too short.
When the hydraulic retention time was higher (Figure 2b), the concentration of erythromycin in the permeate remains constant for 10 minutes; all the new substrate feed in the reactor is degraded. However after this period, like in previous experiment, the degradation rate decreased and an accumulation of erythromycin was observed. The rapid EreB inactivation is probably caused by shear stresses due to the recirculation rate. This problem is generally solved by periodic addition of biocatalysts. Nevertheless, because of the quick inactivation of the free EreB, the frequency of enzyme adding required to maintain a high level of degradation would lead to

huge additional costs.

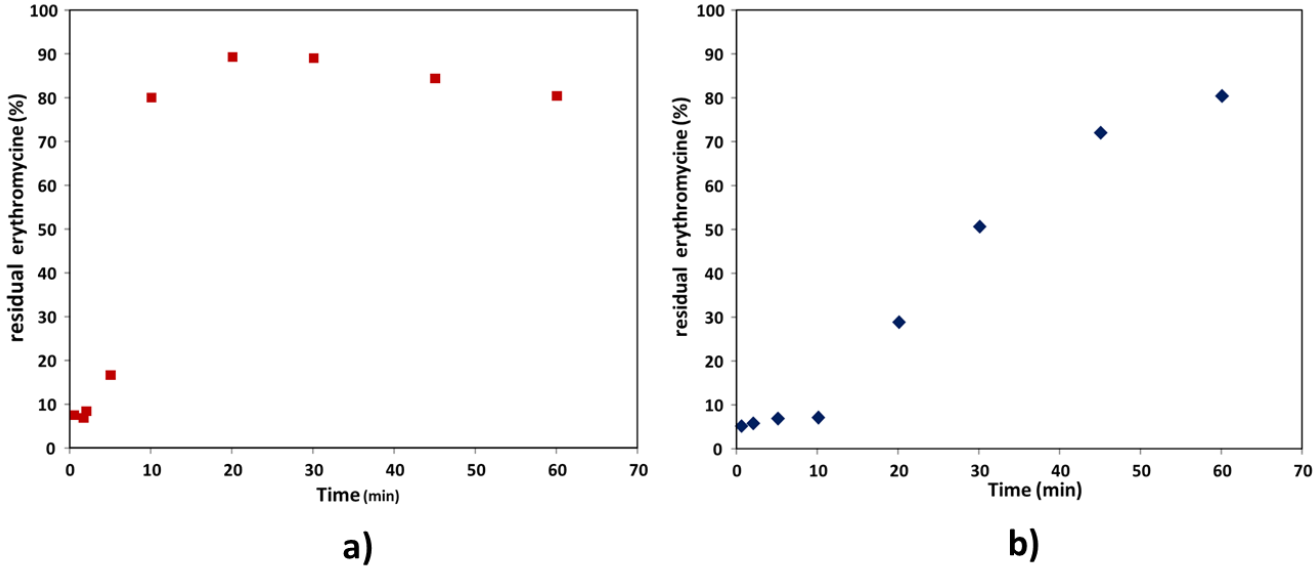

Figure 2. Degradation of erythromycin (100 mg.L^{-1}) by free EreB (1 mg.L^{-1}) in the enzymatic membrane reactor with the permeate valve open (continuous configuration); (a), hydraulic retention time = 6 min; (b) hydraulic retention time = 60 min.

3.1.2 EMR with active membranes

3.1.2.1 Performance and stability

The stability of both types of active membranes (covalent grafting or adsorption) was studied over several degradation cycles of erythromycin in the pilot unit. A same active membrane was used during 4 cycles of 24 hours in batch configuration: permeate valve closed and retentate recycled in the feed tank. It was checked that the membrane permeability was constant during the whole experiment (about 800 L.h^{-1}.m^{-2} under a transmembrane pressure of 5 kPa). The degradation products did not polymerize and their presence did not lead to membrane clogging.

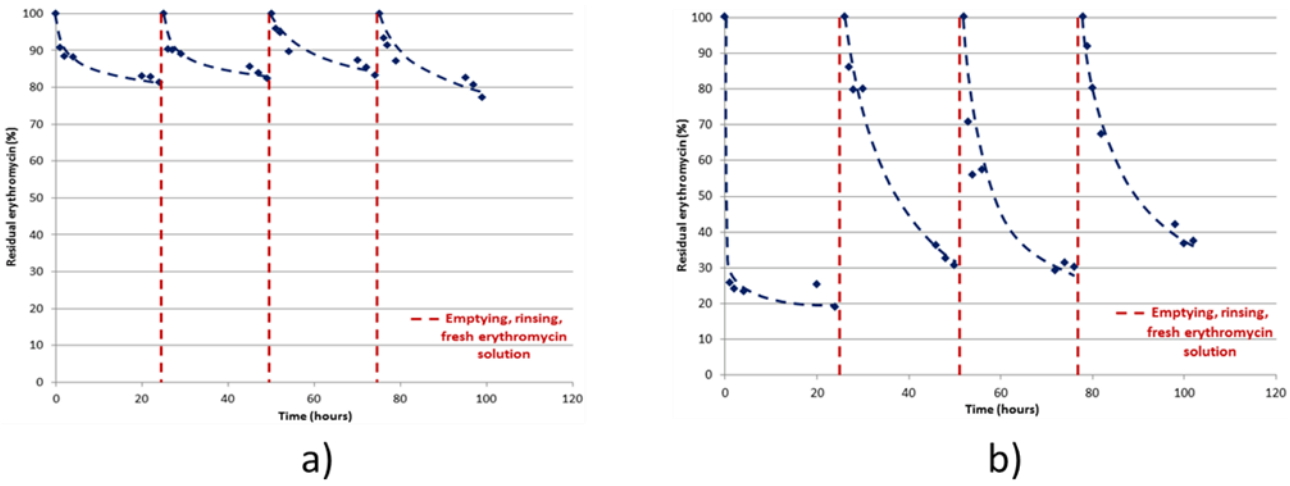

Figure 3. Erythromycin degradation (20 mg.L-1, de-ionised water) in the EMR on a multichannel membrane (Øpore = 1.4 µm, L = 25 cm) at 25°C. a) By EreB covalently grafted b) by EreB adsorbed.

The Figure 3 presents the evolution of residual erythromycin percentage over 4 cycles of degradation with both types of active membranes. As regard membrane prepared by covalent grafting (Figure 3a), the degradation of erythromycin was similar over each cycle. The enzyme activity was stable over 100 hours of reaction and the average degradation speed was equal to 1.19 $mg.h^{-1}$. As it was seen in previous studies [10], covalent grafting could protect the biocatalyst from potential leaching or conformational changes. Thus the active sites remained available to react with the substrate. Nevertheless the erythromycin depletion potential of this EMR is low; after 24h, only 20% of erythromycin was degraded. In the case of the active membrane obtained by adsorption (Figure 3b), an activity loss can be observed between the two first cycles. During the first cycle, the erythromycin concentration decreased rapidly (in less than 1 hour) to 21% of the initial concentration and then leveled off to 20% after 24 hours. In the second cycle, the degradation rate was slower; only 16% of degradation was observed after 1 hour of reaction. Nevertheless 70% of degradation could be achieved after 24h. The activity loss could be due to enzymes leaching because of the recirculation flow; nevertheless no degradation activity could be observed in permeate and retentate samples. In additional experiments 20 $mg.L^{-1}$ of an erythromycin fresh solution was added to samples of permeate and retentate and any enzymatic activity was observed. It is important to notice that after the first 24 hours, the reaction rate remained stable during the other cycles. Its average value over 24 hours was maintained at 15.8 $mg.h^{-1}$.

According to these results, adsorption seems more suited than covalent grafting for EreB immobilization. This type of active membranes was thus chosen in view to study the effect of operating parameters on the degradation of erythromycin in the EMR.

3.1.2.2 Impact of operating parameters

In order to optimize the process, degradation experiments were carried out varying some parameters such as erythromycin concentration, recirculation rate and permeate flow. The reactions were run during 2 hours at 25°C in the pilot unit used in batch configuration (i.e. both permeate and retentate were recycled in the feed tank); the mean specific degradation rates are reported in Table 1.

Erythromycin concentration ($mg.L^{-1}$)	Recirculating flow ($L.h^{-1}$)	Permeate flow ($L.h^{-1}$)	Specific degradation rate ($g.h^{-1}.m^{-2}$)
5	10	0	0.43
20			1.56
50			8.01
100			11.31
200			16.45
20	5	0	1.19
	10		1.56
	20		2.37
20	10	0	1.56
		0.3	2.15
		3	2.18
		8	2.40

Table 1. Impact of operating parameters on erythromycin degradation in the EMR with adsorbed EreB.

As expected, when the substrate concentration was increased, the average degradation rate was higher (Table 1). Indeed the reaction speed rose from 0.43 $g.h^{-1}.m^{-2}$ to 16.65 $g.h^{-1}.m^{-2}$ when the erythromycin concentration was increased from 5 $mg.L^{-1}$ to 200 $mg.L^{-1}$. The substrate concentration represents thus an important limiting factor. Moreover it is important to note that there was no reaction inhibition due to the presence of degradation products. According to Table 1, the recirculation flow is also a key parameter; the mean degradation rate is positively correlated with this parameter. This result was predictable since the enzymes were immobilized by adsorption on the membrane. Therefore the higher the recirculating flow was, the more often the substrate is renewed around the enzyme environment leading to an increase in degradation rate. At recirculating flow of 20 $L.h^{-1}$ (0.25 $m.s^{-1}$) the degradation rate was 2.5 fold higher than the one observed at 5 $L.h^{-1}$ (0.06 $m.s^{-1}$). However

increasing the recirculation flow is thus a proper solution to improve the performance of the reactor as far as the shear stresses induced by the fluid velocity does not lead inactivation or leaching of the enzyme adsorbed at the membrane surface as is currently the case with free enzymes. Finally results reported in Table 1 highlight the interest to implement an active membrane. When the substrate can flow through the membrane, the degradation rate increases and its value is greater as the flow is higher. This result confirms that a part of the enzymes were adsorbed not only on the membrane surface but also within the membrane pores. Each pore can thus be considered as a micro reactor where the contact between the substrate and the biocatalyst is improved leading to higher reaction rate.

4 Conclusions

In this work free and immobilized EreB esterase demonstrated their efficiency to degrade erythromycin at 25°C. The optimal pH and temperature values to degrade erythromycin were not modified after the immobilization step. Free esterase resulted to be more active but less stable than immobilized biocatalyst in a CSTR. Erythromycin degradation experiments with free esterase in an EMR resulted on a fast biocatalyst inactivation when circulated in the reactor. However experiments with immobilized erythromycin by adsorption and implementation in an EMR, show that even if EreB lost initially a part of its activity it was then stabilized and able to degrade continuously 15.8 $mg.h^{-1}$ of erythromycin during 100 hours. EMR with such active membrane appears to be suitable to treat concentrated wastewaters such as pharmaceutical effluents.

Acknowledgements

This project has received funding from the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement n°282818. This work is a collabo-ration between Da Volterra (France), c-LEcta GmbH (Germany), ChiralVision (Netherlands), European Membrane Institute (France), Catalan Institute for Water Research (Spain) and Goethe University (Ger-many).

References

[1] T. Heberer, Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data, Toxicology Letters, 131 (2002) 5-17.
[2] K. Kummerer, Antibiotics in the aquatic environment - A review - Part I, Chemosphere, 75 (2009) 417-434.
[3] D. Martinovic, W. T. Hogarth, R. E. Jones, P. W. Sorensen, Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows, Environmental Toxicology and Chemistry, 26 (2007) 271-278.
[4] N. Kemper, Veterinary antibiotics in the aquatic and terrestrial environment. Ecological Indicators, 8 (2008) 1-13.
[5] F.Baquero, J. L. Martinez, R. Canton, Antibiotics and antibiotic resistance in water environments, Current Opinion in Biotechnology, 19 (2008) 260-265.
[6] K. Kummerer, Antibiotics in the aquatic environment - A review - Part I, Chemosphere, 75 (2009) 435-441.
[7] P. Demarche, C. Junghanns, R. R. Nair, S. N. Agathos, Harnessing the power of enzymes for environmental stewardship, Biotechnology Advances, 30 (2012) 933-953.
[8] M. Arthur, D. Autissier, D. P. Courvalin, Analysis of the nucleotide sequence of the ereB gene encoding the erythromycin esterase type II, Nucleic Acids Research, 14 (1986) 4987-4999.
[9] Sanchez Marcano, J.G., Tsotsis, T.T. Catalytic Membranes and Membrane Reactors, 2002, Wiley VCH, Weinheim, pp. 133-168.
[10] M. de Cazes, M.-P. Belleville, E. Petit, M. Llorca, S. Rodríguez-Mozaz, J. de Gunzburg, D. Barceló, J. Sanchez-Marcano, Design and optimization of an enzymatic membrane reactor for tetracycline degradation, Catalysis Today, 236 (2014) 146-152.