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A new way to conduct enzymatic synthesis in an active membrane using ionic liquids as catalyst support

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Abstract

Several active membranes were prepared by immobilization of *Candida antarctica* lipase B (CALB) onto the surface of ceramic membranes via three different ionic liquids (ILs): [bmim$^+$][PF_6^-], [emim$^+$][NTf_2^-] and [bmim$^+$][NTf_2^-] and compared to an active membrane obtained by simple enzyme adsorption. The performances of these various membranes were evaluated by studying the synthesis of butyl laurate by the acidolysis between butyl acetate and lauric acid in a hexane/water mixture (98:2, v/v). The results obtained show that all the membranes prepared with or without ILs were active and maintained their activity after several runs and during some months.

Keywords: Active membrane; Enzymatic membrane reactor; Immobilised lipase; Ionic liquid; Butyl laurate synthesis

1. Introduction

The enzymatic and selective acylation and deacylation of natural and synthetic compounds to produce different esters (i.e. triglycerides, sugar esters, nucleosides, short and long-chain aliphatic esters, etc.) with lipases is widespread and well documented [1–3]. In a great number of enzymatic reactions immobilized enzymes have been used in order to decrease the production costs and also to enhance the biocatalyst stability [4]. These reactions take place generally in organic media; however, during the last five years enzymatic reactions with lipases using ionic liquids (ILs) as solvents have gained great interest [5]. ILs are salts which are liquid at room temperature and capable of solubilizing a great number of compounds [6]. Contrary to organic solvents, they are not volatile and can form biphasic systems with classical solvents. This last characteristic is very interesting to perform enzymatic reactions with lipases which are well known to be active on interfaces [7]. Besides, ILs are less denaturing than polar solvents and have been considered as over-stabilizing agents, maintaining also the conformation of the enzyme. Thus, they should be considered both as immobilisation support and reaction media [8].

This work concerns the study of new active membrane reactors based on the activation of ceramic supports using ionic liquids like a vector for *Candida antartica* lipase B (CALB) immobilization. Catalytic activities of these membranes were evaluated through butyl laurate production from butyl acetate and lauric acid in hexane and compared to the performance of a membrane obtained by simple enzyme adsorption.

2. Experimental

2.1. Materials

A commercial preparation of thermostable lipase Novozym 525L (EC 3.1.1.3) from *Candida antarctica*

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was provided by Novo Nordisk. The commercial enzyme solution was dia-ultrafiltered to eliminate all the low molecular weight additives using an Amicon (Millipore) system equipped with cellulose acetate membranes (10 kDa cut-off). The final concentrated solution was aliquoted and stored at −20 °C before use. Substrates, solvents and other chemicals were purchased from Sigma–Aldrich–Fluka Chemical Co, and were of the highest purity available. α-Alumina macroporous tubular supports (130 mm in length, 1.5 mm wall thickness, 7 mm i.d., 0.2 μm mean pore size and 28 cm^2 of effective surface) were supplied by PALL-EXEKIA (France).

2.2. 1-Ethyl-3-methylimidazolium triflimide [$emim^+$][Tf_2N^-] synthesis

This ionic liquid was obtained according to the procedure of Bonhôte et al [9]. 13.16 g (68.89 mmol) of $emim^+Br^-$ and 20.5 g of $LiNTf_2$ (68.89 mmol) were mixed in 50 mL of hot water (70 °C). The IL was extracted with dichloromethane (2 × 50 mL), concentrated under reduced pressure and dried under high vacuum (10^{-2} Torr) at 100 °C for 24 h which led to 23.9 g [$emim^+$][Tf_2N^-] (89% molar yield).

2.3. 1-Butyl-3-methylimidazolium hexafluorphosphate [$bmim^+$][PF_6^-] synthesis

This ionic liquid was prepared according to Huddleston et al. [10] as follows: hexafluorophosphoric acid (1.3 mol) was slowly added to a solution of 1-*n*-butyl-3-methylimidazolium chloride ($bmim^+Cl^-$) (176.5 g, 1 mol) in 500 mL of water. After stirring for 12 h, the upper acidic aqueous layer was decanted. The lower ionic liquid portion was washed with water until the washings were no longer acidic. The ionic liquid was then dried under high vacuum (10^{-2} Torr) at 70 °C for 24 h leading to 252.8 g [$bmim^+$][PF_6^-] as a clear oil (89% molar yield).

2.4. 1-Butyl-3-methylimidazolium triflimide [$bmim^+$][Tf_2N^-] synthesis

This ionic liquid was obtained according to the procedure of Bonhôte et al. [9]. 8.72 g (50 mmol) $bmim^+Cl^-$ and 14.35 g $LiNTf_2$ (50 mmol) were dissolved in 50 mL acetone at room temperature. After 6 h, elimination of acetone under vacuum left a residue, which was dissolved in 100 mL of dichloromethane. This solution was washed with water (3 × 25 mL). Concentration under reduced pressure and drying under high vacuum (10^{-2} Torr) at 100 °C for 24 h led to 17.8 g [$bmim^+$][Tf_2N^-] (85% molar yield).

2.5. Enzyme activity assay

The activity of the enzyme solution was estimated by titrating the acid liberated during the hydrolysis of butyl acetate with a NaOH solution at the appropriate concentration using a pH-stat titrator (Schott). One unit of lipase hydrolytic activity (UA) was defined as the amount of enzyme that liberates a titrable amount of acid equivalent to 1 μmol NaOH per minute.

2.6. Enzyme immobilization

The dia-ultrafiltrated lipase solution was diluted to the desired concentration with a 10 mM phosphate buffer (pH 8) and 150 μl of the obtained solution were added to 1 mL of the ionic liquid and strongly stirred. Finally, 4 mL of dichloromethane were added to the mixture under stirring. This mixture was then introduced in the inner compartment of a dry ceramic tubular membrane where one of the ends has been plugged. The mixture which permeated through the membrane was re-introduced until to complete adsorption of the solution by the membrane pores. The membrane was then dried at 40 °C during 40 min and stored under vacuum at room temperature. The enzymatic membranes prepared with the following ILs: [$bmim^+$][PF_6^-], [$bmim^+$][NTf_2^-]) and [$emim^+$][NTf_2^-] were named M2, M3 and M4, respectively. The membranes made without ILs (M1) were prepared according to the same method but in this case 150 μL of the enzymatic solution were diluted in 5 mL of dichloromethane before impregnation. After enzyme deactivation, the support was regenerated following a classical cleaning procedure [11].

2.7. Membrane reactor

The enzymatic membranes were placed in a tubular reactor and the runs were carried out in the pilot unit shown in Fig. 1. All experiments were carried out in dead-end mode by closing the valve V3. The feed solution placed in a 100 mL glass reservoir was pumped at a constant flow rate by an HPLC pump (Gilson 307) (P). The permeate obtained was then recycled to the feed reservoir. Reaction temperature was controlled by keeping the reservoir in a thermostatic bath whereas the membrane reactor was placed in an oven.

2.8. Membrane activity measurement

Membrane activity was evaluated by measuring the production of butyl laurate from butyl acetate and lauric acid in hexane containing 2% (v/v) of water. Firstly, the reactor was fed with hexane containing 2% (v/v) of water during 20 min at a flow rate of 5 mL/min. Then, the reaction mixture composed of a butyl acetate/lauric acid equimolar solution (50 mM) in the same solvent mixture was introduced in the feed reservoir and the activity measurement began. The total volume of the feed solution was 100 mL, feed flow rate and thus permeate flow rate were fixed at 0.5 mL/min and the transmembrane pressure obtained was around 2 bar. Permeate samples of 0.5 mL were withdrawn and analysed by gas chromatography as previously described [12].

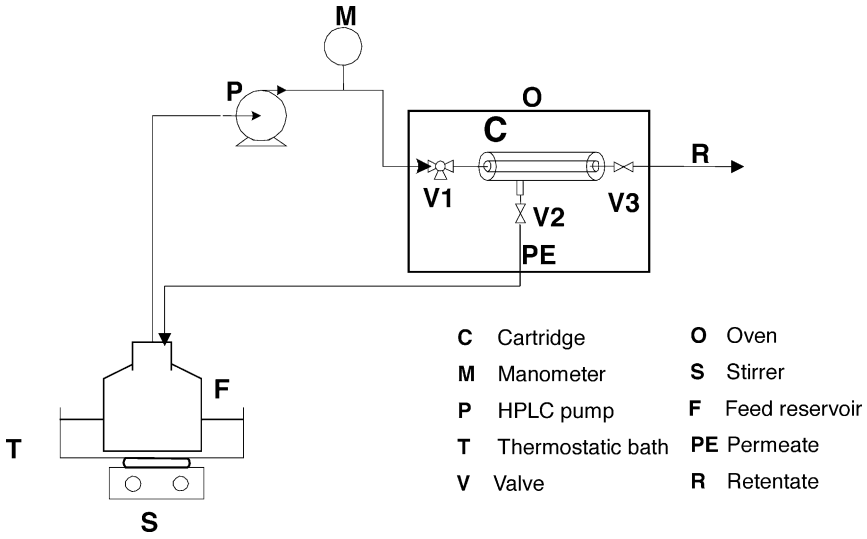

Fig. 1. Experimental set-up of the enzymatic membrane reactor.

3. Results and discussion

3.1. Synthesis of butyl laurate in the enzymatic membrane reactor

In order to evaluate the interest of the ionic liquids in the immobilization process, the performances of membranes M2, M3 and M4 were compared with those of M1. All the membranes were prepared starting from an enzymatic solution with 300 UA. All synthesis reactions were carried out under the same conditions: a continuous and constant (0.5 mL/min) substrate supply (50 mM of butylacetate and lauric acid in hexane with 2%, v/v of water) at 37 °C.

In Fig. 2 we can observe the reaction rate obtained for the different membranes tested. It is important to note that all membranes presented constant butyl laurate production during the experiment time (5 h) and no enzyme activity was found in permeate samples. The results obtained show that all the membranes prepared with ILs are active. The ionic liquids can thus be used as vector of immobilization. Within the framework of our study [bmim$^+$][NTf$_2^-$] appears as the most suitable IL to retain the enzyme and maintain its activity. Nevertheless, the membrane prepared by simple adsorption (M1) shows a butyl laurate production two times higher than that of the membrane M3. In previous works the effect of ILs on the enzymatic membrane activity has been incongruous, in some cases a reaction rate enhancement has been reported [13], but in other cases this rate enhancement has not been observed [14]. Nevertheless, ILs have especially shown to increase the enantioselectivity [14,15] or the enzyme stability [16,17]. Furthermore, it has also been reported that the non-miscibility between the enzyme–IL and hexane results in a biphasic system [18], where the mass-transfer phenomena between phases can produce a decrease in the catalytic efficiency of the active membrane with ILs. The ILs used in this work are non miscible with hexane and lipase solution, it is almost certainly that lipase entities are included in the thin film of ILs or/and in the interface between the IL and the alumina membrane. Indeed, in this case we can note that the mass transfer of the substrates and/or products would be weakening by a higher mass transfer resistance though the very viscous thin layer of ILs.

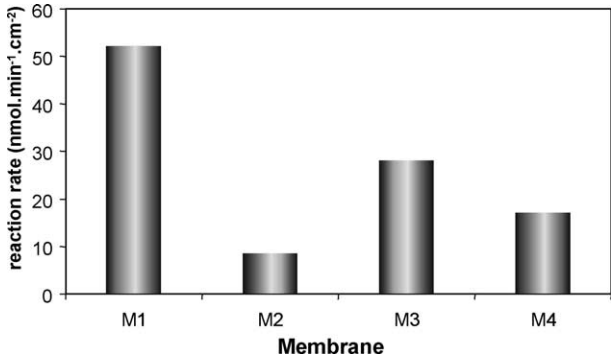

Fig. 2. Production of butyl laurate with enzymatic membranes prepared by direct adsorption (M1) or with ILs: ([bmin$^+$][PF$_6^-$] (M2), [bmin$^+$][NTf$_2^-$] (M3) et [emim$^+$][NTf$_2^-$] (M4)). Reaction conditions: $T = 37$ °C, butyl acetate and lauric acid: 50 mM, hexane/water (98:2), feed flow 0.5 mL/min.

3.2. Reproducibility and storage effect

In order to evaluate the membrane stability with time, several successive reactions were carried out with membranes M1 and M2 during 4 months. After each experiment, the membranes were rinsed with hexane, dried and were stored under vacuum. The evolution of the performances of these active membranes, is represented on Fig. 3. It is noted that for both membranes, the performances are almost constant. There is, thus, neither salting out of enzyme during experiments (already confirmed by the absence of evolution of the samples of permeate), nor deactivation of lipase during the use or storage of the membranes. The results obtained with alumina membranes are surprising because

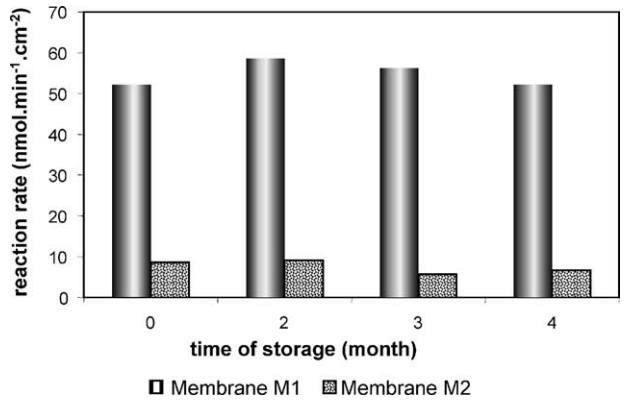

Fig. 3. Reactivity and stability of membranes M1 and M2. Reaction conditions: $T = 37$ °C, butyl acetate and lauric acid: 50 mM, hexane/water (98:2), feed flow 0.5 mL/min.

the lipases are well known by their ability to be adsorbed on the surface or inside the porosity of hydrophobic membranes but not in hydrophilic surfaces.

3.3. Operational parameter effects

In order to improve the rate of production of the butyl laurate, we studied the effect of the operational parameters such as the concentration of the substrates, water content, temperature, and quantity of immobilized enzyme. For this purpose we focused our study on membrane M1. The results of these experiments are shown on Fig. 4. As we could expect, when the concentration of both substrates is multiplied by 10, the rate of production of butyl laurate increases impressively from 56.5 to 280 nmol/(min cm^2). However, if we increase only the lauric acid concentration, the rate of production is unchanged whereas, the butyl acetate concentration enhancement results in an equally good performance reached when both substrate concentrations are increased. The ester concentration is thus a factor limiting the catalytic activity of the membrane.

To avoid problems involved with the precipitation of the lauric acid, the butyl acetate and lauric acid concentrations

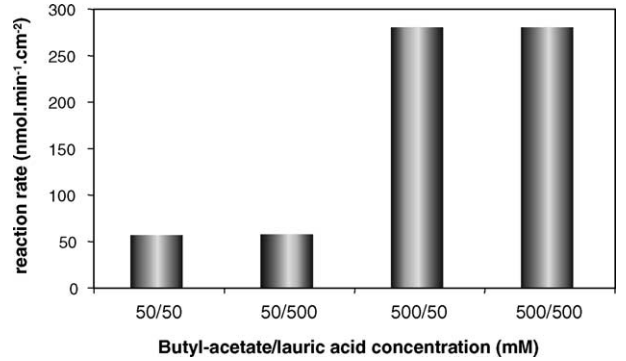

Fig. 4. Rate of butyl acetate production vs. substrate concentration. Reaction conditions: $T = 37$ °C, butyl acetate and lauric acid: 50 mM, hexane/water (98:2), feed flow 0.5 mL/min, membranes prepared like membrane M1.

were fixed at 500 and 50 mM, respectively, for the rest of the experiments.

The water content has a main influence on the reaction yield. Indeed, if dry hexane is used as solvent the reaction rate is almost zero, whereas a water content higher than 2% by volume does not result in better productivity. We observed that the temperature had a negligible influence on the reaction rate. A small variation on the productivity (280–260 nmol/(min cm^2)) was observed when the temperature was varied between 37 and 50 °C. However, at temperatures higher than 50 °C the reaction rate decreases dramatically during the experiment which means that thermal deactivation occurs. The influence of the offered amount of enzyme for membrane preparation on butyl laurate production was also studied, resulting in an enhancement in productivity. Thus, the membranes prepared with solutions presenting 200, 300 and 3000 UA, respectively, had an initial butyl laurate production of 4.9, 8.0 and 13.3 μmol/min, respectively (measured between 1 and 2 h of reaction). We can note that the productivity enhancement is not proportional to the quantity of immobilized enzyme; this result can be explained either considering that a part of enzyme presents steric hindrance which can results on the inaccessibility of the active site or by the fact that the reaction has been carried out in conditions were the substrate is limiting. Indeed, the production rate was constant during 5 h for the membranes prepared at 300 and 200 UA whereas, for the membrane with the highest activity (3000 UA) the productivity rate seems to decrease with the substrate concentration.

The last result presented here concerns the performance of membrane M3 (the lipase was immobilized with the [bmim$^+$][NTf$_2^-$]). The reaction was carried out at a substrates feed of 0.5 mL/min, butyl acetate 500 mM, lauric acid 50 mM, hexane with 2% (v/v) of water and at 37 °C. With those operating conditions the rate of ester production was 130 nmol/(min cm^2). This productivity is higher than the rate obtained when both substrates concentrations are identical (50 mM, for an ester production rate of 28 nmol/(min cm^2), see Fig. 2) but it is much lower than the production rate reached with the membrane prepared by simple adsorption. As it was explained previously, the non-miscibility between the hexane and enzyme–ILs can explain the difference on the reactivity between the membranes prepared with IL and by simple adsorption. Moreover to this explanation, we can consider that in the case of IL a fraction of the lipase is adsorbed at the alumina–IL interface, then the substrate diffusion through the thin film of ionic liquids can be responsible for the lower productivity observed respect to the membrane prepared by simple adsorption.

4. Conclusions

Several active membranes were prepared by immobilization of CALB onto the surface of ceramic membranes via three different ILs: [bmim$^+$][PF$_6^-$], [emim$^+$][NTf$_2^-$] and

[bmim$^+$][NTf$_2^-$], and compared to one obtained by simple enzyme adsorption. The synthesis of butyl laurate starting from butyl acetate and lauric acid in a hexane/water mixture (98:2, v/v) catalyzed by CALB has been chosen as a model reaction. The results obtained showed that all the membranes prepared with ILs were active and stable as function of time. In addition, their performances are maintained after several uses and during several months. An unexpected result was obtained with enzymatic membranes prepared by simple adsorption (without ILs) which resulted to be more reactive when compared with membranes prepared with ILs. Nevertheless, ILs appear to be good vectors for enzyme immobilization on ceramic membranes; their ability to maintain both catalytic integrity of the protein and a stable film-layer onto the membrane surface must be underlined. Within the framework of our study [bmim$^+$][NTf$_2^-$] appears as the most suitable Il to retain the enzyme and maintain its activity.

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