Influence of the Enzyme Concentration in the Phase Behaviour for Developing a Homogeneous Enzymatic Reaction in Ionic Liquid-CO_2 Media

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A homogeneous enzymatic reaction in an ionic liquid (IL)-CO_2 medium integrated with the separation of the product is proposed. It takes advantage of the miscibility switch phenomenon using CO_2, which is able to force two of the immiscible phases to form one homogeneous reaction medium and later on to split the homogeneous fluid phase in two or three phases upon pressure decrease in order to facilitate the product recovery. For this purpose the enzyme *Candida antarctica* lipase B (CaL B) and the IL 1-hydroxy-1-propyl-3-methyl imidazolium nitrate ($HOPMImNO_3$) are used. In this paper the solubility of CO_2 in solutions of CaL B in $HOPMImNO_3$ is experimentally determined using the Cailletet apparatus that operates according to the synthetic method. Concentrations of the enzyme vary from 3 to 12% in weight, and concentrations of CO_2 from 5 to 20 mol % have been investigated in a temperature range from 30 to 75^o C and pressures up to 12 MPa. No precipitation of the enzyme has been observed when dissolving CO_2 in the IL. Keeping constant the ratio CO_2/IL the pressure of the bubble point remains almost unchanged. Other aspects of the process were also investigated. Triacetin tests showed that the reduction of the activity of the enzyme after three months of storage dissolved in the $HOPMImNO_3$ is only of the order of 20%. Recovery and purification of the IL was possible by precipitation of the enzyme using IPA as antisolvent.

INTRODUCTION

In the last years an there has been an increasing interest in studying enzymatic reactions in ionic liquids (IL) in order to replace volatile halogenated organic solvents by non-volatile ionic liquids as solvents for this kind of reactions [1]-[4]. In general it can be said that the ionic liquids (ILs) as solvent for enzymatic reactions ease the product separation and have an over-stabilizing effect on biocatalysts compared to traditional organic solvents without affecting the yield of the process. Most of the enzymatic reactions in ILs have been carried out with immobilized enzymes or free enzymes in suspension, but recently it has been reported that it is possible to have a homogeneous enzymatic reaction in ILs [5]-[9].

Supercritical carbon dioxide ($scCO_2$) was found to be able to extract substances from an IL without any cross-contamination of the IL itself [10]. As an additional feature CO_2 is able to force two or more immiscible phases to form one homogeneous phase upon pressure increase and, in reverse, to split a homogeneous fluid phase in two or three phases upon pressure decrease. This phenomenon is called miscibility switch [11],[12]. It was applied to the enzymatic processes combining ILs and CO_2 [13],[14] to have a two-phase medium in which enzyme is immobilized in the IL rich liquid phase and substrates and products are introduced and extracted from the system dissolved in the $scCO_2$. The protective effect of the IL against the denaturisation effect of CO_2 was clearly observed.

In this work, a homogeneous enzymatic reaction in a IL-CO_2 medium, integrated with a separation of the product by taking advantage of the miscibility switch phenomenon is proposed. A simplified scheme of the process is shown in **Figure 1**. Pressurizing with CO_2 the reaction would take place in a homogeneous phase, and once outside the reactor the pressure would be reduced to separate the mixture into two phases: the IL rich phase with the enzymes dissolved and the CO_2 rich phase containing the products. After a new depressurization step separation of the CO_2 from the products would be carried out. In the case that more than one product is obtained or that the reagents are not completely converted, a fractionate separation is possible. For this purpose the enzyme *Candida antarctica* lipase B (CaL B) and the IL 1-hydroxy-1-propyl-3-methyl imidazolium nitrate ($HOPMImNO_3$) are used. The potential of the HOPMIm cation for dissolving enzymes while maintaining their catalytic activity has been demonstrated by Walker and Bruce [6], [15]. Even when the main aim of this work was studying the solubility of CO_2 in solution with different concentrations of CaL B in $HOPMImNO_3$, some other preliminary aspects about the stability of the enzyme and the recoverability of the IL have been considered.

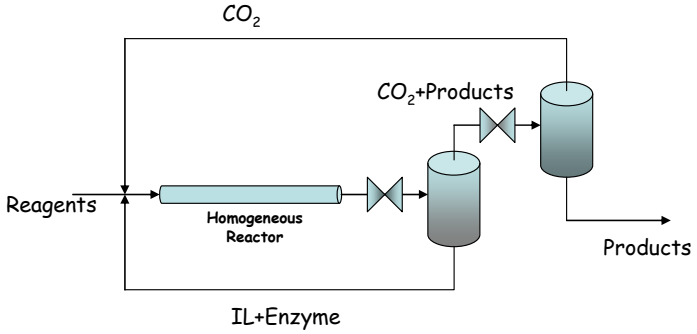

Figure 1: Scheme of the integrated reaction-separation process in a homogeneous CO_2-IL medium

EXPERIMENTAL

The synthesis of the ionic liquid ($HOPMImNO_3$) is described in Bermejo et al. [16]

Aqueous solution of free enzyme CaL B 525 L provided by CLEA Technologies has been used. It has been previously determined that 144 μL of the original solution of the enzyme corresponds to one unit of activity (U). The preparation procedure of the enzyme dissolved in the IL consists of the following steps: 1) Precipitation of a known volume of aqueous enzyme solution using acetone; 2) Drying the precipitated enzyme under vacuum in an exsiccator over P_2O_5. 3) The IL, preserved in an exsiccator under vacuum and in presence of P_2O_5, is dried further in the oven at 90 ^oC under vacuum prior to use. 4) A known amount of the dry $HOPMImNO_3$ is added to the dry enzyme. The dissolution of the enzyme is observed after about 30 minutes under vigorous shaking and heating up between 45 and 60 ^oC. In order to

avoid any loss of the enzyme activity, the prepared solution has been stored in a fridge and placed in the exsiccator only 24 h prior to the preparation of the sample for the phase equilibrium measurements. Just between 15 and 30 minutes before the preparation of the sample the solution is dried in the oven at 90^{o}C under vacuum conditions.

The water content in the IL and in the enzymatic solutions was determined periodically by Karl-Fisher moisture analysis (Mettler DL35 Karl Fischer Titrator). The structure of the cationic part of the IL was confirmed using ^{1}H NMR and ^{13}C NMR in the Unity Inova 300 s of Varian. The same technique has been used to determine the structure of the enzyme in the commercial aqueous solution and to determine the structure of the enzyme dissolved in the IL. ^{13}C NMR of the pure HOPMImNO3 and aqueous solution of CaL B are found in Table 2.

For all the phase equilibrium measurements a Cailletet apparatus was used. It operates according to the synthetic method, i.e., a sample of known overall composition is used. A detailed description of the experimental facility and the experimental procedures as well can be found elsewhere [16].

Hydrolytic activity of CaL B 525 L towards 0.1 M triacetin (99% Acros Organics) in sodium phosphate buffer 0.1 M at pH 7.5 was determined by titration of the formed acetic acid with 0.1 sodium hydroxide as a function of time using a DOSIMAT equipment with pH meter. The assay was performed in a volume of 10 ml.

EXPERIMENTAL RESULTS

Experimental data of the solubility of CO_2 in solutions HOPMImNO$_3$-CaL B

Bubble points for the binary systems CO_2+HOPMImNO$_3$+CaL B have been determined for several CO_2 relative-enzyme free concentrations ranging from 5 to 20 mol % in a temperature region from 30 up to 75 ºC, pressures up to 12 MPa, and for several CaL B concentrations. The CO_2 concentrations of the samples in the ternary systems have been calculated as relative enzyme-free compositions (X_i).

Solutions of 0.5, 1, 1.5 and 2 U/ml HOPMImNO$_3$ have been studied. These solutions correspond to approximately 3, 6, 9 and 12 % by weight of enzyme and approximately 40, 80, 128 and 171 mg/mL IL. It should be remarked that the solubilized amount of CaL B in the HOPMImNO$_3$ is higher than the reported solubilities of enzymes in other ILs. Madeira Lau et al [5] reported a solubility of 3 mg/mL of CalB in [Et$_3$MeN][MeSO$_4$]. Nakashima et al. [8] reported a solubility of 40 mg/mL of poly(ethylene glycol) modified subtilisin Carlsberg in EMImTf$_2$N (containing 2 mg/mL of the enzyme).

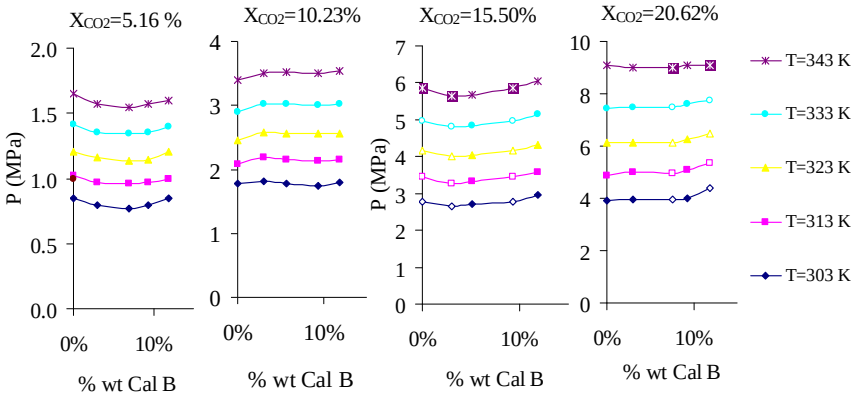

Figure 2. Equilibrium bubble points pressure against CaL B concentration. Open symbols are interpolations. Data of the binary system CO_2+HOPMImNO$_3$ are from Bermejo et al [16].

In Figure 2, the equilibrium pressure is plotted versus CaL B concentration for a fixed relative enzyme-free CO_2 molar fraction. The data with a different concentration of CO_2 has been interpolated and are represented as open symbols. It can be observed that the equilibrium pressure remains almost unchanged with enzyme concentration. The data from the binary system CO_2+$HOPMImNO_3$ have been taken from Bermejo et al. [16].

Structure and aging of CaL B dissolved in the $HOPMImNO_3$:Preliminary study

This study has been performed in order to understand the aging effect of both the enzyme stored in hydrogen-bonding ionic liquids and the IL itself. The NMR spectra and the activity assay were performed in two solutions of 1.5 U/mL $HOPMImNO_3$: a three months old sample and a freshly prepared equivalent solution. The first solution was 3 months old and it had been preserved in an exsiccator at vacuum conditions and in presence of P_2O_5 at room temperature.

Proton and carbon NMR of both solutions were performed and compared to that of the pure IL. No differences in the spectra of the old and fresh solutions of the IL could be observed. The characteristic peaks of the IL were present and the new peaks characteristic of the enzyme were the same in both spectra and in the spectra of the commercial aqueous solution of the enzyme. This indicates first that aging for the IL does not occur and that the structure of the enzyme is the same when dissolved in water and when it is dissolved in $HOPMImNO_3$. The ^{13}C NMR spectra of the fresh and old solution are summarized in

Table 1.

Table 1: ^{13}C NMR (300.2 MHz, D_2O, t-BuOH) of the $HOPMImNO_3$, the aqueous solutions of CaL B and solutions of CaL B in the $HOPMImNO_3$

Pure $HOPMImNO_3$ **δ(ppm)**	**Aqueous solution of CaL B** **δ(ppm)**	**Solution of CaL B in $HOPMImNO_3$ (3 months old)** **δ(ppm)**	**Solution of CaL B in $HOPMImNO_3$ (fresh)** **δ(ppm)**	**Recycled $HOPMImNO_3$** **δ(ppm)**
				24.180
31.946		33.206	33.190	32.076
35.894		37.179	37.187	35.951
46.727		48.003	48.011	46.800
58.183		59.459	59.459	58.207
	62.665	64.038	64.111	
	62.737	64.119	64.434	
	63.045	64.442		
				64.388
	69.881	71.23	71.238	
	71.232	72.67	72.646	
	71.353	72.791		
	72.292	73.698	73.689	
	73.149	74.563	74.555	
122.532		123.816	123.832	122.588
123.866		125.151	125.159	123.923
136.374		137.65	137.650	136.438

In addition, the enzyme activity in the two solutions was determined. Also the activity of the enzyme in the original commercial aqueous solution was evaluated. Duplo measurements were performed with each solution for the equivalent of 50 μL and 100 μL of the original solution. Results are shown in Table 2. The enzyme freshly dissolved in the $HOPMImNO_3$ lost 35% of its activity with respect to the initial activity. Surprisingly, after three months of storing the solution, the enzyme resulted to maintain about 80% of its activity with respect to the fresh solution. This is an excellent result. This indicates that the enzyme stored in $HOPMImNO_3$ keeps its initial activity with a very high percentage, which means that hydrogen bonding ILs stabilizes the enzymes.

Table 2: Results of the activity assays performed to the original solution of the CaL B, and the two solutions in $HOPMImNO_3$, (freshly prepared and 3 months old). The activity is expressed in U/ ml of the commercial solution.

Activity (U/ml)	50 μL	100 μL	Average	Loss of activity respect Free enzyme	Loss of activity with respect fresh solution
Free enzyme CaL B	98.4	121.8	110.1		
Fresh Solution	71.8	70	70.9	-35.6%	
Old solution (3 month old)	60.4	51.9	56.15	-49.0%	-20.8%

Recovery and purification of the IL

For that purpose, a simple and efficient separation procedure is proposed. First the enzyme was precipitated from the $HOPMImNO_3$ using IPA as an anti-solvent. At least 3 x triple aggregations have to be done. The in this manner precipitated enzyme has to be centrifuged. Remaining IPA in the IL solution was evaporated under reduced pressure. We have applied this procedure to two enzymatic solutions. Solution 1: 1.5 U/ml (9.30 % wt CaL B), 41 days old. Solution 2: 0.5 % U/ml (3.03 % wt CaL B), 25 days old.

The obtained NMR spectra of recovered IL in this procedure have been compared to the ones of pure $HOPMImNO_3$ and with that of the starting enzymatic solution. We observed that in solution 2 all the characteristic peaks of the enzyme has disappeared, but in solution 1 still some traces of the enzyme are still present. Traces of IPA were still present in both of them. However they can be easily eliminated by further vacuum distillation. In conclusion, we observe that the NMR of 1 and 2 are almost identical and provide us the information that the IL has been recovered with nearly 100% purity. The C13 NMR of solution 2 can be found in

Table 1.

CONCLUSIONS

Some preliminary aspects for developing an enzymatic reaction separation process using the IL $HOPMImNO_3$ have been studied:

- The phase equilibrium of the system CO_2+$HOPMImNO_3$+CaL B has been studied experimentally. The equilibrium pressure of the bubble points of the system is essentially unchanged in the presence of the enzyme.
- $HOPMImNO_3$ is able to solubilize a much larger amount of enzyme than the amount of enzyme solubilised by other ILs as suggested literature.

- C^{13} and H^{1} and NMR tests did reveal that the structure of the IL is preserved when CaL B is dissolved, and that the structure of the enzyme dissolved in $HOPMImNO_3$ is the same than in aqueous solution.
- Activity assays performed in an aged solution of CaL B in $HOPMImNO_3$ shows that the activity of the enzyme dissolved in the IL is maintained up to 80% of the activity of a fresh solution even after three month of room temperature storage. This demonstrates that this IL is very appropriate for the preservation of enzymes.

In summary, all results presented in this study look as very promising to develop the proposed process using the ionic liquid $HOPMImNO_3$ as a solvent.

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