ENZYMATIC CATALYSIS IN IONIC LIQUIDS AND SUPERCRITICAL CARBON DIOXIDE BIPHASIC SYSTEMS

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INTRODUCTION

The use of both enzyme-catalyzed processes and benign environmental media are key areas for future innovation in Green Chemistry. The technological purposes for which enzymes can be used have greatly increased in scope, and they have been used under anhydrous conditions in organic solvents, mainly for asymmetric synthetic transformations, due to the growing demand for enantiopure pharmaceuticals [1]. In the same context, the use of supercritical fluids [2] and ionic liquids [3] as reaction media has become an important strategy for developing clean manufacturing processes and environmentally friendly technologies of industrial interest.

Ionic liquids (ILs) have emerged as exceptionally interesting non-aqueous reaction media for enzymatic transformations. These neoteric solvents are simply salts and therefore entirely composed of ions that are liquid below 100°C, usually close to room temperature [4,5]. Typical room temperature ionic liquids are based on organic cations, *e.g.* 1,3-dialkylimidazolium, tetraalkylammonium, paired with a variety of anions that have a strongly delocalized negative charge (*e.g.* BF_4^-, PF_6^-, etc), resulting in colourless, low viscosity and materials that are easy to handle with very interesting properties as solvents. Their interest as green solvents resides in their negligible vapour pressure, excellent thermal stability, high ability to dissolve a wide range of organic and inorganic compounds, including gases (*e.g.* CO_2) and non-flammable nature, which can be used to mitigate the problem of volatile organic solvents emission to the atmosphere. By using anhydrous conditions or low water content (< 2% v/v), all the assayed water-immiscible ILs (i.e. $[Bmim]^+$ $[PF_6]^-$, etc.) were shown to be suitable reaction media for biocatalytic reactions, while no enzymatic activity was observed when anhydrous water-miscible ILs were used, with a few exceptions (i.e. $[Bmim]^+$ $[BF_4]^-$, etc) [6]. By using this strategy, the enzymes display a high level of activity and stereoselectivity for synthesizing many different compounds, *e.g.* aspartame, aliphatic and aromatic esters, amino acid esters, chiral esters by kinetic resolution of *rac*-alcohols, carbohydrate esters, polymers, terpene esters, etc. [7]. Furthermore, free enzyme molecules suspended in these media behave as anchored or immobilized biocatalysts because they cannot be separated by liquid-liquid extraction (i.e. with buffer or aqueous solutions) [8]. ILs form a strong ionic matrix and the added enzyme molecules could be considered as being included rather than dissolved in the media, meaning that ILs should be regarded as liquid enzyme immobilization supports, rather than reaction media, since they enable the enzyme-IL system to be reused in consecutive operation cycles [9]. The excellent stability of free enzymes in water-immiscible ILs for reuse has been widely described, and has been demonstrated using spectroscopic techniques. It was demonstrated that these neoteric solvents have a remarkable ability to maintain the secondary structure and the native conformation of the protein towards the usual unfolding that occurs in non-aqueous environments [10].

Additionally, after an enzymatic transformation process in ILs is carried out, the products may be recovered by using supercritical fluids (SCFs), which represents in a clear opportunity towards the development of the integral green design of any chemical process.

The physical properties of SCFs (*e.g.* $scCO_2$) make them an interesting alternative to volatile organic solvents for the development of clean processes and biocatalytic transformations in non-aqueous environments [2, 11]. However, the poor stability exhibited by enzymes in SCFs is probably the main drawback for the application of biocatalytic processes in these solvents. The best approach to protect enzymes against these adverse effects of $scCO_2$ was resulted from the coating or suspension of biocatalysts with ILs. Additionally, it was demonstrated the exceptional ability of $scCO_2$ to extract organic compounds from ILs, which results from the fact that, although $scCO_2$ is highly soluble in the IL phase, the same IL is not measurably soluble in the $scCO_2$ phase [12]. In this context, continuous biphasic biocatalysis systems, based on the enzyme immobilization in one IL phase (catalytic phase), while substrates and products reside largely in a supercritical phase (extractive phase), have been put forward as the first approach of integral green bioprocesses in non-aqueous media, directly providing pure products [13, 14]. The system has been tested for several lipase-catalyzed reactions (*i.e.* synthesis of butyl butyrate, kinetic resolution of *rac*-1-phenylethanol, etc), showing an exceptional level of activity, enantioselectivity (ee> 99.9) and operational stability, even under harsh conditions (i.e. 100 bar and 150 ^{o}C) [15]. A further step towards reducing the amount of IL used in the enzymatic processes in IL/$scCO_2$ biphasic systems has resulted from the development of monolithic solid supports containing a covalently attached IL phase [16]. The adsorption of a lipase onto this linked IL phase provides excellent immobilized biocatalysts with enhanced activity and increased operational stability for the synthesis of citronellyl butyrate in $scCO_2$, compared with the original strategy based on enzymes coated with ILs.

The aim of this work is to push our efforts towards the design of green enzymatic processes in IL/$scCO_2$ biphasic systems. The catalytic system is based on an enzyme (*Candida antarctica* lipase B, CALB) immobilised by non-covalent attachment onto a macroporous polymer to give a supported ionic liquid like phase (SILLP). This was used for a stereoespecific reaction model, such as the kinetic resolution (KR) of *rac*-1-phenylethanol, in continuous process. The presence of acid catalysts, such as zeolites and silica modified with benzenesulfonic acid, to provided *in situ* racemization of the undesired enantiomeric substrate, was also studied in order to enhance the yield in the desired enantiomeric product [17,18]. This approach combines the advantages of supported ionic liquid as a "supported liquid solvent", an immobilised enzyme as a "green" catalyst, and the use of a SCF as a "green" reaction/extraction solvent with the advantages of a continuous flow process, easy product separation and catalyst reuse.

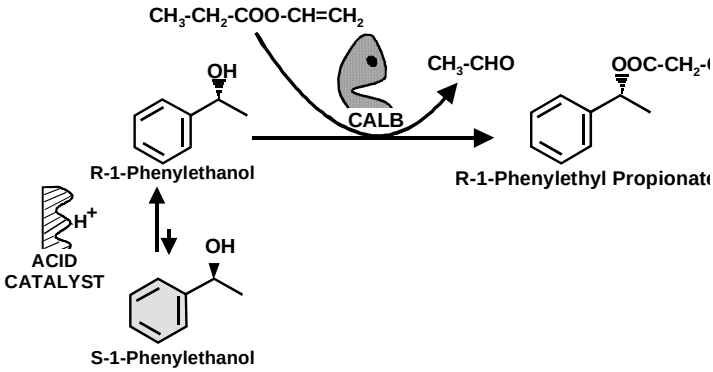

Figure 1. Mechanism of dynamic kinetic resolution (DKR) of *rac*-1-phenylethanol by coupling of both chemical and enzymatic catalysts.

MATERIALS AND METHODS

Materials. Aqueous solution of *Candida antarctica* lipase B (Novozym 525L, EC 3.1.1.3) was a gift from Novozymes S.A. (DK). Silica gel (40-120 μm particle size, 6 nm pore diameter), containing butyl groups (Si-C4) or benzenesulfonic groups (SCX), respectively, were obtained from Applied Separations Inc (Allentown, PA, USA). Acid zeolites CP811C (zelolite beta, 620 m^2/g), CBV720 (zelolite Y, 720 m^2/g) and CP811E (zelolite beta, 600 m^2/g) were obtained from Zeolysts International (Valley Forge, PA, USA). Substrates, solvents and other chemicals were purchased from Sigma-Aldrich-Fluka Co (Madrid, Spain), and were of the highest purity available.

Synthesis of butyl-trimetylammonium bistriflimida [Btma][NTf_2]. The IL [Btma][NTf_2] was synthesized as previously was described in detail [10].

Preparation of the monolith. Protocol for preparing the monolithic-supported ionic liquid phase, containing covalently attached 1-butyl-3-methylimidazolium bistriflimide moieties (SILLP-[Bmim] [NTf_2]), have been previously reported in detail [16, 19].

Enzyme immobilization. Immobilized enzyme derivatives were prepared by simple adsorption of an aqueous solution of CALB (10 mg dissolved in 500 μL water) onto SILLP-[Bmim] [NTf_2] (300 mg, or Si-C4 (1 g). Then, each wet support was stored under controlled Aw (0.11) conditions over LiCl in a desiccator for 48 h at room temperature prior to use.

Racemization of *S*-1-phenylethanol. Racemization reaction was carried out in 1-mL screw-capped vial with teflon-lined septa, containing (S)-1-phenylethanol (37 μL, 0.300 mmol) dissolved in hexane (463 μL). The reaction was started by adding the acid catalyst (5 mg) and run at 50 ºC in a glycerol bath for 5 h. At regular time intervals, 20 μL aliquots were taken and suspended in 480 μL hexane. The biphasic mixture was strongly shaken for 3 min to extract all substrates and product into the hexane phase. Then, 400 μL of hexane phase were collected and mixed with 100 μL of 150 mM internal standard solution in hexane, and finally analyzed by GC.

Dynamic kinetic resolution of *rac*-phenylethanol in $scCO_2$. For the case of CALB-Si-C4 derivative, both the immobilized enzyme preparation (500 mg) and SCX (20 mg) catalysts were separately coated with 1 mL and 150 μL, respectively, of [Btma][NTf_2] in a test-tube, and then strongly shaken for 30 min, to ensure proper coating of all silica particles. Finally, the acid catalyst was packed into a column, while the immobilized lipase powder was divided into two fractions, and then packed into separated columns (see Fig. 2). For the CALB-SILLP case, two fractions (150 mg) of immobilized derivative, and one fraction of acid zeolite (100 mg of CP811C, CBV-720 or CP811E coated with 150 μL of [Btma][NTf_2]) were packed in three separated columns. The reactor was operated in two different configurations: (i) one column (immobilized lipase); and (ii) three columns connected in series (immobilized lipase-acid catalyst-immobilized lipase). The system is equipped with a supercritical pump (JASCO, model PU-1580-CO_2), needle valves, filter and a back-pressure regulator. The reactor was run by the continuous pumping of $scCO_2$ at 10 MPa and 50 ºC through the packed catalysts. The SCF then flowed through the back-pressure regulator, and finally bubbled through a capillary tube (60 ºC) in a controlled amount of hexane placed on ice-bath. The synthetic process was carried out by continuous pumping of an equimolar solution of *rac*-1-phenylethanol and vinyl propionate at 21.2 μmol/min mass flow-rate, by using a HPLC pump (model LC-10AT, Shimadzu Europe, Duisburg, Germany) for 4 h. Collected samples were analyzed by GC. The substrates/products mass- balance from the outlet was consistent with the substrates mass-flow inlet.

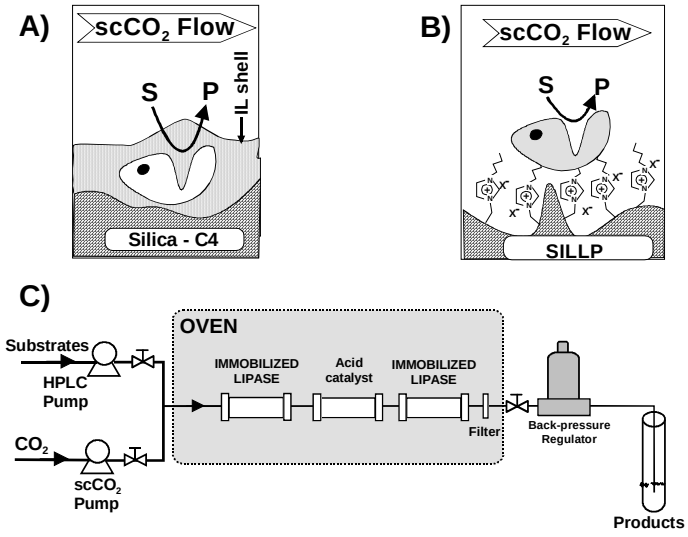

Figure 2. Experimental set-up of the continuous (chemo)enzymatic reactor for DKR in IL-$scCO_2$ biphasic system.

GC analysis. Analysis were performed with a Shimadzu GC-2010 (Shimadzu Europe, Duisburg, Germany) equipped with FID detector. Samples were analyzed on a Beta DEX-120 column (30 m x 0.25 mm x 0.25 μm, Supelco), using He as carrier gas and a FID detector, as described previously [18]. Retention times of compounds are as follows: vinyl propionate (3.1 min; propionic acid (5.6 min), butyl butyrate (internal standard, 7.1 min), *R*-1-phenhylethanol (14.8

min), *S*-1-phenhylethanol (15.5 min), *S*-1-phenylethyl propionate (18.2 min) and *R*-1-phenylethyl propionate (18.6 min).

RESULTS AND DISCUSSION

Immobilized CALB derivatives were demonstrated to have an exceptional ability to catalyze the kinetic resolution of sec-alcohols in IL/scCO_2 biphasic systems. This is mainly due to the high stereoselectivity of the enzyme and the enhanced stability in these non-conventional media [13-15]. However, the main drawback of theses processes is that the chemical yield is limited to 50 %. This can be overcome by combining kinetic resolution with *in situ* racemization of the undesired enantiomer, using so-called dynamic kinetic resolution (DKR) [17,19].

In this context, the ability of solid acid catalysts, such as acid zeolites or benzenesulphonic silica SCX, to carry out the racemization of *S*-1-phenylethanol was previously studied in hexane medium at 50 ºC (see Fig. 3). As can be seen, SCX showed the best racemization activity (246 μmol/min g catalyst) which led to the practically total racemization after 3 h (ee < 8%).

However, all assayed acid zeolites were shown to have a lower racemization activity than SCX, which resulted in a high ee for the 1-phenylethanol enantiomeric mixture after 3 hours of reaction.

As a function of these facts, for DKR experiments in scCO_2 using SCX as chemical catalyst, a 1:25 (w/w) ratio of SCX:CALB-Si-C4, respectively, was assayed to ensure the rapid consumption of *R*-1-phenylethanol by the enzyme, and the shift of the racemization equilibrium to the *R*-substrate.

Fig. 4A depicts both the yield and the enantiomeric excess of *R*-1-phenylethyl propionate for immobilized CALB, obtained by enzyme adsorption onto silica particles modified with butyl chains (CALB-Si-C4), in continuous operation with scCO_2 at 50 ºC and 10 MPa.As it was expected, the immobilized enzyme derivative was able to increase the yield of the enantiomeric *R*-ester product only up to 50 %, reaching the steady state after 120 min of continuous process. The synthetic product *R*-1-phenylethyl propionate was obtained with an ee>99.9, while *S*-ester product was never detected, due to the excellent enantioselectivity of the enzyme towards the *R*-substrate [15]. Furthermore, the hydrolysis of the acyl donor to produce propionic acid did not occur which was clearly related with the continuous flow of dry scCO_2 not drawing out any free water molecules from the immobilized enzyme particles which are capable of acting as a nucleophile acceptor. The simultaneous presence of both immobilized CALB and SCX particles in the reactor resulted in a clear improvement of the DKR process, reaching a time-course profile for *R*-ester product yield with a maximum level at 78 % (Fig. 4B).

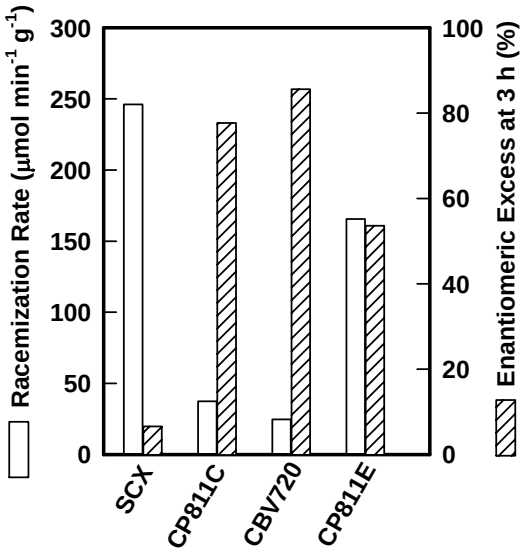

Figure 3. Activity of different acid catalysts for the racemization of *S*-1-phenylethanol in hexane at 50 ºC, and enantiomeric excess of the resulting racemic mixture of 1-phenylethanol at 3 hours reaction time.

The simultaneous presence of both immobilized CALB and SCX particles in the reactor resulted in a clear improvement of the DKR process, reaching a time-course profile for *R*-ester product yield with a maximum level at 78 % (Fig. 4B). It was decided to use separate columns for each catalyst as a consequence of the enzyme deactivation observed in previous experiments where both catalyst particles were mixed together [20]. These results are explained by the ability of SCX to catalyze the racemization of *S*-1-phenylethanol, which is coupled with the consumption of *R*-1-phenylethanol by the immobilized lipase. For this particular reactor configuration, the *R*-ester product yield may only reach 100 % by coupling in series several enzymic and acidic catalytic columns, following a dichotomic progression. It is also worth noting how enantiomeric excess of the *R*-ester product was continuously reduced until 92 %, due to the synthesis of *S*-1-phenylethyl propionate (up 4 % yield). Taking into account the excellent enantioselectivity of the enzyme, this fact is only explained by an uncontrolled transesterification activity of the SCX catalyst under the assayed conditions [11,17,20].

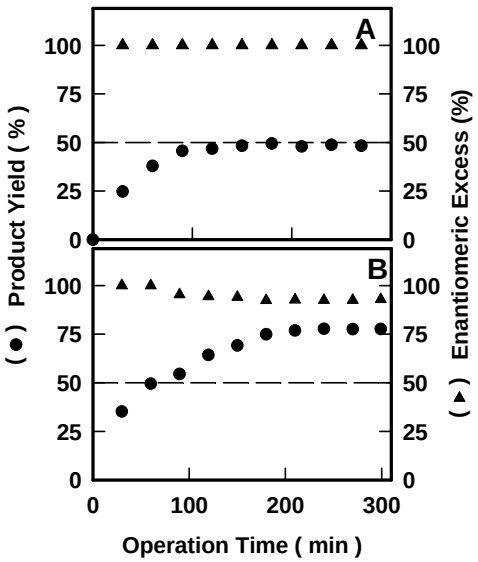

Figure 4. Profiles of *R*-1-phenylethyl propionate yield (●) and enantiomeric excess of ester products (▲) during continuous DKR of *rac*-1-phenylethanol catalyzed by (**A**) CALB-Si-C4/[Btma][NTf_2] and (**B**) CALB-Si-C4/[Btma][NTf_2] plus SCX/ [Btma][NTf_2] systems in $scCO_2$ at 50 ºC and 10 MPa

A second set of DKR experiments were carried out by using both zeolites and CALB-SILLP catalyst at a 1:1.5 (w/w) ratio, respectively, because of the lower racemization activity of zeolites with respect the SCX (see Fig 3). As can be see in Fig. 5, the combination of two columns of immobilized enzyme with one column of zeolites in the proposed reactor configuration (enzyme - zeolite - enzyme) was successful in all cases, where the yield in *R*-ester product ranged from 58 to 71 % for all the time-course profiles. It is necessary to point out how the same sample of CALB-SILLP was maintained in continuous operation for 19 d, where the enzymatic activity remained practically unchanged. This excellent operational stability was in agreement with previous results of enzymes in ionic liquids environments and enzymes immobilized onto SILLP [7,9,16]. The use of different zeolite columns resulted in changes to the ee parameter of the *R*-ester product, because of the differing ability of zeolites to catalyze the non-stereoselective transesterification reaction that produces the *S*-ester. The use of CP811C zeolite reduced the e.e. of *R*-ester product to 92 %, while the best results were obtained for both CBV720 and CP811E zeolites that reached the maximum level in e.e. (100 %).

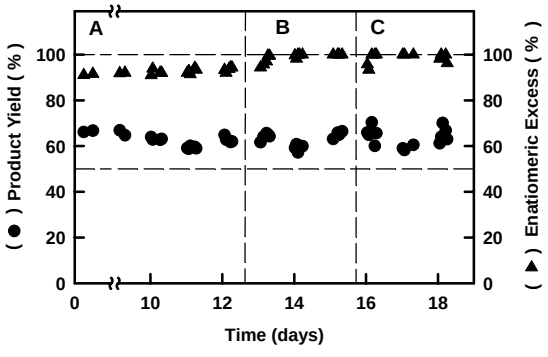

Figure 5. Profiles of *R*-1-phenylethyl propionate yield (●) and enantiomeric excess of ester products (▲) during continuous DKR of *rac*-1-phenylethanol catalyzed by CALB-SILLP and zeolytes CP811C (A), CBV720 (B) and CP811E (C) catalysts in $scCO_2$ at 50 ºC and 10 MPa

Furthermore, the presence of propionic acid was observed in the $scCO_2$ flow when a acid catalyst column was included into the reactor configuration. In spite of the dryness of $scCO_2$ and the coating of these catalyst particles (*e.g*. SCX, zeolites) with the water-immiscible IL [Btma][NTf_2], a residual water content into the porous structure of the catalyst could be the involved in this undesired activity. This shows the necessity for a strict control of the water content of these materials.

As a conclusion, it was found how an IL shell placed around the biocatalyst particle provides a good microenvironment for enzyme activity in $scCO_2$ (Fig. 2A). Furthermore, the covalent attachment of ILs onto a macroporous monolithic material then used for enzyme immobilization is a new way to perform biocatalytic reactions in IL environments, maintaining all the advantages of these neoteric solvents for continuous processes in $scCO_2$ (Fig. 2B). The combination of catalytic steps in continuous operation has been shown as an efficient way to improve synthetic processes. The enormous potential of multi-enzymatic and/or multi-chemoenzymatic processes in ILs/SCFs for synthesizing pharmaceuticals drugs may be open for the green chemical industry.

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