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Continuous green biocatalytic processes using ionic liquids and supercritical carbon dioxide

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Soluble *Candida antarctica* lipase B dissolved in ionic liquids showed good synthetic activity, enantioselectivity and operational stability in supercritical carbon dioxide for both butyl butyrate synthesis and the kinetic resolution of 1-phenylethanol processes by transesterification.

The use of enzymes in organic solvents rather than in aqueous media greatly enhances their technological applications.[1] However, volatile organic solvents (vos) have a detrimental impact on the environment and human health, and it is necessary to develop green and clean reaction media before scaling-up biocatalytic processes of industrial interest. Neoteric solvents, *e.g.* ionic liquids and supercritical fluids, seem to be promising alternative because of their physical and chemical characteristics.[2] Room temperature ionic liquids (ILs), have been shown to be good solvents for many chemical[3–5] and biochemical[6] processes, and to have an exceptional ability to stabilize enzymes during continuous operation.[7] Supercritical CO_2 ($scCO_2$) has been widely described as an excellent solvent for hydrophobic compounds, and is billed as clean technology for a range of industrial extractive processes.[8] However, $scCO_2$ has an adverse effect for enzymatic catalysis, because the enzymes exhibit important deactivation phenomena probably due to local pH changes caused by the CO_2, or conformational changes produced during the pressurization/depressurisation steps.[9]

In this paper, we propose a new concept for continuous biphasic biocatalysis, where a homogeneous enzyme solution is immobilized in one liquid phase (working phase) and substrates and products reside largely in a supercritical phase (extractive phase). This concept is illustrated as follows: an aqueous solution of *Candida antarctica* lipase B (CALB) dissolved in pure 1-ethyl-3-methylimidazolium triflimide ([EMIM][Tf_2N]) or 1-butyl-3-methylimidazolium triflimide ([BMIM][Tf_2N]), was used as biocatalyst. Both the synthesis of butyl butyrate from vinyl butyrate and butan-1-ol, and the kinetic resolution of *rac*-1-phenylethanol by transesterification with vinyl propionate were selected as reaction models. In all cases, the synthetic activity of each enzyme-IL system was continuously tested by operation/storage cycles. During the operation period (4 h d^{-1}) an ISCO 220SX high pressure extraction apparatus was used as a continuous packed bed reactor in the selected conditions (Fig. 1). The packed bed reactor was stored (20 h d^{-1}) under dry conditions over P_2O_5 in a dessicator at room temperature.†

Table 1 summarizes the activity and stability parameters of the CALB–[BMIM][Tf_2N] system for butyl butyrate synthesis under different $scCO_2$ conditions. As can be seen, lipase was able to catalyse the transesterification reaction in all the assayed conditions, the specific activity increasing with temperature; the high selectivity (>95%) observed was due to the low water content (< 4% v/v) used, giving a conversion degree higher than 50%. No activity was observed in the absence of enzyme. The increased selectivity with temperature could be explained by a reduction in water solubility in the ionic liquid fraction. However, the previously mentioned adverse effect of CO_2 was also observed because the specific activity of the CALB–[BMIM][Tf_2N] system with $scCO_2$ was 10-fold lower than that observed without $scCO_2$.[7] The increase in synthetic activity

Table 1 Activity and operational stability parameters of free *Candida antarctica* lipase B dissolved in [BMIM][Tf_2N] for continuous butyl butyrate synthesis in $scCO_2$

Temp./°C	Pressure/ MPa	CO_2 density/g mL^{-1}	Specific activity (*U*/ mg Enz	Selectivity[a] (%)	Half-life time (cycles)[b]
40	15.0	0.792	44 ± 2.1	96 ± 0.8	284
50	12.5	0.618	62 ± 3.8	98 ± 0.5	101
100	15.0	0.336	71 ± 3.9	99 ± 0.9	12

[a] Ratio between synthetic and acyl-donor consumption rates. [b] Cycles: operation (4 h) followed by storage (20 h, 25 °C).

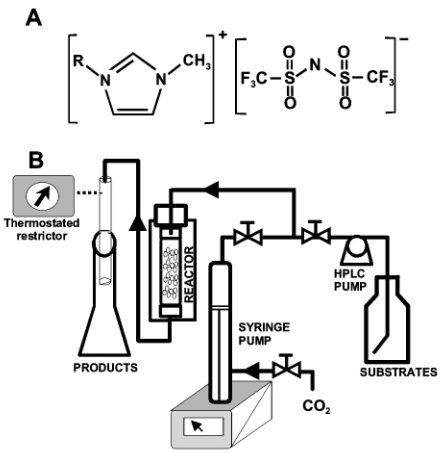

Fig. 1 **A**. Structure of 1-alkyl-3-methylimidazolium triflimide, obtained according to Lozano *et al.*[7] **B**. Experimental set-up.

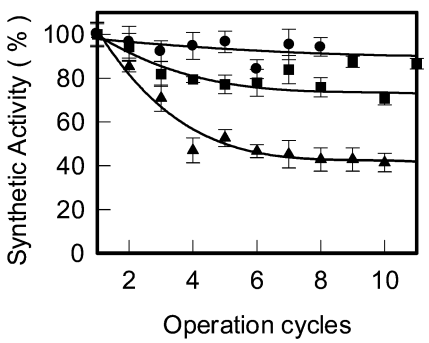

Fig. 2 Deactivation profiles of *C. antarctica* lipase B in [BMIM][Tf_2N] for continuous butyl butyrate synthesis in $scCO_2$ under different conditions (●, 40 °C and 15.0 MPa; ■, 50 °C and 12.5 MPa; ▲, 100 °C, 15.0 MPa).

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Table 2 Activity and operational stability parameters of free *Candida antarctica* lipase B dissolved in ionic liquids for continuous (*R*)-1-phenylethyl propionate synthesis in $scCO_2$ at 15 MPa

Ionic liquid	Temp.°C	Specific activity (*U*/mg Enz.)	Selectivity (%)	Ee (%)	Half-life time (cycles)
[EMIM][Tf_2N]	50	1.6 ± 0.3	86.3 ± 1.3	>99.9	24
	100	1.1 ± 0.1	95.2 ± 1.5	>99.9	16
[BMIM][Tf_2N]	50	1.7 ± 0.2	84.8 ± 3.2	>99.9	22
	100	0.6 ± 0.1	88.1 ± 4.6	>99.9	8
None[a]	50	0.2 ± 0.02	81.5 ± 2.9	>99.9	10

[a] Lyophilised powder of aqueous enzyme solution adsorbed on Celite.

with increasing temperature could also be related to a reduction in $scCO_2$ density. Almeida *et al.*[9] also reported enhanced activity of immobilized CALB (Novozyme) with decreasing $scCO_2$ densities, which they attributed to a reduction in the adverse effects produced by the enzyme solvation with CO_2 molecules. Blanchard *et al.*[6] reported that $scCO_2$ can dissolve (up 0.6 mole fraction for [BMIM][PF_6]) in the IL phase, and so a decrease in $scCO_2$ density could improve the transfer-rate of substrates to the enzyme microenvironment, thus favouring the enzyme action. Fig. 2 depicts activity loss profiles obtained when the CALB–[BMIM][Tf_2N] system was reused in the different supercritical conditions. As can be seen, activity decay was enhanced by the increase in temperature. In all cases, enzyme deactivation followed first-order kinetics. Table 1 shows the half-life time of the enzyme. The protective effect of the IL against thermal and solvent denaturation was clearly observed because the enzyme showed practically the same half-life time with and without $scCO_2$. Additionally, the enzyme exhibited interesting activity and stability levels at 100 °C, a clearly denaturative condition in free $scCO_2$ reaction medium.[7]‡

The synthetic activity of two CALB-IL ([EMIM][Tf_2N] and [BMIM][Tf_2N]) systems in $scCO_2$ was also tested for an enantioselective reaction (see Table 2). As can be seen, all the assayed conditions were able to catalyse the racemic resolution of *rac*-1-phenylethanol. However, the synthetic activity of the enzyme and selectivity of this reaction were lower than in the case of butyl butyrate synthesis. These results were the consequence of a drop in nucleophilic power from butan-1-ol to *rac*-1-phenyltheanol due to the primary position of the hydroxy group in the former and the secondary position in the latter. Furthermore, the suitability of this reaction system operating in a continuous way was demonstrated by the high enantioselectivity exhibited by the enzyme, because the (*S*)-1-phenylethyl propionate isomer was never detected, and the (*R*)-1-phenylethyl propionate isomer was always obtained at a conversion degree higher than 35%. Additionally, the half-life times of these CALB-ILs systems for continuous reuse are shown in Table 2, where it can be seen that they are lower than those observed for butyl butyrate synthesis. Furthermore, it seems that the increase in polarity of IL caused by shortening the alkyl chain on the N3 of the imidazolium ring slightly enhances all the activity and operational stability parameters, probably due to a more adequate microenvironment for the enzyme. Once again, the protective effect of ILs towards enzyme deactivation by temperature and/or CO_2 was demonstrated by the observed decrease in activity and stability of CALB, when it was assayed in the absence of IL.

In conclusion, this work clearly demonstrates the exciting potential of combining ILs with $scCO_2$ for carrying out synthetic biocatalytic processes in anhydrous conditions. ILs provide the enzyme with an adequate microenvironment, allowing high activity, enantioselectivity and stability, together with their possible continuous reuse. Supercritical CO_2 is seen as a suitable solvent for the efficient transport of substrates and easy product recovery. This paper shows how adequately designing the enzyme microenvironment, green and clean enzymatic biotransformations of industrial interest can be carried out.

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Notes and references

† Typical experimental procedure for butyl butyrate synthesis: the soluble enzyme (0.6 mg/65 μL water) was dissolved in 2 mL [BMIM][Tf_2N], and then introduced into the 10 mL cartridge of an ISCO 220SX high pressure extraction apparatus, containing 3 g of glass wool. Reactions were carried out by continuously pumping substrate solution (0.38 M vinyl butyrate and 0.76 M butan-1-ol in hexane) at 0.1 mL min^{-1}, which was mixed with the $scCO_2$ flow of the system. Substrates and products were fully soluble in $scCO_2$, and the reaction mixture was recovered by depressurising through a calibrated heated restrictor (1 mL min^{-1}, 70 °C) every 30 min. Samples were analysed by GC.[7]

‡ *Typical experimental procedure for kinetic resolution of rac-1-phenylethanol*. The soluble enzyme (1.3 mg/150 μL water) was dissolved in 4 mL ionic liquid, and then introduced into the 10 mL cartridge of a ISCO 220SX high pressure extraction apparatus, containing 3 g Celite. The enzyme without ionic liquid was prepared by adsorption of the aqueous enzyme solution (1.3 mg/4 mL) in the same amount of Celite, and then lyophilising. Reactions were carried out by continuously pumping substrate solution (50 mM vinyl propionate and 100 mM *rac*-1-phenylethanol in hexane) at 0.1 mL min^{-1}, and mixed with the $scCO_2$ flow of the system. Samples, containing 10 mM butyl butyrate as internal standard, were analysed by GC using a capillary Beta DEX-120 column (30 m × 0.25 mm × 0.25 μm, Supelco) and a FID, under the following conditions: carrier gas (He) at 1 MPa (205 ml min^{-1} total flow); temperature programme: 60 °C, 10 °C min^{-1}, 130 °C; split ratio, 100:1; detector, 300 °C. Other details are included in the above protocol.

One unit of activity was defined as the amount of enzyme that produced one micromole of product per min.

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