BIOCATALYSIS IN FLUOROUS SYSTEMS AND SUPERCRITICAL CARBON DIOXIDE

Benaissi, K.*, Thomas, N.R., Poliakoff, M.

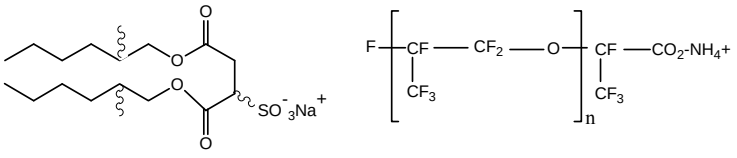
Clean Technologies Group –School of Chemistry
University of Nottingham, University Park, NG7 2RD – Nottingham, U.K.

The activities of solubilised and immobilised enzymes were investigated in fluorous biphasic system (FBS) and supercritical carbon dioxide ($scCO_2$) batch reactors. The kinetic resolution (KR) and dynamic kinetic resolution (DKR) of racemic 1-phenylethanol with an acyl donor were catalysed by *Pseudomonas cepacia* lipase (PCL), either as the suspended native protein (native PCL) or immobilised on ceramic particles (commercial lipase Amano PS CI) in $scCO_2$. These results were compared to similar experiments carried out in hexane. The immobilised lipase PS CI shows excellent activities (48-49 %) and enantioselectivities (98-99 %) to the desired (*R*)-phenylethyl acetate after 2.5 hours reaction at 40 °C in $scCO_2$. Its activity appeared to be increased compared to the reaction carried out in hexane under comparable conditions. Unlike native PCL, the immobilised lipase PS CI was then successfully reused to catalyse the KR with no loss of activity or selectivity observed over four cycles. Transesterification of *N*-acetyl-L-phenyl alanine ethyl ester (APEE) with 1-butanol or 2-butanol was catalysed by protease α-chymotrypsine (CMT) in hexane-perfluoromethylcyclohexane (PFMC) or $scCO_2$ at 40 °C for two hours. Hydrophobic ion pairing (HIP) was used to extract the protein into PFMC using the fluorinated surfactant KDP 4606 and under comparable conditions, the activity of the solubilised protease CMT-KDP in PFMC (6-10 %) was shown to be significantly higher than that of the suspended protease (1-3 %) in both hexane-PFMC and $scCO_2$. CMT-KDP was then successfully reused over four cycles with no loss of activity. The particle size of the solubilised CMT-KDP in PFMC was estimated using dynamic light scattering (DLS) to 25 nm which suggested the formation of an active protein aggregate (of about 100 molecules).

INTRODUCTION

Biocatalysis in non-aqueous media has attracted considerable interest in recent years because fine chemicals such as lipids, esters, acids and alcohols[1] used as starting materials for the commercial production of important pharmaceuticals, cosmetics and food products, are often poorly soluble in water.[2] In addition the field of "green" chemistry, which aims at performing reactions in a more environmentally friendly manner, has recently seen an increase of interest. [1] For these reasons, one can easily see that the combination of these two research areas have the potential to be synergistic. Different strategies[3,4] aimed at increasing the stability of enzymes have been employed to enhance enzymatic activity in organic media, however some of these methods may lead to a loss of enzymatic activity.[4] One of the challenges of using enzymes for organic synthesis is to produce catalysts that are stable in non-aqueous media. [5] Enzymes immobilised on solid supports have demonstrated stability in non-aqueous media and can also be easily recycled, hence these were chosen for enantioselective heterogeneous catalysis[4] in supercritical carbon dioxide ($scCO_2$). Besides, the use of enzymes solubilised in fluorous solvents for homogeneous reactions with organic

substrates in a fluorous biphasic system (FBS) as well as the potential for easy recycling of the catalyst solubilised in the fluorous phase has only been realised recently.[4-6] The technique of hydrophobic ion pairing (HIP) has previously been used to solubilise proteins in organic solvents by forming ion pairs with anionic organic surfactants such as AOT (see Figure 1 for structure) [3] but the scope of application of this method to fluorinated surfactants such as KDP 4606 (see figure 1 for structure) is still to be investigated. The objectives of this research were to investigate the activity and selectivity of different forms of enzymes for reactions carried out in $scCO_2$ and FBS. Thus, the activity of the HIP-complexes formed was studied for homogeneous catalysis in both FBS and $scCO_2$.



Aerosol-OT (AOT), MW = 444

KDP 4606 (n = 7, MW = 1400)

Figure 1: Structures of the anionic surfactants sodium bis (2-ethylhexyl) sulfosuccinate (Aerosol-OT, AOT) and KDP 4606 (Dupont).

MATERIALS AND METHODS

Materials

Lipase from *Pseudomonas cepacia* (PCL, 40 U/mg) was purchased from BioChemika. Lipase from *Pseudomonas cepacia* immobilised on ceramic particles (Amano PS CI 1,000 U/g) and protease α-chymotrypsine were obtained from Aldrich. Chemicals *rac*-1-phenylethanol, N-acetyl-L-phenyl alanine ethyl ester (APEE), n-butanol and 2-butanol were purchased from Aldrich. Vinyl acetate and surfactant sodium bis (2-ethyl-2hexyl)succinate (AOT) were purchased from Fluka. Fluorinated surfactant KDP 4606 was donated by Dupont. Perfluoromethylcyclohexane (PFMC) was purchased from Fluorochem. All organic solvents were purchased at the highest purity available from Aldrich. All commercially available reagents and solvents were used without further purification. High purity carbon dioxide was purchased from CryoService. Gases hydrogen, helium and air were purchased from BOC Gases.

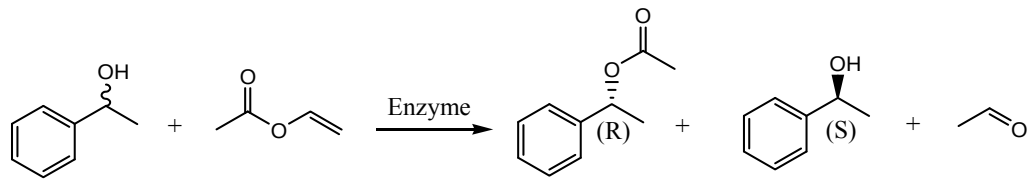
Methods

Enzymatic reactions in organic solvents were carried out in (2 or 5 mL) septum-sealed Supelco glass micro reaction vessels equipped with magnetic stirrer under stirring at 500 rpm in a silicon oil bath thermostated to the indicated temperature using a Yellowline MST basic hot plate equipped with a Yellowline TC1 thermostat. Reactions in $scCO_2$ were performed in a stainless-steel high pressure batch reactor specially designed at the University of Nottingham. The batch reactor (autoclave) equipped with a magnetic stirrer had an internal volume of 8.5 mL and was placed on an IKA Labotecknick RCT basic stirring plate. Solid substrates and reactants and stirrer bar were added into the reactor prior to sealing using a clamp. Liquid substrates and reactants were added using a Gilson Pipetman micropipette into the autoclave then sealed using a safety valve. Heater band, thermocouple, input and output pressure were then connected to the reactor to enable temperature and pressure control of the system. The system was then heated to the desired temperature and liquid CO_2 was pumped into the reactor using high pressure MWA PM-103 Pickel pump until the desired pressure was

achieved. Gas leaks were checked using inert liquid Snoop. The reaction mixture was stirred for the period of time required and the system was then depressurised with dry ice and acetone. The resulting mixture was dissolved in acetone (3 mL) and centrifuged for two min at 8000 rpm. Aliquots (50 μL) were diluted in acetone (1 mL) and substrates and products were analyzed by gas-liquid chromatography (GLC) using a Shimadzu GC 2010 chromatograph equipped with a Shimadzu AOC-20Si autosampler and using helium as a carrier gas. The structure of products was further identified using GLC and GLC-MS by comparing the retention times and fragmentation patterns with those of authentic samples. GLC-MS was performed using a thermo Finnegan Polaris-Q trap GC-MS equipped with a DB- 5 (30 x 0.25 x 0.25 μm film thickness) fused silica column. Helium was used as a carrier gas and sample ionisation was carried out using electron impact (EI) at 70 eV.

Kinetic resolution of *rac*-1-phenylethanol

The transesterification of *rac*-1-phenylethanol with vinyl acetate catalysed by *Pseudomonas cepacia* lipase (PCL, MW = 39,000) to give *(R)*-1-phenylethyl acetate and acetaldehyde was studied at 40 °C for 2.5 hours in $scCO_2$ and compared to that in hexane. The reaction studied is shown in Scheme 1.



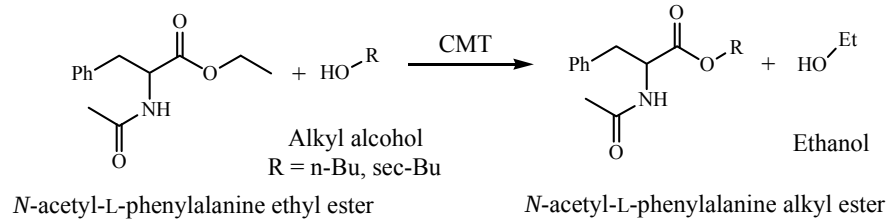
rac-1-phenylethanol vinyl acetate *(R)*-1-phenylethyl acetate *(S)*-1-phenylethanol acetaldehyde

Scheme 1: KR of *rac*-1-phenylethanol (0.14 M) with vinyl acetate (0.28 M) catalysed by *Pseudomonas cepacia* lipase PCL (0.026 mM) to produce *(R)*-1-phenylethyl acetate, *(S)*-1-phenylethanol and acetaldehyde in $scCO_2$ (8.5 mL) and hexane (5 mL) at 40 °C for 2.5 hours.

Substrates r*ac*-1-phenylethanol (1.2 mmol, 142 μL) and vinyl acetate (2.4 mmol, 220 μL) were added to the immobilised enzyme PS CI (0.00022 mmol, 8.5 mg) in the batch reactor (8.5 mL) then pressurised with CO_2 to 100 bar. The system was then heated to 40 °C and the reaction mixture stirred for 2.5 hours. Comparable reactions were set up in hexane (5 mL) and all experiments were performed in triplicate. Aliquots were collected and analyzed as described above. Yields and enantioselective excesses (ee) of substrates and products were estimated using GLC equipped with Supelco Betadex 110 (30 m x 0.25 mm x 0.25 μm film thickness) chiral column.

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with *n*-butanol or 2-butanol was catalysed by protease α-chymotrypsine (CMT, MW = 51,000) either the solubilised protein (CMT-AOT in hexane or CMT-KDP in PFMC) in hexane, hexane-PFMC v/v 1:1 or $scCO_2$; or the suspended native in either solvent at 40 °C for two hours. The reaction studied is shown in Scheme 2.



N-acetyl-L-phenylalanine ethyl ester *N*-acetyl-L-phenylalanine alkyl ester

Scheme 2: Transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) (0.0042 M) with *n*-butanol or 2-butanol (0.45 M) was catalysed by protease α-chymotrypsine (0.01 mM) in hexane or hexane-PFMC v/v 1:1 (2 mL) or $scCO_2$ (8.5 mL) at 40 °C for two hours.

Protease CMT (0.00017 mmol, 1 mg) solubilised in PFMC (1 mL) was added to the substrates *N*-acetyl-L-phenylalanine ethyl ester (APEE) (0.0085 mmol, 2 mg) and *n*-butanol (0.9 mmol, 80 µL) or 2-butanol (0.9 mmol, 80 µL) in hexane or hexane-PFMC v/v 1:1 (2 mL). Comparable reaction was set up in $scCO_2$ 100 bar (8.5 mL) and the system was left stirring at 40° C for two hours. Aliquots were then collected and analyzed as described above. Yields of products were estimated using GLC equipped with RTX5-FAST column (fused silica, crossbond 5 % diphenyl/95 % dimethyl polysiloxane, 10 m x 0.1 mm x 0.1 µm) from Restek.

Dynamic light scattering (DLS)

DLS measurements were carried out at 25 ˚C on a Zetasizer Nano S at Malvern Instruments. The instrument contains a 4 mW He-Ne laser operating at a wavelength of 633 nm. The particle size was taken as a mean value of three measurements. CMT dissolved in aqueous buffer; AOT and CMT-AOT dissolved in hexane; and CMT-KDP and KDP dissolved in PFMC were measured at 0.02 mM, 0.7 mM and 2.3 mM respectively. The data was analyzed with the following fixed parameters: refractive index 1.333, 1.372, 1.277, 1.39., 1.4 and 1.45 (water, hexane, PFMC, AOT, KDP and protein respectively) and viscosity (cP) 1.0019, 0.294 and 1.561 (water, hexane and PFMC respectively).

RESULTS

Kinetic resolution of *rac*-1-phenylethanol

The catalytic activities of PCL suspended native enzyme and PCL immobilised enzyme PS CI was examined for the kinetic resolution (KR) of *rac*-1-phenylethanol carried out for 2.5 hours in $scCO_2$ compared to hexane. The results comparing the activities of the two enzyme forms are represented in Figure 2.

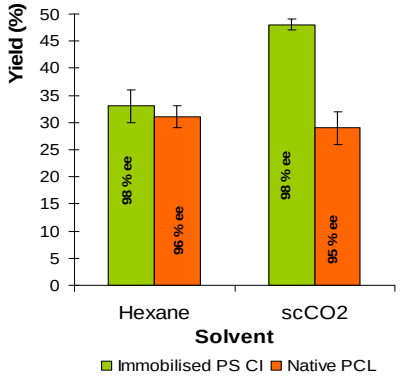


Figure 2: KR of *rac*-1-phenylethanol (0.14 M) with vinyl acetate (0.28 M) catalysed by PCL (0.026 mM) either immobilised (PS CI) or suspended (native PCL) in hexane and $scCO_2$ 100 bar at 40 °C for 2.5 hours. Yields and *(R)*-ester enantioselective excesses (ee) were estimated by GLC equipped with chiral Betadex 110 column.

The immobilised enzyme PS CI exhibited high activities and enantioselectivities for the kinetic resolution of *rac*-1-phenylethanol with vinyl acetate at 40 °C. Excellent yields (48 %) were obtained after only 2.5 hours reaction in $scCO_2$ whereas only 33 % of (R)-ester was produced under comparable conditions in hexane. This higher activity observed in $scCO_2$ might be due to a change in substrate solubility in $scCO_2$ thus leading to a better mass transfer from substrates to enzyme. The immobilised enzyme PS CI also exhibited greater activity than the suspended native enzyme in $scCO_2$ which might due to less aggregation of the immobilised enzyme in $scCO_2$. The reusability of the immobilised lipase PS CI for the KR of 1-phenylethanol catalysed in FBS and $scCO_2$ was also investigated and the activity and enantioselectivity of the enzyme was compared to that of the suspended native enzyme. The results achieved are depicted in Figure 3.

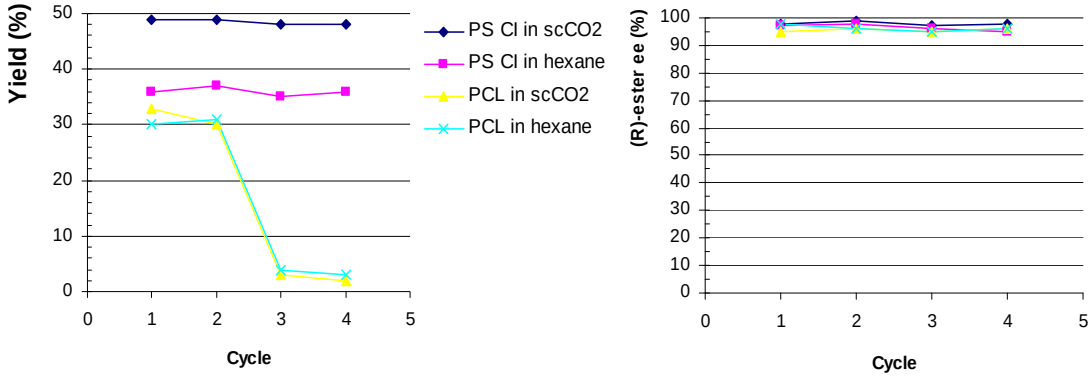


Figure 3: Recycling of PCL either immobilised PS CI or native PCL for the KR of *rac*-1-phenylethanol in hexane and in $scCO_2$ 100 bar at 40 °C for 2.5 hours. Yields (left) and *(R)*-ester enantioselective excesses (ee) (right) were estimated by GLC equipped with chiral Betadex 110 column.

Unlike the suspended native PCL, the immobilised lipase PS CI showed great stability in both hexane and $scCO_2$ since it was successfully reused for the KR of *rac*-1-phenylethanol over four cycles. Also, it is worth noting that both the activity and the enantioselectivity of the immobilised lipase did not appear to be affected by the pressurisation and depressurisation procedures for the reaction carried out in $scCO_2$.

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester

The activity of the protease α-chymotrypsine solubilised in hexane (CMT-AOT) and in PFMC (CMT-KDP) for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with n-butanol or 2-butanol was investigated. The activity of the solubilised protein was compared to that of the suspended native protease (native CMT) and control reactions with only the AOT in hexane or KDP in PFMC were also set up. The results are shown in Figure 4.

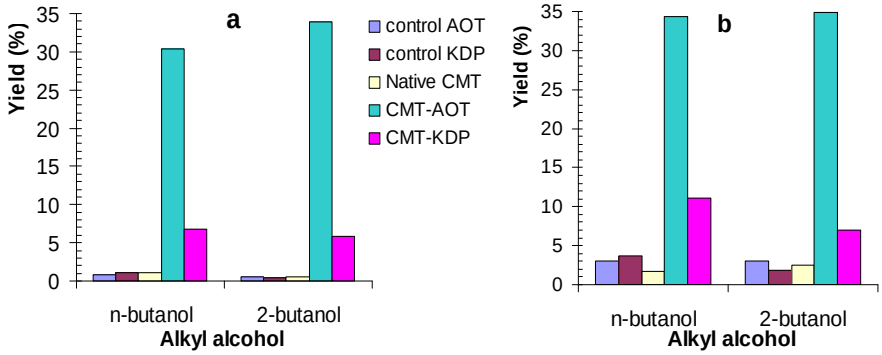


Figure 4: Transesterification of APEE (0.0042 M) with either n-butanol or 2-butanol (0.42 M) catalysed by native CMT or solubilised CMT-AOT or CMT-KDP (0.01 mM) at 40 °C with stirring for two hours in the FBS hexane-PFMC v/v 1:1 in 2 mL septum sealed glass vessel (a) or in $scCO_2$ 100 bar in 8.5 mL high pressure stainless steel batch reactor (b). Yields were estimated by GLC equipped with RTX-5 Fast column.

The HIP solubilised protease CMT exhibited higher activity than the native one for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with the two alcohols tested in both FBS and $scCO_2$. Under homogeneous conditions (hexane-PFMC and $scCO_2$), the dispersion of the enzyme-surfactant complexes into the reaction medium as well as the contact between the enzyme and the substrates are believed to be increased, thus enhancing the yield of the reaction.[31] With both alcohols tested, the yields to the ester products appeared to be slightly higher in $scCO_2$ compared to hexane-PFMC which might be due to a better mass transfer from substrates to enzyme in $scCO_2$. Also CMT-AOT in hexane appears to be around three times more active than CMT-KDP in PFMC and this trend was observed in both $scCO_2$ and hexane-PFMC. It is believed that the protein-surfactant complexes formed adopt an active configuration in both media tested and that the difference in activity observed between CMT-AOT and CMT-KDP might be due to a difference in conformation of the HIP complexes formed. To gain a better understanding of the difference observed, DLS was performed to determine the particle size of the complexes formed. The reusability of CMT-KDP dissolved in PFMC was then investigated for the transesterification of APEE with n-butanol or 2-butanol in the FBS hexane-PFMC and in $scCO_2$ 100 bar for 2 hours at 40 °C. The results achieved are depicted in Figure 5.

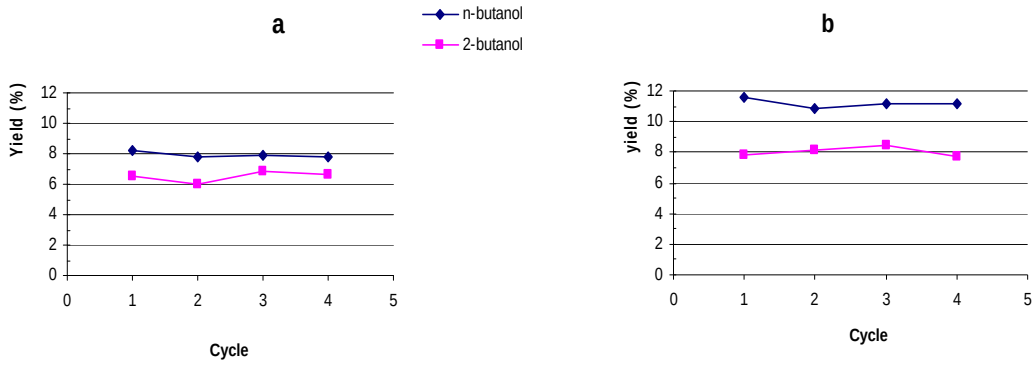


Figure 5: Recycling of CMT-KDP in PFMC for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with n-butanol or 2-butanol in the FBS hexane-PFMC (a) and in $scCO_2$ 100 bar (b) at 40 °C for 2 hours. Yields of were estimated by GLC equipped with RTX5-Fast column.

The solubilised protease CMT-KDP in PFMC was successfully reused for the transesterification of APEE with both alcohols tested and no loss of activity was observed over four cycles in the FBS hexane-PFMC and in $scCO_2$. CMT-KDP in PFMC exhibited a high stability in both media investigated; in particular its activity in $scCO_2$ did not appear to be affected by the pressurisation and depressurisation procedures.

Dynamic light scattering (DLS)

The particle size of CMT solubilised in pH 6.9 buffer, CMT-AOT in hexane and CMT-KDP in PFMC were estimated using DLS; results are shown in Figure 6.

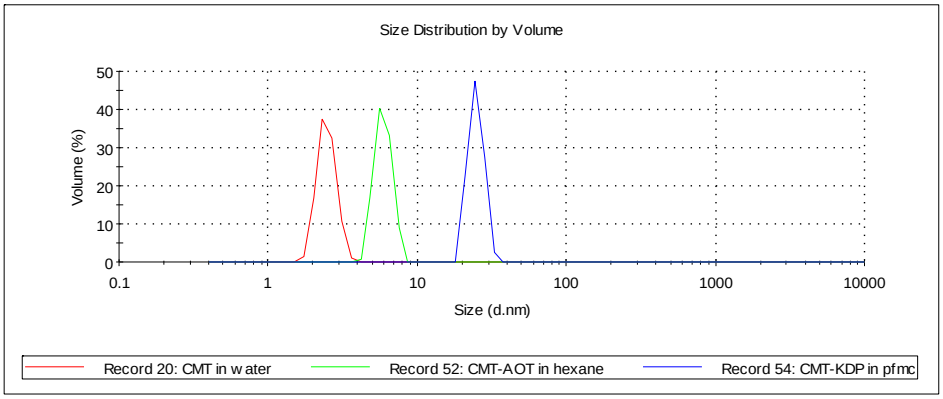


Figure 6: Particle size of CMT in buffer, CMT-AOT in hexane and CMT-KDP in PFMC estimated using DLS.

The particle size of CMT dissolved in aqueous buffer was estimated to 2.5 nm; this was assumed to be the size of a single molecule of CMT. The size of CMT-AOT in hexane was found to be around 6.0 nm which is equivalent to the size of a single molecule of CMT (2.5 nm) and two molecules of AOT (size of AOT 1.2 nm[3]). This suggests the formation of a single CMT particle surrounded by the surfactant AOT in hexane. CMT-KDP in PFMC was found to have a size of 25 nm which would correspond to the formation of a protein aggregate of about 100 molecules surrounded by the surfactant KDP in PFMC. This difference in the particle size might explain the highest activity observed for CMT solubilised in hexane compared to PFMC.

CONCLUSION

The kinetic resolution of *rac*-1-phenylethanol catalysed by immobilised enzymes was investigated in $scCO_2$. Excellent conversions and enantioselectivities were achieved using the immobilised lipase PS CI at 40 °C in 2.5 hours in $scCO_2$. In comparable conditions, the conversions and enantioselectivities of the reaction were significantly higher than the ones in hexane. Also, we have shown that protease α-chymotrypsine solubilised in either hexane or PFMC via hydrophobic ion pairing (HIP) exhibits higher activities than the native one for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester in both FBS and $scCO_2$. Future work will include the use of immobilised enzymes for DKR in a $scCO_2$ continuous flow reactor and the study HIP-solubilised enzymes for enantioselective reactions in both FBS and $scCO_2$ batch reactor.

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