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Enzymes in non-conventional environments

Enzymes are Nature's catalysts. They have evolved over millions of years to perform their tasks, often with exquisite precision, in water at physiological pH and ambient temperature and pressure. While these conditions are ideal for many of the natural reactions that are catalysed by enzymes *in vivo*, they are not always favourable for many synthetic biotransformations *in vitro*. Many organic substrates are sparingly soluble or insoluble in water and some processes, *e.g.* esterification, are not feasible in water (at least in the dissolved state) owing to unfavourable equilibria.

Although it has been known for more than a century that some enzymes could perform their tasks in non-aqueous media, it was the pioneering work of Klibanov in the mid-1980s that drew attention to the use of enzymes for performing a variety of biotransformations in essentially anhydrous organic solvents. This stimulated a surge in research on the use of enzymes in organic synthesis which continues to this day and has resulted in numerous commercial applications. Many enzymes are not only stable in the non-natural environment of organic solvents they are more stable than in water. On the other hand, rates are generally much lower compared to those in aqueous media. Furthermore, the use of many volatile organic solvents is under considerable environmental pressure which has stimulated research on the use of non-conventional media for performing atom efficient catalytic conversions, including bioconversions. These alternative solvents should be relatively non-toxic and non-hazardous, readily separated from the biocatalyst and the product, and recyclable. Added benefits could be enhanced activity, selectivity and stability compared to conventional organic solvents. Hence, there is currently much attention being focused on the use of *e.g.* ionic liquids and supercritical carbon dioxide and mixtures thereof, for conducting biotransformations. This special issue of *Green Chemistry* is devoted to various aspects of this important topic.

The use of enzymes in supercritical carbon dioxide is reviewed by Nakamura and coworkers.[1] Barreiros and coworkers compare the results of conducting an enzymatic transesterification in various non-conventional media: ionic liquids, supercritical ethane, supercritical carbon dioxide and hexane.[2] Other reports concern enhanced enantioselectivities and rates of lipase-catalysed transesterifications in some novel ionic liquids (Itoh *et al.*[3]) and chemoenzymatic dynamic kinetic resolution of alcohols in ionic liquids (Kim *et al.*[4]). An investigation of the lipase-catalysed synthesis of a key intermediate for the drug, Lotrafiban, in ionic liquids is reported by Lye and coworkers.[5] The results compared favourably with those obtained in the industrial process in *tert*-butanol. Sometimes enzymes dissolve in ionic liquids, generally with loss of activity. The dissolution of *Candida antarctica* lipase B in a variety of ionic liquids was followed by FT-IR spectroscopy, showing that loss of activity correlated with denaturation of the enzyme. In contrast, when the enzyme retained its activity on dissolution denaturation had not occurred (Sheldon *et al.*[6]).

The use of enzymes or whole cells at solid–gas interfaces offers interesting benefits, such as high rates and minimum plant sizes, compared to the standard liquid systems. An overview of solid–gas biocatalysis is presented by Legoy and coworkers.[7] Another interesting approach to obtaining high volumetric yields is by using solid-to-solid biocatalysis, which is presented by Ulijn and Halling.[8]

Finally, a wide variety of hyperthermophilic enzymes are now available. Their potential advantages for application in biotransformations are discussed by Kelly and coworkers.[9]

In short, biocatalysis in non-conventional media is scientifically interesting and challenging and has significant commercial potential. We hope that the papers in this special issue of *Green Chemistry* will stimulate further discoveries in this fascinating area.

Roger Sheldon
Gillian Stephens
Ken Seddon

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Highlights

Markus Hölscher reviews some of the recent literature in green chemistry

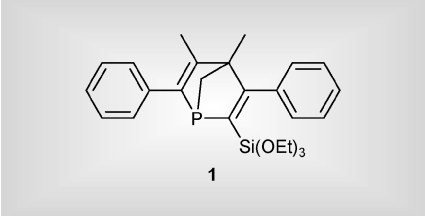
DOI:10.1039/b411901k

Gas–liquid–solid-hydrogenations in a microfluidic device

Multiphase catalytic reactions are often difficult to control because mass transfer between different phases can be very slow, resulting in low reaction rates. Increasing the interfacial area between the different phases usually helps in improving mass transfer. A new device for multiphase reactions was recently developed by Kobayashi *et al.* from the University of Tokyo (*Science*, 2004, **304**, 1305–1308). The central idea is to use a micrometer-sized channel (200 μm width, 100 μm depth, 45 cm length) whose walls are coated with an immobilized catalyst. Using appropriate flow rates and gas pressures it is possible to pass the gas through the center of the channel, whereas the liquid flows along the inner channel surface. At these dimensions molecular diffusion is fast, which should facilitate gas–liquid–solid-reactions. The authors succeeded in coating the channel walls with a polymeric Pd catalyst and by carefully choosing the flow conditions it was possible to hydrogenate a variety of different C–C double and triple bonds quantitatively with residence times of 2 min. Product isolation was accomplished in most cases by simply removing the solvent. According to ICP analyses there was no Pd metal contained in the product solutions, and the microreactors could be used several times without loss of activity.

Hemilabile P^O ligand grafted on mesoporous silicas for bifunctional catalysis

The anchoring of homogeneous catalysts to solid supports is a well known approach for the heterogenization of catalytically active metal complexes, circumventing the general problems of catalyst recyclisation and product separation. During the past decade much research has been devoted to the synthesis of mesoporous silica supports for catalysis and other fields. As knowledge has increased, this heterogenization approach can be expanded to carry out more than one reaction using the same catalyst system in a one-pot setup. Sanchez *et al.* from UMPC-CNRS, Paris, have anchored ligand **1** to two different mesoporous silica materials: one having a pore diameter of 23 Å, while the pore diameter of the second material is 60 Å (*Chem. Commun.*, 2004, 1240–1242).



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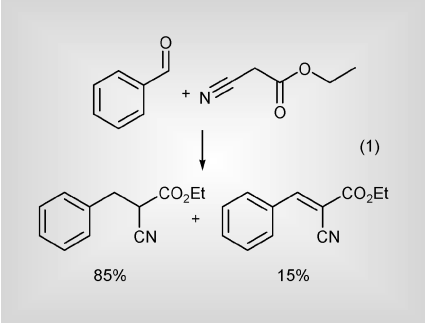
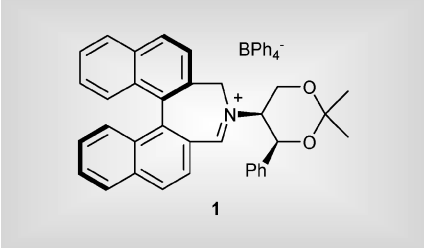
After grafting the ligand to both materials they were reacted with $[Rh(acac)(CO)_2]$ yielding active catalysts for the hydrogenation of 1-hexene. Both catalyst systems were active with the large pore catalyst showing a superior TON (>100 000) to the small pore material (TON = 5600), with TOF's of 48 and 72, respectively (cycles in min^{-1}). The hydrogenation of 1-methyl-1-cyclohexene and imines are also possible in high yields. As an example for bifunctional catalysis the authors chose the Knoevenagel condensation, which made a modified grafting procedure necessary. With this catalyst system the reaction sequence shown in (1) was possible yielding the desired product in 85% yield.



High enantioselectivities in asymmetric epoxidations with iminium salt catalysts

Chiral epoxides are important building blocks in asymmetric synthesis, which explains the interest in asymmetric epoxidations of olefins. Oxaziridinium salts were developed as early as 1976, however in epoxidations using oxone as the oxygen carrier enantioselectivities have remained low to moderate. Page *et al.* from the University of Loughborough focused on the synthesis of chiral iminium salt catalysts with significant differences in the core structure of the molecule. Their efforts yielded active and enantioselective catalysts for epoxidations of olefins (*Org. Lett.*, 2004, **6**, 1543–1546). For instance the epoxidation of 1-phenylcyclohexene with catalyst **1** resulted in the efficient formation of the corresponding (1*S*,2*S*)-configurated epoxide (yield 69%, ee 91%).



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However, their studies also made clear that both catalyst and substrate structure are highly correlated with regard to enantiomeric excess and activity, since deviations in substrate and/or catalyst structure lead either to poor ee values or to inactive catalyst systems.

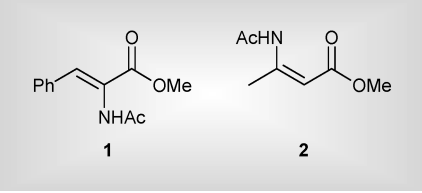
Enantioselective photoadditions of alcohols to olefins in $scCO_2$

Photochirogenesis is an interesting tool to introduce chirality into chemical reactions. Recent studies in this field have shown that both enantiomeric excesses and absolute configurations are significantly affected by temperature, pressure and solvation. Supercritical fluids are therefore interesting reaction media, since their solvent properties (viscosity, density, dielectric constant) can be tuned, which opens up an interesting field with regard to environmentally benign chemistry. Inoue *et al.* from the University of Osaka investigated among others the influence of the pressure of $scCO_2$ in photoadditions of methanol, ethanol and isopropyl alcohol to 1,1-diphenylpropene (*J. Am. Chem. Soc.*, 2004, **126**, 6568–6569). Interestingly the

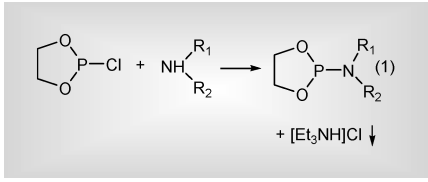
authors found that ee values were low for subcritical conditions but depend linearly on the pressure, with rising pressure yielding higher ee values (*ca.* 5 to 25% depending on the alcohol used). In contrast the ee undergoes a steep jump when the supercritical density of the solvent is reached. Increasing the pressure to higher values shows again a linear behaviour of the ee values, which increase with increasing pressures (*ca.* 20 to 43%). This behaviour has been rationalized by the enhanced clustering of the alcohol at supercritical conditions.

Asymmetric hydrogenation by *in situ*-screening using a phosphoramidite ligand library

Chiral phosphoramidites are excellent ligands for many different metal catalyzed enantioselective reactions. They can be easily synthezised which makes them ideal candidates for a parallel synthesis approach. In the search for ideal ligands for asymmetric hydrogenation of methyl 2-acetamidocinnamate 1 and *Z*-methyl 3-acetamido-2-butenoate **2**, Lefort *et al.* set up a 32-fold parallel synthesis protocol for phosphoramidite ligands and *in situ* catalyst generation/asymmetric hydrogenation of **1** and **2** (*Org. Lett.*, 2004, **6**, 1733–1735).



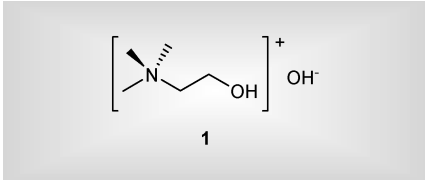
This approach is possible since a simple filtration of the resulting ammonium chloride is the only necessary purification step, due to the fact that phosphoramidite synthesis as shown in (1) goes to completion with good reliability. The authors found hydrogenation of **1** using 32 different ligands to be quantitative in most cases with ee values ranging between 91 and 95% when secondary amines are used for phosphoramidite production. Primary and tertiary amines yield considerably lower enantiomeric excesses.



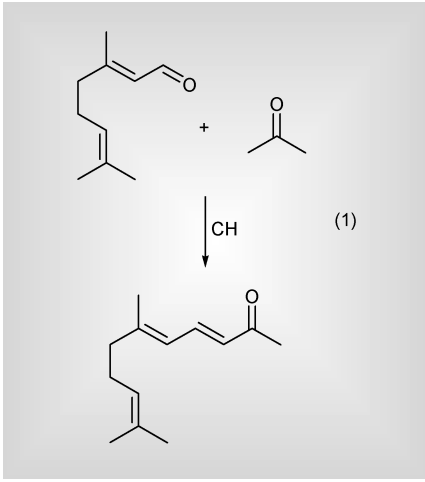
Upon hydrogenation of the more challenging substrate **2** it was found that primary aliphatic amines are best used as the amino part in the ligand (ee values ranging between 88 and 96%), whereas secondary and tertiary amines yielded ligands which were much less effective in terms of activity and enantioselectivity.

Cholin hydroxide as IL and base catalyst

Waste production, corrosion and no catalyst recovery are the most significant disadvantages of conventional Brønsted-type basic catalysis, which explains the current interest in developing green alternatives. As ionic liquids are meanwhile well established reaction media in academia it is not surprising that attempts are made to combine the solvent properties of ILs with their potential as base catalysts. Medina *et al.* from the University Rovira i Virgili, Tarragona, used choline hydroxide (CH) **1** as a base catalyst for aldol condensations and also developed a supported CH catalyst (*Chem. Commun.*, 2004, 1096–1097).



Several carbonyl compounds were subject to aldol condensations and the products obtained are interesting for pharmacological and fragrance industries. As an example the two citral isomers neral and geranial react to the corresponding pseudoionone with excellent selectivity (98%) and a conversion of 93% in 1 h (1).



When CH impregnated on MgO was used, selectivity dropped to 95% whereas conversion was slightly increased to 94%. The comparison with conventional NaOH revealed that only a conversion of 80% and a selectivity of 87% was reached, making CH the superior reaction medium/catalyst.

COMMUNICATION

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Enhanced enantioselectivity and remarkable acceleration on the lipase-catalyzed transesterification using novel ionic liquids

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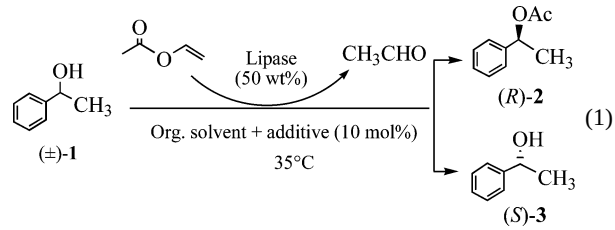
Novel imidazolium salt ionic liquids were prepared derived from polyoxyethylene(10) cetyl sulfate and used as an additive for lipase-catalyzed transesterification in organic solvent; a remarkable enhanced enantioselectivity was obtained when the salt was added in 3 to 10 mol% *vs.* substrate in the *Pseudomonas cepacia* lipase-catalyzed transesterification of 1-phenylethanol using vinyl acetate in a diisopropyl ether or hexane solvent system. There was a remarkable acceleration when the lipase was coated with this novel ionic liquid and used as catalyst for transesterification in *i*-Pr_2O or in hexane.

The value of an enzymatic reaction in organic synthesis is extensively increased by its environmentally friendly aspect.[1] Since only a limited number of lipase-catalyzed reactions are applicable for practical optical resolution, development of a strategy to improve their reaction performance is desirable.[2] Several methods are reported to enhance enzyme enantioselectivity in a nonaqueous solvent system, the most simple being the addition of proper compounds that affect the enzyme activity[2]; several compounds are reported to be effective additives: crown ethers,[3,4] inorganic salts,[5] cyclodextrin,[6] surfactants,[7] and polar solvents.[8] Lipase PS is well respected and is one of the most widely used enzymes applicable for various substrates,[1] however, it was reported that the enantioselectivity was significantly dependent on the solvent system for some substrates.[2,10] For example, excellent enantioselectivity was recorded when (±)-1-phenylethanol (**1**) was acetylated by lipase PS in toluene (Entry 1, Table 1),[7f,7g,10] while a moderate enantioselectivity was obtained when the reaction was carried out in diisopropyl ether (i-Pr_2O) as solvent (Entry 2, Table 1). We were fascinated by this interesting solvent effect in the acetylation of (±)-**1** by lipase PS, and decided to investigate regulation of the enantioselectivity by an additive in the i-Pr_2O solvent system.



(1)

To a solution of (±)-**1** (50 mg, 0.56 mmol), vinyl acetate (56 mg, 0.88 mmol), and an additive (0.56 mmol) in i-Pr_2O (2.0 ml) was added lipase PS (25 mg) and the mixture was stirred at 35 °C . The reaction course was monitored by capillary GC-analysis and silica gel TLC. Acetate (*R*)-**2**[10] and unreacted substrate (*S*)-**1**[10] was obtained by preparative silica gel thin layer chromatography (TLC). The enantioselectivity was determined by HPLC analysis on a chiral column (Daicel OB, hexane : 2-propanol = 200 : 1). We found that a significant enhanced enantioselectivity was obtained when the reaction was carried out in the presence of 10 mol% (*vs.* substrate) of 1,4,8,11-tetrathiatetracyclodecane (14-ane-S4) or dimethylsulfoxide (DMSO) (Entries 3 and 4). It was reported that modified stereoselectivity sometimes occurred when the lipase-catalyzed reaction was conducted in an ionic liquids solvent system,[10,11] and it was established that the lipase activity was dependent on the combination of the cationic part and anionic part of the imidazolium salts.[12] It was also known that some surfactants, such as polyethyleneoxy alkyl ether affected lipase reactivity,[7] so we next tested the lipase PS-catalyzed acetylation of (±)-**1** in the presence of a surfactant, polyethylenoxy(10) cetyl ether (Brij 56)[7f,13] or an ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF_4) or 1-butyl-2,3-dimethylimidazolium tetrafluoroborate ([bdmim]BF_4). Interestingly, enhanced enantioselectivity was recorded for [bdmim]BF_4 (Entry 7), while a significant reduction was obtained by addition of [bmim]BF_4 (Entry 6). On the other hand, a slight enhancement of enantioselectivity was obtained when Brij56 was added (Entry 5). These results prompted us to prepare a hybrid of Brij56 with an imidazolium salt, and to investigate its effect on the lipase activity.

Two types of imidazolium salts, [bmim][cetyl-PEG10-sulfate] (**4**) and [bdmim][cetyl-PEG10-sulfate] (**5**) were synthesized:[14] a mixture of Brij56 with ammonium amidosulfate was stirred for 17 h at 110 °C and dried under reduced pressure at 66.7 Pa at 60 °C for 3 h to give ammonium Brij56 sulfate as a white powder. This was treated with an equivalent amount of imidazolium chloride in acetone with vigorous stirring at rt for 6 h to form ammonium chloride as a white precipitate which was removed by filtration through a glass sintered filter with a celite pad. The resulting filtrate was evaporated and the residue was again dissolved in acetone and filtered through an alumina (neutral type I, activated) short column. The filtrate was evaporated and dried under reduced pressure to

Professor Toshiyuki Itoh was born in Mie, Japan in 1954. He received his PhD in Organic Chemistry from the University of Tokyo under the supervision of Professor Teruaki Mukaiyama in 1986. In 1987 he joined the Faculty of Education of Okayama University as Assistant Professor and he was promoted to Associate Professor in 1990. Between 1990 and 1991 he spent one year at Colorado State University as a post-doctoral fellow under the supervision of Professor A. G. M. Barrett. In 2002 he moved to Tottori University and he was promoted to full Professor in 2004. Four projects are now running in his group: development of enzymatic reactions using ionic liquids as reaction medium, synthesis of optically active partly fluorinated compounds, such as gem-difluorocyclopropanes and point-fluorinated insect pheromones, novel ferric ion catalyzed reactions in an ionic liquid solvent system, and asymmetric synthesis of chiral axis biaryl compounds.

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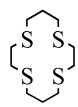
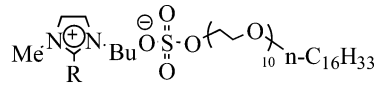
Table 1 Results of lipase-catalyzed enantioselective acetylation of (±)-1-phenylethanol (**1**) in the presence or absence of additive compounds (10 mol% *vs.* substrate)

Entry	Solvent	Additive	Time/h	Yield[a] of (*R*)-2 (ee_p,%)[b]	Yield[a] of (*S*)-3 (ee_s,%e)[b]	% Conv.[c]	*E* value[c]
1	Toluene	none	26	31 (99)	40 (76)	43	≥200[d]
2	i-Pr_2O	none	27	37 (73)	62 (42)	37	10
3	i-Pr_2O	14-ane-S4	28	34 (≥99)	58 (66)	40	≥200
4	i-Pr_2O	DMSO	28	33 (≥99)	50 (71)	41	≥200
5	i-Pr_2O	Brij56	26	15 (92)	66 (33)	26	35
6	i-Pr_2O	[bdmim]BF_4	24	15 (43)	64 (20)	31	3
7	i-Pr_2O	[bdmim]BF_4	24	16 (92)	55 (24)	21	≥200
8	i-Pr_2O	**4**	29	30 (76)	57 (35)	32	10
9	i-Pr_2O	**5**[e]	28	31 (99)	50 (≥99)	50	≥200
10	i-Pr_2O	**5**	28	33 (96)	50 (≥99)	50	≥200
11	i-Pr_2O	**5**[f]	28	16 (≥99)	50 (≥99)	50	≥200
12	Hexane	none	28	16 (85)	63 (29)	25	16
13	Hexane	**4**	29	44 (90)	36 (70)	44	40
14	Hexane	**5**	30	39 (98)	50 (≥99)	50	≥200



14-ane-S4

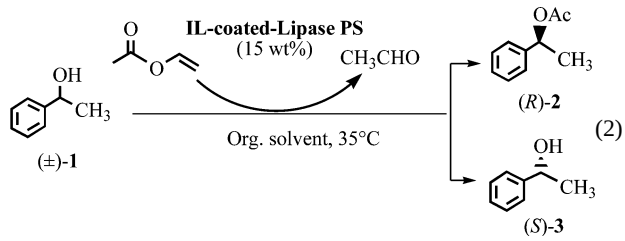


4: R= H
5: R= Me

[a] Isolated yield. [b] Determined by HPLC (Chiralcel OB, hexane : i-PrOH = 200 : 1). [c] Calculated by %ee of **2** (ee_p) and %ee of **3**(ee_s). $E = \ln[(1 - c)(1 + ee_p)]/\ln[(1 - c)(1 - ee_p)]$, here *c* means conv., which was calculated by the following formula: $c = ee_p/(ee_p + ee_s)$. See ref. 9. [d] It was reported that high enantioselectivity was also obtained using THF or acetone as solvent, while no reaction took place using DMF or DMSO as solvent (see, ref. 10). [e] 3 mol% of Brij-IL-Me was added. [f] 30 mol% of Brij-IL-Me was used as additive.

form **4** and **5** with 70 ~ 74% overall yield, respectively.[15] The salts have an amphiphilic property and dissolved easily in many types of both organic solvents and water.

There was a clear contrast in the modification properties of these two ionic liquids in the lipase PS-catalyzed reaction (Entries 7 and 8); a remarkable enhanced enantioselectivity was accomplished when the reaction was carried out in the presence of *ca.* 10 mol% (*vs.* substrate) of **5**, which possessed a methyl group at the 2-position on the imidazolium ring (Entries 10–11), while no marked change of enantioselectivity was recorded if **4**, which has a proton at the 2-position on the imidazolium ring, was used as additive (Entry 8). Ionic liquid **5** worked as an excellent additive to enhance enantioselectivity, and a high *E* value[9] was recorded even when only 3 mol% was used as additive (Entry 9). We believe it might be possible to reduce the amount of additive required for a certain enhanced activity if the reaction is conducted on a larger scale; we tested reactions using 10 mol% of the additive to obtain reproducible results on the present experimental scale. In contrast,



(2)

a slight reduction in the chemical yield of (*R*)-**2** was recorded when 30 mol% of **5** was used as additive due to the difficulty of isolating the reaction product in the presence of a large amount of **5** (Entry 11), though enantioselectivity was perfect. The salt **5** worked better than **4** for the reaction in hexane (Entries 13 and 14). Since the 2-proton of the imidazolium ring of the corresponding imidazolium salt has high acidity, **4** may work as a Brønsted acid catalyst in transesterification, thus causing the reduction in enantioselectivity.

We next prepared ionic liquid-coated lipase PS and investigated its reactivity as a catalyst for the transesterification of (±)-**1** (eqn. 2). Since commercial lipase PS was supported by Celite, we prepared [bdmim][cetyl-PEG10-sulfate]-coated lipase PS (PS-**5**) as follows: to 10 ml of 0.1 M phosphate buffer (pH 7.2) was added 1.0 g of commercial lipase PS (involving *ca.* 10 wt% of the enzyme protein), this was stirred for 30 min at rt, then centrifuged at 3500 rpm for 5 min. The resulting supernatant was mixed with 29 mg (*ca.* 3.1×10^{-2} mmol) of **5** and the solution was lyophilized to give 297 mg of PS-**5**. Celite free lipase PS (CF-PS) was prepared by lyophilization of the supernatant of a mixture of commercial lipase PS (1.0 g) in 10 ml of 0.1 M phosphate buffer (pH 7.2) after centrifugation. The results of the transesterification of (±)-**1** using these ionic liquid-coated enzymes are shown in Table 2. A remarkable acceleration was accomplished using PS-**5** while maintaining good enantioselectivity. *Reaction conversion reached 49% after just 2 h reaction* when PS-**5** was used as catalyst in i-Pr_2O (Entry 5), while it took 26 h at 15% conversion when CF-PS was

Table 2 Results of ionic liquid-coated lipase PS-catalyzed enantioselective acylation of (±)-1-phenylethanol (**1**)

Entry	Solvent	Enzyme	Time/h	Yield[a] of (*R*)-2 (ee_p,%)[b]	Yield[a] of (*S*)-3 (ee_s,%)[b]	% Conv.[c]	*E* value[c]
1	i-Pr_2O	CF-PS	26	9 (87)	72 (15)	15	17
2	i-Pr_2O	CF-PS+Brij56	26	15 (92)	66 (33)	26	35
3	i-Pr_2O	CF-PS+**5**[d]	26	21 (96)	60 (43)	33	75
4	i-Pr_2O	PS-**5**[e]	**2**	27 (94)	44 (89)	49	96
5	Hexane	PS-**5**[e]	**2**	46 (89)	46 (≥99)	53	≥90

[a] Isolated yield. [b] Determined by HPLC (Chiralcel OB, hexane : i-PrOH = 200 : 1). [c] Calculated by ee_p and ee_s. $E = \ln[(1 - c)(1 + ee_p)]/\ln[(1 - c)(1 - ee_p)]$, here *c* means conv., which was calculated by the following formula: $c = ee_p/(ee_p + ee_s)$. See ref. 9. [d] 10 mol% (*vs.* substrate) of Brij–IL–Me was added. [e] PS-**5**: lipase PS coated with [bdmim][cetyl-PEG10-sulfate].

used as catalyst (Entry 1). It was confirmed that these remarkable results were due to the coating effect of **5** upon the enzyme, because no marked acceleration was obtained when the reaction was carried out in the presence of Brij56 or **5** as additive using CF-PS (Entries 2 and 3). Further, it was also found that *coating with ionic liquid* **5** *significantly stabilized lipase PS in the organic solvent system. No drop in reactivity was observed when PS-***5** *was placed in dry hexane for a week at rt*, while the reactivity was completely lost if CF-PS was placed in hexane for the same period.

We currently assume that two factors are involved in the origin of the modification of the present lipase-catalyzed reaction. One is the modified flexibility of the enzyme by ionic liquid **5**. It was established that the flexibility change of the lipase protein reflects the stereoselectivity of the enzyme,[16] and organic media cause modification of the flexibility of the lipase protein.[16b] It is assumed that **5** has a certain impact on the flexibility of the lipase protein. The other factor is the influence of the presence of water molecules that might be involved in **5**. A small amount of water reportedly significantly accelerated the transesterification of lipase in the organic solvent system.[8] Although we dried **4** and **5** under vacuum, it was impossible to remove the water completely from such a polyoxyethylene compound.

In conclusion, we found that the novel ionic liquid **5** caused remarkable enhanced enantioselectivity of lipase PS-catalyzed acetylation of 1-phenylethanol using vinyl acetate as an acyl donor in i-Pr_2O, or in hexane, as solvent. In particular, there was a remarkable acceleration when lipase PS was treated with **5** while high enantioselectivity was maintained. We believe that this work represents not only a significant advance in the manner of preparation of optically active compounds using an enzymatic reaction but also provides a new aspect in application of an ionic liquid to an enzymatic reaction. Further investigation on the stabilization of enzymes by the ionic liquid coating is ongoing.

Acknowledgement

The authors are grateful for the financial support from Asahi Glass Foundation for this study.

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15 Some spectroscopic properties of **5**: mp 35 ~ 37 °C; IR (neat) 2916, 2851, 1468, 1350, 1252, 1115, 951, 845 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.80 (3H, t, J = 7.4 Hz), 0.88 (3H, t, J = 7.3 Hz), 1.10–1.30 (32H, m), 1.29–1.31 (2H, m), 1.47–1.50 (4H, m), 1.70–1.73 (2H, m), 2.61 (3H, s), 3.36 (4H, t, J = 5.1 Hz), 3.49–3.62 (34H, m), 3.81 (3H, s), 4.00 (2H, t, J = 5.1 Hz), 4.07 (2H, t, J = 7.8 Hz), 7.31 (1H, s), 7.45 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 9.48, 13.20, 13.77, 19.23, 22.30, 25.73, 28.98, 29.11, 29.23, 29.31, 31.35, 31.54, 35.06, 48.01, 61.20, 65.78, 69.68, 69.05, 70.19, 71.12, 72.26, 120.17, 122.65, 143.57 ppm.

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TUTORIAL REVIEW

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Organic synthesis using enzymes in supercritical carbon dioxide

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Enzymes have been used in supercritical carbon dioxide ($scCO_2$) rather than in the conventional media to make enzyme reactions greener. This review introduces some enzymatic asymmetric synthesis in $scCO_2$ such as esterification by a lipase and reduction by an alcohol dehydrogenase. A carboxylation by a decarboxylase is also described.

Introduction

Supercritical carbon dioxide ($scCO_2$), CO_2 above its critical point (T_c = 31.0 °C, T_p = 7.38 MPa, 72.8 atm), has been used for extraction, chromatography, and as a solvent for chemical catalysis due to its environmentally benign nature, nonflammability, low toxicity, high availability and high functionalities, such as the tunability of the physical properties by the manipulation of pressure and temperature.[1] Enzymatic catalysis has also been conducted in supercritical carbon dioxide ($scCO_2$)[2] rather than in the conventional media to make enzyme reactions greener. Moreover, the greenness of the enzymatic reaction in $scCO_2$ also comes from the fact that enzymes themselves are environmentally benign catalysts because enzymes are reproducible resources and have high chemo-, regio-, and enantioselectivities, so production of wasteful side products can be minimized. The first reports on enzyme catalyzed reactions in $scCO_2$ were in 1985 by Randolph *et al.*,[2a] Hammond *et al.*,[2b] and in 1986 by Nakamura *et al.*.[2c] More recently, the benefit of using supercritical fluids for enzymatic reactions has been demonstrated by Mori *et al.*[2d,e] and Kamat *et al.*,[2f–i] *e.g.* improved reaction rates, control of selectivities by pressure, *etc.* This short review introduces some enzymatic asymmetric syntheses in $scCO_2$ such as esterification by a lipase[3] and reduction by an alcohol dehydrogenase.[4] A carboxylation by a decarboxylase is also described.[5]

Enantioselective esterification

Lipase catalyzed esterification is usually conducted in an organic solvent because hydrolytic reactions are favoured in aqueous media. By replacing the organic solvent with $scCO_2$, the amount of organic waste is decreased. We have examined the enantioselective acetylation of racemic 1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol (*R*/*S*)-**1** with lipases and vinyl acetate[6] in $scCO_2$ as shown in Scheme 1.[3] The fluorinated compound was chosen because optically pure fluorinated alcohols have received much attention for the synthesis of ferroelectric liquid crystals or bioactive compounds.[7] We found that the enantioselectivity of the reaction catalyzed by Novozym lipase can be controlled by adjusting the pressure and the temperature of $scCO_2$. The role of water activity, which had been examined before,[2l] was not examined here.

First, we screened various lipases for the enantioselective acetylation of (*R*/*S*)-**1** with vinyl acetate in $scCO_2$ at 40 °C and at two extreme values of pressure. The results are listed in Table 1. To evaluate the enantioselectivity of this reaction, the ratio of the specificity constants of the enantiomers, *E*-value,[8] was used. In all

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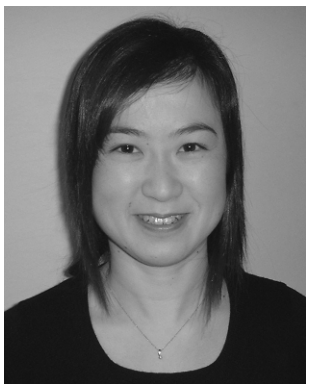

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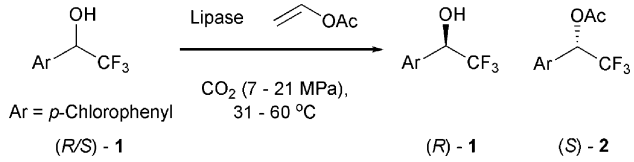


Scheme 1 see ref. 3.

Table 1 Screening of lipases for enantioselective acetylation of (*R*/*S*)-**1** in $scCO_2$[3b]

	Low pressure conditions (9.1 MPa)		High pressure conditions (14.5 MPa)	
Lipase	Yield (%)	E^a	Yield (%)	E^a
LPL (*Pseudomonas aeruginosa*)	52	12	38	16
AY (*Candida rugosa*)	8	1[b]	2	2
AH (*Pseudomonas cepacia*)	3	29	0	—
PS-D (*Pseudomonas cepacia*)	0	—	0	—
PS-C (*Pseudomonas cepacia*)	43	8	22	17
Lipozyme (*Rizomucor miehei*)	0	—	0	—
Novozym (*Candida antarctica*)	25	38	24	23

Reaction conditions: 40 °C, 4 h.[a] Enantiomeric ratio, *E* value,[8] was used to evaluate enantioselectivity. $E = (V_A/K_A)/(V_B/K_B)$ where V_A, K_A and V_B, K_B denote maximal velocities and Michaelis constants of the fast- and slow-reacting enantiomers, respectively. The (*S*)-enantiomer reacted faster than the (*R*)-enantiomer. [b] In this case, the (*R*)-enantiomer reacted slightly faster than the (*S*)-enantiomer.

cases but one the (*S*)-enantiomer reacted faster than the (*R*)-enantiomer, affording (*S*)-acetate (*S*)-**2** and the remaining (*R*)-alcohol (*R*)-**1**. The highest enantioselectivity (*E* = 38) was obtained using Novozym at 9.1 MPa. Interestingly, the enantioselectivity was significantly affected by the pressure.

The effect of pressure on enantioselectivity was investigated in more detail by carrying out the esterification at pressures ranging from 8 and 19 MPa and at different reaction times, while maintaining the temperature at 55 °C. As shown in Fig. 1, the *E* value decreased from 50 to 10 continuously when the pressure was changed from 8 to 19 MPa, regardless of the reaction time.

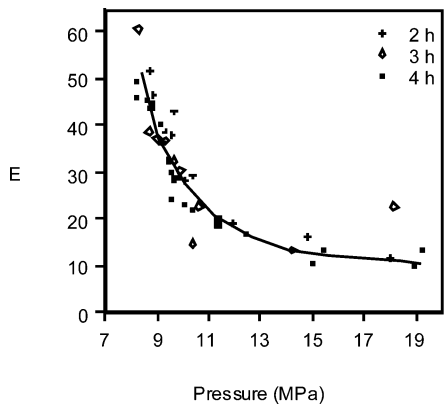


Fig. 1 Effect of pressure on enantioselectivity of acetylation of (*R*/*S*)-**1** by Novozym in $scCO_2$ at 55 °C[3a] (*E*: Enantiomeric ratio described in Table 1[8]).

The effect of pressure on enantioselectivity is indeed noteworthy, although the reason is not clear at present. When the pressure of $scCO_2$ was changed, there was no significant change in the polarity evaluated as dielectric constant.[1h] and log *P* (at 50 °C; 1.4 at 8 MPa and 1.9 at 11 MPa).[9] It is not clear whether this small change in polarity has a large effect on this reaction. On the other hand, the density of $scCO_2$ does change from 0.20 to 0.42 g mL^{-1} when the pressure is changed from 8 to 11 MPa at 55 °C.[1h,i] Ikushima explained the high enantioselectivity of lipase in a very limited pressure range at 304.1 K as resulting from interaction between CO_2 and enzyme molecules.[2m,n] We also propose that the large change in density could significantly change the interaction between CO_2 and the enzyme by the formation of carbamates from CO_2 and the free amine groups on the surface of the enzyme.[2l] This can also occur by CO_2 adsorption on the enzyme, as reported for other proteins,[10] and/or by CO_2 incorporation in the substrate-binding pocket of the enzyme, in analogy to the incorporation of organic molecules in enzymes.[11] These interactions may gradually change the conformation of the enzyme in response to pressure, resulting in a continuous change in enantioselectivity.

The effect of pressure on the enantioselective acetylation of (*R*/*S*)-**1** with vinyl acetate in $scCO_2$ by Novozym was also investigated at 31, 40 and 60 °C (Fig. 2). As in the case at 55 °C, the *E* value

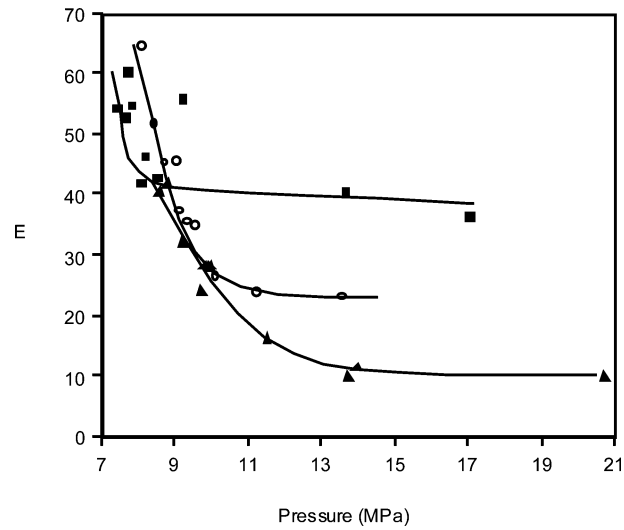


Fig. 2 Effect of pressure on enantioselectivity of acetylation of (*R*/*S*)-**1** by Novozym in $scCO_2$ at 31 °C (square), 40 °C (circle) and 60 °C (triangle)[3b] (*E*: Enantiomeric ratio described in Table 1[8]).

changed continuously according to the pressure. This is most probably due to the change of $scCO_2$ density, as described above. This explanation is in agreement with the observation that at lower temperatures (31 and 40 °C) the decrease of the *E* value measured at pressures below 10 MPa is steeper whereas at higher temperatures the *E* values decrease more gradually. These changes correlate well with the change in density as shown in Fig. 3.[1h,i]

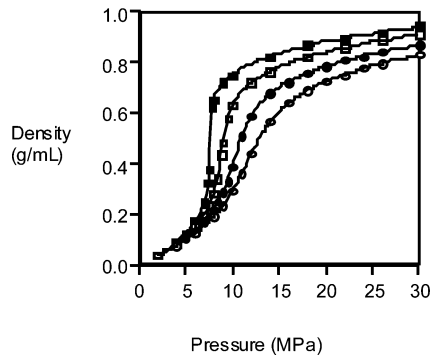


Fig. 3 Tunability of density of CO_2 by the temperature and pressure (closed square: 32 °C, open square: 40 °C, closed circle: 50 °C, open circle: 60 °C).[1h]

However, when *E*-values obtained at the same density but at different temperatures were compared, a significant effect of temperature became evident. Therefore, the enantioselectivity is determined not only by the density but also by the temperature. In a reaction under ambient conditions, the enantioselectivity of a kinetic resolution is temperature-dependent and obeys a modified Eyring equation:[12]

$$\ln E = -(\Delta\Delta H^{\ddagger}/R)(1/T) + (\Delta\Delta S^{\ddagger}/R) \quad (1)$$

Therefore, according to the Eyring equation, a modulation of the stereoselectivity of enzymatic catalysis is possible through temperature variation. Using enzymatic reactions performed at temperatures ranging from 30 °C to −50 °C, Sakai *et al.* showed the first experimental evidence supporting the theory of the effect of temperature on stereochemistry.[12b,c] Here, we examined whether the theory is applicable to the reaction in $scCO_2$. At a density of 0.75 g mL^{-1} (31 °C at 9.5 MPa, 35 °C at 11.2 MPa, 40 °C at 13.2 MPa, 45 °C at 15.3 MPa, 50 °C at 17.5 MPa, 55 °C at 19.6 MPa, and 60 °C at 21.8 MPa), ln *E* was plotted against 1/*T*. As shown in Fig. 4, the Eyring plot was found to be linear throughout this range and

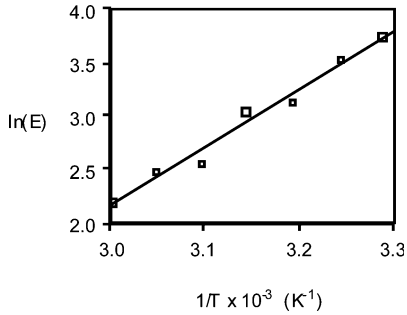


Fig. 4 Effect of temperature on enantioselectivity of acetylation of (*R*/*S*)-**1** by Novozym in $scCO_2$ at 0.75 g mL^{-1} [3b] (*E*: Enantiomeric ratio described in Table 1[8]).

thus indicates the conformational stability of the transition state. The differences in enthalpy and entropy values calculated from the above graph are as follows. The values are comparable to those for the reaction in organic solvent.[2w]

$$\Delta\Delta H^{\ddagger} = -11 \text{ kcal mol}^{-1} \qquad \Delta\Delta S^{\ddagger} = -28 \text{ cal K}^{-1} \text{ mol}^{-1} \quad (2)$$

Finally, the reaction in $scCO_2$ was compared to the reaction in conventional media in terms of reactivity, enantioselectivity, continuity of the enantioselectivity change, and the possibility of examining the solvent effect without changing the kind of solvent. The reaction rates largely depended on the kinds of organic solvent or $scCO_2$ conditions and did not differ significantly between conventional and supercritical. With respect to the enantioselectivity, the highest value was obtained when benzene, a poisonous solvent, was used for the reaction. Large differences between conventional and supercritical appeared when the continuity of the enantioselectivity change and the possibility of examining the solvent effect without changing the molecular shape of the solvent were compared; both of them were possible using the supercritical solvent, but impossible using conventional solvents. Continuity of the enantioselectivity change was not observed using conventional organic solvents for the same reaction as shown in Fig. 5.[3a]

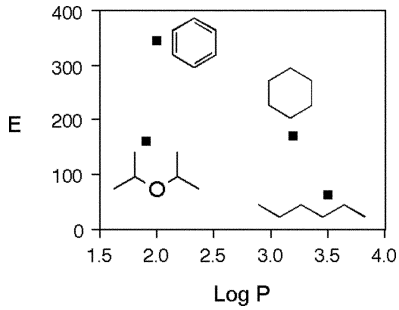
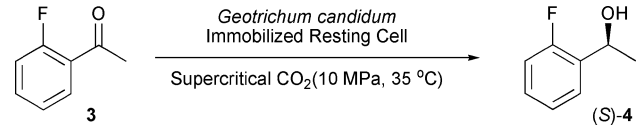


Fig. 5 Effect of organic solvent on enantioselectivity of acetylation of (*R*/*S*)-**1** by Novozym[3a] (*E*: Enantiomeric ratio described in Table 1[8]).

Moreover, it is unclear whether the *E* values depended only on the polarity of the solvent, because a polarity change is inevitably accompanied by a change in the molecular structure of the solvent (cyclic or acyclic) and the molecular structure of the solvent, as well as its polarity, affects the enantioselectivity of the reaction. On the other hand, by using CO_2, the solvent properties can be changed simply by altering the pressure or temperature.

Asymmetric reduction

Asymmetric reduction using alcohol dehydrogenases is usually conducted in aqueous media. The difficulties encountered in such reactions are the extraction of products which dissolve in aqueous media at low concentration, and organic solvent is usually used. However, by using $scCO_2$ this becomes unnecessary because CO_2 transforms into a gas as the pressure decreases. Therefore, asymmetric reduction of various ketones using alcohol dehydrogenases was conducted in $scCO_2$. Whole resting cells of *Geotrichum candidum* were used for the reduction so that the addition of expensive coenzymes was avoided. Moreover, the solubility of the coenzymes in $scCO_2$ did not need to be considered. The cell was immobilized on a water-absorbing polymer[13c] to spread it on the large surface of the polymer. At first, the reduction of *o*-fluoroacetophenone **3** in $scCO_2$ at 10 MPa was conducted using 2-propanol as a reductant (hydrogen donor), which afforded (*S*)-1-(*o*-fluorophenyl)ethanol (*S*)-**4** in 81% yield (determined by GC) after 12 h (Scheme 2). The time course of the reaction shows



Scheme 2 see ref. 4.

that the yield increased with the reaction time, which proved that the alcohol dehydrogenase catalyzed the reduction even under supercritical conditions.

The substrate specificity was investigated and, as listed in Table 2, the enzymatic reduction in $scCO_2$ proceeded for various ketones.

Table 2 Reduction of various ketones by *Geotrichum candidum* in $scCO_2$[4a]

Substrate	Yield (%)	ee (%)	Config.
	51	> 99	*S*
	81	> 99	*S*
	53	> 99	*S*
	11	97	*S*
	96	96	*R*
	22	> 99	*S*
	61	> 98	*S*
	96	—	—

Acetophenone, acetophenone derivatives, benzyl acetone and cyclohexanone were used as substrates, and it was found that all of them were reduced by the alcohol dehydrogenase in $scCO_2$ with 2-propanol. The effects of fluorine substitution at the *ortho*, *para* and α positions of acetophenone were obvious. Compared with the unsubstituted analogue, substitution at the *ortho* or α position increased the yield, whereas substitution at the *para* position decreased the yield.

With respect to the enantioselectivity, very high values (> 99% ee) were obtained for the reduction with the majority of the substrates tested, while slightly lower enantioselectivities (96, 97% ee) were observed for a few of them. The enantioselectivities obtained in this system are superior to or at least equal to those for most other biocatalytic and chemical systems.[13,14]

The immobilized resting-cell of *G. candidum* was also used as a catalyst for the reduction of *o*-fluoroacetophenone **3** and cyclohexanone in a semi-continuous flow process using $scCO_2$.[4b] The apparatus is shown in Fig. 6. With flow reactors, the addition of a

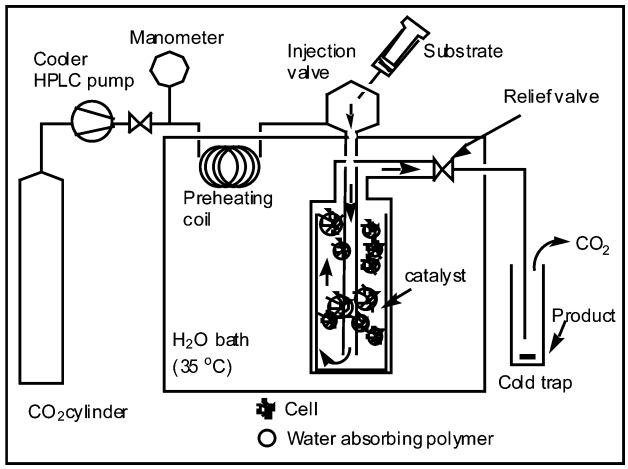
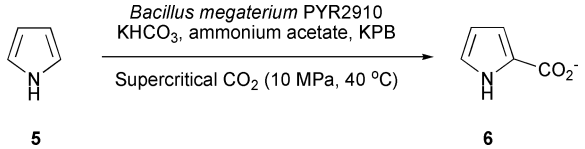


Fig. 6 Apparatus for biocatalytic reduction with a flow process using $scCO_2$.[4b]

substrate to the column with a catalyst yielded the product and CO_2, which is a gas at ambient pressure, whereas, with the batch reactor, separation of the product from the biocatalyst was necessary after depressurization. Therefore, the flow type was superior to the batch type for achieving virtually no solvent reaction. Moreover, the size of the reactors using the flow process to generate an amount of product comparable with the corresponding batch reactors is smaller, which is particularly attractive for a supercritical fluid system.[15] This reaction using a semi-continuous flow process also resulted in a higher space-time yield than that of the corresponding batch process.

Carboxylation

The development of CO_2 fixation reactions on organic molecules is one of the challenges in synthetic chemistry since the use of $scCO_2$ as a raw material is very attractive. An increasing number of chemical CO_2 fixation reactions[16] have been reported, especially using $scCO_2$, such as the synthesis of urethane,[16a] dimethyl carbonate,[16b] styrene carbonate,[16c] and methyl ethanoate.[16d] Some enzymatic CO_2 fixation reactions have also been reported including the central CO_2 fixation reaction in photosynthetic organisms catalyzed by ribulose-1,5-diphosphate carboxylases,[17] the reduction of CO_2 to formic acid or methanol by dehydrogenases,[18] the reductive CO_2 fixation on 2-oxoglutarate and pyruvate by isocitrate[19] or malate[20] dehydrogenases, and the CO_2 fixation on pyrrole and phenolic compounds (phenol and catechol) by decarboxylases from *Bacillus megaterium*[21] or *Clostridium hydroxybenzoicum*,[22] respectively. This section describes the CO_2 fixation catalyzed by cells of *B. megaterium* in $scCO_2$.[5] As shown in Scheme 3, CO_2 was fixed on pyrrole **5** to produce pyrrole-



Scheme 3 see ref. 5.

2-carboxylate **6** at 10 MPa and 40 °C. The yield of the carboxylation reaction in supercritical CO_2 was 12 times higher than that under atmospheric pressure.

The cells of *B. megaterium*[21b] were employed for the CO_2 fixation reaction. The reaction was conducted by adding CO_2 at 10 MPa to a mixture containing the substrate **5**, the cells, $KHCO_3$, and NH_4OAc in potassium phosphate buffer. For the reaction at atmospheric pressure (0.1 MPa), the evolved CO_2 was released to keep the pressure atmospheric. As shown in Table 3 and Fig. 7,

Table 3 Carboxylation of **5** by *Bacillus megaterium* in $scCO_2$[5]

Entry	Pressure/MPa	Cells/mL[a]	pH	Time/h	Yield (%)[b]
1	0.1 (Atmospheric)	0.5	5.5	1	7
2	10 (Supercritical)	0.5	5.5	1	54
3	10 (Supercritical)	0.0	5.5	3	0
4	10 (Supercritical)	1.0	5.5	3	59
5	10 (Supercritical)	0.5	7.0	1	59

[a] OD_{610} = 32; decarboxylation activity for **6** = 0.024 mmol/min/ml. [b] Yield is the percentage of **6** based on the starting amount of **5**.

yields are much higher for reactions carried out in $scCO_2$ than at atmospheric pressure. It was also confirmed by the control experiment without the cells that the non-biocatalytic carboxylation

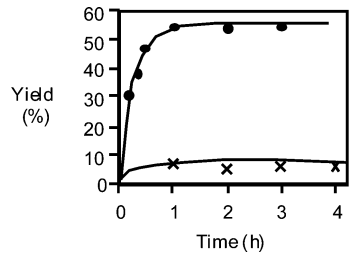


Fig. 7 Time course of carboxylation of **5** by *Bacillus megaterium* (●: 10 MPa (supercritical), ×: 0.1 MPa (atmospheric)).[5]

of **5** did not proceed (Table 3, Entry 3). A control reaction using heat treated cells afforded no carboxylation product, either, which indicates that a biocatalyst is at work and that the carboxylation is not an unexpected process promoted by non-enzymic constituents of the cell. The doubling of the quantity of cells (Table 3 Entry 4) as well as the change in the initial pH value from 5.5 to 7.0 to prevent a pH decrease caused by CO_2 (Table 3 Entry 5) did not have any significant effect on the equilibrium position.

The effect of pressure on the carboxylation of **5** was also investigated, and the result is shown in Fig. 8. The maximum yield

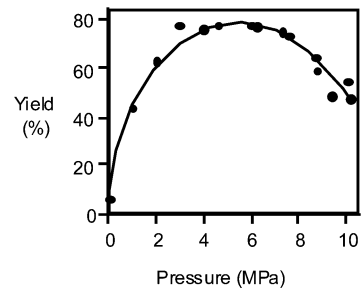


Fig. 8 Effect of pressure on carboxylation of **5** by *Bacillus megaterium*.[5]

was between 4 and 7 MPa; the yield obtainable at values slightly above the $scCO_2$ critical pressure is about 12 times that at atmospheric pressure (0.1 MPa). Similar pressure dependencies of the yield were also observed using an increased quantity of the cells, shorter reaction times and different temperatures. At present, it is not clear why pressure values far above the critical pressure did not favorably shift the carboxylation equilibrium.

Conclusion

As a solvent for lipases, alcohol dehydrogenase and decarboxylase reactions, $scCO_2$ was used instead of the conventional media to improve the greenness of the enzyme reactions. This is the beginning of the investigation of novel reaction systems which are

in harmony with the natural environment. We believe that it opens up new possibilities for synthesis employing various types of enzymes in $scCO_2$, a natural, easily-removable, and highly-functional solvent.

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CRITICAL REVIEW

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Solid/gas bioreactors: powerful tools for fundamental research and efficient technology for industrial applications

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Solid–gas biocatalysis appears today to be a promising technology for fundamental research and for the development of new cleaner industrial processes. The use of enzymes or whole cells at the solid–gas interface now appears concurrent to liquid processes and presents some very interesting features since total thermodynamic control of the system can be achieved easily. Moreover, from a technological point of view, solid–gas systems offer very high production rates for minimal plant sizes, allow important reduction of treated volumes and permit simplified downstream processes. These advantages result from the ability to precisely control all the thermodynamic parameters influencing not only the kinetics of the reactions performed, but also the stability of the biocatalysts working with biological catalysts at elevated temperatures. In this article, an overview of some existing systems and application of solid–gas technology to fundamental studies related to the influence of the microenvironment on biocatalysts is given. The potential of this peculiar system, and examples of applications that should benefit from the technology are presented herein.

Introduction

Non conventional enzymology can not only be applied to the use of enzymes in monophasic organic systems. During the last few decades, new technologies in the field of enzymatic catalysis have been developed, such as multiphasic systems, micro emulsions and micellar systems. Lately, the use of supercritical fluids or solid–gas catalysis have been tested and implemented for new biotechnological processes.

Solid–gas catalysis presents many advantages compared to other systems (*i.e.* liquid, mono or biphasic ones). Its strength results from the possibility of obtaining very high conversion yields compatible with a high production rate for a minimal plant scale, considering that: mass transfers are more efficient at the solid–gas interface, diffusional limitations are less important due to the low viscosity of gases and high diffusion coefficients compared to liquids, enzymes and cofactors are more stable in systems with restricted water availability, problems of solubility of substrates and products do not exist and the use of solvent can be avoided.

Moreover, because solid–gas catalysis is often synonymous with the use of higher temperatures and lower water availability, microbial contamination of the bioreactor can be avoided.

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Finally, the downstream process is simplified due to the absence of a solvent phase and the scale-up operation for such a process is simpler due to the use of a gaseous circulating phase.

Nevertheless, the field of applications of solid–gas systems remains limited, compared to non conventional liquid systems, since it is based on the volatile character of the substrates and products of the reaction. Physical and chemical properties of substrates and products of a reaction are of crucial importance for a solid–gas system since they condition the efficiency of the technology. This is probably why, although this system presents very interesting features, the technology in which biocatalysts are suspended in mixtures of substrates and water vapors remains relatively unexplored in contrast to those where enzymes are placed directly in aqueous or non aqueous solvents.

Nature offers only one example of an enzyme acting on gaseous substrates. Hydrogenase is a unique enzyme whose substrate is gaseous hydrogen. Yagi and collaborators[1] have clearly demonstrated that hydrogenase in the dry state binds the hydrogen molecule and renders it activated, resulting in parahydrogen–orthohydrogen conversion, whereas, aqueous protons do not participate in the reaction mechanism.

In a subsequent paper Kimura *et al*,[2] proved that, using purified hydrogenase, it was possible to obtain not only the conversion and exchange reactions, but also the reversible oxido-reduction of the electron carrier, cytochrome c3 with H_2.

Nevertheless, from the middle of the 1980s, examples of gas–solid systems using either entire cells or isolated enzymes were reported in the literature. These systems were involving either isolated enzymes or whole cells for mono or multi steps reactions. Different isolated enzymes were tested successfully such as the horse liver dehydrogenase,[3] the *Sulfolobus solfataricus* dehydrogenase,[4] the *Pischia pastoris* alcohol oxidase,[5,6] the baker's yeast alcohol dehydrogenase[7] and lipolytic enzymes.[8–12]

From the beginning of the 1990s, the use of entire cells in solid–gas systems such as *Methylocystis sp*,[13] *Pseudomonas putida*,[14] *Saccharomyces cerevisiae*[15] or *Rhodococcus erythropolis*,[16] was also reported for applications concerning mainly the bioremediation of VOCs polluted gases.

The demonstration that solid–gas biocatalysis was possible with enzymes usually acting on liquid substrates opened a new research area that led to the definition of new continuous cleaner processes for single or multi steps biotransformations, involving either enzymatic solid–gas bioreactors or microbial set-ups. As well, since solid–gas systems allow control and independent variation of the thermodynamic activity of substrates and other added components, they offer the possibility to modulate and to study the effect of each component present in the microenvironment of the catalyst.

Solid–gas systems now constitute an efficient tool to understand and rationalize the effects of the microenvironment of a biocatalyst on its activity and on its stability. Some examples of the benefits of this thermodynamic approach and control of enzymatic reactions will be discussed as well as industrial application and further development of the technology.

Operating solid–gas systems: the water activity paradox.

The impact on biocatalysis of very high substrate concentrations or of the addition of organic solvents in liquid systems have been extensively studied in the last few decades. Numerous publications reported the effect of physico-chemical modifications of the microenvironment on catalysis and stability of enzymes in non conventional media, and consequently highlighted the importance of water on enzymatic catalysis.[17–19]

In 1985, Drapron[20] recommended that the thermodynamic state of water, described by the water activity (a_w), be taken into account in studying enzymes in low water content media in order to rationalize the observed effects.

Water activity is a thermodynamic equilibrium parameter that describes the (equilibrium) amount of water available for hydration of the different components of a system. It can be easily determined by measuring the partial pressure of water on the top of a system and by calculating the ratio of this value over the saturation pressure of water at the temperature of the system. Subsequently, Halling[21] described the relationships existing between the water activity and the hydration/solvation state of the different parts of a multiphasic system and made some recommendations concerning the use of water activity for non aqueous enzymology in order to rationalize the observed effects of water onto biocatalysts.

Thus, water activity was reported to be the key parameter in non conventional biocatalysis and, consequently, the effect of water activity on catalysis and stability constituted, in most of the published works, the core of all the studies devoted to solid–gas biocatalysis.

Ethanol oxidation in the gaseous phase in batch reactors using *Pichia pastoris* alcohol oxidase, reported by Barzana *et al*,[5,6] showed that dehydrated enzyme immobilized on DEAE cellulose or on controlled pore glass beads was able to oxidize methanol and ethanol vapors at elevated temperatures, in absence of water in the gas phase. Nevertheless, the study on the effect of the water activity showed that enzyme activity in the gas phase increases by several orders of magnitude, whereas the thermostability decreases drastically when water activity is increased from 0.11 to 0.97.

Similar conclusions were drawn from the studies on alcohol dehydrogenases in a solid–gas bioreactor for alcohol and/or aldehyde production,[3,4] using alcohol dehydrogenase and NAD^+ (or $NADH,H^+$) co-immobilized into albumin-glutaraldehyde porous particles in batch and continuous fed column reactors. In these experiments, acetaldehyde reduction was coupled to butanol oxidation performed by the same enzyme, in order to regenerate the cofactor.

Water activity was the key parameter in studies concerning other types of enzymes tested in solid–gas reaction such as lipases or esterases as well. *Candida rugosa* lipase and other esterolytic enzymes coated on glass beads[22] and suspended in mixtures of substrates and water vapors over a range of water activity from 0.56 to 1 showed the ability to perform different reactions, and the extent of reaction was classified in the following order, for decreasing a_w values: hydrolysis > alcoholysis > ester exchange > esterification.

Robert *et al*[12] showed the relationships existing between water activity, the hydration state characterized by the sorption isotherm of the catalytic preparation and the activity/stability of a lipase used in a continuous solid–gas system for a transesterification reaction. In regard to the isotherm sorption curve of their enzymatic preparation, showing that beyond a water activity of 0.7, solvent water was present, they concluded that the best stability and activity were obtained for a catalyst having a complete first hydration layer without free water that was responsible of a rapid denaturation of the enzyme once the water activity was higher than 0.7.

Water activity effects were also extensively studied for solid–gas systems involving whole cells as catalysts. Fifteen years ago, the epoxidation of alkenes by bacteria had received much attention, in view of the possible production of certain epoxides by mean of a biotechnological process. A solid–gas bioreactor has been used to promote a rapid and continuous removal of the toxic ethylene oxide from the environment of the immobilized cells of *Mycobacterium* Py 1 in alginate beads and on sand.[23] The conversion of propylene or ethylene to the corresponding oxide was performed by a monooxygenase system needing molecular oxygen and $NADH,H^+$ or $NADPH,H^+$. The cofactor regeneration was reported to be possible through endogenous respiration. The influence of immobilization and reduced water activity was studied, on gaseous-alkene oxidation by the same type of organism in a solid–gas bioreactor by Hamstra *et al*.[24] Similarly, continuous production of propylene oxide was successfully demonstrated using a simple solid–gas

heterogeneous bioreactor with *Methylosinus sp* CRL 31 coated onto porous glass beads[25] working at 40 °C at an optimal water activity of 0.7.

VOC removal in polluted gases was the second major research topic developed involving solid–gas catalysis. Trichloroethylene, one frequent gaseous contaminant, has been shown to be degraded into metabolic intermediates and used as carbon source afterwards by immobilized resting cells of *Methylocystis* sp M in the same kind of reactor.[13] The catalytic activity was found to decrease rapidly as water activity was lowered since the cellular viability was rapidly decreasing.

Alcohol oxidase activity of extruded pellets presenting different moisture content (20, 30 and 50% w/w) of *Pichia pastoris* has been tested for vapor phase oxidations of ethanol to acetaldehyde in the presence of oxygen by Duff and Murray,[26] to the end of production or removal of organics. They noticed an important effect of the initial moisture content on catalytic activity and on stability: the higher the water content, the higher the activity but the lower the stability. More recently, Erable *et al*[16] reported the use or *Rhodococcus erythropolis* cells for the dehalogenation of chloro and bromo alkanes and studied the effect of water activity on stability and catalysis.

All the examples briefly listed above, dealing with the influence of water activity on biocatalysts in solid–gas systems, constitute the water activity paradox: while the authors in their majority noted that the degree of conversion, the observed reaction rates, and the catalyst stability were highly influenced by the hydration state of the biocatalyst and concluded the crucial impact of the water activity, all the other constituents (substrates, products, effectors…) were characterized in terms of volumetric concentrations or partial pressures but rarely in terms of thermodynamic activity.

Surprisingly, for many years the use of the thermodynamic activity was recommended for quantifying substrate availability in non-conventional media, such as liquid organic media.[27,28] Indeed, in such media, the "availability of substrate to the enzyme" cannot be quantified using its concentration, mainly because substrate solvation is modified as the reaction medium changes. Replacement of concentrations of species by their thermodynamic activities in liquid media requires the knowledge of their activity coefficients (γ values). This is far from straightforward, as (1) γ values depend on the molar ratios of other species present in the medium and (2) the methods used to estimate these values, such as the UNIFAC group contribution method,[29] are often called into question as sources of inaccuracy.[30–32]

In solid–gas technology, however, the thermodynamic activity of each compound can be precisely determined by calculating the ratio of the partial pressure of the compound in the gas to its saturation vapor pressure at the working temperature. As explained below, the partial pressure is obtained from the molar composition of the inlet gas and the total pressure, assuming that an inert inlet gas with a low organic content can be considered as an ideal gas.

Moreover in solid–gas reactors the molar fraction of the different species in the inlet gas can be chosen independently. As a result the thermodynamic activities of the different species can be fixed independently very easily. This constitutes a significant advantage over solid–liquid systems, in which reaction species and solvent molar fractions are linked together and for which liquid solvents that are totally inert towards solvation of substrates, products and catalyst do not exist.

Since total thermodynamic control was easily achievable in solid–gas systems, the concept of working at a controlled thermodynamic activity for all the constituents only aroused in the mid 1990s.[8,9] Thus, this thermodynamic approach allows improvement of the control and then the understanding of solid–gas systems. It is now clear that the ability to control each thermodynamic activity independently constitutes the major advantage of solid–gas systems, as it will be discussed later in this paper.

Then, solid–gas systems represent a powerful tool of investigation concerning the relationships existing between biocatalysts and their microenvironment, and its influence on catalytic activity and stability.

Theory and practice

The advantage of solid–gas catalysis: a system which permits independent control of the thermodynamic activity of the different chemical species in the microenvironment of the catalyst.

If one considers a solid–gas bioreactor based on a packed bed, the following representation can be done, as depicted in Fig. 1. Then, very simple parameters, controlling the thermodynamic activity of each compound can be easily identified.

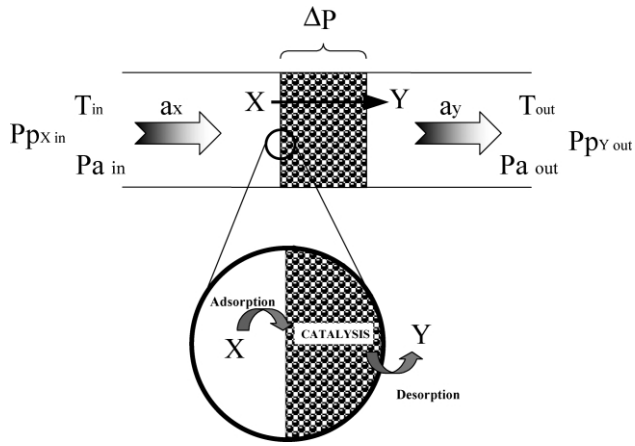


Fig. 1 Schematic diagram of solid–gas catalysis (reaction X→Y) in a packed bed reactor. The inlet gas is characterized by the thermodynamic activity of X (a_X), resulting from the different parameters: T_{in}, the inlet temperature of the gas, Pp_X, the inlet partial pressure of X and Pa_{in} , the absolute pressure of the inlet gas. The outlet after reaction over the catalytic bed generating a pressure drop ΔP is characterized by the thermodynamic activity of the product (a_Y), resulting from the different parameters: T_{out}, the outlet temperature, Pp_Y, the partial pressure of Y and Pa_{out}, the absolute pressure of the outlet gas.

The inlet gas will be characterized by its molar composition, the inlet temperature and the total pressure of the system. For an inert gas having a low content of organics, if one assumes that it can be considered as an ideal gas, these few parameters allow a complete definition of the thermodynamic parameters of the gas entering the reactor.

While the molar composition of the inlet gas and the total pressure are known, it is possible to calculate easily the partial pressure of each compound (Pp_X), according to *eqn. 1*, assuming that n_X is equal to the number of moles of X in a finite gas volume containing a total of n_{total} moles at the absolute pressure of *Pa*.

$$Pp_X = \frac{n_X}{n_{total}} Pa = \frac{\dot{Q}_X}{\dot{Q}_{total}} Pa \ (atm) \qquad (1)$$

Molar flow rates $\dot{Q}$ can be used instead of n in continuous systems. Then, the thermodynamic activity (a_X) can be calculated according to *eqn. 2*, by using the saturation pressure of the pure compound, obtained from the saturation curves at the temperature of the gas stream (T_{in}) as described in Fig. 2 and using the following equation:

$$a_X = \frac{Pp_X}{Pp_{sat_X}} \text{ (dimensionless)} \qquad (2)$$

Two main strategies can be used for creating a gas with fixed thermodynamic activity, based either on saturation by an organic compound of a carrier gas or by vaporization of liquid molecules using a liquid–gas flash operation in an inert carrier flow. The reactors described in the literature, in the majority, use the first approach, and simple calculations and controls allow a precise control of all the operating parameters of solid–gas systems.

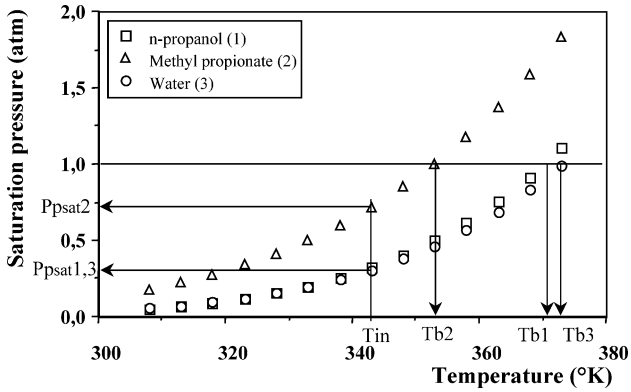


Fig. 2 Example of partial pressure saturation curves (VLE equilibriums). $Tb_{1,2\text{ and }3}$, refer to the boiling points of compounds 1, 2 and 3 at atmospheric pressure (1 atm). $Pp_{\text{sat 1, 2 and 3}}$ refer to the saturation pressure of compounds 1, 2 or 3 at a temperature T_{in}.

If one considers the following bioreactor (depicted in Fig. 3) in which different saturated gases are mixed for the realization of the final gas, the control of operational parameters can be realized as indicated below.

Creating a gas: the saturated gases mixing technique

One can assume that a carrier gas, after bubbling in a substrate solution at controlled temperature, is in equilibrium with the liquid phase since a sufficient contact time can be achieved so that the partial pressure of the substrate in the gas leaving the saturation apparatus is equal to the vapor pressure corresponding to the saturation pressure above the pure compound at a given temperature. In order to calculate the composition of the gas, the different molar flow rates for each compound (carrier + substrates + water) have to be known. The molar carrier flow rate in each line $\dot{Q}^n_{N2}$ can be calculated from *eqn. 3* when the volumetric normalized flow rates $\dot{Q}v^n_{N2}$*normalized* (l/h at 273.15 K, 1 atm) of the carrier gas used for saturation are known:

$$\dot{Q}^n_{\text{N}_2} = \frac{\dot{Q}v^n_{\text{N}_2}\,normalized}{RT}\ (\text{mol h}^{-1}\ \text{for}\ T = 273.15\ \text{K}) \qquad (3)$$

Then, with knowledge of the molar flow rate of the carrier gas it becomes possible to calculate the different molar flow rates leaving the saturation flasks by using the saturation pressure Pp_{sat} determined at the temperature of saturation, according to *eqn. 4.*

$$\dot{Q}^n_{\text{X}} = \dot{Q}^n_{\text{N}_2}\frac{Pp_{\text{sat}^n_{\text{X}}}}{(Pa - Pp_{\text{sat}^n_{\text{X}}})}\ (\text{mol h}^{-1}) \qquad (4)$$

After mixing the different lines, the partial pressure of each compound Pp^n_{X} in the gas entering the bioreactor is determined using *eqn. 5*

$$Pp^n_{\text{X}} = \frac{\dot{Q}^n_{\text{X}}}{\sum_1^n(\dot{Q}^n_{\text{N}_2} + \dot{Q}^n_{\text{X}})}Pa\ (\text{atm}) \qquad (5)$$

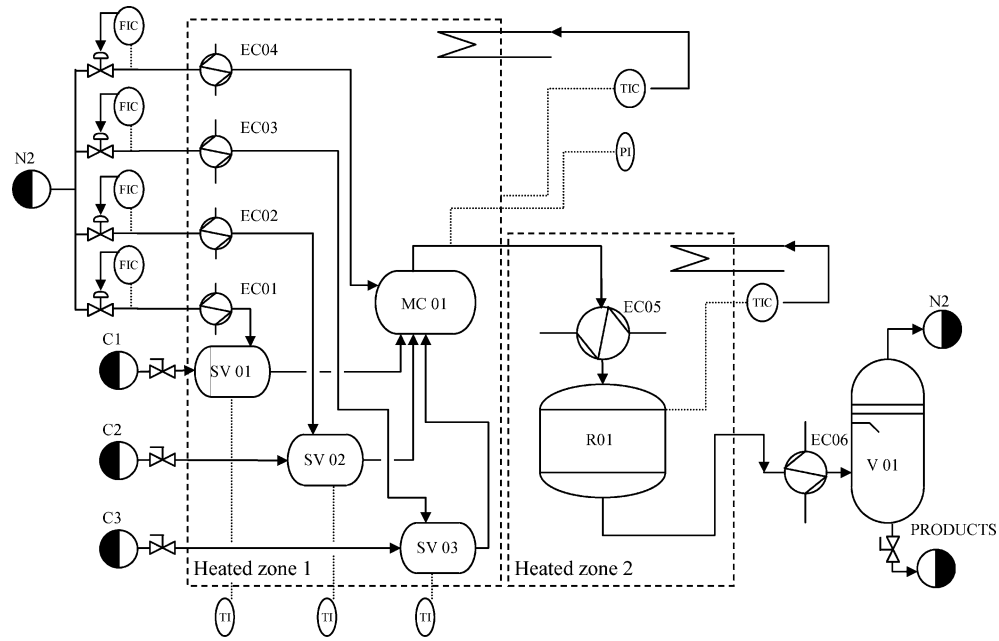
and the activity of each compound a_{X} in the reactor stage is calculated according to *eqn. 6* with the respective saturation pressure Pp_{sat}

$$a_{\text{X}} = \frac{Pp^n_{\text{X}}}{Pp_{\text{sat}^n_{\text{X}}}}\ (\text{dimensionless}) \qquad (6)$$

Assuming that the applied conditions are far from the critical temperature and the critical pressure for each compound, and by applying the ideal gas law $PV = nRT$, one can calculate a good estimation of the total volumetric flow rate $\dot{Q}v_{\text{total}}$ in the bioreactor using *eqn. 7*.

$$\dot{Q}v_{\text{total}} = \frac{RT\sum_1^n(\dot{Q}^n_{\text{N}_2} + \dot{Q}^n_{\text{X}})}{Pa}\ (\text{l/h at the temperature of the bioreactor in °K}) \qquad (7)$$

Although this method is simple for creating a gas with controlled partial pressure for each compound, it may in some cases present some limitations. By mixing the different saturated gases, it is difficult to obtain a final gas with a partial pressure for each component close to its saturation pressure. Nevertheless, for one who wants to realize a gas with very high thermodynamic activity for each component, the gas can be cooled after its realization in order to increase the thermodynamic activity of each component



SV01/02/03: carrier gas saturation vessel
MC01: gas mixing chamber
R01: packed bed bioreactor
V01: phase separator
EC01/02/03/04: carrier gas heater (gas/gas exchanger)
EC05 feed gas heater/cooler (gas/gas exchanger)
EC06: exhaust gas cooler

Fig. 3 Schematic diagram of a packed bed solid gas bioreactor working at atmospheric pressure based on saturation of a carrier by organic compounds in separate lines. The different lines are then mixed and partial pressures of the final gas may be adjust by a fourth line of pure carrier gas.

without changing the composition of the gas, but just by lowering the Pp_{sat} values.

Creating a gas: the vaporization of organics in a carrier gas technique

When a liquid–gas flash vaporization technique is chosen, a precise control of the different molar flow rates of each molecule contributing to the composition of the gas must be realized. Fig. 4 describes a solid–gas setup in which liquid substrates, water and nitrogen are injected in a flashing vessel (flash evaporator) for realization of the gas.[10]

Then, thermodynamic control is realized by calculating the different partial pressures based on the different molar flow rates. While $\dot{Q}_{N2}$ and $\dot{Q}^n_X$ are well known, then total molar flow rate $\dot{Q}_{total}$ can be determined using *eqn. 8* and resulting partial pressures can be calculated easily according to *eqn. 9*.

$$\dot{Q}_{total} = \dot{Q}_{N_2} + \sum_1^n \dot{Q}^n_X \text{ (mol h}^{-1}\text{)} \quad (8)$$

$$Pp^n_X = \frac{\dot{Q}^n_X}{\dot{Q}_{total}} Pa \text{ (atm)} \quad (9)$$

Thermodynamic activities are then calculated according to eqn. 6.

Although the system allows better control of the molar flow rates of substrates compared to the saturated gases mixing technique previously described, care must be taken in order to insure complete vaporization of the different molecules injected in the system. To this end, saturation pressure curves (Vapor Liquid Equilibriums) have to be known for defining the minimal temperature that has to be used for a correct liquid–gas flash evaporation. This point can be considered as a bottleneck since, in some cases, the use of very high temperatures can represent a serious constraint. Nevertheless, this problem can be solved by performing solid–gas catalysis under reduced pressure. This strategy offers multiple advantages for the use of longer chain length compounds or for the improvement of production rate. The use of reduced pressure allows an important enrichment of the gaseous phase in reactants while minimizing greatly the carrier gas needed and the required flashing temperature.

Variations of working absolute pressure: an easy way to improve solid–gas systems

As an example, one can compare the effect of pressure on a simple theoretical transformation.

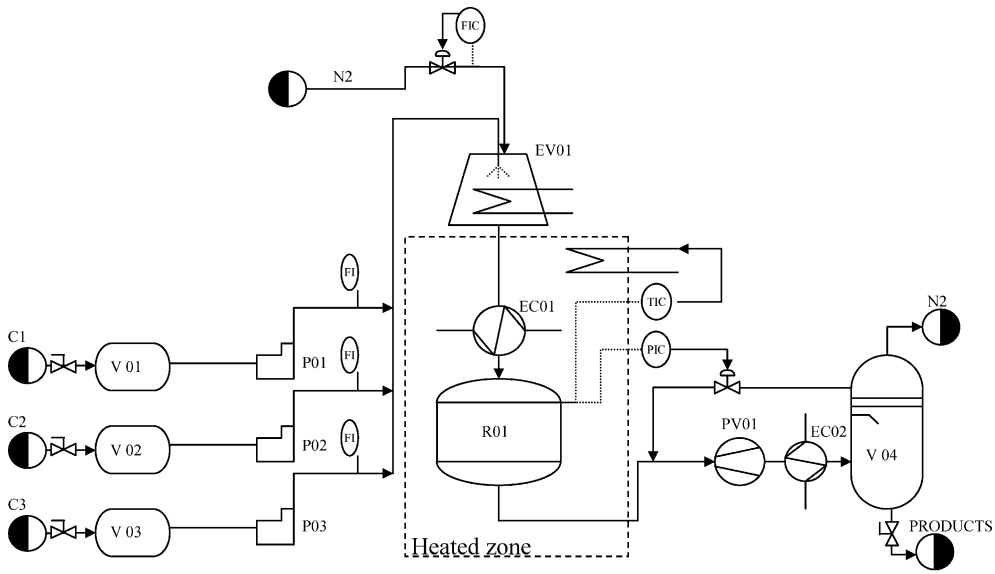
Considering a simple transformation of X to Y carried out at 100 °C and $a_X = a_w = 0.1$, with a saturation pressure of X at 100 °C of $Pp_{sat,\,X} = 0.2$ atm and $Pp_{sat,\,H_2O} = 1$ atm; boiling point of X is 200 °C at 1 atm, then, Pp_X will be set to 0.02 atm and Pp_{H_2O} will be 0.1 atm in the inlet gas. The total molar flow rate will be 100 mol h^{-1}, and the reaction will be carried out at two absolute pressures, 0.2 atm and 1 atm.

The characteristics of the two gases are summarized in Table 1.

The benefit of a reduced absolute pressure is therefore clearly established since the gas at 0.2 atm is highly enriched in X molecules, while the nitrogen consumption is divided by a factor of 2.2 for a production rate increased by a factor of 5 compared to the system functioning at atmospheric pressure. Of course, in order to insure a production rate increased by a factor of 5, the amount of

Table 1 Compositions and characteristics of two gases for the same reaction in terms of thermodynamic activity performed at two different absolute pressures

Absolute working pressure/atm	1.0	0.2
Minimal flashing temperature required/°C	200	100
Reactor inlet gas partial pressures: $Pp_X/Pp_{H_2O}/Pp_{N_2}$/atm	0.02/0.10/0.88	0.02/0.10/0.08
Reactor inlet molar flow rates: $\dot{Q}_X/\dot{Q}_{H_2O}/\dot{Q}_{N_2}$/mol h^{-1}	2/10/88	10/50/40
Production rate : $\dot{Q}_Y$/mol h^{-1}	2	10
Organic content of the gas (molar %)	12%	60%
Pp_Y at the outlet/atm	0.02	0.02
Pp_Y at the inlet of the condenser/atm	0.02	0.1
Condensation efficiency (%)	50	90
Production rate after condensation/ mol h^{-1}	1	9
Loss of Y after condensation step (%)	50	10



V01/02/03: substrates storage drums
EC01: feed gas cooler (gas/gas exchanger)
EC02: exhaust gas cooler
P01/02/03: feeding metering pumps
R01: packed bed bioreactor
V04: phase separator
EVO1: evaporator (Flashing unit)
PV01: vacuum pump

Fig. 4 Schematic diagram of a packed bed solid gas bioreactor working at reduced pressure based on the flash vaporization of liquid substrates in an inert carrier gas.

catalyst required for performing this operation will be roughly 5 times higher for the system working under reduced pressure.

The flashing temperature is also lowered by using a reduced pressure, thus minimizing the energy required for this operation.

Nevertheless, if one compares the two systems functioning at the same production rate, the advantages of the reduced pressure are still significant. While total volumetric flow rate and molar flow rates for X and water will be the same in both cases, the required amount of nitrogen will now be 5 times lower at reduced pressure. Thus, nitrogen consumption is divided by a factor of 11 compared to a system at atmospheric pressure for the same production rate.

Moreover, the use of solid–gas systems functioning at reduced pressure is of great importance considering the step following the catalysis in which the products are recovered. If one considers now that, after catalysis, the gas stream is cooled at 10 °C under an absolute pressure of 1 atm in both cases for the recovery of the product Y, and that the saturation pressure of Y at 10 °C is 0.01 atm, then the system depicted in Fig. 4 allows a better recovery of Y, while the recovery is very low for the system depicted in Fig. 3.

If all X is transformed into Y then, in both systems, the gas at the outlet of the reactor will have the following characteristics at the steady state: Pp_{H_20} = 0.1 atm, Pp_X = 0 atm, Pp_Y = 0.02 atm.

Prior to the cooling operation, the gas in the system described in Fig. 4 will have an absolute pressure at the outlet of the vacuum pump of 1 atm. Then, in this case, the characteristics before the inlet of the condenser will be: Pp_{H_20} = 0.5 atm, Pp_X = 0 atm, Pp_Y = 0.10 atm since *Pa* is multiplied by a factor of 5.

Once cooled at 10 °C, the condensation will take place, while the partial pressure of Y is higher than the saturation pressure of Y at 10 °C. Thus, the efficiency will be completely different in the two cases. For the reactor working at atmospheric pressure, only 50% of Y will be condensed $\left(\frac{Pp_Y - Pp_{sat_Y}}{Pp_Y} = \frac{0.02 - 0.01}{0.02} = 0.5\right)$ while 90% of Y will be condensed $\left(\frac{Pp_Y - Pp_{sat,Y}}{Pp_Y} = \frac{0.1 - 0.01}{0.1} = 0.9\right)$ for the system working at 0.2 atm.

Absolute pressure therefore appears as crucial as temperature for optimizing such systems since the thermodynamic properties of gases are highly dependent on pressure and temperature parameters. As a result, the use of any carrier gas can be avoided by working at an absolute pressure equal to the sum of the partial pressures of the organic compounds.

Thermodynamic activity and the hydration/solvation equilibrium of the catalyst

When using thermodynamic activity, care must be taken since this is an equilibrium parameter. Halling[21] illustrated clearly the equilibriums existing in a multiphasic system. In solid–gas systems, the equilibrium between the solid phase and the gaseous phase is crucial, since it defines the true solid–gas character.

Any solid phase, when placed in a gaseous environment containing a chemical species at a given thermodynamic activity, will be able, depending on its physico-chemical properties to adsorb molecules until it reaches an equilibrium.

Like water, any compound can compete for adsorption sites on a biocatalyst, thus having different interaction states and levels.

Experiments performed by Yang and Rupley[33] related to the interactions of water molecules with solid proteins highlighted numerous points concerning the processes involved. They showed that the hydration of lysozyme was a sequential process, with a controlled distribution of water molecules onto the protein. By using a micro calorimetric technique, they followed the evolution of the specific heat (C_p) of a powder of lysozyme under different hydration conditions. The results, presented in Fig. 5, allow us to distinguish between 4 regions.

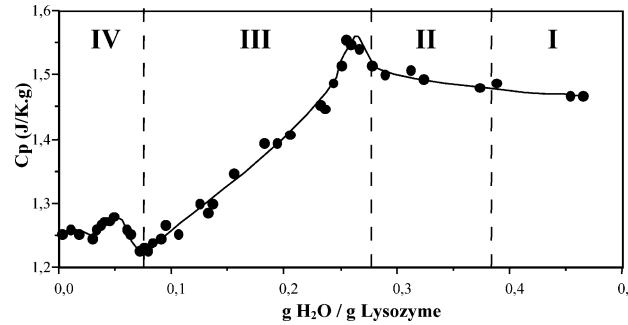


Fig. 5 From data from Yang and Ruppley.[33] Variation of lysozyme specific heat as a function of its hydration state.

By taking into account the difference between the global C_p and the C_p of lysozyme and water, they gave the following description of the sequence of events taking place when raising the water content of the powder:

• in region IV, the C_p of water is very close to that of ice, indicating a very high degree of organization. They also observed a lot of proton exchanges between water and the ionic groups of the protein.

• region III corresponds to the binding of water molecules onto amines and carboxylic groups. The variation of C_p close to 0.25 g H_2O/g protein corresponds to the complete coverage of sites involved in H bonds, and to the simultaneous appearance of less structured water molecules.

• in phase II water still continues to cover the protein but without any specific interactions.

• then, region I corresponds to a protein in aqueous solution, with apparition of solvent water (water molecules constituting the solvent water do not interact with the protein, but solely with other water molecules).

These interactions can also be characterized by determination of the isotherm sorption curve of the solid preparation (Fig. 6), by plotting the variation of water content *versus* the water activity, as reported by Drapron[20] and Hahn-Hagerdäl.[34]

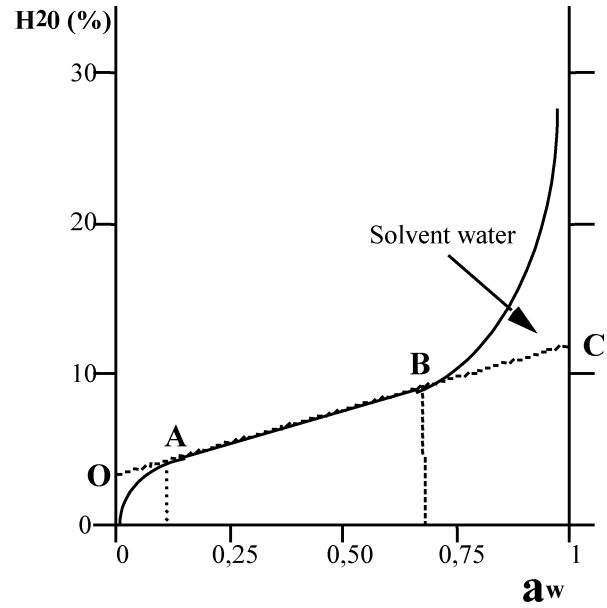


Fig. 6 Schematic diagram of an isotherm sorption curve.

Typically, isotherms present two characteristic break points (A and B) corresponding to two important states of water.

• Before point A, the water is highly structured. Water molecules are located on ionic groups and polar groups. They constitute what was earlier called the first hydration "layer". Point O corresponds to tightly bound water (or structural/buried water).

• Between A and B, the water content changes in a linear way while increasing the a_w. It corresponds to the formation of new

hydration layers. The protein dissolves molecules of water, thus forming a solid solution.

• Point B corresponds to the appearance of free water. Once the water activity is higher than the abscissa value of point B, an aqueous phase exists, with lots of water molecules having no interaction with the protein. Point C corresponds to the total quantity of non solvent water, *i.e.* structured by the solid sample.

Since each molecule present in the gas phase will be able to compete for adsorption onto the catalyst, isotherms resulting from the use of multi component systems may vary greatly depending on the thermodynamic activities used in the gaseous phase. This competition for adsorption onto the solid seems to be the key to understanding the observed effects on catalytic activity and stability, as will be discussed in the following part of the present paper.

Moreover, considering that thermodynamic activity is an equilibrium parameter, care must be taken in order to insure that the exchanges of molecules between the different phases are in a steady state, and that a set of thermodynamic values do not lead to the appearance of a liquid phase by modifying the global isotherm of the preparation, resulting in the definition of a solid–liquid–gas system. Some systems, reported as solid–gas systems were in fact solid–liquid–gas systems.[35]

Fundamentals applications of solid–gas technology

The possibility of determining intrinsic kinetic and activation thermodynamic parameters

Liquid enzymatic reaction systems can be said to consist of three components: the substrate, the protein and the solvent. These three components interact and it is their overall interactions that determine the efficiency of the catalysis. The differential activation parameters from each of these components, having effects on catalysis, are complex and highly interdependent.

In solid–gas enzymatic systems the addition of solvent is avoided and the microenvironment of the enzyme is solely composed of substrate and product molecules. It is therefore possible to measure enzyme activity in the absence of a predominant species, contrary to liquid systems in which the solvent constitutes by far the most important molar fraction. The presence of such large quantities of solvent molecules in liquid media renders the interpretation of kinetic and thermodynamic measurements quite hazardous. Indeed, it is now well established that solvent molecules can directly interact with amino acids of the active site.[36,37] The result is an important effect of solvents on enzyme catalysis: numerous examples of modification of substrate specificity,[38] enantioselectivity,[39,40] thermodynamic and kinetic parameters[41] can be found in the literature. Therefore the ability offered by solid–gas technology to determine the kinetic and thermodynamic parameters of an enzyme in the absence of solvent is very interesting when looking for the individual properties of an enzyme.

Moreover, diffusion in the gaseous phase is more efficient than in solution because of very low viscosity coefficients and very high molecular diffusion coefficient values. Thus the reactions are less likely to be limited by diffusion.

For these reasons kinetic and thermodynamic parameters obtained in solid–gas reactors can be considered as "intrinsic parameters".

To test the ability of the solid–gas system to determine intrinsic parameters of enzymes, we have performed a complete kinetic study of alcoholysis of methyl propionate and *n*-propanol catalysed by immobilised *Candida antarctica* lipase B in a continuous solid–gas reactor.[42] The kinetics obtained were suggested to fit a Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol (Fig. 7), as is the case in liquid organic media and in supercritical carbon dioxide.[43,44] Even if mechanism determination cannot rest on kinetic data alone, these results strongly suggest that the mechanism of lipase-catalysed alcoholysis is unchanged over a wide range of non conventional reaction media. The kinetic constants inherent to this model were calculated and the values obtained were compared with the ones obtained in liquid 2-methyl-2-butanol. The use of thermodynamic activities to quantify

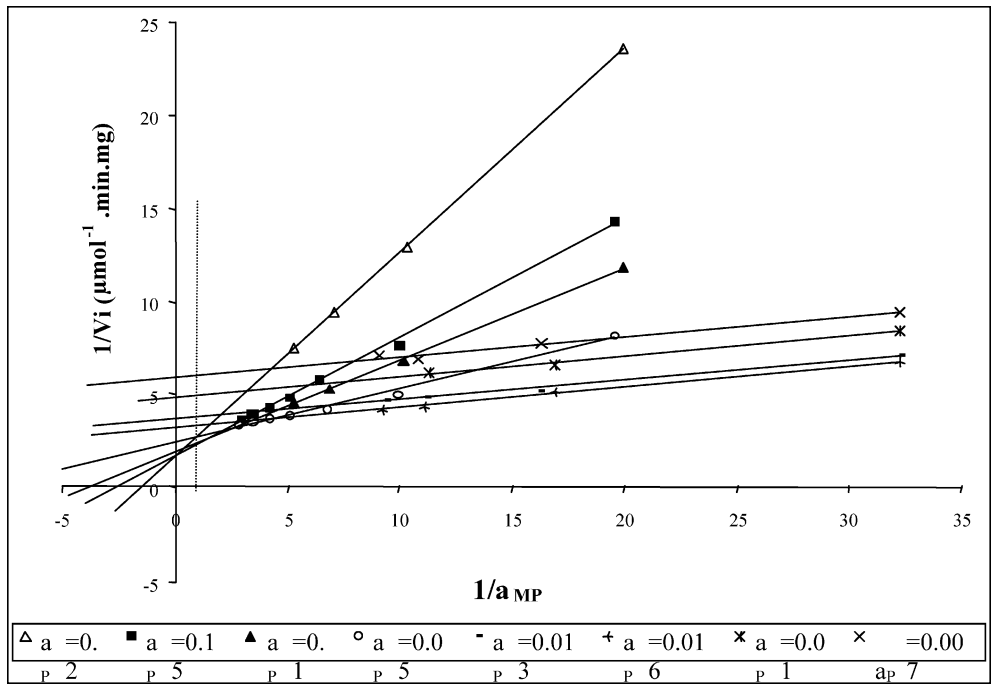


Fig. 7 Reprinted from M. P. Bousquet-Dubouch, M. Graber, N. Sousa, S. Lamare, and M. D. Legoy, *Biochim. Biophys. Acta: Protein Struct. Mol. Enzymol.*, 2001, **1550**, 90–99, with permission from Elsevier. Reciprocal initial rates *versus* reciprocal a_{MP} for different a_P values. Synthesis was carried out in a solid–gas bioreactor at 70 °C, at a molar flow rate of 1 mmol min^{-1} in the presence of 15 mg of CALB adsorbed onto Chromosorb AW DMCS. Water activity was set near to 0. For low a_P values (from 0.007 to 0.016), the plotted profiles were parallel with a decrease of the $1/V$ axis intercept. For higher a_P values, the plots converged to a limit of $1/V_{max}$ on the *y*-axis corresponding to $1/a_{MP}$ equal to 1. Indeed, in the present case, as thermodynamic activities are used instead of classical concentrations, the $1/V_{max}$ axis is not the usual *y*-axis (for $1/a_{MP}$ values equal to zero) but a parallel axis corresponding to $1/a_{MP}$ values of 1, corresponding to the highest possible value of a_{MP}. These results agree with the Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol.

substrate availability for the enzyme in both media permitted us to express all kinetic constants in terms of thermodynamic activities and to compare them. This methodology permitted us to point out a significant difference between results determined in the two media: values obtained for the methyl propionate dissociation constant and the propanol inhibition constant were found to be, respectively, 15 and 10 times lower in the gaseous phase than the ones obtained in organic medium. Experimental studies are currently in progress to explain this difference; it already seems that the inhibition of the enzymatic activity afforded by solvent molecules can largely explain the apparent lower affinity of the enzyme for its substrates in liquid organic media. Indeed, several authors have explained part of the solvent effects on enzyme kinetics by competitive inhibition of the solvents.[30,45]

Concerning the determination of intrinsic enzymatic activation energies, the solid–gas system, which allows the measurement of the activation energy of enzymatic reactions in the absence of added solvent, also appears to be an interesting tool. Indeed, the reaction thermodynamics are strongly influenced by substrate solvation and the ability of solvents to stabilize an enzyme's transition state.[46,47] In an attempt to determine the intrinsic effects of solvent polarity on subtilisin catalysis activation energies, Kim *et al.*[46] used a transfer free energy approach to calculate differences in activation energies between several organic solvents and acetone. The observed differences in activation energies were corrected for substrate desolvation, *i.e.* ground state stabilisation of substrate in a given solvent relative to acetone (the quantitative measure of this last effect was provided by saturation solubility experiments). This enabled "intrinsic enzymic activation energies" to be calculated, which resulted in a linear free energy relationship with respect to solvent polarity. This result was considered to be consistent with the charged nature of subtilisin's transition state. In this study, the calculation of differential activation energies required the choice of a particular solvent as a standard state (acetone in this case), despite the fact that it affects substrate solvation and the enzyme's transition state stabilisation. In this kind of investigation the solid–gas biocatalysis would afford an interesting standard state for enzymatic activation energy in the absence of solvent *(provided that the substrate can be vaporised).*

A powerful tool for the determination of the role of water on biocatalysis

The behaviour of biocatalysts in non-conventional media is usually greatly affected by the level of water present in the medium. Numerous attempts have been made to investigate this dependence. As is the case for the substrates, water availability in non-conventional media cannot be quantified using its concentration because the capacity of the medium to dissolve water varies considerably with its polarity. The influence of water is best analyzed in terms of its thermodynamic activity (a_w).[48,49]

Concerning the role of water on enzyme kinetics, as mentioned by many authors, the only way to assess the influence of water on enzyme kinetic behaviour is to perform a full kinetic description based on properly determined k_{cat} and K_M values at different water activities.[50] Indeed this leads to the true maximal enzyme activity, and not substrate concentration-dependent activity.

If we focus on a particular class of enzymes, *i.e.* lipases, it appears that k_{cat} obtained in liquid organic media generally increases with a_w.[51–53] This corresponds to the activation of the enzyme by hydration, involving a general role of water in making the enzyme structure more flexible and mobile, increasing the turn-over rate. However high water levels have also been shown to reduce the rate of lipase-catalysed esterification or transesterification.[51,52] This decline cannot be attributed to water competition with the alcohol because k_{cat} is obtained at a saturating substrate concentration, but might be due to physical aggregation of enzyme, reducing interfacial area and limiting mass transfer.[52,54] All these studies were performed in organic media, which makes the sole role of water impossible to assess. Indeed it was shown that at constant a_w, k_{cat} varies with the organic solvent used. For example in the case of PEG-lipase catalysed transesterification k_{cat} decreases with increasing solvent hydrophobicity.[52]

As far as the K_M values are concerned, it is shown in several studies that K_M obtained at constant a_w in different organic solvents vary with the solvent used, but as they are expressed in molar concentration instead of thermodynamic activity, it is impossible to distinguish between the variation ascribed to different partitioning of the substrate and water between the enzyme and the organic phase or to the influence of the solvent alone.[52] In another study, K_M values obtained in different solvents were subjected to treatment with the UNIFAC group contribution method, to be expressed in thermodynamic activity, but this attempt failed to give adequate alignment of the K_M in the different solvents.[53] This would indicate that solvent has its own specific effect on this parameter.

As mentioned above, the solid–gas process permits us to limit the microenvironment of the enzyme to substrates/products/water since no species are predominant, contrary to synthesis in organic solvent. As a consequence, the sole role of water can be better assessed. Moreover the thermodynamic activity of water can be continuously controlled by incorporating water in the inlet gas at the correct molar fraction.

We have studied the influence of water on the kinetics of alcoholysis of methyl propionate and *n*-propanol catalysed by immobilised lipase B from *Candida antarctica* in a continuous solid–gas reactor.[55] Results obtained concerning the substrate dissociation constants and propanol inhibition constants at different a_w are summarised in Table 2.

Table 2 Effect of a_w on dissociation constants of propanol-enzyme, enzyme-methyl propionate and acyl-enzyme-propanol complexes respectively (K_I, K_{MP} and K_P)

	a_w	K_I	K_{MP}	K_P
Gas phase	0	0.019	0.047	0.022
	0.1	0.009	0.009	0.015
	0.2	0.052	0.044	0.021
	0.3	0.161	0.161	0.013
Organic phase[a]	0	0.160	0.651	0.020

[a] Results from Bousquet-Dubouch *et al.*[42]

It appears that water affects mainly the first step of the reaction, *i.e.* the acylation step, by first decreasing the dissociation constants between both enzyme and acyl substrate (K_{MP}) and enzyme and alcohol (K_I) when a_w increases from 0 to 0.1. This can be related to the general water-induced enzyme activation, observed when biocatalysis is performed in anhydrous medium.[36,56] From a_w = 0.1 upwards water increases both K_I and K_{MP}. It was suggested that water, as polar alcohols, binds to the enzyme through polar interactions and thereby blocks the access to the active site. These results suggest that addition of water in the solid–gas reactor mimicks an increase of solvent polarity in organic media. Indeed Garcia-Alles and Gotor[57] studied the kinetics of immobilised CALB-catalysed transesterification in non-aqueous organic media. They measured how the solvent affects the first substrate (ester) binding constant and the nucleophile inhibition constant. It was shown that these constants were significantly increased when the polarity of the solvent increased; these variations could not be explained using thermodynamic arguments (solvation of the ester or the inhibitor). The solvent itself could thus lead to a decrease of the affinity between enzyme and ester or nucleophile by an effect other than solvation of these molecules, like a direct hindrance effect of the active site for example. It was shown that this direct hindrance effect increases with the polarity of the solvent.

It appears in Table 2 that K_P is not influenced by a_W nor by the state of the medium (gas or organic phase). This seems to mean that the second step of the reaction (deacylation) is not influenced by the

microenvironment, contrary to the first step (acylation step) which is both influenced by the water activity and the state of the medium.

As far as the role of water on activation thermodynamic parameters is concerned, we have shown that the solid–gas technology afforded interesting results, again because of the possibility of performing catalysis in the absence of solvent.

We have determined the enthalpic and entropic contributions of water to the Gibbs free energy of activation in the alcoholysis of methyl propionate and *n*-propanol catalysed by immobilised *Candida antarctica* lipase B, both in a continuous solid–gas reactor and in an organic liquid medium (2-methyl-2-butanol).[58] It appears that ΔH^* and ΔS^* variations with a_w are opposite in the two media. Upon increasing a_w, the effects involved in the gas phase were a decrease of the reaction energetic barrier (ΔH^*) and an increase of enzyme flexibility (ΔS^*), which means that the ground state ES complex occupies a broader distribution of conformational states. In the organic medium, the predominant phenomenon affecting thermodynamic parameters was an increased polarity of the solvent at high a_w, leading to an increase of the reaction energy of activation and to a decrease of the entropy level of the ground state ES complex.

Thus it appears that the influence of water on activation parameters measured in liquid media is affected remarkably by the solvent itself. On the contrary, the effects observed thanks to the solid–gas reactor can really be ascribed to water.

An appropriate tool for the investigation of the role of organic species on biocatalysis

Another component, like an organic compound, that differs from the reaction species, can be added to the solid–gas biocatalysis system by incorporating this additional species in the inlet gas. This offers the possibility of assessing the role of this component at different chosen levels of availability.

As an example, we have studied the effect of 2-methyl-2-butanol and hexane at a fixed thermodynamic activity of 0.3 on kinetic parameters of the alcoholysis of methyl propionate by 1-propanol, catalysed by immobilised lipase B from *Candida antarctica*.[59]

It has to be emphasized that the possibility of determining kinetic parameters at constant a_{2M2B} or a_{hexane} by fixing the partial pressure of added 2-methyl-2-butanol or hexane in the carrier gas at the adequate value, and simultaneously varying a_{MP} and a_P to perform the full kinetic study, is a specific advantage of solid–gas reactors. As a comparison, we have calculated the variation of 2-methyl-2-butanol thermodynamic activity inherent in the variations of substrate thermodynamic activities necessary to perform a full kinetic study in liquid 2-methyl-2-butanol as solvent: this involves variations of a_{2M2B} between 0.33 and 0.96! These a_{2M2B} values are obtained by calculations from molar fractions using the UNIFAC group contribution method, and correspond for $a_{2M2B} = 0.33$ to the highest substrate quantities used ($a_P = 0.5$ and $a_{MP} = 0.3$) and for $a_{2M2B} = 0.96$ to the lowest substrate quantities used ($a_P = 0.01$ and $a_{MP} = 0.05$). This very important variation of a_{2M2B} makes the interpretation of solvent effect difficult and renders kinetic parameters obtained in liquid solvents questionable, when keeping in mind the own specific effect of solvent on enzyme catalysis.

Results obtained thanks to solid–gas technology concerning the effect of 2-methyl-2-butanol, hexane and water ($a_w = 0.3$) on the inhibition constant of propanol are given in Table 3.

It appears that inhibition by propanol is stronger (K_I lower) as the hydrophobicity of the added component increases (K_I hexane < K_I 2-methyl-2-butanol < K_I water). These results indicate that these components interfere with the binding process between substrate and immobilized enzyme. As mentioned above, these components, usually used as solvents in liquid media, would act as competitive inhibitors.

In the solid–gas reactor it is possible to study the effect of increasing thermodynamic activities of an added compound on enzyme activity by keeping availability of substrates for the enzyme constant. This experiment is shown in Fig. 8 and clearly demonstrates the inhibitory character of water, 2-methyl-2-butanol and hexane. The more polar the component is, the more important the inhibitory character. This has to be related to the effect of these components on the intrinsic value of K_I, indicating that the inhibition by propanol is stronger in the presence of a hydrophobic component like hexane. It is thus clearly demonstrated that the effect of added organic components on alcoholysis catalyzed by lipase B from *Candida antarctica* is related to their inhibitory character. As reported by Uppenberg *et al.*,[60] the region around the catalytic serine (Ser-105) is polar in nature so hydrophilic components may bind to this region to form a dead-end complex. This explains the differences of the K_I intrinsic values obtained in the presence of an apolar component like hexane, of a moderately polar tertiary alcohol such as 2-methyl-2-butanol and in presence of water.

Table 3 Effect of water, 2-methyl-2-butanol and hexane on dissociation constants of propanol-enzyme complex (K_I) in the solid–gas reactor

Added component	Thermodynamic activity	K_I
None	0	0.019[a]
Water	0.3	0.161[b]
2-Methyl-2-butanol	0.3	0.045
Hexane	0.3	0.018

[a] Results from Bousquet-Dubouch *et al*. (2001).[42] [b] Results from Graber *et al*. (2003)[55]

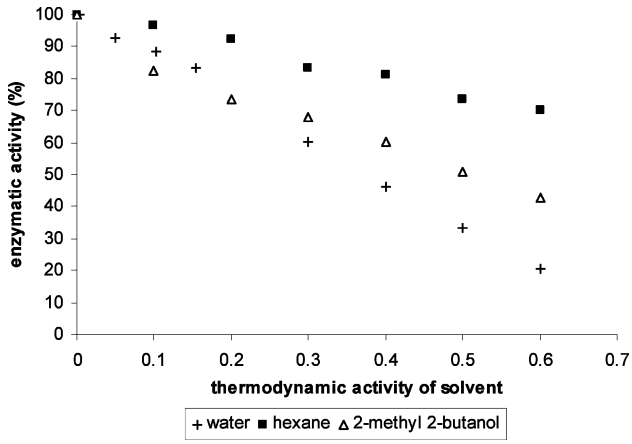


Fig. 8 Reprinted from F. Letisse, S. Lamare, M.-D. Legoy, and M. Graber, *Biochim. Biophys. Acta: Proteins Proteomics*, 2003, **1652**, 27–34, with permission from Elsevier. Effect of water, hexane and 2-methyl-2-butanol thermodynamic activity on initial rates. Reaction was carried out at 70 °C, at a molar flow rate of 1 mmol min^{-1} in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Methyl propionate and propanol thermodynamic activities were fixed at 0.15 and thermodynamic activity of solvents was varied from 0 to 0.6.

Technological applications of solid–gas biocatalysis

Enzyme technology: production of natural flavoring substances

Esters represent an important class of aroma compounds. They often possess fruity odors and are widely used by the food and the fragrance industries. Many of the interesting compounds are alkyl esters of acetic, propionic and butyric acids. Most of these compounds are obtained either by chemical synthesis for non-natural labeled molecules or by extraction from natural sources, which is dependent on seasonal, climatic and geographical variations and often requires a large amount of raw materials.

A biotechnological process can be an alternative solution for producing naturally labeled esters, minimizing the constraints

encountered in natural extraction, and offering economical costs closer to the ones of the chemical processes.

Thanks to the thermodynamic approach we developed, quick optimization of solid–gas enzymatic catalysis to perform esterification reactions was achieved and direct esterification of natural kosher alcohols and acids by Novozym 435 has been transferred successfully to industrial scale for the production of fragrances and aromas.[61]

The methodology constituted different steps, all of them deriving from the determination of the $\Delta G°$ of the target reaction by determination of the K_{eq} under different conditions in terms of the thermodynamic activities of the substrates and water. For all syntheses, three different inlet conditions were applied for obtaining the correct value of $\Delta G°$.

Once the $\Delta G°$ was obtained, *in silico* optimization was realized, after experimental determination of thermodynamic barriers. These barriers were defined by kinetic experiments and stability tests and corresponded to the upper limits of thermodynamic activities for alcohols, acids or water affecting either the stability or the reaction rate of the catalyst. Cross effects (especially concerning water and acid moieties) were also studied.

Taking into account these barriers, further optimization was realized taking into account the process constraints (liquid and gaseous flow rates, power consumption, absolute pressure on the different stages).

Tests using the defined set of parameters were then performed at the lab scale for sample production at the kilogram level. This last step also permitted us to validate the stability of the catalyst over periods varying from 50 to 100 hours. Then, results were directly transferred to the pilot scale for validation.

The industrial pilot used for real production tests is depicted in Fig. 9. This setup is developed on a closed loop of nitrogen, circulating in three different zones, thus reducing the nitrogen consumption to zero during continuous operation.

This loop is realized between a low pressure zone (flashing unit, heat exchanger and bioreactor), a medium pressure zone (gas–liquid separator) and a high pressure zone (final condenser).

Liquid substrates (acid and alcohol), water and nitrogen are injected into a flashing unit using a pressurized loop with a mass controlled leak going to the flashing unit of each liquid substrate. Once the liquid–gas flash is realized in the flashing unit, the gas enters in a heat exchanger and its outlet temperature is set to the working temperature of the bioreactor. Then, the thermodynamic activities in the gaseous stream are obtained at this stage by controlling each partial pressure as explained previously. A packed bed type reactor follows the heat exchanger, and all this part of the system is maintained under a regulated vacuum using the liquid ring vacuum pump and the vacuum regulation valve. After catalysis, the end of the process is solely dedicated to the removal of condensable molecules from the nitrogen fraction, and is performed by a two stage condensation operation involving a cooling of the gas stream coupled to an increase of absolute pressure performed by the liquid ring vacuum pump itself and a compressor for finishing the condensation process. Then, clean nitrogen at the outlet of the pressurized heat exchanger can be depressurized and recycled to the flashing unit.

In order to control proper operation of the installation, an automated sampling system was developed for gas chromatograph analyses. Thus, recycled nitrogen, inlet and outlet gaseous streams on the bioreactor were sampled and analyzed for on line monitoring purposes. Complete automated supervision was developed, in order to define a process able to operate without any human supervision. Fig. 10 gives an overview of the overall plant.

Fig. 11 shows an example of butyric alkyl ester synthesis realized at a production rate of 2.5 kg h^{-1} of ester. Because the esterification reaction is exergonic ($\Delta G°$ varying from −14 to −9,5 kJ mol^{-1} for the different syntheses), the removal of heat produced during the reaction over the packed bed on the pilot installation was found to be insufficient.

As a result, a temperature gradient exists over the catalytic bed, as a function of the degree of conversion and total molar flow rate passing over the bed. Thus, the temperature at the outlet of the reactor was found to be 15 to 20 °C higher in the majority of the tests performed at production rates of 3 to 5 kg of ester per hour.

As shown in the example in Fig. 11, since the inlet temperature was 80 °C and the production rate was set at 2.5 kg h^{-1} of ester, the temperature of the gas at the outlet was under steady state stabilized around 89 °C. The main effect of this variation of temperature on the catalytic bed was a slight decrease of the maximal conversion of the acidic moiety of 1.5% as shown in the measured conversion. Since no rapid denaturation of the catalyst was observed by a rapid decrease of the acidic conversion, heat produced by the catalysis was found to be responsible for the decrease of the conversion observed during the first hours. Calculation of the theoretical

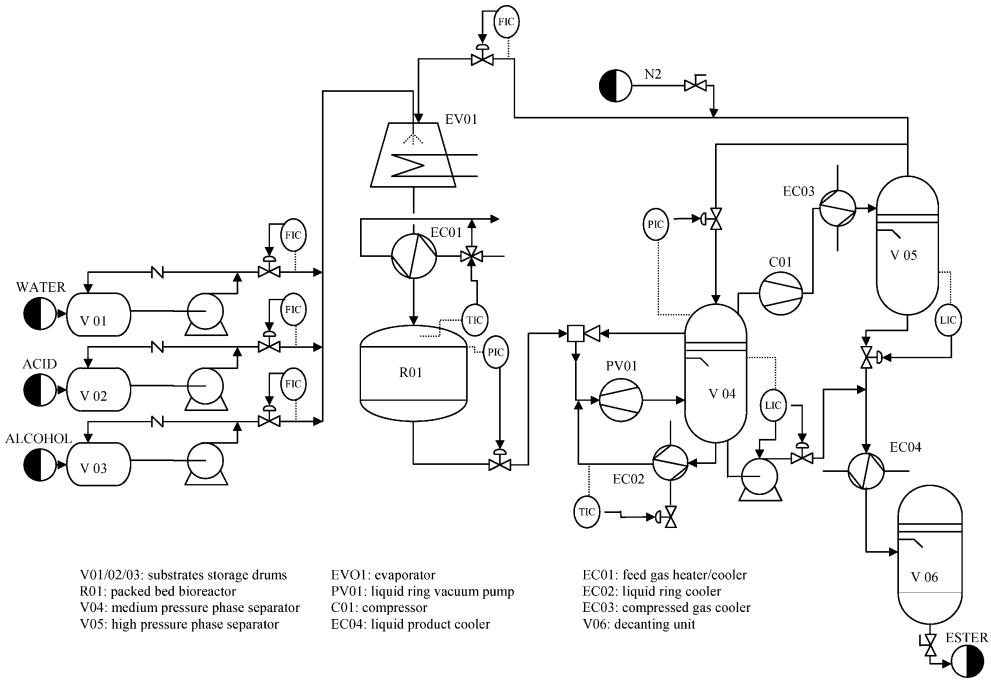


Fig. 9 Schematic diagram of the industrial continuous solid–gas bioreactor developed for the production of natural esters as a closed nitrogen loop.

Fig. 10 Industrial solid–gas bioreactor (University of La Rochelle).

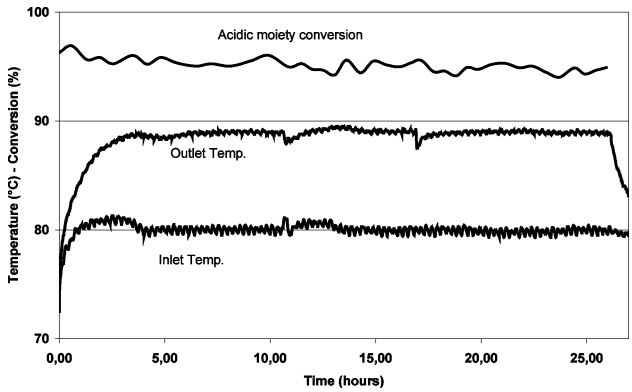


Fig. 11 Evolutions of inlet/outlet temperatures over the catalytic bed and acidic moiety conversion for a production run of a butyric acid alkyl ester realized at 80 °C (inlet gas temperature). The production rate of ester was set at 2.5 kg of ester per hour.

equilibrium point at 89 °C gave the same value as the one that was measured during the experiment indicating therefore that the temperature of the catalyst was in equilibrium with the gas leaving the bioreactor and that the increase of temperature was solely responsible for the lower conversion achieved, by changing the thermodynamic activities of the different chemical species, thus modifying the composition of the gas at equilibrium. Stability was checked for the different syntheses taking into account the heat exchange over the catalytic bed. The half-life time of the reactors for the different syntheses was found to vary from 850 to more than 2000 hours for outlet temperatures ranging from 90 to 120 °C. More detailed analysis of the behavior of the catalytic bed strengthens the conclusion that stability in this system is mainly governed by the acid thermodynamic activity. As a result, the reactor is not homogeneous in terms of stability and the catalyst is more subject to denaturation in its first part (lower temperature, higher acid thermodynamic activity) than in the last part (higher temperature, lower acidic thermodynamic activity). Different syntheses coupling C1 to C4 carboxylic acids and C1 to C9 alcohols for the industrial production of natural aromas were optimized and production tests were performed. Table 4 summarizes some examples of the

Table 4 Examples of ester production rates and acid conversion achieved on the preindustrial scale bioreactor for different optimized syntheses of acetic, propionic, butyric or iso-butyric acid alkyl esters

Acidic moiety	Number of alcohols tested	Acid conversion (%)	Ester production rate/kg h^{-1}	Catalyst charge/kg
Formic	2	94.0–95.4	1.5–2.0	2.0–3.0
Acetic	5	95.0–97.4	3.8–4.0	2.0–3.0
Propionic	5	95.6–98.0	3.0–4.5	2.0–3.0
Butyric	5	97.5–98.1	2.6–3.2	2.0
Iso-butyric	5	94.0–97.8	2.2–3.5	2.0

production rates of the system that can be achieved with an acidic conversion higher than 95% and a maximal excess of alcohol of 2 (mole per mole) in the worst cases ($\Delta G° > -11$ kJ mol^{-1}) for a residence time commonly observed to be as low as 0.5 to 0.15 second.

If the production of natural esters is the first example of a large scale application of solid–gas biocatalysis, many other systems are being studied today with the view of short or medium term development.

Lipases, offer important potential for applications since they are able to carry out reactions of esterification as well as transesterification (acidolysis or alcoholysis), inter esterification or hydrolysis. Moreover, their specificity and/or selectivity is suitable for the production of high added value molecules (stereospecific alkylation, acylation or hydrolysis for the resolution of racemic mixtures of acids, alcohols or esters).

Dehydrogenases also represent a class of interesting enzymes since chiral reduction of ketones can lead to the production of enantiomerically pure alcohols for fine chemistry, and since it has been demonstrated that the regeneration of the cofactor is effective in a solid–gas system by addition of a second substrate.[3]

Many other applications can be envisaged, but one must take into account that solid–gas biocatalysis can only be applied to transformations involving compounds presenting a criteria of volatility, thus limiting the fields of applications to smaller molecules, compared to the ones accessible by other systems (liquid or supercritical ones).

The next challenge: using whole cells

Solid–gas biocatalysis must not only remain synonymous for use of isolated and purified enzymes. Whole cells are of particular interest, on the one hand by lowering the costs related to the catalyst compared to the use of purified enzymes, and on the other hand due to the possibility of coupling several enzymatic systems, contained within the cell itself, in cascade in the same micro reactor. One of the major applications that should benefit from this technology has a strong environmental impact and concerns applications related to the bioremediation of gas effluents.

Different strategies can be envisaged for the bioremediation of polluted gases and two main approaches can be defined:

• either a simple modification of the pollutant for facilitating its trapping in a second step (reduction of aldehydes or ketones to produce the corresponding alcohols presenting a lower volatility, dehalogenation of chloro alkanes, leading to the formation of epoxides or alcohols, acylation or alkylation for increasing the chain length or modifying the hydrophilic/hydrophobic balance…),

• or performing a cascade of reactions insuring a catabolic degradation of the pollutant.

Although some work was initiated at the end of the 1980s on the subject, no real development has resulted from the reported works.

Nevertheless, some works were recently published in the scope of providing new solutions for bioremediation purposes.[13,14,16]

Alcohol dehydrogenases are of particular interest since they are able to catalyze reactions of oxido-reduction with different specificities according to their origin towards primary or secondary alcohols. Nevertheless, they present a major disadvantage which is the use of a relatively expensive nicotinamidic cofactor.

Thus the use of whole cells used as an enzymatic bag containing enzymes and cofactor has received much attention over past decades in liquid systems in order to develop processes insuring an efficient *in situ* cofactor regeneration.

However, the majority of the systems were confronted with the loss of the cofactor which was withdrawn by the circulating liquid phase. Solid–gas catalysis can constitute an interesting alternative for solving this problem, since the washing of the cofactor by a circulating gas is impossible because of its non volatile character.

Maugard *et al.*[62] showed that the reduction of an aldehyde to the corresponding primary alcohol by *Saccharomyces cerevisiae* lyophilized cells was possible in a solid–gas system with an effective cofactor regeneration insured by the oxidation of ethanol.

However, this work and results published by Goubet *et al.*[15] highlighted an important effect of the cellular matrix, which completely modifies the conditions of hydration necessary for the expression of catalytic activity. Tests performed on isolated and purified baker's yeast ADH showed that the enzyme was active at a water activity as low as 0.1, whereas a minimal water activity of 0.4 was necessary for obtaining an enzymatic activity when whole cells were used.

Haloalkane dehalogenase is an enzyme capable of catalyzing the conversion of short-chain aliphatic halogenated hydrocarbons to a corresponding alcohol. Because of their broad substrate specificity and cofactor independence, haloalkane dehalogenases are attractive biocatalysts for gas-phase bioremediation of pollutant halogenated vapor emissions.

Work recently published on haloalkane dehalogenase activity from *Rhodococcus erythropolis* cells for the hydrolysis of chloro and bromo alkanes also highlighted the importance of the cellular matrix constituting the macroenvironment of the enzyme.[16] Since a higher water activity is required for liberating the activity in whole cells compared to that necessary for the purified enzyme, the authors showed that proton exchange was effective in the system, due to the production of HCl after hydrolysis of chloro alkanes. Addition of a volatile Lewis base (triethylamine), coupled to treatment of the cell wall by lysozyme showed that inhibition by HCl could be prevented efficiently. Some similar results were reported previously by Dravis *et al.*[63] concerning the use of purified haloalkane dehalogenase from *Rhodococcus rhodochrous* in the dehalogenation reaction of 1-chlorobutane or 1,3-dichloropropane. Nevertheless, the *Rhodococcus rhodochrous* dehalogenase was found to require a water activity of 1 (a water activity of 0.5 led to a decrease of 80% in enzymatic activity). Triethylamine addition, altering the electrostatic environment of the enzyme *via* a basic shift in local pH, under such conditions of hydration allowed a tenfold increase in activity.

Whole cells appear to be suitable catalysts for solid–gas biocatalysis. Nevertheless, while technological feasibility has been demonstrated, the optimization of these systems involving cells remains a hard task. Some routes have been identified, and a better comprehension of the effects due to macro- and microenvironment of the enzyme and the factors affecting the hydration/solvation of the catalytic material clearly constitutes the key for maintaining these systems active and stable for proper operation.

Concerning the realization of a cascade of reactions, no consistent data are available in the literature, proving that multiple steps reactions can take place in the same cell.

Some work reported by Barzana *et al.*[6] showed that two enzymatic systems were able to be active at the same time. The oxidation of primary alcohol by cells of *Pischia pastoris* involving an alcohol oxidase can be enhanced in terms of stability by the addition of catalase or peroxidase, by elimination of the activated species of oxygen produced during the reaction. However these two enzymatic systems are not both located in the cell.

Following the work reported by Hou,[25] concerning the methane monooxygenase from *Methylosinus sp* CRL 31 for the production of epoxides, we tested the degradation of trichloroethylene by freeze-dried cells of *Methylosinus trichosporium* (unpublished results). According to the degradation pathway presented in Fig. 12, the unique action of the methane monooxygenase results in 3 possible products: the epoxyde, the dichloroacetate or the chloral hydrate.

Further degradation requires the action of dehydrogenases or other monooxygenases in cascade to degrade trichloroethylene either in metabolic intermediates, or in CO_2 and HCl in the case of a complete degradation. The residual concentration of TCE measured at the outlet of the reactor over a period of 24 hours

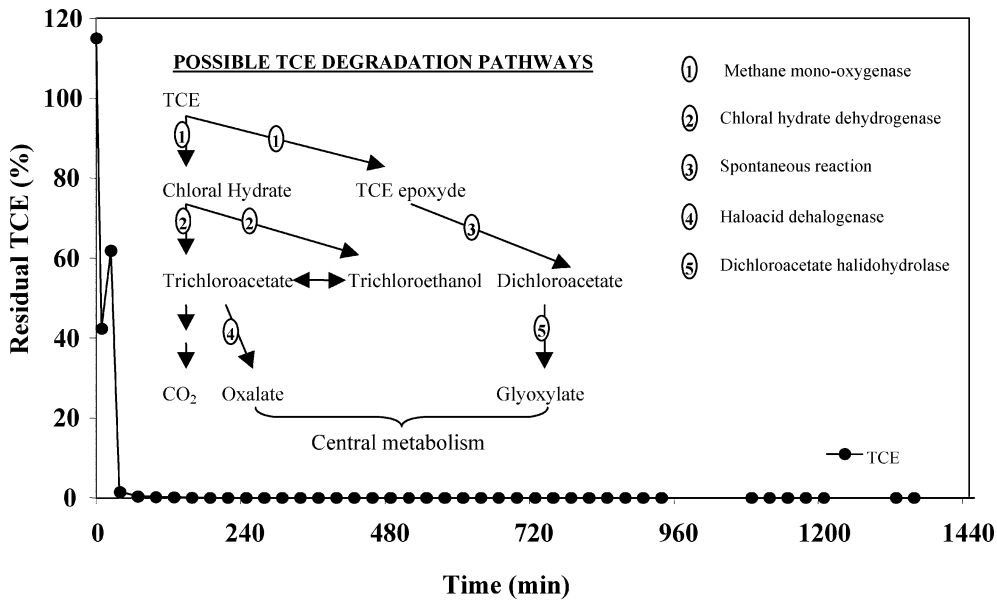


Fig. 12 TCE removal in a solid–gas bioreactor by *M. trichosporium* freeze-dried cells. 50 mg of dehydrated cells are placed in a packed bed reactor at 40 °C. Inlet gas contains water, TCE and methanol at the following levels: a_w = 0.7, a_{TCE} = 0.1, $a_{methanol}$ = 0.1. The total gaseous flow rate is set at 500 μmol min^{-1}. The possible pathways for degradation of TCE are summarized in the figure with the main enzymatic activities involved.

showed that freeze-dried cells of *M. trichosporium* were able to degrade all the TCE in a continuous way. The presence of any product of the action of methane monooxygenase (epoxide, chloral hydrate or dichloroacetate) was not detected in the outlet gas, suggesting a possible re-use by a second (or several) enzymatic system(s) since all three products have a sufficient saturation vapor pressure at 40 °C to be removed continuously by the gaseous stream.

Therefore, a cascade of reaction may occur in whole cells, but the complete characterization of the different tested systems still has to be done, and more fundamental studies appear necessary if one wants to ensure the development of new efficient biofilters.

Conclusion

Solid–gas systems appear to be a powerful tool for studies devoted to the effect of hydration/solvation and more generally for studies on the effect of modification of the microenvironment on biocatalytic activity and stability. The main reason is that solid–gas systems allow a very accurate control of the microenvironment of the biocatalyst by a complete independent control of thermodynamic parameters.

Nevertheless, a lot of questions are still without clear answer, and a lot of work still remains to be done in order to answer the following questions:

• Does the biocatalysis occur directly with molecules in the gaseous state or does the biocatalyst need to be solvated by the substrates first?

• What are the energetics in solid–gas biocatalysis compared to liquid systems?

• How do the mechanisms of enzymes working on gaseous substrates differ from those in solution? How do they differ concerning the binding step and the catalytic process?

• What is the exact influence of water and organic molecules on the enzyme structure? Could its effects on properties such as selectivity, affinity, binding constants and catalytic constants be predictable by controlling the hydration/solvation state?

• What is the partition of water and organic inside more complicated systems such as whole cells, and how can the physicochemical properties of the macro and micro environments affect this distribution?

As previously underlined, solid–gas biocatalysis, because of its peculiarities enables a more accurate approach for studying the effect of the microenvironment on enzymatic activity and stability. This allows access to intrinsic parameters, thus providing additional information for a molecular understanding of enzyme catalysis in general. Finally, solid–gas catalysis appears as probably the most appropriate and the most complementary experimental tool for validating molecular modelization experiments.

From a more applied point of view, biocatalysis in the gas phase seems today more promising than ten years ago, since the demonstration of its ability to face new biotechnological processes, not only in terms of technical feasibility but also in terms of production rate and competitiveness, has been successfully performed.

Nevertheless, important bottlenecks still exist, and the next challenge will really be the use of whole cells as micro reactors for multi steps transformations and the development of technological tricks allowing the number of compounds usable by the technology to be enlarged.

Moreover, solid–gas technology represents a clean technology, since, compared to the organic liquid systems, the use of organic solvent can be reduced to zero. Its use shall fulfil the definition of "green processes" and numerous applications should benefit from this technology concerning either the production of biomolecules or the bio-treatment of waste gases for toxic removal or modification.

Beside these application fields, biosensors and analytical techniques should benefit from the technology. Some examples have already been described concerning the use of a biological part constituting the body of detection, and a transmitter for detection in gaseous media[35] The detection of formaldehyde by a formaldehyde dehydrogenase coated onto a piezoelectric crystal was performed at the ppm level. Detection of pesticides and organophosphorous compounds at ppb levels has been rendered possible by the same technique involving either acetylcholinesterase or butyrylcholinesterase. And lastly, very specific biosensors were developed with the same approach by using antibodies directed against parathion, or against the benzoyl ecognine for the specific detection of cocaine.

Concerning analytical developments, one can imagine easily that affinity gas chromatography can be envisaged seriously, as well as the realization of on-line specific derivatization precolumns for GC analysis.

Abbreviations

R	Ideal gases constant (8.314 J mol^{-1} K^{-1})
T	Temperature (K)
Pa	Absolute pressure in the system (atm)
$\dot{Q}v^{n}_{N2}normalized$	Normalized volumetric flow rate of carrier gas in line n (l/h at 273 K and 1 atm)
$\dot{Q}^{n}_{N2}$	Molar flow rate of carrier gas in line n (mol h^{-1})
$\dot{Q}^{n}_{X}$	Molar flow rate of substrate X in line n (mol h^{-1})
$Pp_{\mathrm{sat}X}{}^{n}$	Saturation pressure of compound X in line n (atm)
Pp^{n}_{X}	Partial pressure of compound X in the gas entering the bioreactor (atm)
a_{X}	Thermodynamic activity of compound X in the bioreactor (dimensionless)
$\dot{Q}v_{\mathrm{total}}$	Total volumetric flow rate in the bioreactor (l/h)
$\dot{Q}_{\mathrm{total}}$	Total molar flow rate in the bioreactor (mol h^{-1})

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CRITICAL REVIEW

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Strategic biocatalysis with hyperthermophilic enzymes

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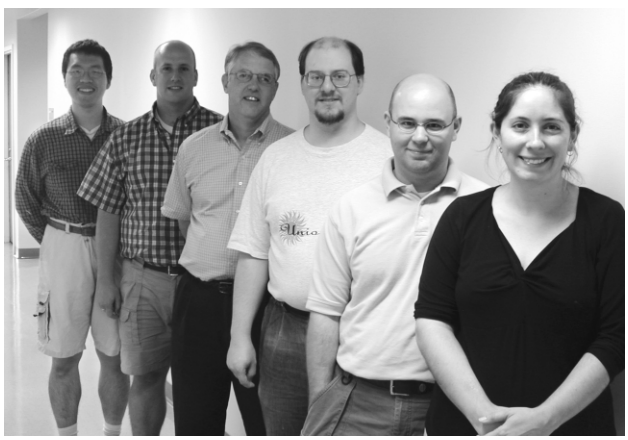
With the advent of genome sequence information, in addition to capabilities for cloning and expressing genes of interest in foreign hosts, a wide range of hyperthermophilic enzymes have become accessible for potential applications for biocatalytic processes. Not only can these enzymes be useful for strategic opportunities at high temperatures, but there may also be advantages that derive from their relatively low activity at suboptimal temperatures. Examples of several possible ways in which hyperthermophilic enzymes could be used are presented, including cases where they could serve as environmentally benign alternatives in existing industrial processes.

Introduction

Enzymes have become an increasingly attractive alternative to chemical catalysts because of their environmentally benign attributes.[1] Not only are they highly specific to the molecular features of substrates, enzymes are also highly catalytic in terms of reaction rate acceleration. Unlike chemical catalysts, enzymes rarely require toxic metal ions for functionality and, while in some cases they have been found to catalyze reactions in organic solvents,[2–4] they typically work best under aqueous conditions at moderate temperatures. If enzymes were able to operate over a wider range of conditions than has historically been considered for biocatalysis, the opportunities for their utilization could be expanded.[5] Even though methodologies exist for expanding the functional ranges of enzymes (*e.g.*, directed evolution[6–8]), it is often desirable to identify wild-type enzymes for particular applications as starting points for optimization efforts and because of regulatory concerns. In this regard, enzymes from extremophiles, which are intrinsically stable in otherwise biologically-challenging environments, should be considered.

Extremophilic enzymes are derived from extremophiles—organisms that grow optimally under otherwise biologically harsh conditions (*e.g.* high temperature, low temperature, high acidity or alkalinity, high salinity, *etc.*).[9–11] Extremophilic enzymes are frequently resistant to the environmental situation in which the host organism thrives, offering novel sources of robust biocatalysts that can withstand the demands of industrial processes.[12,13] Thus, extremophilic enzymes offer the advantage that they could be integrated into existing processes with little need to change normal operating parameters. Hyperthermophiles, extremophiles growing at temperatures of 80 °C and above, produce biocatalysts that are intrinsically thermostable and thermoactive.[14] In some instances



The Hyperthermophile Group at NC State University (authors left to right): Chung-Jung Chou, Matthew Johnson, Robert Kelly, Kevin Epting, Donald Comfort, and Shannon Conners. Not pictured: Swapnil Chhabra, Kristen Jones, and Amitabh Sehgal.

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they also offer another unique advantage: at suboptimal temperatures, hyperthermophilic enzymes either have reduced activity or essentially no activity.

Discussed here are opportunities for biocatalysis that make strategic use of hyperthermophilic enzymes which function at high temperatures, in addition to examples that capitalize on their reduced or modified function at suboptimal temperatures.

Oil and gas well stimulation—enzyme breakers

Galactomannans are used in the pharmaceutical industry for drug delivery,[15–17] in the food industry as thickening agents,[18] and in the oil industry for oil/gas well fracturing.[19–21] Galactomannan is a polysaccharide frequently found in the seed endosperm of leguminous plants, and comprises a mannose backbone linked by β-1,4 glycosidic bonds and decorated with α-1,6-bonded galactose residues.[22] The rheological properties and solubility of galactomannan are determined by the degree of backbone decoration with galactose and the length of the mannan backbone.[23] These properties can be modified through the enzymatic action of glycoside hydrolases, enzymes that break glycosidic linkages, liberating sugar molecules or saccharide chains. Enzymes known to act on galactomannan are endo-β-D-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), and α-galactosidase (EC 3.2.1.22). β-Mannosidases are exo-acting β-mannanases, which typically hydrolyze the terminal glycoside group on the polymer backbone, as shown in Fig. 1. Endo-acting β-mannanases cleave the internal β-1,4-mannosidic bond, reducing the chain length, while α-galactosidase removes the side chains from the mannan backbone. While α-galactosidase does not reduce the viscosity of galactomannan, it enables complete hydrolysis of the galactomannan polysaccharide by β-mannanase and β-mannosidase.[19]

The enzymatic modification of galactomannan can be applied to the oil industry to fracture oil/gas well and stimulate flow for petroleum extraction.[19–21,24] Oil and gas well fracturing stimulates the well by creating cracks through which oil and gas can seep through the bedrock perpendicular to the well bore.[25] To stimulate the well, a fracturing fluid, consisting of proppant (*i.e.*, sand particles) suspended in a matrix, is pumped into the well at sufficient pressure to fracture the well, as shown in Fig. 1. After fracturing, the fluid must be broken down so that it can be pumped out, and the well can start to produce. One common fracturing fluid matrix is guar gum, a common galactomannan, which can be hydrolyzed either by chemical oxidation or enzymatic action. The viscosity in the guar solution is needed to suspend the proppant in the fluid being injected into the well. Enzymes or chemical oxidizers used for this purpose are referred to as "breakers".[19]

Chemical oxidizers, such as persulfates, can react before the fracturing process is complete, resulting in reduced well stimulation.[19] Alternatively, enzymatic breakers can be incorporated into the fracturing fluid and the guar hydrolyzed *in situ*. The proppant remains in the well to hold open crevices and allow the oil or gas to

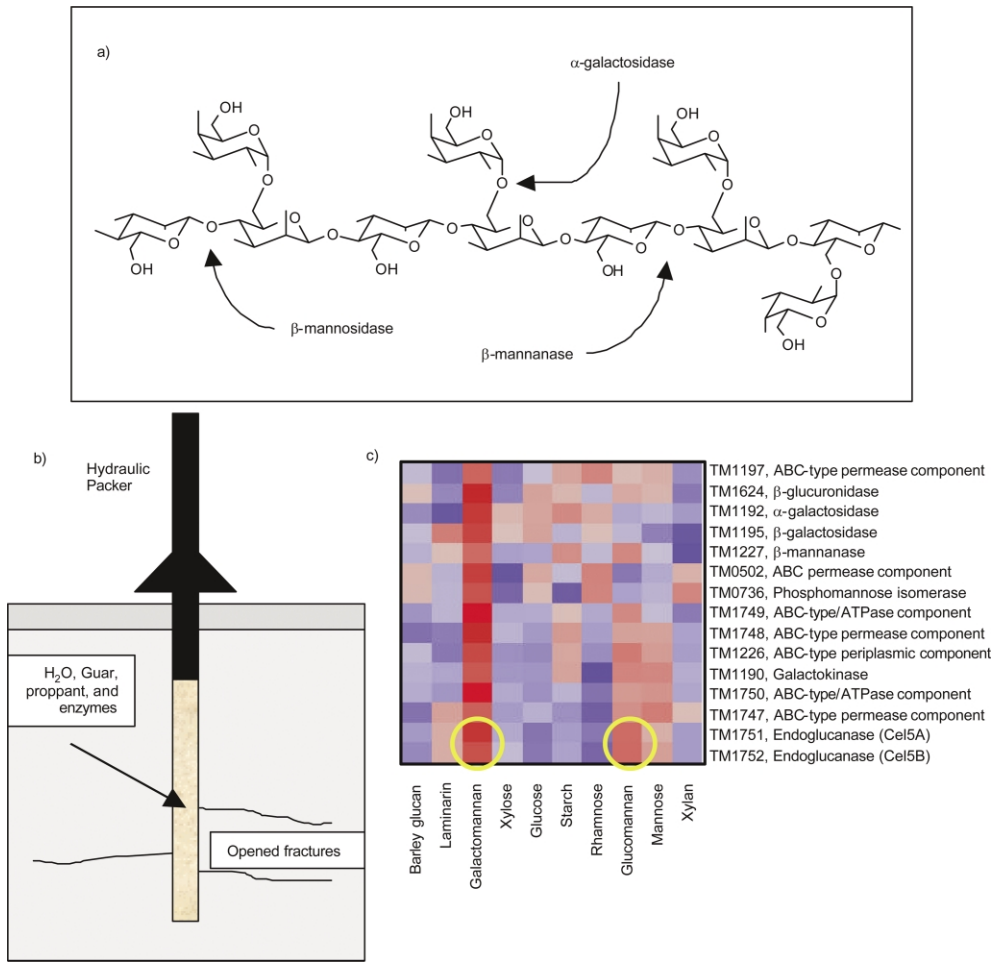

Fig. 1 a) Galactomannan is hydrolysed by the action of the galactomannanases α-galactosidase, β-mannanase, and β-mannosidase, reducing the galactomannan to oligo- and monosaccharides. Hydroxyl groups at positions 2, 3, and 4 have been omitted for figure clarity. b) Galactomannan can be used as the supporting matrix for hydraulic fracturing of oil/gas wells to prop open crevices allowing oil and gas to flow. c) Microarrays can be used to identify differentially expressed genes corresponding to desirable biocatalysts. Shown is a differential expression heat plot for the hyperthermophile *Thermotoga maritima* grown on a variety of carbohydrates, including galactomannan. In addition to the expected up-regulation (red) of genes encoding β-mannanase (TM1227) and α-galactosidase (TM1192), mixed model statistical analysis of differential expression data also showed up-regulation of two genes encoding putative endoglucanases *Tm*Cel5A (TM1751) and *Tm*Cel5B (TM1752) on mannan polysaccharides (circled in yellow).[99] These genes were cloned and expressed in *E. coli* and found to be highly active on galactomannan.

flow. As wells are drilled deeper, temperatures at the lower reaches will be hotter, with temperature gradients over 30 °C km^{-1}.[26] Therefore, the biophysical properties of the enzymatic breakers are important because of the increased temperature that can occur in the deeper reaches of the well. Enzymes from hyperthermophilic organisms that are active at high temperatures are ideal for use in these environments. In addition, it is desirable to have enzymes that have little activity at surface temperatures (35–40 °C), because this would result in premature hydrolysis of the guar. To this end, hyperthermophilic enzymes are potentially important reagents for the oil/gas well stimulation process. Hyperthermophilic enzymes have extremely low activity at low temperatures, but their thermoactivity would be activated at higher temperatures such that efficient hydrolysis of guar in deep wells occurs.[20,21] In fact, hyperthermophilic organisms have been isolated from deep well environments. The hyperthermophile *Thermococcus sibiricus* has been cultured from a high temperature oil well in Siberia,[27] indicating that the temperature range within the wells is within the range of hyperthermophilic enzymes.

From genome sequence information as well as from more conventional enzyme discovery approaches, hyperthermophilic galactomannanases and α-galactosidases have been identified.[28,29] There are a number of hyperthermophilic β-mannanases that are active over a broad range of temperature and pH. For instances, the β-mannanases from *Thermotoga maritima* and *Thermotoga neapolitana* both have optimal activity at 90 °C and pH 7.0.[29,30] Another thermophilic β-mannanase from *Rhodothermus marinus* is optimally at 85 °C, but under acidic conditions (pH 5.4).[31] There are also a number of α-galactosidases with optimal temperatures ranging between 90 and 95 °C from the hyperthermophiles *Thermus brockianus* ITI360,[32] *T. maritima*,[33] and *T. neapolitana*.[34] Furthermore, the pH optima vary from 5.0 for *T. maritima* to 7.0 for *T. neapolitana*, thus providing a wide range of operating conditions for these α-galactosidases.

The potential for use of hyperthermophilic enzymes for oil and gas recovery has been examined in laboratory settings. *T. neapolitana* β-mannosidase, β-mannanase and α-galactosidase have all been evaluated for viscosity reduction of galactomannan solutions at elevated temperatures.[19] It was shown that β-mannanase substantially reduced the viscosity of galactomannan solutions. When β-mannanase was combined with α-galactosidase the residual particulate material was minimized, indicating that these enzymes could be used as enzymatic breakers for oil and gas well recovery.[19] Based on these results, opportunities for hyperthermophilic enzymes for this application have been proposed.[20,21] As the demand of oil and gas increases and these resources become more difficult to recover, hyperthermophilic enzyme-based approaches for oil and gas recovery efforts should be examined more closely for both environmental and production reasons.

Bioethanol production

Oil is expected to be the primary energy source world-wide until 2025, during which time the consumption of oil is expected to rise by 54%.[35] In this same period, the greenhouse gas carbon dioxide (CO_2) is expected to increase from 23.9 to 37.1 billion metric tonnes per year.[35] To reduce CO_2 emissions and save natural resources, alternative energy sources must be utilized in place of fossil fuels. One possible alternative is an ethanol-based fuel economy. Hydrous ethanol as an auto fuel was used extensively in Brazil in the early 1990's, but gasoline has since replaced it there as the most common auto fuel. Still, there are more than 3.5 million operating vehicles in Brazil that use hydrous ethanol as fuel.[36] In the US, ethanol is currently used as an oxygenate in gasoline, which allows it to burn cleaner and improves the octane rating.[37] With recent concern over the health effect of methyl *tert*-butyl ether (MTBE) as a ground water contaminant, alternative oxygenates including ethanol are being utilized.[37,38] Ethanol has also been proposed as a stable source of protons for fuel cells.[39] Ethanol as an auto fuel or fuel cell proton source is environmentally attractive. The carbon dioxide released during ethanol combustion is recently fixed CO_2 and part of the active carbon cycle, so new greenhouse gas release can be significantly reduced.[36]

To be a green process, ethanol must be produced from a renewable resource. Fermentation of sugars to ethanol is one such method. There are many current processes for producing ethanol *via* fermentation using a variety of carbohydrate sources. The most common source in the US is corn, which is processed by milling, enzyme treatment (liquefaction, saccharification), fermentation, and distillation, as shown in Fig. 2. Another sugar source that has been studied is potatoes and potato waste.[40] The potatoes are ground into small particles, heated and treated with enzymes to hydrolyze the starch to dextrin, and fermented by yeast to ethanol, which is then recovered. A similar strategy can potentially be used to ferment starch from other tubers, such as sweet potatoes. The common sweet potato (*Iponea babatas*), in fact, has an intrinsically higher dry mass, over 40% for *I. batatas* as compared to under 10% for potatoes (*Solanum tuberosum)*.[41]

The enzymes necessary for complete hydrolysis of the starch to glucose or maltose include α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41), α-glucosidase (EC 3.2.1.20), and glucoamylase (EC 3.2.1.3). These enzymes work synergistically to remove the branched chains, hydrolyze the linear polysaccharide, and hydrolyze maltooligosaccharides to glucose. The starch is hydrolyzed through the processes of liquefaction and saccharification, shown in Fig. 2. The starch hydrolysis process can be improved by using hyperthermophilic glycosidases, allowing processing to be carried out at elevated temperatures where the solubility of starch is greater and substrate and product diffusional rates are higher. Higher temperatures are also beneficial in reducing contamination of the sugar mixture, which can serve as an excellent medium for unwanted organisms incapable of producing ethanol. To date, many of the required amylolytic enzymes needed for starch hydrolysis have been identified and/or characterized from hyperthermophilic organisms.[42–46]

Another property of hyperthermophilic enzymes is that they exhibit significantly reduced activity at low temperatures. One approach to increase the processing efficiency of tuber and root starch into ethanol involves developing transgenic plants that produce hyperthermophilic amylolytic enzymes *in situ*. For example, sweet potatoes grow below ground level at about 18 °C, a temperature at which hyperthermophilic enzymes are virtually inactive. Thus, there should be minimal interference with normal plant development. Furthermore, hydrolytic activity of the hyperthermophilic enzymes can be activated as the sweet potatoes are mashed and heated. Incorporating genes encoding hyperthermophilic enzymes directly into the plant has considerable potential for saving time and money, since the enzymes are produced as a consequence of plant growth. Using this technique, less enzymes need to be added, in contrast to traditional starch processing shown in Fig. 2. Ultimately, the liquefaction and saccharification steps could be combined to hydrolyze the starch in a single "one-pot" process.

Production of α-amylase using genetically modified potatoes has been demonstrated: a fusion gene of α-amylase from *Bacillus stearothermophilus* and glucose isomerase from *Thermus thermophilus* has been inserted into a potato.[47] The starch from the potato was hydrolyzed to glucose and converted to fructose by glucose isomerase. The insertion of thermophilic archaeal genes into plants has also been shown to be possible for the expression of α-glucosidase from *Sulfolobus solfataricus* in a transgenic tobacco plant that produced the active enzyme capable of surviving tissue preservation.[48]

The final step in bioethanol production is the fermentation of glucose into ethanol, followed by distillation to purify the ethanol. To reduce purification expenses, it is desirable to ferment the saccharification mixture to generate the greatest concentration of ethanol. One method of increasing the ethanol concentration is very

high gravity fermentation, which has produced 23.8% v/v ethanol from wheat mash containing 38% dissolved solids.[49] Continuing research in very high gravity fermentation has investigated supplementing the media with nitrogen additives[50] and utilizing multistage continuous fermentors,[51] both of which generated about 17% v/v ethanol.

High fructose corn syrup production

In addition to production of fuel ethanol from transgenic plants, other processes that take advantage of hyperthermophilic enzymes can be envisioned, such as the production of high fructose corn syrup (HFCS), a sweetener used in soft drinks, teas, condiments and baked goods. The HFCS process currently utilizes corn as a source of starch for the production of fructose. While other sources of starch, such as potatoes, wheat, and tapioca can be used for producing fructose syrup, corn is the most economical choice.[52] The production of HFCS was one of the first large scale applications of immobilized enzymes.[14]

The HFCS process consists of three main steps, liquefaction, saccharification, and isomerization. The starch is processed by liquefaction and saccharification at high temperatures to produce low glucose, as shown in Fig. 2. In the liquefaction process, starch granules are gelatinized in a jet cooker at 105 to 110 °C and pH 5.8–6.5 for 5–8 minutes and then treated with a thermostable α-amylase at 95 °C for 1–2 hours. After treatment, the average oligosaccharide chain length is 10–13 glucose residues.[53] This process must be run at low pH in order to prevent byproduct and color contamination.[14] The saccharification process utilizes glucoamylase and pullulanase to complete the hydrolysis of starch to glucose. This step occurs at 55 to 60 °C and a lower pH (4.2–5.0), which requires acidification of the mixture. In the last step, isomerization, the glucose is converted into fructose by immobilized xylose isomerase (often referred to as glucose isomerase because it can convert glucose to fructose). The immobilized xylose isomerase columns typically operate at 60 °C and pH 7.5–8.0 for times of 1–4 hours.[53] Under these operating conditions, a mixture containing 42% fructose is produced.[52] However, 55% fructose is required to have the same sweetness as sucrose from cane sugar. Enriched syrup of 90% fructose is prepared using strong acid cation-exchange chromatography, which is then combined with the 42% fructose to achieve the desired 55% fructose blend.[54]

There has been much work in recent years attempting to discover, or create through mutagenesis techniques, thermally

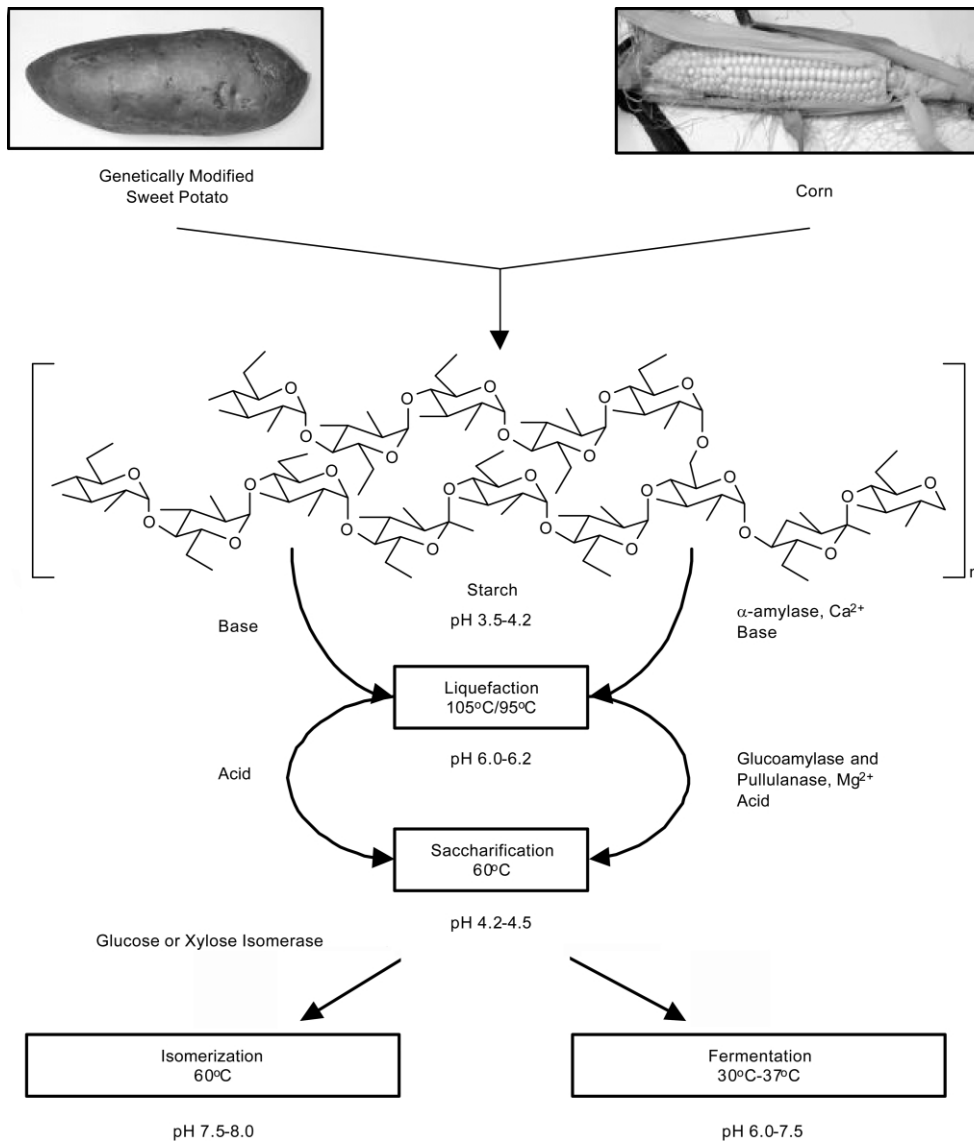

Fig. 2 The saccharification process is similar for various sources of starch. Starch is hydrolysed by α-amylase during the liquefaction process, which occurs at 95 °C. The starch is further hydrolysed by glucoamylase and pullulanase after acidification. The resulting glucose can then be utilized in subsequent processes, such as fermentation to ethanol or isomerization to high fructose corn syrup. If transgenic plants containing genes encoding starch processing enzymes are utilized, addition of biocatalysts from other sources may be unnecessary.[53] Hydroxyl groups at positions 2, 3, 4, and 6 have been omitted for clarity.

stable, low pH active xylose/glucose isomers enzymes.[55–63] This work is driven by the desire to eliminate the chromatography purification step from the HFCS process by achieving 55% fructose content through adjustment of the equilibrium concentration of fructose by raising the temperature. Because the glucose–fructose equilibrium favors fructose as temperature increases,[64] operation at higher temperatures could potentially eliminate the need for chromatographic enrichment.[65] At the current operating temperature of 60 °C, the theoretical equilibrium concentration of fructose is 50.7%, whereas if the process is operated at 90 °C, the equilibrium rises to 55.6% fructose.[14] A comparison of the *Thermotoga* xylose isomerases and the xylose isomerase from *Streptomyces murinus* (an enzyme currently used in the industrial production of fructose from glucose) determined that for production at 90 °C, the estimated productivity for *S. murinus* isomerase dropped to 200 kg fructose per kg enzyme, whereas the *T. neapolitana* isomerase had a lifetime productivity of 1000 kg fructose per kg enzyme.[66] One concern when operating at these elevated temperatures is byproducts resulting from the Maillard reaction. In order to reduce these byproducts, the reaction would need to be operated under acidic conditions and at rapid processing rates to minimize enzyme–substrate exposure.

There are a number of possibilities for streamlining the HFCS process by incorporating extremophilic enzymes. The liquefaction step occurs at pH 6.2, which has to be obtained by neutralizing the starch solution from the natural pH of about 4.5.[53] If a thermally stable α-amylase that has activity at pH 4.5 can be directly incorporated into the process, it will eliminate the need to change the pH to 6.2 and then back to 4.5 for the saccharification step. One such α-amylase has been generated by directed evolution *via* DNA shuffling of three enzymes, and was screened from over 19 000 possible candidates.[67] These candidates were screened for activity at pH 4.5 and 100 °C without Ca^{2+} and compared to current wild-type enzymes. The enhanced thermostability of this enzyme resulted in a greater degree of starch hydrolysis at 115 °C at pH 4.5.[67] If acidophilic, thermostable pullulanases and glucoamylases are discovered or evolved, the saccharification step can be performed at the same temperature and pH as the liquefaction step eliminating the need to cool the liquefied stream or saccharified stream. This assumes that a thermally stable xylose isomerase can also be integrated into the process. There are already several potential glucose isomerase candidates for thermal starch processing, including enzymes from *T. thermophilus* (pH_{opt} 7.0 and T_{opt} 95 °C),[68] *T. maritima* (pH_{opt} 6.5–7.5 and T_{opt} 105 °C),[69] and *T. neapolitana* (pH_{opt} 7.1 and T_{opt} 95 °C)[70] More work is needed to determine if a single reaction vessel could be used to produce fructose from a starch starting material.

Thermophilic esterases

The resolution of stereoisomers is one of the most difficult purification challenges[71,72] and essential drugs marketed as pure enantiomers, such as Naproxen. Naproxen is a popular nonsteroidal anti-inflammatory drug (NSAID) which is distributed solely as the (*S*)-enantiomer.[73] This is unique within the NSAID family because most others are marketed as racemic mixtures.[74] Resolution of Naproxen is accomplished by combinations of asymmetric synthesis, chromatography, and diastereomeric crystallization.[74]

To simplify purification of chiral molecules, the synthetic reaction can sometimes be designed to favor one enantiomer by the addition of protective groups.[75] The dielectric constant of the solvent used in chiro-selective molecular recognition may also have a profound effect, allowing resolution of one chiral selector by changing solvent.[76] For example, the use of ionic solvents was able to improve enantioselectivity of the lipase-catalyzed reaction of 1-phenylethanol.[77] An alternative method to purifying racemic mixtures under environmentally benign conditions is to exploit the natural enantioselectivity of enzymes.[78] Esterases, for example, can discriminate between the (*R*)- and (*S*)-enantiomers of a particular ester and selectively hydrolyze only one form, thereby creating a separation potential. The selectivity of these enzymes can be dependent upon the presence of organic solvents in the resolution of racemic mixtures.[79]

Enzyme flexibility has been shown to be a function of an enzyme's reaction environment.[80] By relaxing conformational restraints within an enzyme by addition of a co-solvent, there is often a corresponding loss of enantioselectivity.[80] Temperature is another means of affecting the stereoselectivity of an enzymatic reaction.[81] One would expect the stereoselectivity of a reaction to increase at lower temperatures because of decreased conformational flexibility, but this is not always the case.[82] For the secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus*, the conversion of 2-butanone to (*R*)-2-butanol was favored when temperatures were above 26 °C while (*S*)-2-butanol was favored at temperatures below 26 °C.[83] In another example, increasing the temperature from 28 °C to 55 °C increased the selectivity ratio (*E*) from 24 to 130, respectively, for the kinetic resolution of racemic 1-*N*-(propen-1-yl)-3-*N*-(phenylacetoxy)-aminoazetidin-2-one by Penicillin G-Acylase.[84]

Hyperthermophilic proteins offer the unique advantage that they have a large soluble temperature range below the optimum activity temperature and that they are frequently resistant to denaturation by organic chemicals.[12] Sehgal and Kelly[85,86] showed that enantioselectivity with hyperthermophilic esterase could be manipulated in the presence co-solvent or by running reactions at suboptimal temperatures. When the reaction of racemic Naproxen methyl ester was conducted at 50 °C using an esterase from *S. solfataricus* (*Sso* EST1, T_{opt} > 95 °C) the enzyme was able to selectively hydrolyze the (*S*)-enantiomer of Naproxen methyl ester, producing 95% (*S*)-Naproxen,[87] as illustrated in Fig. 3. It was also shown that a co-

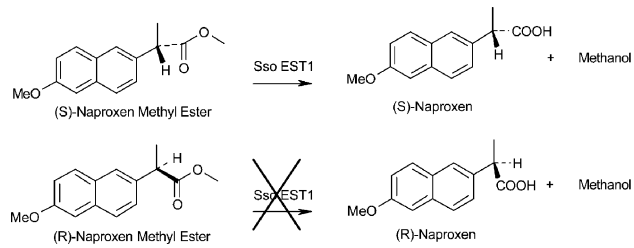

Fig. 3 The hydrolysis of racemic Naproxen by *Sso* EST1 preferentially produces (*S*)-Naproxen at 50 °C.[87]

solvent increased the activity of a hyperthermophilic esterase at suboptimal temperatures,[85] probably the result of increased conformational flexibility. Using circular dichroism and NMR, the *Sso* EST1 showed increased flexibility when the co-solvent DMSO was added at 3.5% (v/v) compensating for the thermal activation of the enzyme.

Enzyme discovery

The need for enzymes which meet ever-expanding processing criteria (*e.g.*, thermostability, specificity, *etc.*) continues to drive the search for new enzymes either from traditional sources, newly discovered organisms, or through directed evolution. One approach that is being utilized is cloning the metagenome,[88] *i.e.*, a collection of genomes of all the organisms present in an environmental sample. Until recently, this concept had been combined with screening to explore genetic diversity and discover new enzymes.[89] This approach has recently been expanded in the Sargasso Sea project in which surface water samples were collected, genomic DNA extracted, and cloned into genomic libraries with insert sizes of 2 to 6 kb. Whole-genome shotgun sequencing techniques were applied to this genomic library and led to identification of an estimated 1800 genomic species based on sequence similarity, of which 148 were unknown bacteria. The sequencing project also revealed over 1.2 million previously unknown genes.[90] Approaches

such as this one promise to be important for discovering enzymes from unculturable organisms.

For organisms that are culturable, advances in molecular biology and increased amounts of bioinformatic data provide tools to identify gene products that can be used for processes. Prior to recombinant expression of proteins, researchers were limited to discovering proteins one at a time from organism cultures. With the advances of PCR and molecular cloning, the rate of protein discovery has been increased by the ability of high-throughput robotics and screening.[91–93] Advances in molecular biology have also led to methodologies for determining differential expression of gene using microarrays or real-time PCR. Both techniques quantitatively determine the amount of messenger ribonucleic acid (mRNA) that is produced for a particular gene under various conditions. Full-genome microarrays have been utilized to put together sugar utilization pathways,[94] identify novel putative interactive sites for anticancer therapy,[95] and discover new wood processing enzymes.[96]

The utility of microarrays for identifying promising new biocatalysts is just starting to be recognized. In some cases, differential gene expression data has revealed unexpected enzyme activities on substrates of interest. For example, BLAST searches[97] showed that the *T. maritima* enzyme Cel5A (TM1751) was most similar to the characterized endo-1,4-glucanase B (EngB) from *Clostridium cellulovorans* (28% identity).[98] Based on sequence alignments alone, *Tm*Cel5A would be expected to be most active on polysaccharides containing the β-glucan linkage; however, the purified enzyme displayed comparable activity on glucomannan, galactomannan, and β-glucan.[94] Subsequent experiments analyzing the differential gene expression in *T. maritima* on various saccharide growth substrates confirmed the expression of *Tm*Cel5A on galactomannan and glucomannan,[99] as shown in Fig. 1. If not for the information obtained through differential expression experiments, potentially important galactomannanases for application such as oil and gas discovery would have gone unnoticed. As additional microbial genomes are sequenced, microarrays should become an increasingly important tool for identifying new enzymes for biotechnological applications.

Final comments

There are many areas in which enzymes from hyperthermophiles can be considered for use in technologically important biocatalytic processes. Some examples are presented here. The combination of power of biocatalysis and the expanded functional temperature range intrinsic to hyperthermophilic enzymes should open up numerous opportunities as the production of chemicals and biochemicals moves increasingly towards environmentally benign processes.

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PAPER

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A comparative study of biocatalysis in non-conventional solvents: Ionic liquids, supercritical fluids and organic media

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The catalytic activities of cutinase immobilized on zeolite NaY and *Candida antarctica* lipase B immobilized on an acrylic resin (Novozym 453) were measured in a model transesterification reaction in three imidazolium cation-based ionic liquids (RTILs), supercritical ethane (sc-ethane), sc-CO_2 and *n*-hexane, at a water activity (a_W) of 0.2 and 0.7. The transesterification activity of cutinase was highest and similar in 1-*n*-butyl-3-methylimidazolium hexafluorophosphate ([C_4mim][PF_6]), sc-ethane and *n*-hexane, more than one order of magnitude lower in sc-CO_2, and increased with an increase in a_W. Hydrolysis was not detected in sc-fluids and *n*-hexane, and was observed in RTILs at a_W 0.7 only. Both initial rates of transesterification and of hydrolysis of Novozym decreased with an increase in a_W. sc-CO_2 did not have a deleterious effect on Novozym activity, which was as high as in sc-ethane and *n*-hexane. The low reaction rates obtained in this case in RTILs suggested the existence of internal diffusion limitations absent in the cutinase preparation where the enzyme is only adsorbed at the surface of the support. sc-CO_2 did not adversely affect the catalytic activity of cutinase suspended in [C_4mim][PF_6], suggesting a protective effect of the RTIL. In the case of Novozym, a marked increase in the rate of transesterification was obtained in the [C_4mim][PF_6]/sc-CO_2 system, compared to the RTIL alone. This may reflect improved mass transfer of solutes to the pores of the immobilization matrix due to a high concentration of dissolved CO_2 and a reduction in viscosity of the RTIL.

Introduction

The use of room-temperature ionic liquids (RTILs) or of supercritical fluids (sc-fluids) can be a good strategy to meet the increasing demand for the introduction of clean technologies in industrial processes.[1] RTILs have no effective vapor pressure and sc-fluids can be easily eliminated through venting. In addition to being able to comply with environmental concerns, RTILs and sc-fluids have properties perceived as novel, on which their potential to compete with more conventional solvents for industrial applications is based, that have led to the designation "neoteric" solvents.[2]

The first reports on biocatalysis in sc-fluids[3] followed closely the publication of the paper that twenty years ago[4] triggered the since then ever increasing interest in nonaqueous biocatalysis. The first reports on biocatalysis involving an RTIL appeared much more recently, and dealt with the use of the RTIL as the dispersion medium in an enzyme catalyzed reaction,[5] or in a water-RTIL biphasic system with whole cells.[6] But the number of similar accounts is increasing steadily, as summarized in recent reviews.[7,8] In fact, the advantages afforded by the use of enzymes in organic solvents[9] can also be realized in ionic liquids. The choice of solvent, an important aspect of the medium engineering approach in nonaqueous biocatalysis, may in the case of RTILs be truer to the word by involving the design of the RTIL itself.[10] Recent developments include the design of RTILs that are chiral, functionalized or synthesized from renewable sources.[11,12] The important issue of the toxicology of RTILs is also being investigated with a view to the sustainable development of RTIL-based technology.[13]

The combination of RTILs with sc-fluids may be a way to circumvent the use of organic solvents to recover solutes from RTILs.[14] A study of the phase behavior of RTIL–CO_2 systems with some commonly used RTILs has shown that the latter did not significantly expand even when they held large quantities of dissolved CO_2, and all the while the solubility of the RTIL in the CO_2-rich phase remained immeasurably small.[15] A high solubility of the sc-fluid in the RTIL not only facilitates contact with the solutes of interest but also decreases the viscosity of the RTIL, thus improving mass transfer. RTIL–CO_2 systems have been successfully applied for chemical synthesis,[16] homogeneous and biphasic catalysis[17] and biocatalysis.[18–22] The study by Reetz *et al.*[22] is a good demonstration of the potential of coupling a biotransformation in an RTIL with extraction by sc-CO_2 in an environmentally benign and efficient reaction/separation process.

Cutinase is a versatile enzyme that catalyzes synthetic and hydrolytic reactions on a wide range of substrates. It has been applied as a lipolytic enzyme in detergents to remove fats, other applications including the hydrolysis of milk fat in the dairy industry or the synthesis of structured triglycerides.[23] Lipase B

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from *Candida antarctica* is probably the most commonly used lipase in biocatalysis. It has been applied in the production of soaps, creams and lubricants *via* esterification reactions.[24] It has been shown to be an excellent biocatalyst for the resolution of *sec*-alcohols.[25] In the present study, we compare the activity of cutinase immobilized on zeolite NaY and of *Candida antarctica* lipase B immobilized on a macroporous acrylic resin (Novozym 435) at fixed water activity (a_W) in three classes of solvents: the RTILs 1-*n*-butyl-3-methylimidazolium hexafluorophosphate ([C_4mim][PF_6]), 1-*n*-octyl-3-methylimidazolium hexafluorophosphate ([C_8mim][PF_6]) and 1-*n*-butyl-3-methylimidazolium tetrafluoroborate ([C_4mim][BF_4]), sc-ethane and sc-CO_2, and *n*-hexane, a commonly used organic solvent. We also explore the combination of [C_4mim][PF_6] with sc-CO_2 in a continuous flow reactor.

Results and discussion

Water activity (a_W) is the most convenient parameter for correlating enzymatic activity in nonaqueous media.[26] The correspondence between water concentration and a_W in the various RTILs tested was found by equilibrating the RTILs through the vapor phase with saturated salt solutions (Fig. 1). [C_4mim][BF_4] is water-miscible

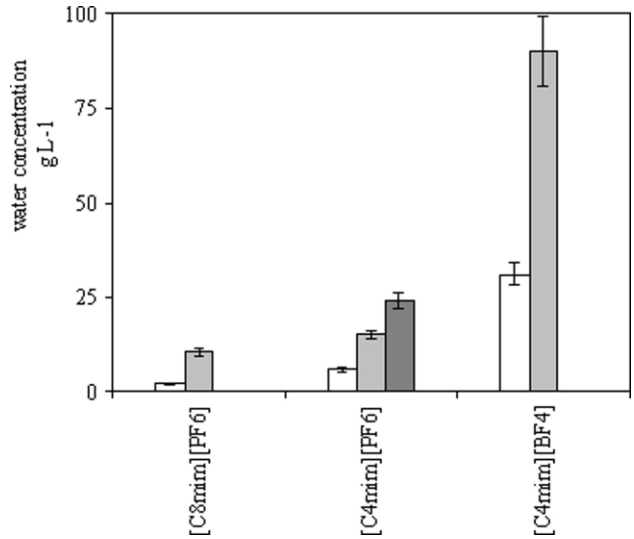


Fig. 1 Water concentration in RTILs at a_W = 0.2 (white bars), a_W = 0.7 (light gray bars), and saturation (darker gray bar), at T = 25 °C.

and requires larger amounts of water to reach the required a_W. The values shown for [C_8mim][PF_6] and [C_4mim][PF_6] are in good agreement with those presented by Anthony *et al.*[27] Water solubility data for the latter solvent has also been reported by Berberich *et al.*[28] In this case, the authors compared the results obtained when using saturated salt solutions or salt hydrate pairs *in situ*. The two sets of data were in good agreement, also with the data of Anthony *et al.*[27]

An increase in a_W from 0.2 to 0.7 had a positive impact on the rates of transesterification of cutinase immobilized on zeolite NaY in all the solvents (Fig. 2). The enzyme exhibited highest transesterification activity in sc-ethane, *n*-hexane and [C_4mim][PF_6] that dropped by more than one order of magnitude in sc-CO_2. Calculated activity coefficient data for the substrates[29] showed that both the alcohol and the ester are better solvated in *n*-hexane than in sc–CO_2 and therefore are less available for reaction in *n*-hexane. *i.e.* the impaired activity of cutinase in sc-CO_2 does not result from a decreased driving force towards partitioning into the active site, but rather from a direct negative effect of sc-CO_2 on the enzyme. Only a similar thermodynamic analysis for the RTILs tested might elucidate to what extent the observed differences in Fig. 2 are due to differences in the solvation of the substrates. We note however that rates of transesterification do not provide a full

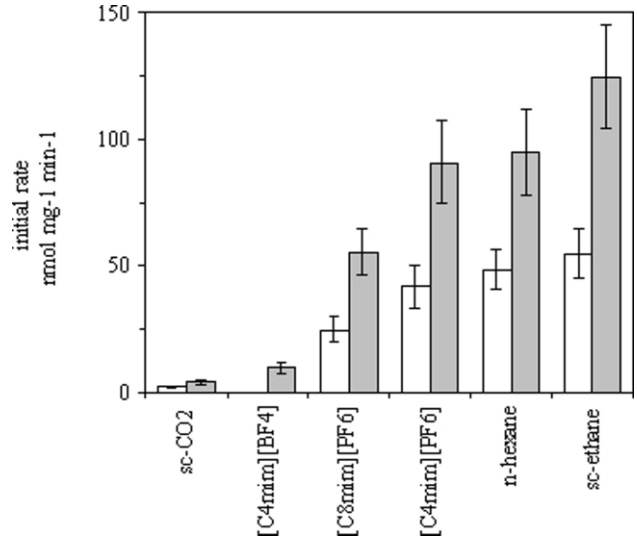


Fig. 2 Initial rates of transesterification for cutinase immobilized on zeolite NaY in RTILs, sc-fluids and *n*-hexane at a_W = 0.2 and a_W = 0.7. Code for bars as in Fig. 1. T = 35 °C. P = 100 bar (sc-fluids). [2-Phenyl-1-propanol] = 60 mM. [Vinyl butyrate] = 170 mM. [Immobilized cutinase]: 6 g L^{-1} (ionic liquids), 1 g L^{-1} (sc-ethane and *n*-hexane), 4 g L^{-1} (sc–CO_2). Rates given per mg of immobilized enzyme preparation.

measure of cutinase activity. The catalytic mechanism of the two enzymes used in the present study involves the binding of the ester in a first step and proceeds *via* the binding of the nucleophile. Depending on the amount of water available in the medium, competitive inhibition of water against the alcohol may occur. Hydrolysis was not detected in sc-ethane, *n*-hexane or sc-CO_2 at the two a_W values tested, and was observed in the RTILs at a_W = 0.7 only (Fig. 3). Because the solubility of water is higher in

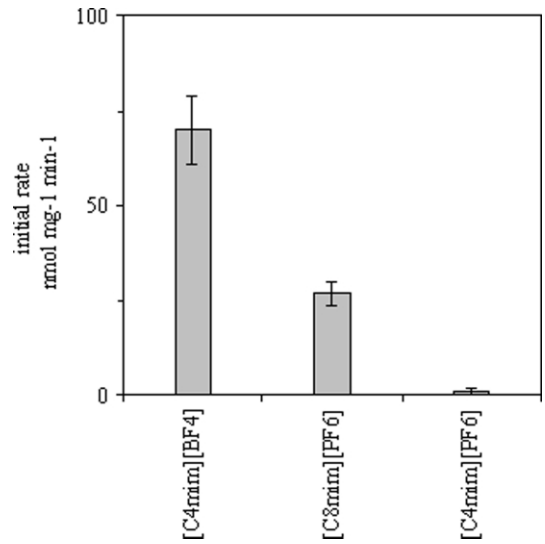


Fig. 3 Initial rates of hydrolysis for cutinase immobilized on zeolite NaY in RTILs at a_W = 0.7. Reaction conditions as in Fig. 2.

[C_4mim][BF_4], at fixed a_W there will be a larger number of water molecules available to the enzyme in this RTIL than in the other two. In fact, rates of hydrolysis were higher in [C_4mim][BF_4]. However, the solubility of water in [C_4mim][PF_6] exceeds that in [C_8mim][PF_6], and yet hydrolysis was more pronounced in the latter solvent. By taking hydrolysis into account, one can see that although initial rates of transesterification were clearly higher for cutinase immobilized on zeolite NaY in [C_4mim][PF_6] at a_W = 0.7 than in the other two RTILs, total initial rates (transesterification + hydrolysis) were very similar in the three RTILs. At low a_W there was a higher discrimination between solvents. *e.g.* as seen in Fig. 2,

no ester product was detected at a_W = 0.2 in [C_4mim][BF_4], and the initial rate of transesterification in [C_4mim][PF_6] was almost twice as high as in [C_8mim][PF_6].

Unlike with cutinase, the transesterification activity of Novozym decreased with an increase in a_W (Fig. 4). Similar findings for this enzyme have been reported by Berberich *et al.*[28] and Lozano *et al.*[19]

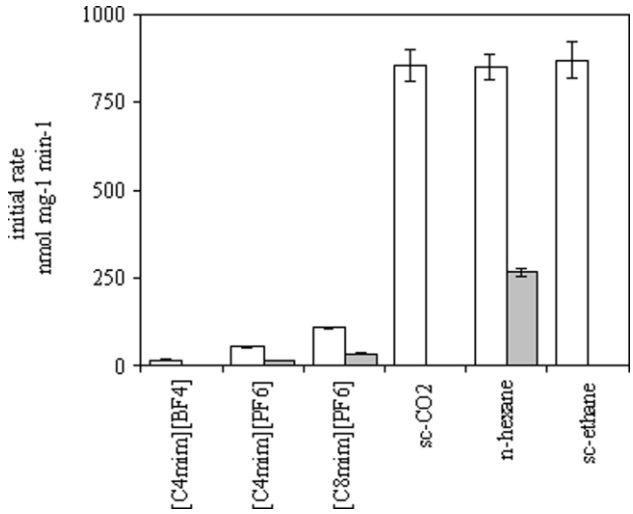


Fig. 4 Initial rates of transesterification for Novozym in RTILs, sc-fluids and *n*-hexane at a_W = 0.2 and a_W = 0.7 (no data collected at a_W = 0.7 in sc-fluids). [Novozym]: 2 g L^{-1} or 4 g L^{-1} (ionic liquids). Other reaction conditions as in Fig. 2. Rates given per mg of Novozym.

for different transesterification reactions or by Peres *et al.*[30] and Chamouleau *et al.*[31] for esterification. Hydrolysis was observed in all the solvents (Fig. 5). The extent of hydrolysis at a_W = 0.2 was

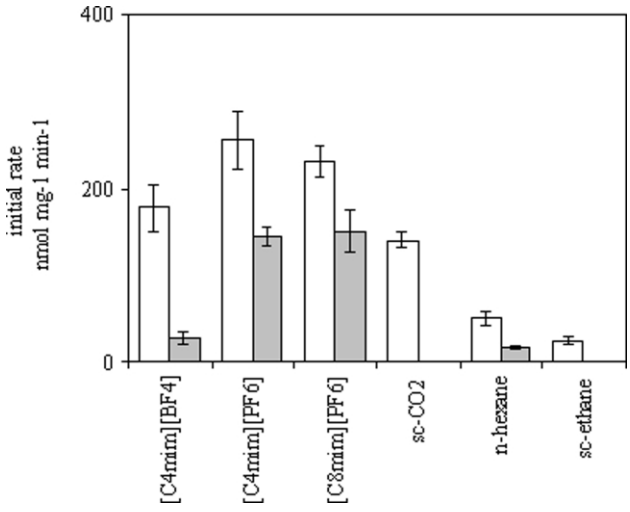


Fig. 5 Initial rates of hydrolysis for Novozym in RTILs, sc-fluids and *n*-hexane at a_W = 0.2 and a_W = 0.7 (no data collected at a_W = 0.7 in sc-fluids). Reaction conditions as in Fig. 4.

much higher than that observed with cutinase at a_W = 0.7, showing that Novozym was less selective than cutinase for transesterification. Interestingly, the negative impact of increasing a_W on Novozym activity led to a decrease in rates of hydrolysis from low to high a_W as well. A striking feature of Fig. 4 when compared to Fig. 2 is the comparable transesterification activity of the enzyme in sc-ethane or *n*-hexane and in sc-CO_2. This must have to do with the enzyme itself but also with the type of immobilization. Immobilization on zeolites offers cutinase no protection against CO_2, unlike other types of immobilization.[32] Zeolites have a microporous structure and cutinase is adsorbed only at the external surface of the support.[33] Novozym, on the other hand, has a macroporous structure where the enzyme is entrapped. Evidence for a less pronounced deleterious effect of sc-CO_2 on Novozym than on other proteases has been reported previously.[30,34] Another notable difference between Figs. 2 and 4 are the low reaction rates obtained with Novozym in RTILs when compared to cutinase. Although Schöfer *et al.*[35] reported very low conversions for this enzyme in [C_4mim][PF_6] and [C_4mim][BF_4], Park and Kazlauskas,[36] Lau *et al.*[37] and Sheldon *et al.*[7] obtained reaction rates in those RTILs that were similar to the rates measured in commonly used organic solvents. Given that the availability of the substrates is the same in a given solvent and the fact that reaction rates for cutinase in two of the RTILs tested were similar to those found in sc-ethane and in *n*-hexane, the reason for low reaction rates for Novozym in the RTILs cannot be found in arguments based on substrate solvation. We believe that our results in RTILs may reflect the existence of internal diffusion limitations arising from the higher viscosity of the solvents[12,38] and thus increased difficulty for the substrates to penetrate the pores of Novozym. This difficulty may have been circumvented by the substrate concentrations used by Berberich *et al.*[28] who report similar rates of transesterification for this enzyme in *n*-hexane and [C_4mim][PF_6] in the a_W range of the present study. We note that in our case Novozym exhibited higher activity in [C_8mim][PF_6] than in [C_4mim][PF_6], as opposed to cutinase. [C_8mim][PF_6] is the more highly viscous of the three RTILs (viscosity almost twice as high as that of [C_4mim][PF_6]).[12,38] Therefore, the rate profile observed for Novozym in RTILs must reflect the influence of some other factor in addition to diffusion limitations.

As mentioned earlier, the combination of RTILs with sc-fluids has been applied successfully.[18–22] Here we used a continuous flow process with the enzyme suspended in [C_4mim][PF_6] and sc-CO_2 acting as the vehicle for carrying substrates and products to and from the enzyme. Reaction conversion increased in the beginning but soon assumed a constant value. The results obtained were remarkable. In the case of cutinase, the rate of transesterification measured was (130 ± 15) nmol min^{-1} mg^{-1}. The presence of the RTIL avoided the pronounced deleterious effect that direct contact of cutinase with sc-CO_2 had on this enzyme (Fig. 2). This suggests that the microenvironment of the enzyme is mainly determined by the RTIL, even at the high concentrations of CO_2 achieved in [C_4mim][PF_6].[39] A protective effect of the RTIL against CO_2 is one of the reported advantages of this type of system.[19,21] The reaction rate value given above, measured at a less favorable a_W (a_W = 0.12) than used in batch experiments, suggests that an additional effect must have contributed to the observed cutinase performance in the continuous flow experiments. This effect could be the impact of the high concentration of CO_2 dissolved in the RTIL on substrate solvation *via* higher activity coefficients than those in the RTIL alone, and thus a higher availability of the substrates for reaction. The stability of the reaction system was also tested. A reaction was run for about four hours, after which the system was washed with sc-CO_2 and kept pressurized with this solvent for two days before restarting the addition of substrates, and the process was repeated yet another time; *i.e.* the enzyme was kept in the RTIL under pressurized CO_2 for six days and performed three reactions. The three transesterification rates obtained were in good agreement. Evidence for a high stability of cutinase in sc-CO_2 alone has been presented previously.[40] The rate of transesterification measured in the continuous flow apparatus for Novozym was (412 ± 45) nmol min^{-1} mg^{-1}. In this case, the lower a_W at which the continuous flow experiments were performed should be beneficial for enzyme activity, as shown in the study by Berberich *et al.*,[28] although not sufficient to explain the marked improvement in reaction rate by comparison with the RTIL alone (Fig. 4). We believe this result again reflects the influence of dissolved CO_2 in the RTIL that causes a decrease in the viscosity of the latter, leading to facilitated mass transfer and alleviated diffusion limitations.

The enantioselectivity of the two enzymes towards 2-phenyl-1-propanol was low (*E* values between 1.5 and 2.5 for cutinase, between 2 and 3 for Novozym) at all the reaction conditions tested. A rationalization of the low *E* values obtained in the case of cutinase has been presented, based on molecular modeling studies.[41]

Conclusions

Enzyme activity in nonaqueous media can be markedly dependent on factors such as water activity, substrate solvation, immobilization conditions and direct solvent–enzyme interactions, as shown in the present study. Assaying enzymes at comparable conditions in RTILs, sc-fluids and organic solvents allows for a more global perspective of the relevant parameters at play in such diverse classes of non-conventional media, and provides a sounder basis for optimizing enzyme behavior in those media and for a better evaluation of the options available. As shown previously,[18–22] and also in the present study, RTIL/sc-CO_2 systems have great potential for biocatalysis. Advantages include protection afforded by the RTIL against a possible deleterious effect of CO_2 on the enzyme and the alleviation of mass transfer problems often encountered with immobilized enzyme preparations. Most importantly, RTIL/sc-CO_2 systems allow the design of environmentally friendly integrated biocatalysis/separation processes.

Experimental

Materials

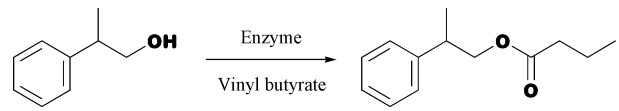
Fusarium solani pisi cutinase was produced by an *Escherichia coli* WK-6 (a gift from Corvas International, Ghent, Belgium) and purified at Centro de Engenharia Biológica e Química, Instituto Superior Técnico.[23,42] Immobilized *Candida antarctica* lipase B (Novozym 435) was a gift from Novo Nordisk Bioindustrial, Spain. (*R*,*S*)-2-phenyl-1-propanol (97% purity), (*R*)- and (*S*)-2-phenyl-1-propanol (98% purity), and zeolite NaY molecular sieves (in powder form) were from Aldrich, vinyl butyrate (99% purity) was from Fluka, tridecane and sodium chloride were from Merck, potassium acetate and Hydranal Coulomat A and C Karl–Fischer reagents were from Riedel de Häen. (*R*,*S*)-2-phenyl-1-propyl butyrate was prepared as described elsewhere.[41] Substrates and tridecane were stored over molecular sieves (3 Å beads from Merck). The RTILs [C_4mim][PF_6], [C_8mim][PF_6] and [C_4mim][BF_4] were prepared following general reported procedures[36,38,43] and neutral pH conditions were confirmed before use. Ethane, CO_2, and nitrogen were supplied by Air Liquide and guaranteed to have purities of over 99.95 mol% (ethane) and 99.995 mol%.

Cutinase immobilization

Cutinase was immobilized by deposition.[33] The lyophilized enzyme was dissolved in a 50 mM sodium phosphate buffer solution (10 mg mL^{-1} of enzyme) at pH 8.5. The support was added to the solution (25 mg of cutinase per g of support) and after vortex mixing for 1 min, the preparation was dried under vacuum for at least 24 h. The average yield of immobilization was (72 ± 12)% for zeolite NaY, as determined by a modified Lowry method.[44]

Batch enzyme assays

For reactions in sc-fluids, we used variable volume stainless steel cells (reaction mixture volume of about 12 cm^3 for most experiments) equipped with a sapphire window and with loading and sampling valves. Details of the high pressure apparatus and experimental technique have been given elsewhere.[45] Reactions in ionic liquids and in *n*-hexane were performed in glass vials (reaction volume up to 3.5 cm^3) placed in a constant temperature orbital shaker set for 450 rpm. Experiments for the two enzymes in [C_4mim][PF_6] in which stirring was provided by a magnetic stirring bar at high speed yielded the same results as those performed in the orbital shaker, indicating the absence of external diffusion limitations. With the exception of sc-fluids, all the reaction mixture components were pre-equilibrated to the a_W of the experiment through the vapor phase with saturated salt solutions at 25 °C for about 3 days, to achieve the values a_W = 0.22 (potassium acetate) and a_W = 0.75 (sodium chloride), taken from the literature.[46] To reach a_W = 0.7 in sc-CO_2, water was added directly to the reactor prior to pressurization. To reach a_W = 0.7 in sc-ethane, the required amount of water was added to sc-ethane in a chamber prior to admission into the reactor, to avoid hydration hysteresis effects[47] and/or aggregation. The reaction studied (Scheme 1) was the



Scheme 1

transesterification of (*R*,*S*)-2-phenyl-1-propanol (60 mM) by vinyl butyrate (170 mM). The reaction was started by adding the latter substrate. Tridecane (15 mM) was used as external standard for GC analysis. Water concentration was measured by Karl–Fisher titration. In sc-fluids, a_W values were calculated by dividing the water concentration in the reaction mixture by the water concentration in the same mixture at saturation.[48]

Continuous flow assays

A stream of sc-CO_2 with dissolved substrates (flow rate = 1 mL min^{-1}, T = 35 °C, P = 100 bar, a_W = 0.12, [2-phenyl-1-propanol] = 60 mM, [vinyl butyrate] = 170 mM) was allowed to flow through [C_4mim][PF_6] (5 mL) with the enzyme suspension (6 g L^{-1} for immobilized cutinase, 4 g L^{-1} for Novozym) that was placed at the bottom of a cylindrical high-pressure reactor mounted vertically (Fig. 6). Sampling of the CO_2 stream that entered and exited the

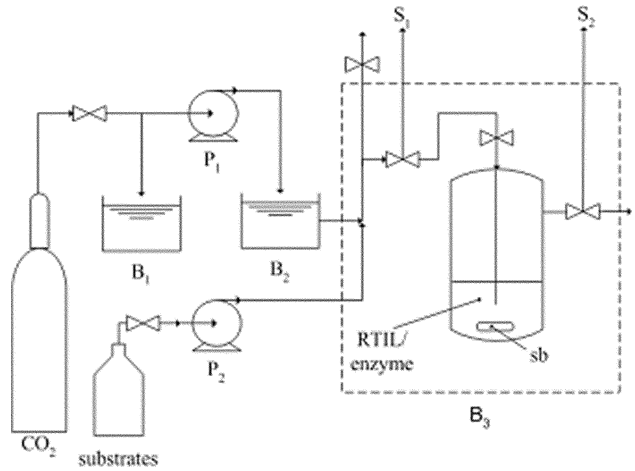


Fig. 6 Apparatus for continuous flow assays. P_1, P_2 = HPLC pumps. B_1, B_2, B_3 = thermostatic baths. S_1, S_2 = sampling. sb = stirring bar.

reactor allowed the calculation of reaction conversion and the monitoring of a_W.

Analysis

Both reaction conversion and the enantiomeric excess of the remaining alcohol substrate (ee_S) were measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph. Column: 30 m × 0.32 mm i.d. home-made fused silica capillary column coated with a 0.25 μm thickness film of 15% heptakis-(2,3-di-O-methyl-6-O-*tert*-butyldimethylsilyl)-ß-cyclodextrin in SE 52 (*DiMe*). Oven temperature program: 90 °C for 5 min, 90–136 °C ramp at 0.8 °C min^{-1}, 200 °C for 5 min. Injection temperature:

250 °C. Flame ionization detection (FID) temperature: 250 °C. Carrier gas: helium (2.0 cm^3 min^{-1}). Split ratio: 1 : 20. No products were detected in assays carried out without enzyme. The enantiomeric ratio, *E*, was calculated from the expression $E = \{\ln[(1 - c)(1 - ee_S)]\}/\{\ln[(1 - c)(1 + ee_S)]\}$, where *c* is the conversion[49] and ee_S is given for the (*R*)-enantiomer, by using data obtained for the racemic substrate along the time course of the reaction (when possible, up to a conversion of 0.5). The data given are the average of at least two measurements.

Acknowledgements

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PAPER

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Dynamic kinetic resolution of secondary alcohols by enzyme–metal combinations in ionic liquid†

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Dynamic kinetic resolutions (DKRs) of secondary alcohols by lipase–ruthenium or subtilisin–ruthenium combo-catalysis were successfully accomplished in an ionic liquid, [BMIM]PF_6 ([BMIM] = 1-butyl-3-methylimidazolium), in the presence of an acyl donor to provide (*R*)- or (*S*)-esters of high optical purities in good yields. Ionic liquid as solvent was essential for the successful performance of the DKRs at room temperature since its use enhanced the activity of the racemizing ruthenium catalyst.

Introduction

Dynamic kinetic resolution (DKR) of racemic substrates provides a useful method for the preparation of enantiomerically enriched compounds. Several groups, including ours, have been developing new procedures for DKR, in which an enzyme catalyzes the enantioselective transformation of one enantiomer of a racemic substrate with simultaneous racemization of its opposite enantiomer catalyzed by a metal catalyst.[1] The enzyme–metal combo-catalysis has been particularly successful in the DKR of secondary alcohols in organic solvents (Scheme 1). The (*R*)-selective DKR[2] of a wide range of secondary alcohols can be achieved by the combination of lipase and ruthenium and its (*S*)-selective counterpart[3] by the combination of subtilisin and ruthenium. We herein wish to report for the first time that DKRs by enzyme–metal combo-catalysis can be successfully done in ionic liquid as well.

In the previous DKRs of secondary alcohols, several ruthenium complexes have been employed as the racemizing catalyst. Two of them, which are practically more useful, are **1**[4] and **2**[2] (Fig. 1). The former catalyzes the racemization rapidly at ambient temperature while the latter requires an elevated temperature (70 °C) for reasonably good racemizing activity. Both of them are commercially available but rather expensive. Accordingly, we turned our attention to more readily available **3** which had been previously shown to display satisfactory racemizing activity toward some activated alcohols such as methyl 3-phenylallyl carbinol in organic solvent at ambient temperature.[5] We have discovered that the cymene-ruthenium complex catalyzes the racemization of simple secondary alcohols more efficiently in ionic liquid[6] at room temperature. Subsequently, we have explored the DKRs of secondary alcohols by coupled enzyme and metal catalysis in ionic liquid.

† This paper is dedicated to Professor Dong Han Kim on the occasion of his retirement.

Results and discussion

The racemizing efficiency of the cymene-ruthenium complex **3** was examined with (*S*)-1-phenylethanol as the substrate in three organic solvents and two ionic liquids. The racemization reactions were carried out with substrate (> 99% ee, 0.2 mmol, 0.25 M) and **3** (4 mol%) in the presence of Et_3N for 12 h at 25 °C and then the enantiomeric excess of the substrate was analyzed by chiral HPLC. The data given in Table 1 clearly indicate that the racemization took place more rapidly in ionic liquids (entries 4–5) than organic solvents (entries 1–3). The racemization was almost complete in ionic liquids while it was far from completion in organic solvents. The most efficient racemization took place in the hydrophobic ionic liquid [BMIM]PF_6 (entry 5).

Ten substrates **4a–j** were examined for DKR in [BMIM]PF_6 with the cymene-ruthenium complex as the racemizing catalyst. Two

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Prof. Jaiwook Park was born in Korea in 1957. He received his PhD in organometallic chemistry from Princeton University in 1988. He carried out research work at MIT, USA, in 1988, and at Colorado State University, USA, in 1997. In 1989 he joined Pohang University of Science and Technology (POSTECH) as an assistant professor. Currently he is a full professor at POSTECH. His current research interests are functional nanomaterial and catalysis of oxidation, reduction, and C–C coupling reactions.

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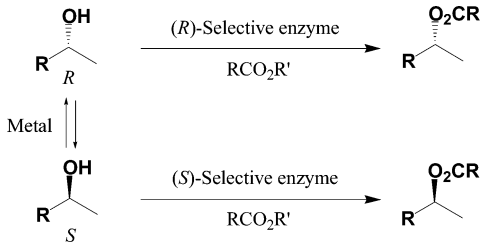


Scheme 1 DKR of secondary alcohols by enzyme–metal combo-catalysis.

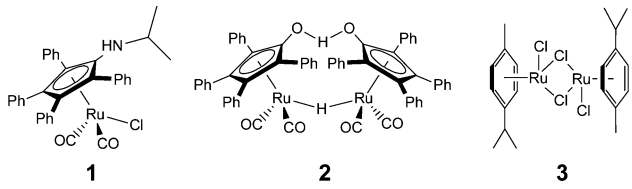
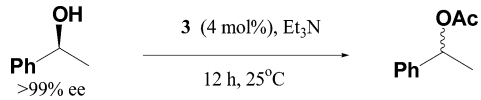


Fig. 1 Ruthenium complexes employed in DKR.

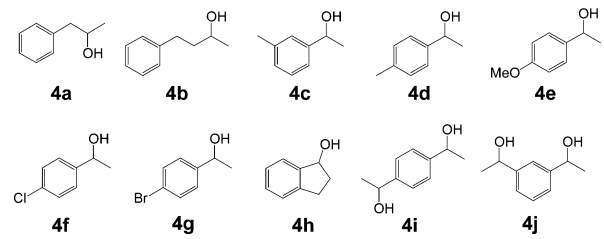
Table 1 Racemization of (*S*)-1-phenylethanol by cymene-ruthenium complex **3**



Entry	Solvent	ee (%)[a]
1	THF	92
2	Toluene	88
3	CH_2Cl_2	25
4	[BMIM]BF_4[b]	5
5	[BMIM]PF_6[b]	3

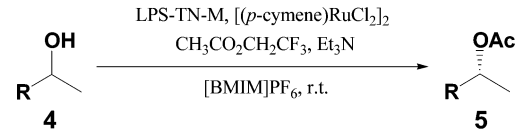
[a] Determined by HPLC equipped with a chiral column. [b] [BMIM] = 1-butyl-3-methylimidazolium.

enzymes were chosen as the catalysts for the enantioselective resolution: lipase for the (*R*)-selective and subtilisin for the (*S*)-selective resolution. The DKR reactions of **4a–j** were carried out at room temperature by stirring a mixture of a substrate (0.3 mmol), an acyl donor (trifluoroethyl acetate for lipase-catalyzed DKRs and trifluoroethyl butyrate for subtilisin-catalyzed DKRs, 0.9 mmol), Et_3N (0.3 mmol), **3** (8 mol%) and an immobilized enzyme (45 mg for lipase, 20 mg for subtilisin) in 1 mL of [BMIM]PF_6. In the DKRs of substrates **4i** and **4j**, the amounts of the catalysts, base and acyl donor were increased 2-fold since they carry two hydroxy groups.



The results from the (*R*)-selective DKRs of secondary alcohols by lipase–ruthenium combo-catalysis are given in Table 2. Most of the reactions reached completion in 2–3 days. The yields ranged from 85 to 92% and, in most cases, the optical purities (enantiomeric excess, ee) of the products reached 99%. In general, the yields and optical purities of products were similar for the DKRs of both arylalkyl carbinols (**4a–b**) and aryl carbinols (**4c–h**) and not affected significantly by the electronic effect of the substituents on the phenyl ring. The DKRs of diols **4i–j** required the addition of a hydrogen donor 2,6-dimethyl-4-heptanol (1 equiv.) to suppress the formation of half-oxidized by-products (hydroxyl

Table 2 DKR of various alcohols by lipase–ruthenium combo-catalysis



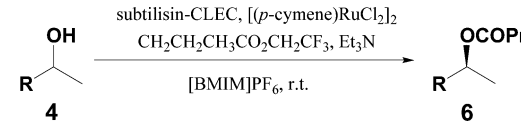
Substrate	Time/d	Conv. (%)	Yield (%)[a]	ee_p (%)[b]
4a	2	88	85	99
4b	2	90	85	99
4c	3	97	87	98
4d	3	94	87	99
4e	2	98	85	99
4f	2	91	87	99
4g	3	95	92	99
4h	2	91	85	99
4i	4[c]	—	87	99 (de 99%)
4j	4[c]	—	86	99 (de 97%)

[a] Determined by ^{1}H-NMR. [b] Determined by HPLC equipped with a chiral column. [c] 1.0 equiv. of 2,6-dimethyl-4-heptanol was added.

ketones) and increase the yields. In both cases, good yields were obtained with high ee and excellent de.

The results from the (*S*)-selective DKRs of secondary alcohols by subtilisin–ruthenium combo-catalysis are given in Table 3. The

Table 3 DKR of various alcohols by subtilisin–ruthenium combo-catalysis



Substrate	Time/d	Conv.(%)	Yield (%)[a]	ee_p (%)[b]
4a	6	89	89	97
4b	6	95	90	97
4c	6	97	90	85
4d	6	97	90	85
4e	6	> 97	80	99
4f	6	> 97	92	87
4g	6	96	91	82
4h	6	> 97	84	86
4i	6	—	78	86 (de 52%)
4j	6	—	83	96 (de 63%)

[a] Determined by ^{1}H-NMR. [b] Determined by HPLC equipped with a chiral column.

subtilisin-catalyzed DKRs required relatively long reaction times (6 days) due to the low enzyme activity. The yields were good but the optical purities were less than satisfactory in some cases. Three (**4a,b,e**) of all the substrates examined were efficiently resolved to give high optical purities (97–99%) while the rest were less efficiently resolved to give markedly reduced ee (82–87%). The (*S*)-selective DKRs of diols **4i–j** were significantly less efficient than the corresponding (*R*)-selective DKRs to afford moderate de (52–63%). The ee and de values indicate that subtilisin is less selective than lipase in ionic liquid.

Conclusion

This work has demonstrated that the racemization of secondary alcohols take places more efficiently in ionic liquid than organic solvent, leading to their successful DKRs by enzyme–metal combo-catalysis in ionic liquid at room temperature. The (*R*)-selective DKRs are accomplished by the combination of lipase and a cymene-ruthenium complex and the complementary (*S*)-selective DKRs by replacing lipase with subtilisin. In most cases, the yields are good and the optical purities are good to excellent. In general, lipase-catalyzed DKRs give higher optical purities than subtilisin-catalyzed DKRs. The efficiency of the DKRs in ionic liquid is comparable to that in organic solvents:[3,4] the DKRs in both solvent

systems provide similar yields and optical purities. The use of ionic liquid, however, allows us to use more readily available racemizing catalyst at room temperature. It is concluded that a pair of complementary DKR procedures have been developed as alternatives for the transformations of racemic alcohols to enantiomerically enriched (*R*)- and (*S*)-esters at ambient temperature.

Experimental

The LSP-TN-M was prepared according to the literature[7] from porous ceramic particle Toyonite 200-M (trade name, Toyo Denka Kogyo Co., Japan) and lipase from *Pseudomonas* species (trade name Lipase-PS, Amano Enzyme Inc., Japan). Subtilisin-CLEC and the ionic liquid were purchased from Altus Biologics Inc. (USA) and C-TRI (Korea), respectively.

General procedure for (*R*)-selective DKR

The procedure for **5a** is described as the representative. A mixture of **4a** (0.3 mmol), 1,1,1-trifluoroethyl acetate (0.9 mmol), Et_3N (0.3 mmol), $[(p\text{-cymene})RuCl_2]_2$ (8 mol%) and LPS-TN-M (45 mg) in 1 mL of $[BMIM]PF_6$ was stirred at room temperature under Ar atmosphere. After the completion of the reaction, the mixture was extracted with diethyl ether. The extract was subjected to ^{1}H-NMR and HPLC analysis. The purification of the products was performed on a flash chromatograph (silica gel, *n*-hexane/ethyl acetate = 4)

5a 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{26.6}$ = −5.61 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.35–7.13 (m, 5H), 5.11 (q, *J* = 6.4 Hz, 1H), 2.92 (dd, J_1 = 13.6 Hz, J_2 = 6.7 Hz, 1H), 2.74 (dd, J_1 = 13.6 Hz, J_2 = 6.5 Hz, 1H), 1.99 (s, 3H), 1.21 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.6, 137.9, 129.6, 128.6, 126.7, 71.7, 42.6, 21.4, 19.7; HRMS (EI+) $C_{11}H_{14}O_2$ calcd 178.1000, found 178.0999.

5b 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{26.4}$ = +7.82 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.34–7.09 (m, 5H), 4.98–4.88 (m, 1H), 2.68–2.60 (m, 2H), 2.03 (s, 3H), 1.83–1.76 (m, 2H), 1.24 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.8, 141.8, 128.6, 128.5, 126.2, 70.8, 37.8, 32.1, 21.4, 20.2; HRMS (FAB+) $C_{12}H_{16}O_2 + H^+$ calcd 192.1200, found 192.1227.

5c 97% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{26.0}$ = +215 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.31–7.09 (m, 4H), 5.84 (q, *J* = 6.6 Hz, 1H), 2.36 (s, 3H), 2.07 (s, 3H), 1.52 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.4, 142.0, 138.3, 128.8, 128.6, 127.1, 123.4, 72.6, 22.3, 21.6, 21.5; HRMS (EI+) $C_{11}H_{14}O_2$ calcd 178.1000, found 178.0994.

5d 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{25.4}$ = +115.8 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.25 (d, *J* = 7.9 Hz, 2H), 7.15 (d, *J* = 7.9 Hz, 2H), 5.85 (q, *J* = 6.6 Hz, 1H), 2.34 (s, 3H), 2.05 (s, 3H), 1.52 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.4, 138.9, 137.8, 129.4, 126.3, 72.4, 22.3, 21.6, 21.3.; HRMS (EI+) $C_{11}H_{14}O_2$ calcd 178.1000, found 178.0994.

5e 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{26.5}$ = +121.7 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.29 (d, *J* = 8.7 Hz, 2H), 6.88 (d, *J* = 8.8 Hz, 2H), 5.84 (q, *J* = 6.6 Hz, 1H), 3.80 (s, 3H), 2.04 (s, 3H), 1.51 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.6, 159.5, 133.9, 127.8, 114.0, 72.2, 55.5, 22.1, 21.6; HRMS (EI+) $C_{11}H_{14}O_3$ calcd 194.0900, found 194.0943.

5f 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{25.1}$ = +68.8 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.34–7.26 (m, 4H), 5.83 (q, *J* = 6.6 Hz, 1H), 2.07 (s, 3H), 1.51 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.4, 140.4, 133.9, 128.9, 127.7, 71.8, 22.4, 21.5; HRMS (EI+) $C_{10}H_{11}O_2Cl$ calcd 198.0400, found 198.0434.

5g 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{22.9}$ = +91.2 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.47 (d, *J* = 8.5 Hz, 2H), 7.23 (d, *J* = 8.4 Hz, 2H), 5.82 (q, *J* = 6.6 Hz, 1H), 2.07 (s, 3H), 1.50 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.2, 141.1, 131.9, 128.1, 122.0, 71.8, 22.2, 21.4; HRMS (EI+) $C_{10}H_{11}O_2Br$ calcd 241.9900, found 241.9945.

5h 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 99 : 1, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{26.0}$ = +87.6 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 4.43–7.20 (m, 4H), 6.20 (q, *J* = 3.3 Hz, 1H), 3.15–3.07 (m, 1H), 2.93–2.83 (m, 1H), 2.56–2.44 (m, 1H), 2.15–2.04 (m, 1H), 2.06 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 171.3, 144.6, 141.3, 129.2, 126.9, 125.8, 125.0, 78.6, 32.5, 30.4, 21.5; HRMS (EI+) $C_{11}H_{12}O_2$ calcd 176.0800, found 176.0834.

5i >97% ee, 99% de by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{22.6}$ = +157.5 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.33 (s, 4H), 5.86 (q, *J* = 6.6 Hz, 2H), 2.06 (s, 6H), 1.52 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.3, 141.5, 126.5, 72.1, 22.2, 21.4; HRMS (EI+) $C_{14}H_{18}O_4$ calcd 250.1200, found 250.1209.

5j >97% ee, 97% de by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{22.3}$ = +124.6 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.36–7.24 (m, 4H), 5.88 (q, *J* = 6.5 Hz, 2H), 2.07 (s, 6H), 1.53 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 170.3, 142.3, 128.9, 125.8, 124.1, 72.5, 22.4, 21.4; HRMS (EI+) $C_{14}H_{18}O_4$ calcd 250.1200, found 250.1221.

General procedure for (*S*)-selective DKR

The procedure for **6a** is described as the representative. A mixture of **4a** (0.3 mmol), 1,1,1-trifluoroethyl butyrate (0.9 mmol), Et_3N (0.3 mmol), $[(p\text{-cymene})RuCl_2]_2$ (8 mol%) and subtilisin-CLEC (20 mg) in 1 mL of $[BMIM]PF_6$ was stirred at room temperature under Ar atmosphere. After the completion of the reaction, the mixture was extracted with ether. The extract was subjected to ^{1}H-NMR and HPLC analysis. The purification of the products was performed on a flash chromatography (silica gel, *n*-hexane/ethyl acetate = 4)

6a 97% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{20.8}$ = +9.4 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.31–7.18 (m, 5H), 5.13 (q, *J* = 6.4 Hz, 1H), 2.92 (dd, J_1 = 13.6 Hz, J_2 = 6.8 Hz, 1H), 2.76 (dd, J_1 = 13.6 Hz, J_2 = 6.4 Hz, 1H), 2.22 (t, *J* = 7.4 Hz, 2H), 1.63–1.53 (m, 2H), 1.21 (d, *J* = 6.3 Hz, 3H), 0.88 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 173.8, 138.4, 130.1, 129.0, 127.1, 71.8, 43.0, 37.2, 20.2, 19.1, 14.3; HRMS (FAB+) $C_{13}H_{18}O_2 + H^+$ calcd 207.1307, found 207.1382.

6b 97% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{20.6}$ = −3.1 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.30–7.15 (m, 5H), 4.98–4.92 (m, 1H), 2.68–2.59 (m, 2H), 2.26 (t, *J* = 7.4 Hz, 2H), 1.94–1.78 (m, 2H), 1.70–1.62 (m, 2H), 1.24 (d, *J* = 6.3 Hz, 3H), 0.95 (t, *J* = 7.4Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 174.0, 142.3, 129.1, 129.0, 126.6, 70.9, 38.4, 37.3, 32.5, 20.8, 19.2, 14.4; HRMS (FAB+) $C_{14}H_{20}O_2 + H^+$ calcd 221.1463, found 221.1545.

6c 85% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{23.3}$ = −37.8 (*c* 1.00, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, ppm) 7.25–7.08 (m, 4H), 5.85 (q, *J* = 6.5 Hz, 1H), 2.35 (s, 3H), 2.30 (t, *J* = 7.2 Hz, 2H), 1.72–1.59 (m, 2H), 1.51 (d, *J* = 6.6 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 173.0, 142.2, 138.3, 128.7, 128.6, 127.0, 123.3, 72.3, 36.8, 22.4, 21.6, 18.7, 13.8; HRMS (EI+) $C_{13}H_{18}O_2$ calcd 206.1307, found 206.1312.

6d 75% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{23.3}$ = −44.5

(c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.24 (d, J = 8.1 Hz, 2H), 7.14 (d, J = 7.8 Hz, 2H), 5.86 (q, J = 6.5 Hz, 1H), 2.33 (s, 3H), 2.29 (t, J = 8.2 Hz, 2H), 1.68–1.61 (m, 2H), 1.51 (d, J = 6.6 Hz, 3H), 0.92 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 173.0, 139.2, 137.7, 129.3, 126.3, 80.3, 72.1, 36.8, 22.3, 21.3, 18.7, 13.8; HRMS (EI+) $C_{13}H_{18}O_2$ calcd 206.1307, found 206.1306.

6e > 99% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{20.9}$ = −61.0 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.29 (d, J = 8.6 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 5.86 (q, J = 6.6 Hz, 1H), 3.80 (s, 3H), 2.28 (t, J = 7.5 Hz, 2H), 1.68–1.57 (m, 2H), 1.51 (d, J = 6.6 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 173.1, 159.5, 134.2, 127.7, 114.0, 71.9, 55.4, 36.8, 22.2, 18.7, 13.8; HRMS (EI+) $C_{13}H_{18}O_3$ calcd 222.1256, found 222.1257.

6f 87% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 95 : 5, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{20.5}$ = −72.0 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.33–7.29 (m, 4H), 5.85 (q, J = 6.6 Hz, 1H), 2.30 (t, J = 7.4 Hz, 2H), 1.68–1.58 (m, 2H), 1.50 (d, J = 6.6 Hz, 3H), 0.92 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 172.9, 140.7, 133.8, 128.9, 127.7, 71.5, 36.7, 22.4, 18.6, 13.8; HRMS (EI+) $C_{12}H_{15}O_2Cl$ calcd 226.0761, found 226.0764.

6g 86% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 99 : 1, flow rate = 0.5 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{20.5}$ = −47.0 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.41–7.20 (m, 4H), 5.85 (q, J = 3.1 Hz, 1H), 3.16–3.06 (m, 1H), 2.93–2.88 (m, 1H), 2.57–2.47 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.13–2.04 (m, 2H), 1.70–1.60 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 174.2, 129.3, 127.1, 125.9, 125.2, 78.5, 36.9, 32.8, 30.6, 18.9, 14.1; HRMS (EI+) $C_{13}H_{16}O_2$ calcd 204.1200, found 204.1150.

6h 82% ee by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 98 : 2, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{22.7}$ = −50.6 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.39 (d, J = 8.3 Hz, 2H), 7.15 (d, J = 8.4 Hz, 2H), 5.75 (q, J = 6.6 Hz, 1H), 2.22 (t, J = 7.3 Hz, 2H), 1.65–1.51 (m, 2H), 1.42 (d, J = 6.6 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 172.9, 141.2, 131.9, 128.0, 121.9, 71.5, 36.7, 22.3, 18.7, 13.8; HRMS (EI+) $C_{12}H_{15}BrO_2$ calcd 270.0255, found 270.0255.

6i 86% ee, 52% de by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{24.0}$ = −39.3 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.34 (s, 4H), 5.88 (q, J = 6.6 Hz, 2H), 2.30 (t, J = 7.3 Hz, 4H), 1.74–1.55 (m, 4H), 1.51 (d, J = 6.6 Hz, 6H), 0.93 (t, J = 7.5 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 172.9, 141.6, 126.4, 71.9, 36.7, 22.3, 18.6, 13.8; HRMS (FAB+) $C_{18}H_{26}O_4$ + H^+ calcd 307.1911, found 307.1903.

6j 96% ee, 63% de by HPLC (Whelk-O1, *n*-hexane : 2-propanol = 90 : 10, flow rate = 1.0 mL min^{-1}, UV = 217 nm); $[\alpha]_D^{23.1}$ = −26.6 (c 1.00, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, ppm) 7.35–7.26 (m, 4H), 5.90 (q, J = 6.6 Hz, 2H), 2.31 (t, J = 7.4 Hz, 4H), 1.75–1.60 (m, 4H), 1.52 (d, J = 6.6 Hz, 6H), 0.93 (t, J = 7.4 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) 172.9, 142.4, 128.8, 125.7, 123.9, 72.0, 36.7, 22.4, 18.7, 13.8; HRMS (FAB+) $C_{18}H_{26}O_4$ + H^+ calcd 307.1911, found 307.1903.

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PAPER

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Lipase catalysed resolution of the Lotrafiban intermediate 2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester in ionic liquids: comparison to the industrial *t*-butanol process

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The *Candida antarctica* lipase B (Novozyme 435) catalysed resolution of 2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349), a key Lotrafiban intermediate, has been investigated in six ionic liquids including [BMIM][PF_6] and [BMIM][$N(SO_2CF_3)_2$]. The initial rate and final yield of the reaction have subsequently been determined in [BMIM][PF_6] as a function of initial substrate concentration (5–40 g L^{-1}), temperature (25–100 °C) and initial water content (3–15% H_2O w/w). In each case the results have been compared to those obtained for the optimised industrial process operated in *t*-butanol (88% v/v). Simply replacing the organic solvent with an ionic liquid under otherwise identical reaction conditions reduced the rate of conversion. However, exploiting the increased solubility of the substrate in ionic liquids and the ability to operate at higher temperatures increased the overall rate of reaction by a factor of four while maintaining the same overall yield of 47%. In each case the ee of the product was 99%. Further experiments demonstrated the ability to re-use the enzyme over 10 reaction cycles and suggested that solute mass transfer in ionic liquids might be an issue for reactions carried out at larger scale. Overall the results suggest that ionic liquids can be very favourable reaction media for industrial bioconversion processes, which also overcome many of the safety and environmental concerns of conventional organic solvents.

1 Introduction

Biological catalysts are increasingly being exploited in industry because of the significant advantages they possess over certain chemical catalysts.[1] These include the ability to operate under mild conditions and their outstanding stereo-, regio- and positional specificity. For industrial bioconversion processes, many of the substrates and/or products of interest have low aqueous solubilities. This has led to the development of bioconversions operated in 'non-conventional' media, usually an organic solvent or a water-solvent biphasic mixture, in order to effect bioconversions at higher overall concentrations.[2] A considerable amount of work has been per-

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Gary Lye was born in the UK in 1967. He received his PhD in Biotechnology from the University of Reading in 1992. Between 1993 and 1996 he was successively a Research Fellow and then Lecturer in Chemical Engineering at Imperial College London and the University of Edinburgh respectively. In 1996 he joined University College London where he is currently a Reader in Biochemical Engineering having become a Chartered Engineer in 2003. His current research interests focus on the design of bioconversion processes (novel solvent systems, multiphase reactors, downstream processing) and the integration of biochemical, chemical and engineering techniques in industrial organic syntheses.

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formed to understand solvent effects on the structure and function of both whole cells and isolated enzymes in order to select more stable biocatalysts.[3] Most organic media have a number of generic disadvantages however, including toxicity to the environment and the fact that they are a potential explosion hazard due to their volatile and flammable nature.[4]

Ionic liquids, also known as molten salts, are solutions composed entirely of ions.[5] They are relatively polar solvents and promote the dissolution of a vast array of pharmaceutical intermediates and final drug substance molecules.[6] The replacement of conventional solvents in biocatalytic processes by ionic liquids could therefore overcome many of the disadvantages associated with organic solvents as initially described for the whole cell–nitrile hydratase catalysed hydration of 1,3-dicyanobenzene.[7] The structures and properties of some common room temperature ionic liquids, as used in this study, are shown in Table 1. Also of interest is the ability to readily alter the physicochemical properties of these solvents by simple structural modifications to the cations or changes in anion.[5a] This has led to ionic liquids being termed "designer solvents"[8] with the potential to design more biocompatible ionic liquids having now been demonstrated.[9]

A number of recent publications have shown the potential to carry out enzymatic bioconversions in ionic liquids and there are already a number of reviews.[10] The first publication on enzyme catalysis in ionic liquids concerned the synthesis of *Z*-aspartame by the protease thermolysin.[11] This was followed by publications concerning other proteases[12] and also galactosylation reactions.[13] Sheldon and co-workers first demonstrated lipases to be active in anhydrous ionic liquids.[14] They examined the activity of *Candida antarctica* lipase B (CALB) in ionic liquids for alcoholysis, ammoniolysis and perhydrolysis, discovering similar reaction rates compared to reactions performed in organic solvents such as propan-2-ol and butan-1-ol.

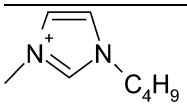
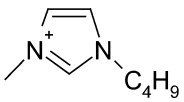
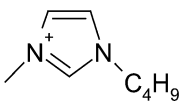
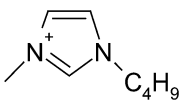
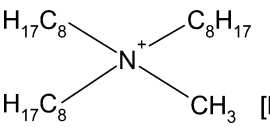
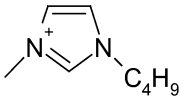
Subsequently other groups have investigated lipases in ionic liquids, due to the widespread use of these enzymes in industry.[1,15] They have generally reported good enzyme activity and stability.[16] Parks and Kazlauskas[17] investigated the regioselective acylation of glucose in ionic liquids and obtained a much higher yield and selectivity than in commonly used organic solvents. This was due to the high solvation properties of ionic liquids for both hydrophobic and hydrophilic reactants. Furthermore, this group also demonstrated the influence of an additional washing regime, following ionic liquid synthesis, to remove impurities and enhance enzyme activity. This is of particular significance when silver salts (*e.g.* of BF_4 and PF_6) are used to prepare ionic liquids, silver being a well-known enzyme inhibitor.[17] Other groups have demonstrated the importance of controlling the water content in ionic liquids in order to achieve higher conversions.[18]

Lotrafiban, as shown in Fig. 1, is a potent non-peptidic glycoprotein antagonist that inhibits platelet aggregation.[19] Phase III clinical trials of Lotrafiban have been undertaken with a view to determine whether the repeat of secondary thrombotic events such as heart attack or stroke could be prevented.[20] The molecule has the (*S*)-stereochemistry and only this enantiomer is active. CALB was identified as an enzyme which was highly selective for hydrolysis of the (*S*)-enantiomer of SB-235349 to give the (*S*) acid, a key Lotrafiban intermediate, with greater than 99.5% chiral purity. The anhydrous nature of the reagents led to Novozyme 435 (a CALB preparation immobilised on acrylic resin) being selected as the preferred catalyst.[21]

Industrial process research and development for Lotrafiban focused on finding the optimum conditions for the enzymatic resolution of SB-235349 with respect to (i) chiral purity of the product, (ii) reaction rate and yield, and (iii) re-use of the resin. Novozyme 435, at a catalogue price of £500 kg^{-1}, would contribute significantly to the cost of goods and maximum re-use would be required. Commercially available *t*-butanol (88% v/v) was identified as the best reaction solvent, having the advantage of being liquid at temperatures above −10 °C. This contrasts to the use of anhydrous *t*-butanol, which has a melting point of 25 °C and is therefore impractical for industrial use. Increasing the reaction temperature was found to cause a decrease in the re-usability of the Novozyme 435, but was necessary to dissolve the substrate at satisfactory levels. Therefore 50 °C was finally selected as the most appropriate reaction temperature.[22] To date the enzymatic resolution has been operated under these conditions at scales of up to 3000 L.

In this work the Novozyme 435 lipase catalysed resolution of SB-235349 is examined in a range of ionic liquids. The rate, yield and chiral purity of the reaction are reported as a function of reaction temperature, substrate concentration and water content of the ionic liquids. The re-use of the enzyme is also explored. The

Table 1 Structure and physical properties of the various ionic liquids used in this study

Ionic liquid structures	Summary of physical properties	References
 [BMIM][BF_4]	Hydrophobic Melting point −71.0 °C Miscible with water Miscible with acetonitrile	23*a–c*
 [BMIM][PF_6]	Hydrophobic Melting point 6.0 °C Immiscible with water Miscible with acetonitrile	23*c*,24*a,b*
 [BMIM][$N(SO_2CF_3)_2$]	Hydrophobic Melting point −6.0 °C Immiscible with water Miscible with acetonitrile	23*c*,24*b*
 [BMIM][CF_3SO_3]	Hydrophilic Melting point 17.0 °C Miscible with water Miscible with acetonitrile	23*c*,33
 [Oc_3MeN][$N(SO_2CF_3)_2$]	Hydrophobic Melting point −70.0 °C Immiscible with water Miscible with acetonitrile	34
 [BMIM][$MDEGSO_4$]	Hydrophilic Miscible with water Miscible with acetonitrile	35

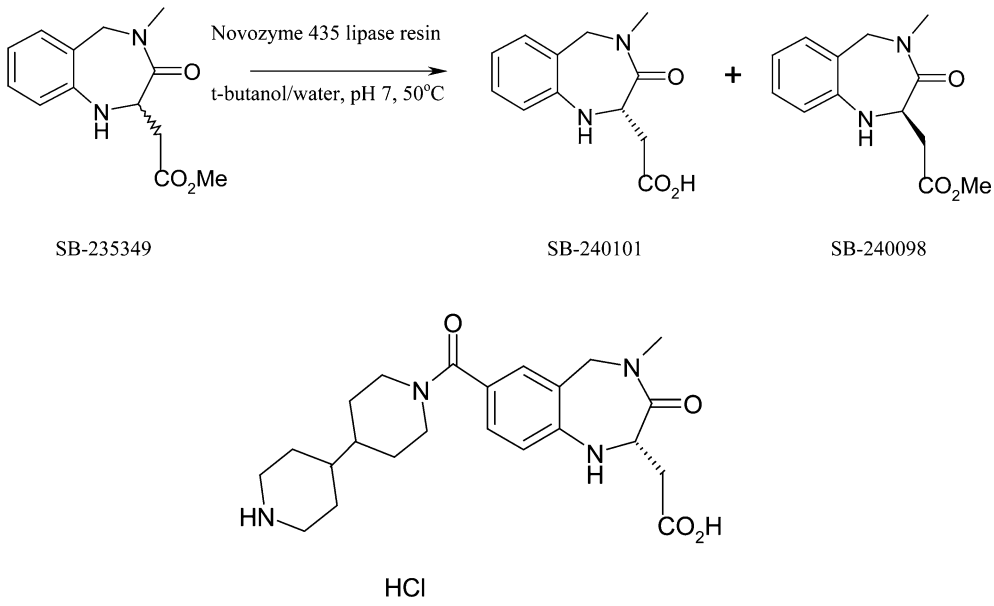


Fig. 1 Details of the industrial process for the lipase catalysed resolution of (2*S*)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349) to (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid (SB-240101) leaving the un-reacted (2R)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester (SB-240098). SB-240101 is a key intermediate in the synthesis of the glycoprotein antagonist Lotrafiban.[22]

results obtained are compared to those for the previously optimised industrial *t*-butanol process. It is shown that by utilising the solvent properties of ionic liquids, namely the ability to operate at higher substrate concentrations and temperatures, significant improvements in reaction rate can be obtained while maintaining the required yield and chiral purity of the product.

2 Results and discussion

2.1 Novozyme 435 catalysed resolution of SB-235349 in *t*-butanol

Preliminary experiments in *t*-butanol examined the activity of the batch of Novozyme 435 lipase used throughout this work in order to provide a basis for comparison with subsequent ionic liquid studies. Experiments were performed under the optimum conditions previously established for the industrial resolution of SB-235349.[22] Typical bioconversion results are shown in Fig. 2.

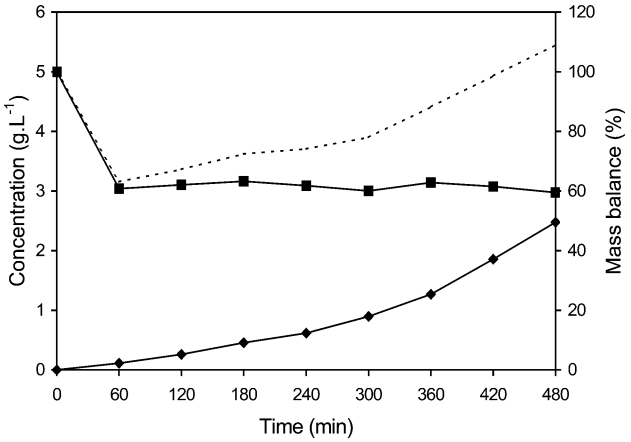


Fig. 2 Kinetics of the CALB catalysed resolution of SB-235349 in *t*-butanol: (♦) SB-240101 concentration, (■) SB-235349 concentration, (**---**) liquid phase mass balance. Reaction conditions as described in Section 4.3: solvent = *t*-butanol (88% v/v), [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, temp. = 50 °C, no pH control.

Measurements of the liquid phase solute concentration show a rapid initial decrease in SB-235349 concentration from 5 to 3 g L^{-1}. This is attributed primarily to the adsorption of substrate onto the Novozyme 435 resin on which the CALB is immobilised. By comparison there is a steady, almost linear increase in the concentration of SB-240101 up to 180 min, which best represents the apparent rate of product synthesis. The final concentration of SB-240101 of 2.3 g L^{-1} after 8 hours corresponds to a 47% conversion as reported for the industrial process.[22] The liquid phase mass balance (Fig. 2) indicates an initial drop to ~60% after 60 min corresponding to the decrease in SB-235349 concentration due to adsorption. This is followed by a steady increase to ~100%, in line with SB-240101 formation, which implies that there is no significant binding of the acid product to the resin.

Experiments were also performed with and without pH control as shown in Table 2. These results showed a small increase in the apparent initial rate of product formation in *t*-butanol but no change in the overall yield of SB-240101. Any pH change induced by the hydrolytic resolution is thus considered to be small under these conditions and it is well known that the Novozyme 435 CALB preparation has a reasonably broad pH optimum between 5–8.[22]

Table 2 Effect of pH control (pH 7) on the initial rate of SB-240101 formation in *t*-butanol (88% v/v) and various ionic liquids (12% H_2O w/w). Reaction conditions as described in Section 4.3: [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, temp. = 50 °C

Medium	Apparent initial rate/g L^{-1} min^{-1}	pH control
t-Butanol	4.6×10^{-3}	No
t-Butanol	4.7×10^{-3}	Yes
[BMIM][$N(SO_2CF_3)_2$]	1.0×10^{-2}	No
[BMIM][$N(SO_2CF_3)_2$]	1.0×10^{-3}	Yes
[BMIM][PF_6]	3.8×10^{-3}	No
[BMIM][PF_6]	5.3×10^{-4}	Yes

2.2 Screening of ionic liquids as bioconversion media

Initial experiments with ionic liquids aimed to establish if the CALB catalysed resolution of SB-235349 would actually occur in an ionic liquid and, if so, which type of ionic liquid was most suitable for the reaction. For these experiments a limited range of ionic liquids were chosen that exhibited different physicochemical

properties as shown in Table 1. All were tested under conditions identical to those used in Fig. 2 except for replacement of the *t*-butanol with the appropriate ionic liquid.

The first ionic liquid tested was [BMIM][BF_4] which is considered hydrophilic and is miscible with water.[23] As shown in Fig. 3 the substrate rapidly disappeared, however no SB-240101

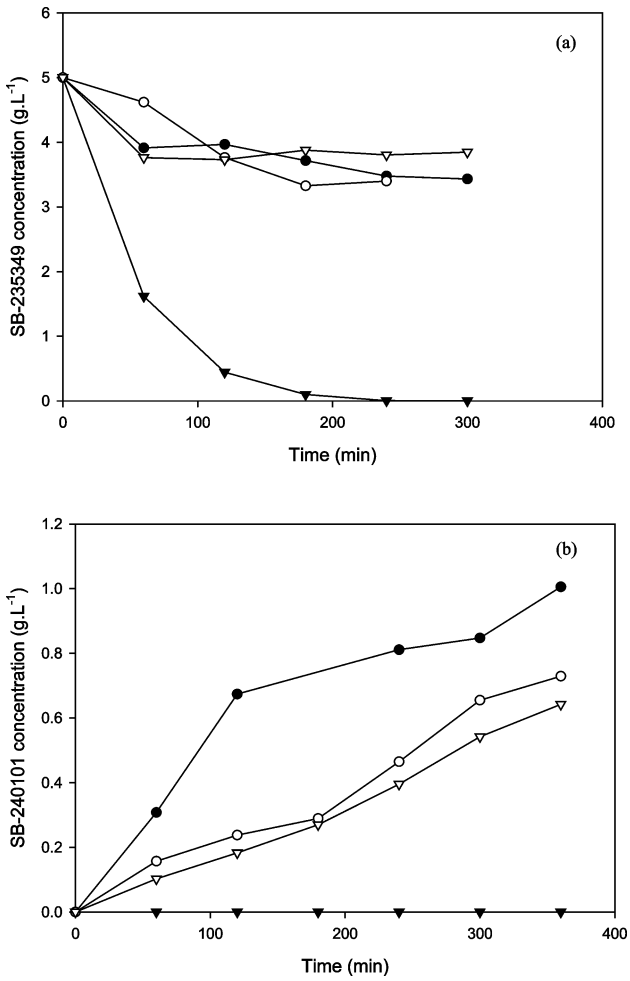


Fig. 3 Kinetics of CALB catalysed resolution of SB-235349 in a range of ionic liquids: (a) SB-235349 concentration, (b) SB-240101 concentration. (○) [BMIM][PF_6], (▼) [BMIM][BF_4], (●) [BMIM][$N(SO_2CF_3)_2$], (▽) [BMIM][CF_3SO_3]. Reaction conditions as described in Section 4.3: solvent = ionic liquid (12% H_2O w/w), [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, temp. = 50 °C, no pH control.

could be detected. With [BMIM][PF_6], which is considered to be more hydrophobic,[23c,24] a relatively slow decrease in the substrate concentration was found with the corresponding production of SB-240101. After six hours the SB-240101 concentration was 0.7 g L^{-1}, approximately half that produced in the *t*-butanol system. The results for [BMIM][$N(SO_2CF_3)_2$] considered hydrophobic and [BMIM][CF_3SO_3] hydrophilic (Table 1) show similar results to those for [BMIM][PF_6], with SB-240101 concentrations of 1 g L^{-1} and 0.65 g L^{-1} respectively being attained after 6 hours. The anions of these three ionic liquids [CF_3SO_3], [PF_6] and [$N(SO_2CF_3)_2$] show increasing hydrophobicity corresponding to increased product concentrations of 0.65 g L^{-1}, 0.7 g L^{-1} and 1.0 g L^{-1} respectively over six hours under optimised *t*-butanol conditions.

Other ionic liquids tested (results not shown in Fig. 3) include methyl-trioctyl ammonium bis(trifluoromethylsulfonyl)imide) [Oc_3MeN][$N(SO_2CF_3)_2$], which is water immiscible, and 1-butyl-3-methyl-imidazolium diethyleneglycol monomethylethersulfate [BMIM][$MDEGSO_4$] which is water miscible (Table 1). The [BMIM][$MDEGSO_4$] showed a rapid decrease in SB-235349 concentration, however there was no SB-240101 formation. The HPLC traces of the reaction in [Oc_3MeN][$N(SO_2CF_3)_2$] showed no peaks of any kind. In summary, screening of a very limited range of ionic liquids yielded a number with different properties that could act as useful media for the CALB catalysed resolution but at rates and conversions below those for the previously optimised *t*-butanol system.

Although the use of immobilised CALB is preferable in order to facilitate recovery and re-use of the enzyme, the resolution of SB-235349 in [BMIM][PF_6] was also performed using a lyophilised preparation of the free enzyme.[3d] At the same biocatalyst concentration the free lipase exhibited a 2.5-fold higher rate of reaction, with 1.85 g L^{-1} of SB-240101 formed after 6 hours compared to 0.7 g L^{-1} with the Novozyme 435. The higher reaction rate with the free enzyme than the immobilised Novozyme 435 is attributed to the lower activity load of the immobilised form but shows that the free enzyme is active and stable.[25] During these experiments it was observed that the free enzyme when added to the [BMIM][PF_6] formed a dispersed suspension of fine particles within the ionic liquid.

2.2.1 Influence of pH control and agitation on reaction rate. As with the *t*-butanol process, a number of the promising ionic liquid bioconversions were performed with pH control. The physical meaning of a pH value measured in an ionic liquid is clearly going to be different to that measured in aqueous solutions,[26] however we were interested to see what benefits, if any, there might be of operating in pH-stat mode. Table 2 shows the apparent initial rates of reaction carried out with and without control of pH at pH 7. In contrast to the results obtained for *t*-butanol, there is a seven-fold decrease in the rate of reaction with [BMIM][PF_6] and a ten-fold decrease with [BMIM][$N(SO_2CF_3)_2$]. These marked decreases were attributed to the large initial volume of ammonia solution added to the ionic liquids, compared to *t*-butanol, which would have a negative effect on the activity of the CALB enzyme. All subsequent experiments in ionic liquids were thus carried out without pH control.

Experiments were also performed in [BMIM][PF_6] with increasing stirrer speeds from 1000–3000 rpm (data not shown). These indicated an increase in the initial rate of SB-240101 formation with stirrer speed, up to 2000 rpm.[3d] This suggests that the measured activity of the CALB is diffusion controlled at the lower agitation rates.[27] Compared to the *t*-butanol system, however, the initial rate of reaction in [BMIM][PF_6] under non-mass transfer limited conditions (3000 rpm) was ~28% slower. This decrease is probably explained by the difference in solvent viscosity as described in Section 4.4.5. The viscosity of the saturated ionic liquid was noticeably less than that of the pure anhydrous ionic liquid[28] but much greater than the *t*-butanol (88% v/v).

2.3 Effect of substrate concentration on SB-240101 formation in [BMIM][PF_6]

Having established that the resolution of SB-235349 was possible in ionic liquids the solvent properties of selected ionic liquids were explored further as were the conditions under which the resolution was performed. In the case of the widely used [BMIM][PF_6][23c,24a,b] studies on the solubility of SB-235349 in this ionic liquid (as described in Section 4.3.2) showed an eight-fold increase in substrate solubility at 50 °C compared to *t*-butanol (88% v/v). As shown in Fig. 4, Novozyme 435 catalysed resolutions were subsequently performed in [BMIM][PF_6] at 50 °C and various initial SB-235349 concentrations of up to 40 g L^{-1} (0.16 Molar). The apparent initial rate of the reaction increased dramatically as the substrate concentration was increased from 5 g L^{-1} until around 25 g L^{-1} where it became comparatively constant. The rate of SB-240101 formation in [BMIM][PF_6] at 40 g L^{-1} and 50 °C showed an increase which was three-fold higher than that obtained under optimal conditions in the *t*-butanol system. For all the substrate concentrations investigated in [BMIM][PF_6] there was no evidence of substrate inhibition at these high solute concentrations.

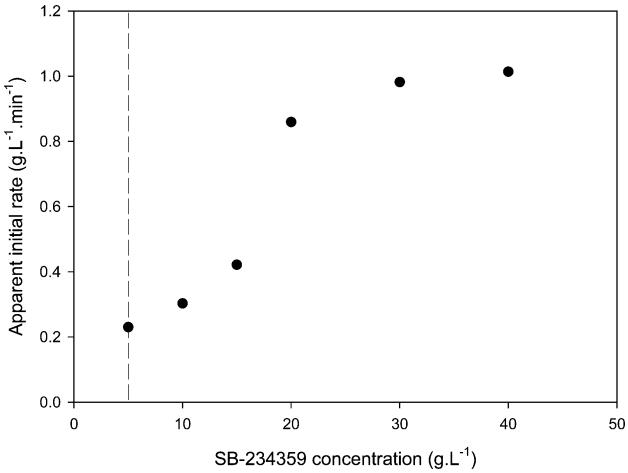


Fig. 4 Effect of SB-235349 concentration on the apparent initial rate of SB-240101 formation in [BMIM][PF_6]. Dashed line indicates solubility limit of SB-235349 in *t*-butanol at 50 °C. Reaction conditions as described in Section 4.3: solvent = ionic liquid (12% H_2O w/w), [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 −40 g L^{-1}, temp. = 50 °C, no pH control.

2.4 Effect of temperature on SB-240101 formation in [BMIM][PF_6]

Although the previous experiments were performed at 50 °C, the kinetics of the resolution were also explored at a range of temperatures from 25–100 °C. For the *t*-butanol system a reaction temperature of 50 °C was necessary to achieve acceptable substrate solubility but at the expense of adverse affects on the kinetics and stability of the Novozyme 435 biocatalyst.[22] As shown in Fig. 5

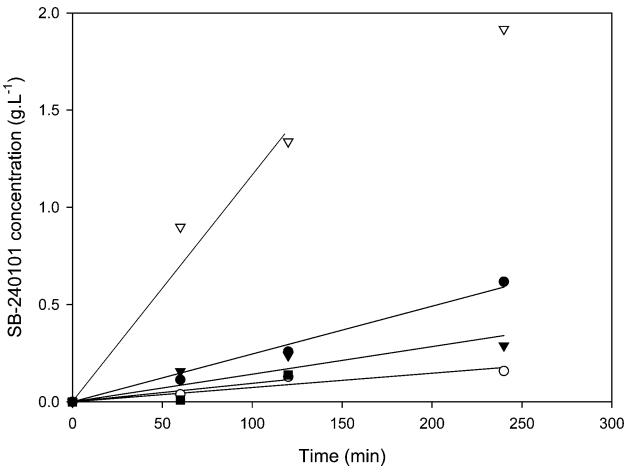


Fig. 5 Effect of temperature on the apparent initial rate of SB-240101 formation: (●) *t*-butanol at 50 °C, (○) [BMIM][PF_6] at 25 °C, (▼) [BMIM][PF_6] at 50 °C, (▽) [BMIM][PF_6] at 75 °C, (■) [BMIM][PF_6] at 100 °C. Reaction conditions as described in Section 4.3: solvent = ionic liquid (12% H_2O w/w), [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, no pH control. Solid lines fitted by linear regression.

with the ionic liquid [BMIM][PF_6], SB-240101 was produced at temperatures up to 75 °C with an associated increase in the apparent initial rate of product formation. At 75 °C there is a four-fold enhancement in the initial rate of product formation compared to the *t*-butanol system at 50 °C. In each case the final conversion obtained was 47%, and no side reactions were observed according to HPLC analysis of reaction samples. Above 75 °C [BMIM][PF_6] is known to degrade releasing acidic species and potentially HF.[29] Experiments performed at temperatures above 75 °C not surprisingly showed a significant decrease in initial reaction rate (final conversion yield not determined). What is remarkable, however, is the sustained enzymatic activity over a period of four hours seen at the elevated temperatures of 75 °C and above (Fig. 5). This suggests that the CALB has a significantly greater thermal stability in [BMIM][PF_6] compared to aqueous or organic solvents.[30] The stability of the substrate and product molecules does not seem to be an issue at elevated temperatures in this ionic liquid since control experiments at 50 and 75 °C (in the absence of enzyme) showed no change in their chromatographic behaviour with time.

2.5 Effect of water content on SB-240101 formation in [BMIM][PF_6]

As the resolution of SB-235349 is a hydrolytic reaction (Fig. 1), water is an important reactant and must be present in order for the reaction to occur. In the case of the *t*-butanol process the commercial source of the solvent already contained 12% H_2O v/v water, sufficient to drive the reaction to completion.[22] The ionic liquids used in this work are supplied as anhydrous or with very low water contents. Experiments with ionic liquids were therefore performed utilising a range of added water concentrations. As described in Section 4.2.1 the preparation of hydrophobic ionic liquids like [BMIM][PF_6] involved multiple extraction stages and thus these were saturated with water before use. The saturation levels of water in the various ionic liquids used were determined by Karl–Fisher titration as described in Section 4.4.3. The saturation water content of [BMIM][PF_6] was determined as 3.07% H_2O w/w (which is in good agreement with the value of 3.18% H_2O w/w reported by Wong *et al.*[31]). For [BMIM][CF_3SO_3], [BMIM][BF_4], and [BMIM][$N(SO_2CF_3)_2$] the saturation concentrations were found to be 0.28, 0.61, 0.17% H_2O w/w respectively. Due to the requirement for water in the resolution studied here, higher initial water contents were used compared to those reported by other groups who frequently dry the ionic liquids after washing to levels around 0.03% H_2O w/w.[12]

Fig. 6 shows the effect of added water content in [BMIM][PF_6] on the initial rate of reaction. For these experiments the overall

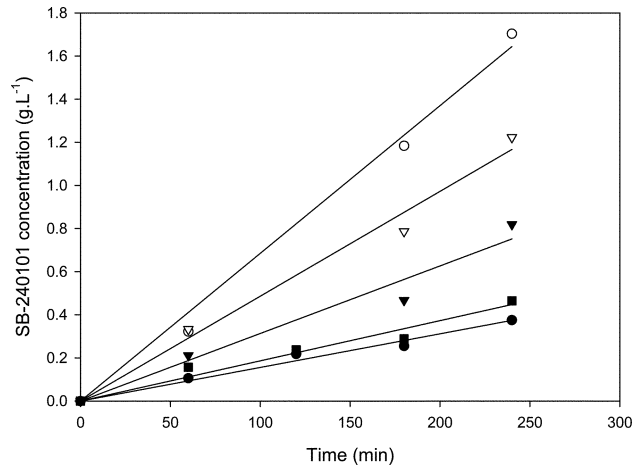


Fig. 6 Effect of initial total water content in [BMIM][PF_6] on the apparent initial rate of SB-240101 formation: (■) 15% H_2O w/w, (○) 8% H_2O w/w, (▽) 6% H_2O w/w, (▼) 4% H_2O w/w, (●) 3% H_2O w/w. Reaction conditions as described in Section 4.3: solvent = [BMIM][PF_6], [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, temp. = 50 °C, no pH control. Solid lines fitted by linear regression.

water content is calculated to include the saturation level of 3.07% H_2O w/w. As the water content is increased up to 8% H_2O w/w the apparent initial rate of product formation increases significantly. At higher water contents of ~15% H_2O w/w, the rate subsequently decreases to a value below that observed with 4% H_2O w/w water. For water concentrations of 8% H_2O w/w and 6% H_2O w/w the final conversion obtained was 47% (final conversions were not determined for the other water contents due to the low rates of reaction). These results with varying water contents are comparable to our previous studies on the hydration of 1,3-dicyanobenzene in [BMIM][PF_6] using a whole cell nitrile hydratase biocatalyst[5c] and suggest that there is an optimum water concentration for hydrolytic

reactions in ionic liquids. Laszlo and Compton[12] have obtained similar trends with the free form of α-chymotrypsin in [BMIM][PF_6] showing that the enzyme activity increased up to a water content of 0.5% (v/v) and then decreased at higher levels.

Although the saturation level of water in [BMIM][PF_6] was determined as 3.07% H_2O w/w, it was observed that bioconversions containing up to 4% H_2O w/w existed as a single liquid phase. This suggests some water could also be adsorbing onto the Novozyme 435 resin or the immobilised CALB. When the water content was increased further a separate water phase was observed which was rapidly dispersed in the form of small droplets when mixed. Scale-down studies[32] on the hydrodynamics of ionic liquid-aqueous dispersions and the implications for solute mass transfer rates are currently underway in our laboratory.

2.6 Evaluation of optimised bioconversion conditions in [BMIM][PF_6]

Having investigated separately the effects of substrate concentration, temperature and water content on the CALB-catalysed resolution of SB-235349 in ionic liquids the final experiments aimed to identify optimum overall reaction conditions. As shown in Fig. 7 at a substrate concentration of 25 g L^{-1} in [BMIM][PF_6], a

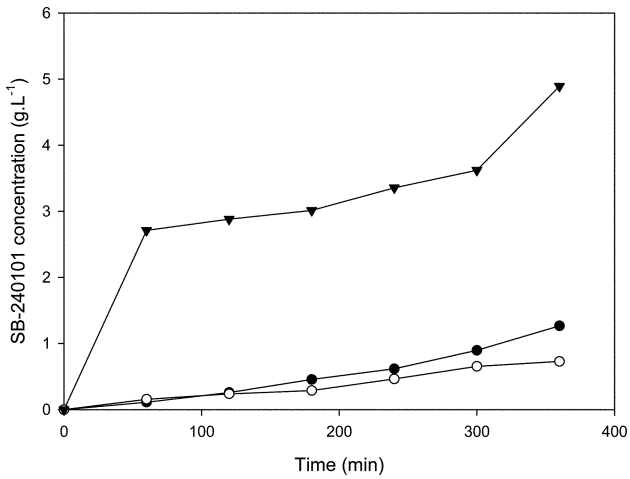


Fig. 7 Kinetics of SB-240101 formation under optimised conditions in various solvent systems: (●) *t*-butanol (88% v/v), initial [SB-235349] = 5 g L^{-1}, temp. 50 °C, (○) [BMIM][PF_6] (12% H_2O w/w), [SB-235349] = 5 g L^{-1}, temp. 50 °C, (▼) [BMIM][PF_6] (8% H_2O w/w), [SB-235349] = 25 g L^{-1}, temp. 75 °C. Other reaction conditions as described in Section 4.3: [Novozyme 435] = 1.4 g L^{-1}, no pH control.

temperature of 75 °C and with a total water content of 8% H_2O w/w, a remarkable twenty seven-fold increase in the concentration of SB-240101 was obtained after 60 min compared to the optimised *t*-butanol system. In each case the final conversion obtained was 47%, and the overall increase in the rate of conversion was calculated to be four-fold greater. Importantly, analysis of the chiral purity of the SB-240101 produced by the CALB-catalysed resolution in [BMIM][PF_6] showed that the product had an ee of 99%, identical to that obtained in the industrial *t*-butanol process.[22]

2.7 Evaluation of enzyme re-use

As stated previously the high cost of the Novozyme 435 CALB preparation requires that there is recycling of the enzyme over a number of batches. In the *t*-butanol process the use of Novozyme 435 over 10 cycles has been reported.[22] Experiments here examined if the Novozyme 435 could be re-used over 10 cycles in [BMIM][PF_6]. Table 3 shows that recycling of the enzyme was possible in the ionic liquid however the initial reaction rate dropped significantly to ~40% of its initial value after six reaction and wash cycles. This was followed by a further gradual decline such that the Novozyme 435 preparation retained ~20% of its initial activity after 10 cycles. In contrast results for enzyme re-use in the *t*-butanol system showed a greater retention in activity with 48% activity achieved over 10 cycles. In the case of the ionic liquid it is supposed that the residual activity after each cycle could be improved by avoiding washing the Novozyme 435 preparation with water between each reaction however this would require further investigation. Although the initial rate of reaction decreased after each cycle an overall conversion of around 47% w/w could be obtained in both ionic liquid and *t*-butanol by simply increasing the reaction time.

Table 3 Biocatalyst recycle and re-use in [BMIM][PF_6]. Values in brackets are for the industrial *t*-butanol process and represent the average rate of product formation (calculated from Atkins *et al.*[22]). Reaction conditions as described in Section 4.3: solvent = [BMIM][PF_6] (12% H_2O w/w), [Novozyme 435] = 1.4 g L^{-1}, initial [SB-235349] = 5 g L^{-1}, temp. = 50 °C, no pH control. The Novozyme 435 resin was recovered and cleaned after each recycle as described in Section 4.3.1

Reaction cycle	Apparent initial rate of SB-240101 formation/g L^{-1} min^{-1}	Fraction of original rate (%)
1	3.8×10^{-3}	100 (100)
2	3.7×10^{-3}	97 (68)
3	3.3×10^{-3}	87 (70)
4	2.2×10^{-3}	58 (78)
5	2.3×10^{-3}	60 (69)
6	1.5×10^{-3}	39 (63)
7	1.2×10^{-3}	32 (74)
8	1.3×10^{-3}	34 (74)
9	1.4×10^{-3}	37 (69)
10	7.4×10^{-4}	19 (48)

3. Conclusions

The results presented in this work show that the *Candida antarctica* lipase B catalysed resolution of SB-235349 occurs in a range of ionic liquids. The ionic liquid systems offer several process advantages such as the ability to work at higher overall substrate concentrations and the ability to safely operate at elevated temperatures due to the non-flammable and non-volatile nature of ionic liquids. In addition the stringent control of the water content in the ionic liquid allows optimum reaction rates to be achieved. Overall the advantages displayed with the use of ionic liquids in this reaction indicate a positive future for the industrial application of ionic liquids as replacements for conventional organic solvents. For application in the pharmaceutical sector, however, there remain many validation issues to be addressed such as the purity of the final product and confirmation that it is entirely free of ionic liquid contamination. Quality control criteria to enable recycling of the ionic liquid must also be established.

Work in our laboratory is now examining downstream processing methods for this bioconversion to facilitate the recovery of pure SB-240101 with high yields. Additionally, the recovery and racemisation of the undesired enantiomer SB-240098 is under investigation as are methods for cleaning and recycling the ionic liquid between successive reaction cycles.

4. Experimental

4.1. Materials

The substrate (SB-235349) and product (SB-240101) standards were generously donated by GlaxoSmithKline (GSK). Ionic liquids used were either synthesised in-house or were obtained from Acros Organics (Geel, Belgium) or Solvent Innovation GmbH (Koln, Germany). All other chemicals and biochemicals were purchased from Sigma Chemical Co. (Dorset, UK) and were of the highest purity available. The CALB enzyme pre-immobilised on a polystyrene resin as Novozyme 435 and the lyophilised version of the same enzyme were also purchased from Sigma.

4.2. Synthesis, purification and preparation of ionic liquids

4.2.1 1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6]. [BMIM][PF_6] was obtained from Solvent Innovation GmbH, at a purity of 97%. To ensure the ionic liquid was saturated with water before use, and to remove any remaining extractable acidic components, the ionic liquid supplied was washed 10 times with an equal volume of reverse osmosis (RO) water. The pH of the water phase after the final wash was measured to ensure a pH of 6.5 before the ionic liquid was used. pH measurements after each wash stage indicated an increase in pH from low initial values, ~pH 2.5, to a steady value of ~pH 6.5. After washing the saturation water content of the ionic liquid was measured as described in Section 4.4.3. For reactions at higher water contents RO water was added to give overall concentrations of between 3–15% H_2O w/w water.

4.2.2 1-Butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4]. [BMIM][BF_4] was obtained from Solvent Innovation GmbH at a purity of 99%. Since this ionic liquid is miscible with water it was used without further purification. The water content as supplied was measured as described in Section 4.4.3 and RO water was added to give an overall water content of 12% H_2O w/w before use.

4.2.3 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [BMIM][$N(SO_2CF_3)_2$]. [BMIM][$N(SO_2CF_3)_2$] was prepared in the laboratory: [BMIM]Cl (200 g, 1.145 moles) was mixed with $LiN(CF_3SO_2)_2$ (237.12 g, 1.15 moles) in distilled H_2O in a round bottomed flask and stirred for 24 h at room temperature. After this time a two-phase mixture had formed. The upper aqueous phase was decanted off and the residual ionic liquid dissolved in dichloromethane (DCM). The DCM–ionic liquid mixture was then washed with ~200 mL distilled H_2O (~4 times) in a separating funnel. After the final wash, $MgSO_4$ was added, to remove any excess water, and shaken with the DCM–ionic liquid mixture. The $MgSO_4$ was then removed by filtering through an S4 (porosity 4) filter. To remove the remaining DCM the mixture was evacuated on a rotary evaporator with heating (~50 °C) until no further solvent was condensed. The retained crude ionic liquid was then immersed in an oil bath and attached to a high-vacuum line incorporating a trap system, each loop being cooled in liquid nitrogen. The oil bath was heated to ~50 °C and the ionic liquid was exposed to these conditions for 6 h. After this time the residual mixture was removed from the oil bath and left to cool. Purity of the ionic liquid was assessed using NMR analysis in d-chloroform.[3d] The water content of the ionic liquids was measured as before and RO water added to give an overall concentration of 12% H_2O w/w.

4.2.4 1-Butyl-3-methylimidazolium trifluoromethanesulfonate [BMIM][CF_3SO_3]. [BMIM][CF_3SO_3] was obtained from Acros Organics at a purity of 99%. Since this ionic liquid is miscible with water it was used without further purification. The water content of the ionic liquid was measured as before and RO water added to give an overall water content of 12% H_2O w/w.

4.2.5 Methyl-trioctyl-imidazolium bis(trifluoromethylsulfonyl)imide [Oc_3MeN][$N(SO_2CF_3)_2$]. [Oc_3MeN][$N(SO_2CF_3)_2$] was obtained from Solvent Innovation GmbH at a purity of 98%. Although this ionic liquid is immiscible with water it was used without further purification. The overall water content of the ionic liquid was ~12% H_2O w/w with additional RO water.

4.2.6 1-Butyl-3-methylimidazolium diethyleneglycol monomethylether [BMIM][$MDEGSO_4$]. [BMIM][$MDEGSO_4$] was obtained from Solvent Innovation GmbH at a purity of 98%. Since this ionic liquid is miscible with water it was used without further purification. The overall water content of the ionic liquid was ~12% H_2O w/w with additional RO water.

4.3 Novozyme 435 resolution of SB-235349 to SB-240101

All reactions were performed in a small, jacketed cone-shaped stirred reactor (h = 8 cm, d_{top} = 3.5 cm, d_{base} = 1 cm). This was fitted with a single flat-bladed impeller having a diameter of 0.4 cm, which was situated 1 cm from the base of the reactor and coupled to an overhead stirrer operated at 3000 rpm. The reactor was charged with fresh Novozyme 435 resin (1.4 g L^{-1}) and SB-235349 (5–40 g L^{-1} depending on the particular experiment) dissolved in either *t*-butanol (88% v/v) or various ionic liquids (each with 12% H_2O w/w). The reaction vessel jacket was pre-heated *via* a circulating water bath, which controlled the temperature to 25–75 ± 0.1 °C depending on the particular experiment. A 2 μL sample of the reaction mixture was taken every hour and analysed as described in Section 4.4.1. Once the reaction was deemed to be complete (conversion > 47% based on HPLC peak area ratio of SB-235349 to SB-240101) the Novozyme 435 resin was removed by filtration. In certain experiments a radiometer autotitrator was used to maintain a constant pH of 7.0 in the reaction mixture (this is discussed in Section 2.2.1), by automated addition of 1.5 M ammonia in either *t*-butanol (88% v/v) or ionic liquid (12% H_2O w/w).[22]

4.3.1 Examination of enzyme re-use. The re-use of the Novozyme 435 was investigated over 10 reaction cycles. Initially 1.4 g L^{-1} of the immobilised enzyme was added to the reaction vessel containing SB-235349 (5 g L^{-1}) in ionic liquid (12% H_2O w/w). The reaction was then repeated over 10 cycles as described in Section 4.3 at 50 °C. After each resolution was deemed complete the reaction mixture was filtered through an S4 filter. The immobilised enzyme was then washed with water, re-weighed, and added to a fresh batch of substrate solution.

4.3.2 Determination of solubility limit. The solubility limit of the substrate in [BMIM][PF_6] was estimated by adding increasing quantities to a known volume of ionic liquid. The suspensions were then placed in a heater water bath set at 50 °C and mixed with a magnetic stirrer after each addition for up to 24 hours until the suspension became clear. The point at which no more substrate would dissolve was taken as the solubility limit. The integrity of the dissolved solutes was confirmed by HPLC.

4.4 Analytical techniques

4.4.1 Quantification of SB-235349 and SB-240101. Bioconversion samples in either *t*-butanol or ionic liquids were analysed by HPLC (Dionex PeakNet system) using a Phenomenex 5 μm C18 column (150 × 4.6 mm) as described by Atkins *et al*.[22] Eluents used were A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile. The mobile phase flow rate was 1 mL min^{-1} with a gradient of 25–75% B over 10 minutes with UV detection at 254 nm. Typical retention times for SB-235349 an SB-240101 were 6.1 and 4.4 minutes respectively. The maximum coefficient of variance for the quantification of SB-235349 and SB-240101 concentrations was 2.5 and 2% respectively.

4.4.2 Confirmation of chiral purity of SB-240101. The chiral purity (ee) of the product SB-240101 was determined by chiral HPLC as described by Atkins *et al*.[22] The column used was a 5 μm CHO-3981 (250 × 4.6 m) operated at 25 °C. The mobile phase consisted of 98% hexane and 2% IPA which was pumped isocratically at 0.7 mL min^{-1} with UV detection at 215 nm. Typical retention times of the *R*-ester (SB-240098) and *S*-ester were 32.2 and 30.2 minutes respectively. Samples of *t*-butanol reactions were injected directly onto the column. Samples from ionic liquid reactions were first extracted into an equal volume of the HPLC mobile phase before injection.

4.4.3 Determination of ionic liquid water content. The water content of the ionic liquids were determined by Karl–Fischer titration. Measurements were performed at the GSK Ulverston site according to standard methods.[3d] The maximum coefficient of variance of this assay was 4%.

4.4.4 Density measurements. Gravimetric analysis was used to determine the liquid density (ρ); 10 mL liquid volumes were measured into density bottles and weighed on a Mettler-Toledo AB54 electronic balance (Leicester, UK). All measurements were performed in triplicate. The values for pure [BMIM][PF_6], ρ = 1380 kg m^{-3} and *t*-butanol (88% v/v), ρ = 795 kg m^{-3} were in line with literature values. The density of the water saturated [BMIM][PF_6] was 1316 kg m^{-3}.

4.4.5 Viscosity measurements. Liquid viscosity measurements were made using a Contraves Rheomat 115 rheometer (Contraves AG, Zurich, Switzerland). All measurements were performed in triplicate. The values for pure [BMIM][PF_6], μ = 207 mPas and *t*-butanol (88% v/v), μ = 5 mPas were in line with literature values. The viscosity of the water saturated [BMIM][PF_6] was 85.05 mPas. The [BMIM][PF_6] was shown to have Newtonian rheology by a straight-line plot of shear stress against shear rate when pure and saturated (data not shown).

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PAPER

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Dissolution of *Candida antarctica* lipase B in ionic liquids: effects on structure and activity

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The effects of ionic liquid media on the activity of *Candida antarctica* lipase B in a simple transesterification reaction were studied. In ionic liquids containing alkylsulfate, nitrate and lactate anions, which dissolved CaLB, the reaction was at least ten times slower than in [BMIm][BF_4]. Only [Et_3MeN][$MeSO_4$] was an exception, as dissolved CaLB maintained its activity in this solvent. By means of FT-IR spectroscopy, denaturation of CaLB was observed upon dissolution in ionic liquids in which the activity was low, whereas the conformation of enzyme dissolved in [Et_3MeN][$MeSO_4$] closely resembled the native one.

Introduction

The use of ionic liquids as reaction media for enzymatic transformations is a rapidly expanding field.[1] Most of these applications involve dispersions of enzymes in low-coordinating strength ionic liquids, such as the by now archetypical [BMIm][PF_6] and [BMIm][BF_4]. Any hopes that ionic liquids would provide an easy way to combine enzyme solubility and

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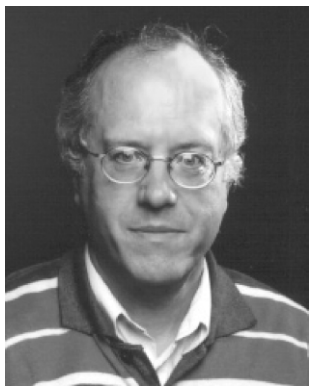

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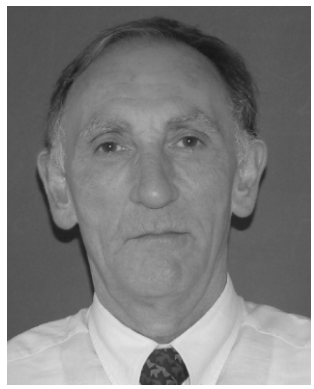

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activity in non-aqueous media were extinguished when it became clear that the small fraction of thermolysin that dissolved in [BMIm][PF_6] containing 5% water was not active, whereas the dispersed solid enzyme maintained its activity.[2]

It has recently been demonstrated that the ability to solvate and dissolve molecules depends on several ionic liquid properties, but mainly on the hydrogen bond-accepting properties of the anion.[3] We recently have published preliminary results indicating that ionic liquids containing a strongly coordinating anion, such as nitrate or lactate, indeed dissolved *Candida antarctica* lipase B (CaLB) but also effected its (partially reversible) deactivation.[4] Kaar *et al.* have accordingly found that *C. rugosa* lipase was not active in ionic liquids that contain a nitrate or acetate anion, whereas activity was maintained in [BMIm][PF_6].[5] The cellulase from *Trichoderma reesei* likewise was inactive when dissolved in [BMIm][Cl]; a fluorometric study showed that the enzyme had denaturated, presumably due to interactions with the—strongly coordinating—chloride ion.[6] It would seem that anions that interact with solid enzymes sufficiently strongly to break the *inter*molecular bonds and effect dissolution also interfere with the *intra*molecular bonds to such a degree that unfolding takes place.

We continued our investigations of the issue of enzyme solubility *vs.* activity in ionic liquids and have studied the structural changes of CaLB upon dissolution in ionic liquids using FT-IR spectroscopy. The results will be presented and discussed in the present paper.

Results and discussion

Activity of CaLB in ionic liquids

We selected the alcoholysis (transesterification) of ethyl butanoate with 1-butanol (see Fig. 1) as a suitable test reaction to measure the

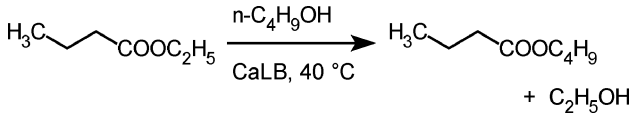


Fig. 1 Transesterification of ethyl butanoate with 1-butanol.

effects of the medium on the activity of Novozym 435, an immobilised preparation of CaLB. When the reaction was performed in [BMIm][BF_4] or [BMIm][PF_6], the reaction rate was comparable with that in *tert*-butyl alcohol.[7] When the reaction was performed in ionic liquids that contained a nitrate, ethylsulfate or lactate anion, in which CaLB is soluble (see below), the reaction rate was at least 10 times lower (see Table 1). Hence, it would seem that ionic liquids that interact with the protein sufficiently strongly to effect its dissolution also induce structural changes that lead to loss of activity.

Table 1 *C. antarctica* lipase B in ionic liquids: activity in transesterification, solubility and activity recovery upon rehydration

Medium[a]	Conversion[b] (%, 24 h)	Dissolution	Recovery from the supernatant[c] (%)
[BMIm][BF_4]	78	no	0
[BMIm][PF_6]	71	no	0
[Et_3MeN][$MeSO_4$]	26	see text	n.d.
[EMIm][$EtSO_4$]	7	yes	53
[BMIm][lactate]	5	yes	42
[$EtNH_3$][NO_3]	5	yes	33
[BMIm][NO_3]	3	yes	73
tert-Butyl alcohol	74	no	0

[a] For ionic liquid nomenclature see Table 3. [b] Reaction conditions: ethyl butanoate (60 mM), 1-butanol (120 mM), Novozym 435 (15 mg) in solvent (0.5 mL) at 40 °C for 24 h. [c] Hydrolytic activity recovered from the supernatant after 24 h incubation at 40 °C; original activity = 100%.

Solubility of free CaLB and recovery of activity

We investigated the related issues of enzyme solubility and denaturation more closely by incubating dry solid CaLB (SP525) in various ionic liquids for 24 h at 40 °C. Enzyme dissolution was observed with [BMIm][NO_3], [BMIm][lactate], [EMIm][$EtSO_4$] and [$EtNH_3$][NO_3], but not with [BMIm][BF_4] or [BMIm][PF_6]. The supernatants were separated, diluted with water and subjected to a triacetin test. The transesterification activity of CaLB in these ionic liquids was low, as noted above, but up to 73% of the original hydrolytic activity was recovered upon rehydration (Table 1). Hence, the activity loss is partially reversible, indicating that the enzyme refolds into the active conformation upon rehydration. A similar reconstitution has been observed with alkaline phosphatase[8] and lysozyme[9] in [$EtNH_3$][NO_3].

CaLB in [Et_2MeN][$MeSO_4$]

The behaviour of CaLB in [Et_3MeN][$MeSO_4$] did not correspond with the simple pattern set out above, as a modest transesterification activity was maintained even though dissolution of the biocatalyst was observed. For a closer examination, we performed transesterification experiments with varying amounts of dry solid CaLB (see Table 2). The conversion increased with the amount of enzyme, as expected, although the reaction was always faster in *tert*-butyl alcohol. To our surprise, a reaction was also observed with 1.2 mg mL^{-1} of CaLB in [Et_3MeN][$MeSO_4$], which was seen to dissolve. To confirm this latter observation, a reaction was performed with a, presumably saturated, solution of CaLB in [Et_3MeN][$MeSO_4$]. On the basis of the conversion (Table 2), it would seem that [Et_3MeN][$MeSO_4$] dissolves approx. 3 mg mL^{-1} of CaLB.

Table 2 Transesterification of ethyl butanoate with CaLB in [Et_3MeN][$MeSO_4$][a]

	SP525/mg mL^{-1}, Conversion in %				
Solvent	1.2	3	6	20	Supernatant[b]
[Et_3MeN][$MeSO_4$]	14[c]	19	46	54	25
tert-Butyl alcohol	18	46	63	68	0

[a] Reaction conditions: ethyl butanoate (60 mM), 1-butanol (120 mM), SP525 in solvent (0.5 mL) at 40 °C for 24 h. [b] SP525 (20 mg mL^{-1}) was incubated in ionic liquid for 24 h at 40 °C and centrifuged; the transesterification was carried out in the supernatant. [c] All enzyme dissolved.

The inescapable conclusion from these results is that dissolution of CaLB in an ionic liquid is not incompatible with catalytic acitivity, in seeming contradiction with the lack of activity of CaLB in [EMIm][$EtSO_4$] (Table 1), which is presumably caused by unfavourable interactions of alkylsulfate anions with CaLB. Formate dehydrogenase and the β-glycosidase from *B. circulans*, in contrast, exhibit some tolerance for methylsulfate.[10] It would seem that the, only mildly H-bond accepting, $MeSO_4^-$ anion is a borderline case and that, perhaps, the H-bond acidity of the Et_3MeN^+ cation exerts a stabilising effect.

Structural changes of CaLB upon dissolution in ionic liquids

Conformational changes are an obvious explanation for the activity loss of CaLB upon dissolution in ionic liquids that we wished to confirm experimentally. While fluorescence spectroscopy and circular dichroism could not be used, because of low signal intensity and excessive background noise, infrared spectroscopy proved suitable to study the conformation of CaLB in ionic liquids.

Infrared spectroscopy is a well-established technique for conformational analysis of proteins.[11–14] Thanks to the possibility of analysing the sample in different physical states, FT-IR has been employed to monitor protein conformation in water, after freeze-drying (in the absence and in the presence of additives)[15–19] and

even after solubilising or suspending them in non-aqueous media.[14,20] The amide I mode of the peptide bond at approximately 1600–1700 cm^{-1} (mainly due to the C=O stretching vibration) is particularly relevant for protein analysis since it is conformationally sensitive. Indeed, this band consists of several overlapping components that can be assigned to different secondary structure elements. In order to identify such individual components and to monitor their variations in the different ionic liquids tested, we analyzed the second-derivative amide I spectra.[13,14,16,21]

In Fig. 2 the second-derivative infrared spectra of CaLB in several ionic liquids are compared with the spectrum of the protein in water, as being representative of native CaLB in a fully hydrated and dissolved state. However, since CaLB was added to the ionic liquid as a dried solid without any preceding dissolution in water, the dried solid sample was also analysed. It can be observed that the second-derivative spectrum of dried solid CaLB shows some differences compared to that of the protein in water. This result is in agreement with that of Vecchio *et al.*, who have shown that lyophilized CaLB has a different secondary structure pattern compared to the protein dissolved in water.[19] We found that the second derivative spectrum of the dried solid sample compared to the sample in water has a new component at around 1673 cm^{-1} (which could be assigned to non hydrogen bonded β-turns) and broader components at around 1619 cm^{-1} and 1690 cm^{-1}, which might be assigned to intermolecular hydrogen-bonded antiparallel β-sheets, which are typically observed with protein aggregates.[22] Nevertheless, in spite of such spectral variations it can be observed that the components at around 1640, 1649, 1656, 1660 (only as shoulder in the dried solid), 1667, and at 1681 cm^{-1} are present with CaLB in water as well as with the dried powder. The presence of these common components that could be assigned to, in the order, β-turns (with internally hydrogen-bonded carbonyls), random coil conformations, α-helix, β-turns (both the bands at 1660 and 1667 cm^{-1} could be assigned to the non-hydrogen-bonded carbonyls of β-turns)[23–26] and to β-sheets (this latter component was assigned in agreement with Mizutani *et al.*[27]), shows that CaLB in the dried state preserves, at least in part, the native conformation.

The spectra of CaLB in $[EtNH_3][NO_3]$, [BMIm][lactate] and $[BMIm][NO_3]$, in contrast, were much less similar to that in water. In particular, the complete absence of the bands above 1650 cm^{-1} in the case of CaLB in $[EtNH_3][NO_3]$ and the presence of only a peak at around 1658 cm^{-1} with CaLB in [BMIm][lactate] and $[BMIm][NO_3]$, instead of the two peaks at around 1656 and 1660 cm^{-1} observed with CaLB in water indicate a loss of α-helix and β-turns structures due to protein unfolding. The broader components below 1650 cm^{-1} in the spectra of CaLB dissolved in these ionic liquids compared with the enzyme in water, might be due to an increased presence of random coil structures and to hydrogen bonded carbonyls (*e.g.* in the case of $[EtNH_3][NO_3]$ hydrogen bonded to the NH_3 groups of the solvent).

A different trend was observed with CaLB in $[Et_3NMe][MeSO_4]$. In fact, in this case, especially in the region 1640–1670 cm^{-1}, the protein second derivative IR spectrum has an equal number of peaks and at the same wave numbers as those observed in the spectrum of CaLB in water. These data suggest that the enzyme preserves its secondary structure in this ionic liquid, even though the broad peaks at approx. 1624 cm^{-1} and 1690 cm^{-1} may indicate the presence of aggregates[22] that presumably result from incomplete dissolution.

Summarising, the FT-IR data indicate that the loss of activity in $[EtNH_3][NO_3]$, [BMIm][lactate] and $[BMIm][NO_3]$ is caused by changes of the secondary structure of the enzyme which appear much more profound than those caused by the freeze-drying process. In contrast, the similarity of the second-derivative infrared spectrum of CaLB in $[Et_3NMe][MeSO_4]$ with that in water,

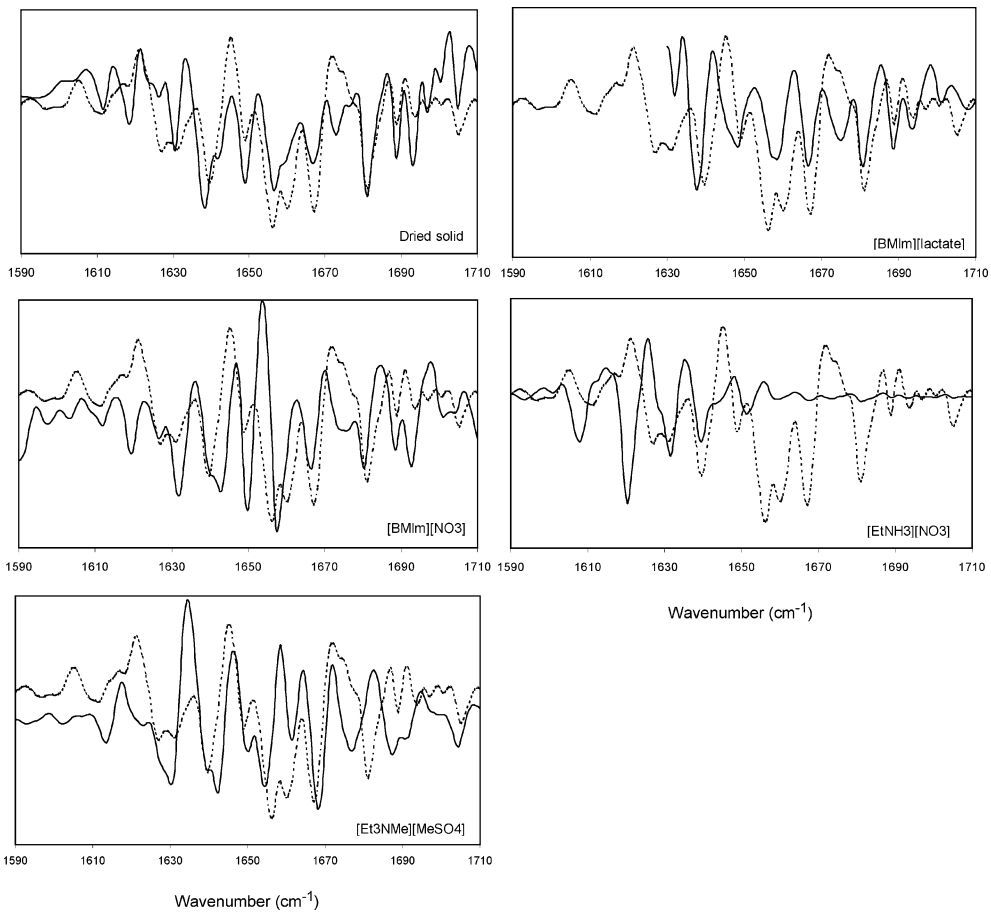


Fig. 2 Comparison of the second derivative spectra of CalB in aqueous solution (**---**) and in the dried state or in an ionic liquid. The spectrum of [BMIm][lactate] was truncated at 1630 cm^{-1} because of a very low signal to noise ratio at lower wave numbers.

especially in the region 1640–1670 cm^{-1}, indicates that the enzyme preserves its native structure in this ionic liquid, in agreement with the fact that it also preserves its catalytic activity.

Concluding remarks

It has become clear that ionic liquids with very similar structures differ greatly in their behaviour towards enzymes. The parameters that quantify interactions of proteins and aqueous electrolytes, such as cosmotropicity *vs.* chaotropicity[28] and the Jones–Dole B constant, do not seem particularly relevant for understanding enzymes in anhydrous ionic liquids. Consider, for example, CaLB which dissolved in [BMIm][NO_3] with loss of activity, but maintained its activity while suspended in [BMIm][BF_4] or [BMIm][PF_6]. These phenomena are not at all reflected in the Jones–Dole constants of the anions (NO_3^-: −0.043; BF_4^-: −0.093; PF_6^-: −0.21),[29] which classify these ions as chaotropic and protein-destabilising.

Loss of essential water is sometimes put forward as a possible cause of enzyme deactivation in anhydrous hydrophilic ionic liquids. This latter explanation seems unlikely in the case of CaLB, which stays active under strictly anhydrous conditions,[30] such as drying over phosphorus pentoxide.[31]

Hydrogen bonding could be the key to understanding the interactions of proteins and ionic liquids. Water is a powerful hydrogen bonding medium and an ionic liquid must mimic water in this respect to dissolve proteins, in particular as regards the hydrogen bond-accepting properties of the anion.[3] The interaction should not be too strong, however, because otherwise dissociation of the hydrogen bonds that maintain the structural integrity of the α-helices and β-sheets will cause the protein to unfold. The lactate anion, in particular, could easily form stable hydrogen bonds with the polypeptide backbone (see Fig. 3).

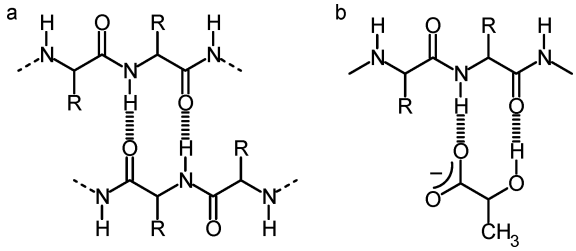


Fig. 3 (a) Schematic representation of hydrogen bonding in a β-sheet; (b) putative hydrogen bonds between a peptide chain and a lacate ion.

In conclusion, we suggest, on the basis of our results, that to maintain the activity of ionic liquid-dissolved enzymes, a balance of mild hydrogen bond-accepting and donating properties is required. Sterically demanding ions, which do not easily penetrate the protein matrix as this would require the dissociation of many intermolecular hydrogen bonds to create a few new ones, could also contribute to maintaining activity.

Experimental

Materials

Novozym 435 (immobilised *Candida antarctica* lipase B), SP525 (dry solid *Candida antarctica* lipase B) and purified CaLB lyophilisate were kindly donated by Novozymes. The ionic liquids [BMIm][lactate] and [EMIm][$EtSO_4$] were received from Prof. K.R. Seddon, Queen's University of Belfast, as a gift. [BMIm][BF_4] and [BMIm][PF_6] were synthesised according to standard procedures[32] and checked for the absence of chloride and acid.[33] Structural details of the ionic liquids used are listed in Table 3. All other compounds were purchased from ACROS.

Analytical methods

NMR spectra were measured on a Varian Unity Inova-300 instrument.

The transesterification of ethyl butyrate was monitored by HPLC on a Waters custom-packed 8 × 100 mm 5 μm Symmetry C_{18} column; eluent 65 : 35 (v/v) MeOH–aqueous 0.05 M acetate buffer pH 4.5 at 1.0 mL min^{-1} with refractive index detection (Shodex SE-51 RI).

Synthesis of ionic liquids

[BMIm][NO_3][34]. To a stirred and ice-cooled aqueous solution (50 mL) of 1-butyl-3-methylimidazolium chloride (8.73 g, 0.046 mol) an aqueous solution (30 mL) of silver nitrate (8.48 g, 0.05 mol) was added dropwise. The solution was slowly brought to room temperature and the white precipitate was removed by filtration. The filtrate was concentrated *in vacuo*, yield 9.3 g (93%).

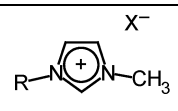
^{1}H NMR ($CDCl_3$): δ = 0.9 (t, 3H, CH_2CH_3), 1.3 (m, 2H, $CH_2CH_2CH_3$), 1.9 (m, 2H, $CH_2CH_2CH_2$), 4.0 (s, 3H, NCH_3), 4.2 (t, 2H, NCH_2), 7.5 (d, 2H, $HC=CH$), 9.7 (s, 1H, CH).

[$EtNH_3$][NO_3][35]. Ethylamine (26.4 g, 70% aqueous solution) was slowly added to nitric acid (18.1 g, 65% aqueous solution) while stirring and cooling in ice-ethanol to maintain a temperature of less than −5 °C. Water and excess ethylamine were evaporated; the residue was further dried *in vacuo* over P_2O_5. The resulting ethylammonium nitrate (20.3 g, 46%) was anhydrous according to ^{1}H NMR.

^{1}H NMR (DMSO-d_6): δ = 1.1 (t, 3H, CH_3), 2.9 (q, 2H, CH_2), 7.7 (s, 3H, H_2C-NH_3).

[Et_3NMe][$MeSO_4$][36]. Dimethyl sulfate (20 mL, 0.211 mol) was added dropwise to a solution of triethylamine (27.5 mL, 0.211 mol) in toluene (100 mL), which was cooled in an ice-bath under nitrogen, at such a rate that a temperature below 0° C could be maintained (the reaction is highly exothermic). The formation of the ionic liquid was immediate and caused the initially clear solution to become opaque, followed by the separation of the denser

Table 3 Structures of ionic liquids



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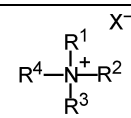

2

Abbreviation	Type	R				X⁻
[BMIm][BF_4]	**1**	n-C_4H_9				BF_4^-
[BMIm][PF_6]	**1**	n-C_4H_9				PF_6^-
[BMIm][NO_3]	**1**	n-C_4H_9				NO_3^-
[BMIm][lactate]	**1**	n-C_4H_9				$CH_3CH(OH)COO^-$
[EMIm][$EtSO_4$]	**1**	C_2H_5				$C_2H_5OSO_3^-$
		R^1	R^2	R^3	R^4	
[Et_3MeN][$MeSO_4$]	**2**	CH_3	C_2H_5	C_2H_5	C_2H_5	$CH_3OSO_3^-$
[$EtNH_3$][NO_3]	**2**	C_2H_5	H	H	H	NO_3^-

ionic liquid phase from the toluene solution. When the addition of dimethylsulfate was complete, the reaction mixture was stirred at room temperature for 1 h. The upper, organic, phase was decanted and the lower, ionic liquid, phase was washed with toluene (50 mL) and dried by heating *in vacuo* to remove residual organic solvents. $[Et_3MeN][MeSO_4]$ was obtained as a colorless ionic liquid; yield: 37.8 g (79%).

^{1}H-NMR (DMSO-d_6): δ = 1.18 (m, 9H, CH_2CH_3); 2.87 (s, 3H, OCH_3); 3.25 (m, 6H, CH_2CH_3); 3.37 (s, 3H, N-CH_3).

Transesterification of ethyl butyrate with butanol

The transesterification of ethyl butyrate (0.06 M) with *n*-butanol (0.12 M) was carried out in 0.5 mL of ionic liquid as the solvent. Novozym 435 (15 mg) and 1,3-dimethoxybenzene (internal standard, 0.06 M) were added and the reaction was carried out at 40 °C. The reactions were monitored for 24 hours by taking 50 μL samples that were diluted with 100 μL of HPLC eluent.

Solubility of CaLB

SP525 (15 mg) was added to 500 μL of ionic liquid and was incubated for 24 hours at 40 °C. The supernatant was separated from the solid enzyme by filtration followed by centrifugation. The lipase activity in the supernatant was assayed in triacetin hydrolysis.

Activity test of CaLB

A 0.2 mL sample of ionic liquid supernatant was diluted with 10 mL of 0.1 M sodium phosphate buffer pH 7.5. Triacetin (0.1 M) was added and the reaction was monitored by titration with 0.118 M KOH.

Fourier Transform Infrared (FT-IR) measurements

FT-IR spectra were recorded using a Jasco 610 instrument equipped with a DTGS detector as the average of 32 scans at 2 cm^{-1} resolution. The system was managed by Jasco software. Interference of water vapour and CO_2 was avoided by purging the sample and detector compartments with N_2, at a flow of 6 L min^{-1}.

Solutions of pure CaLB in water (30 mg mL^{-1}) or in ionic liquids (10 mg mL^{-1}) were loaded in a demountable CaF_2 cell (Hellma) with optical path length of 6-μ or 12-μ (in the case of water) for FT-IR measurements. A spectrum of purified dried solid CaLB was measured by mixing a 1 mg sample with 200 mg of KBr in a mortar; the resulting mixture was pressed into a pellet using a Perkin-Elmer kit and a 10 ton hydraulic press. It has been previously shown that the use of KBr pellets does not affect the protein integrity.[16] The spectra were corrected by subtraction of the background spectrum (water, ionic liquid, KBr blank as appropriate).

The original protein spectra were smoothed with a 9-point Savitsky–Golay method to remove white noise. Second-derivative spectra were obtained with the Savitsky–Golay algorithm for a 9 data point window with Spectra Analysis software (Jasco). The spectra of CaLB obtained as dried powder or in a given ionic liquid were normalized for area and overlaid for comparison.[14]

Acknowledgements

Generous gifts of ionic liquids by Prof. K. R. Seddon, Queen's University of Belfast, are gratefully acknowledged. The authors wish to thank Novozymes (Bagsvaerd, Denmark) for gifts of Novozym 435 and SP525, as well as a purified sample of *C. antartica* lipase B.

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PAPER

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Solid-to-solid biocatalysis: thermodynamic feasibility and energy efficiency

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Enzymes can catalyse solid-to-solid condensation reactions in highly concentrated aqueous substrate suspensions. Reaction products precipitate from the reaction mixture and very high conversion yields can be obtained in low volume reactors. Solid-to-solid biocatalysis combines the advantages of using enzymes in aqueous media with the high conversion yields that are typically associated with non-aqueous biocatalysis. In this article, methods are presented for the calculation of the Gibbs free energy changes and heats of reaction of condensation reactions to form amides. The overall enthalpy change of the enzymatic reaction was compared to that of the conventional chemical methods and it was found that the enzymatic reaction produces a third of the heat with better atom efficiency.

1. Introduction

A common aim in green chemistry is to reduce or eliminate the use of environmentally unfriendly or toxic solvents. Usually this is addressed by replacing them with less harmful solvents, such as water. A more fundamental approach is to ask why we need a solvent at all, or at least why we want to dissolve all reactants completely. One reason for dissolving reactants is to allow for reactant diffusion to and from the site of reaction. It may however be sufficient to have only a small fraction of reactants in solution at any one time. A second reason for using a liquid solution as reaction mixture is to allow for good heat transfer to prevent exothermic reactions from heating up. However, in order for a process to be labelled as 'green', highly exothermic reactions should be avoided when possible. In many cases it may only be tradition that leads to the search for a solvent to dissolve all the reactants. A greener (and more effective) process may be possible by using only small amounts of solvent, so that most of the reactants remain as undissolved solids in 'solid-to-solid' or 'precipitation driven' reactions (Fig. 1).[1–3]

In solid-to-solid reaction systems only a small proportion of the reaction mixture will be in solution at any one time. Starting materials will dissolve progressively, passing fairly briefly through solution as they are converted to products, which then precipitate and drive the reaction to high yields of solid reaction product.

The idea of exploiting product precipitation to drive a reaction in a desired direction is much older than commonly thought. The method was applied to protease-catalysed peptide synthesis as early as the 1930's. More recently systems have been studied in which most of the starting materials are initially also in the solid phase, progressively dissolving as the conversion proceeds. Examples of successful solid-to-solid reactions included the enzymatic synthesis of amides, esters and glycosides.[1–3] Precipitation driven reactions are also applied commercially, for example in the production of the synthetic sweetener aspartame (Asp–Phe–OMe). There, the reaction product of a protease catalysed coupling (Z–Asp–Phe–OMe) forms a poorly soluble salt with the D-enantiomer of the racemic Phe–OMe starting material.[4] Most examples of solid-to-solid reactions that have been described involve enzymatic conversions (biocatalysis) that require a small liquid phase where the enzymatic

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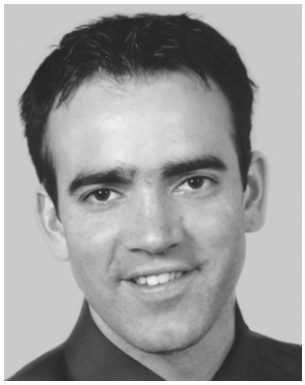

Peter Halling was born in England in 1951. He received a PhD in Biochemistry from Bristol University (UK) in 1975. He joined Unilever Research as a scientist in 1978, and the University of Strathclyde in 1983, where since 1996 he has been Robertson Professor of Bioprocess Technology. His current research interests are fundamental questions related to the use of enzymes or whole cells as industrial catalysts, especially on their action in low water reaction mixtures, and the biophysical chemistry of their behaviour. His work often involves collaborations at the triple interface of chemistry, bioscience and process engineering.

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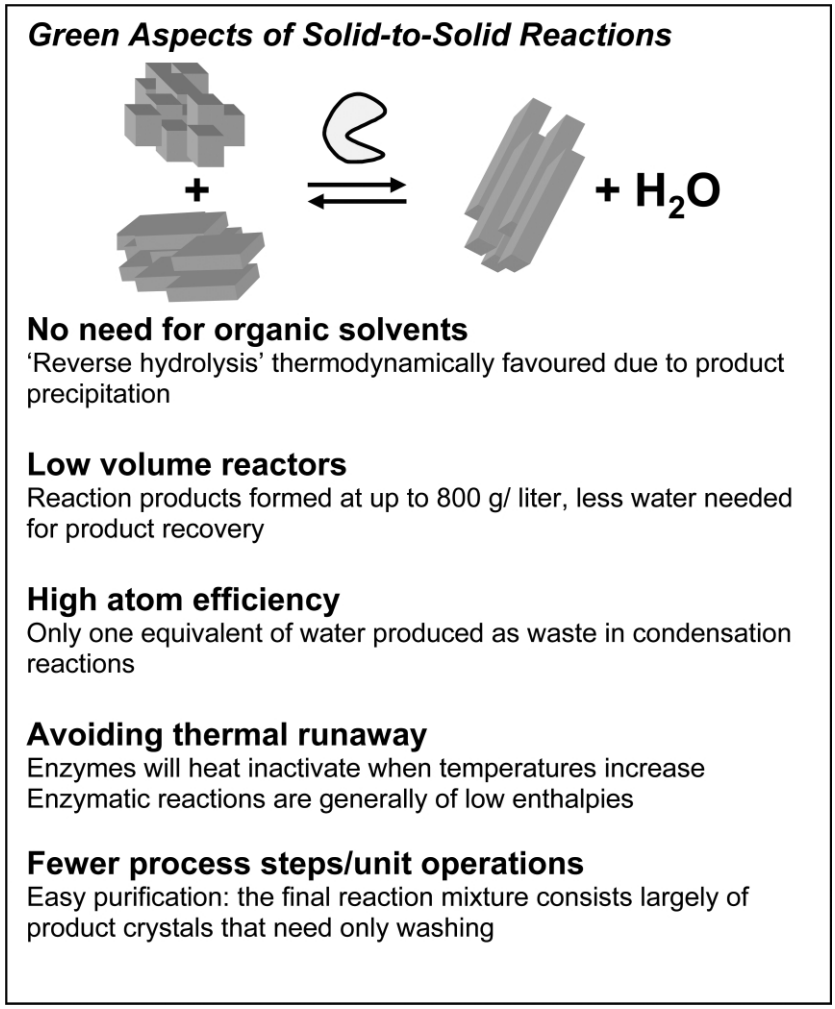

Fig. 1 Green aspects of solid-to-solid biocatalysis.

reaction takes place. Non-enzymatic solid-to-solid (or solvent free) reactions are also increasingly studied as green alternatives to conventional organic solvent systems.[5]

In the first part of this article we will discuss methods to predict the Gibbs free energy changes related to the solid-to-solid synthesis of amides from free acids and primary amines. Using these methods, the thermodynamic feasibility of the solid-to-solid synthesis of a target compound can be predicted. Examples are also included of reactions where one or more of the reactants are present as salts. In the second section, reactions involving zwitterions are discussed. It is demonstrated how zwitterions can be exploited to favour solid-to-solid synthesis by using charged protecting groups. In the third section, the energy efficiency of solid-to-solid reactions are calculated and compared to that of conventional methods of peptide synthesis.

2. Theoretical model, results and discussion

Calculation of Gibbs free energy of solid to solid reactions

The most extensively studied reaction type in enzymatic solid-to-solid synthesis is that of amide formation, usually the coupling of an N-protected amino acid to a C-protected amino acid catalysed by a protease [eqn. (1)].

$$\text{R–COOH (s)} + \text{H}_2\text{N–R}' \text{ (s)} \leftrightarrows \text{R–CONH–R}' \text{ (s)} + \text{H}_2\text{O} \quad (1)$$

Product precipitation during such a reaction is only expected when the equilibrium concentration of the reaction product (R–CONH–R′) is higher in value that its solubility, so that precipitation occurs before equilibrium is reached. With solid starting materials present, the reaction will then necessarily continue until one or both reactants (R–COOH and/or H_2N–R′) completely dissolve and as a consequence very high yields are observed.[1] By contrast, if product precipitation is not favoured the reaction product will only be formed in the low volume liquid phase, leading to very low yields. Because of these two possible situations, the thermodynamics of solid-to-solid reactions have been named 'switch-like'. It is clearly useful to be able to predict whether a particular solid-to-solid conversion is energetically feasible (thermodynamically favourable) or not. This would avoid wasting time on fruitless experimental study. Such a prediction may be made using the thermodynamic cycle shown in Fig 2 that allows for the estimation of the overall feasibility of the conversion. This thermodynamic cycle makes use of an established approach for the prediction of aqueous solubilities based on the near ideal solubility of most bio-organic compounds in octanol and partitioning between octanol and water (log *P*) as described by Yalkowsky *et al.*,[6] and a consensus value for the equilibrium constant in aqueous solution.[7–9] Equations may be written in terms of solubility ratios, as previously presented.[10] An alternative equivalent presentation will be given here, that allows for the direct calculation of Gibbs free energy contributions. As Fig. 2 indicates, the overall $\Delta G_{\text{solid-to-solid}}$ can be calculated as the sum of 4 contributions:

1. Fusion of starting materials (S1) and solidification of the product (P1). We require the $\Delta\Delta G_{\text{fusion}}$; that is the difference between values for starting materials and products of ΔG_{fusion} of the solid to a supercooled liquid at reaction temperature.

2. Mixing (S2) and de-mixing (P2) of these supercooled liquids with *n*-octanol. The required contribution is $\Delta\Delta G_{\text{mixing}}$; again a difference in mixing values between starting materials and products.

3. Partitioning of starting materials (S3) and products (P3) between *n*-octanol and water. We require $\Delta\Delta G_{\text{partitioning}}$, the

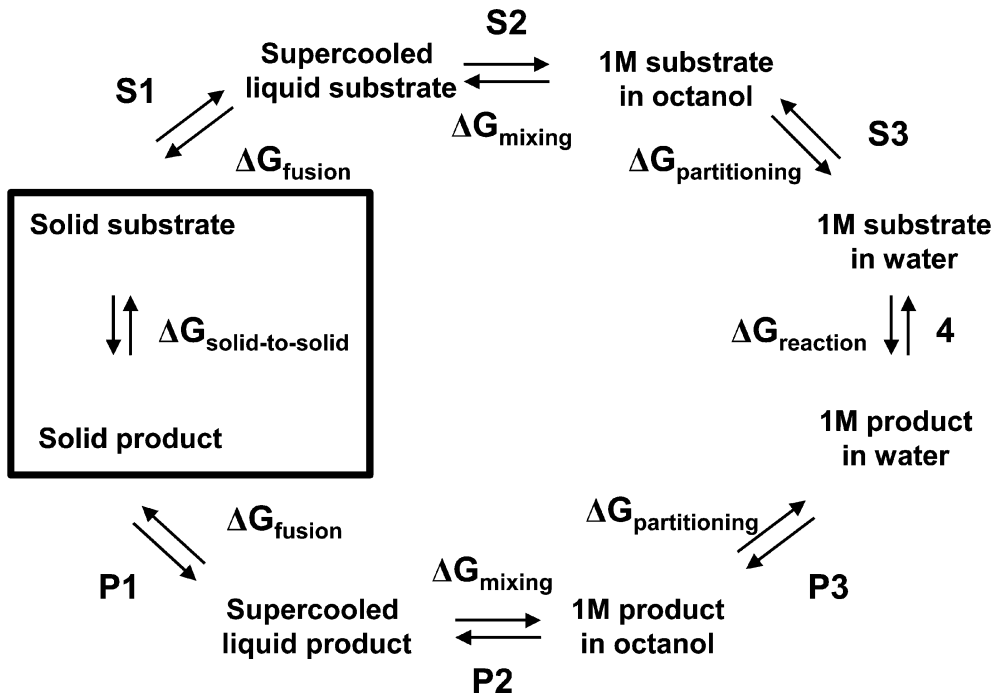

Fig. 2 Thermodynamic cycle to predict the free energy change of a solid-to-solid conversion based on the reaction in aqueous solution.

difference between octanol–water partitioning values for starting materials and products.

4. The $\Delta G_{\text{reaction}}$ in water (4), for the actual conversion of starting materials to products.

Step 1: fusion and solidification. For the melting of the solid at the reaction temperature to form a supercooled liquid we can write:

$$\Delta G_{\text{fusion}} = \Delta S_{\text{fusion}}(T_{\text{m}} - T) \quad (2)$$

where T_{m} is the melting point and T the reaction temperature. The expression makes the assumption that both ΔH_{fusion} and ΔS_{fusion} are temperature independent (*i.e.* there is no specific heat change on fusion). This assumption is not exactly correct, but often a good approximation. In turn ΔS_{fusion} can be estimated from:[11]

$$\Delta S_{\text{fusion}} = 50 \text{ J mol}^{-1} \text{ K}^{-1} + R1\text{n } 2.85^{\tau} \quad (3)$$

where R is 8.314 J mol^{-1} K^{-1} and τ is the number of conformationally significant torsion angles in the molecule. Evaluation of τ in the molecular structure involves a count of sp3, sp2 hybridised atoms and ring structures in the molecule according to:

$$\tau = \text{sp3} + 0.5\text{sp2} + 0.5\text{ring} - 1 \quad (4)$$

Rules for the calculation of τ were presented previously by Ulijn *et al.*[10] and Dannenfelser and Yalkowsky.[11] $\Delta\Delta G_{\text{fusion}}$ is then calculated as the difference between ΔG_{fusion} values for the starting material(s) and product(s). In the case of amide synthesis, this will consist of an acid and amine component, minus the amide product.

Step 2: mixing and de-mixing. For mixing with *n*-octanol, it is found that most organic liquids mix approximately ideally with this solvent.[6] In step 4 a constant value for the $\Delta G_{\text{reaction}}$ is used that corresponds to the reaction with all reactants present at 1 M concentrations. Hence, we need a value for ΔG_{mixing} to give a 1 M solution, given by

$$\Delta G_{\text{mixing}} = RT1\text{n } V_{\text{oct}} \quad (5)$$

where V_{oct} is the molar volume of octanol (0.15 L mol^{-1}). The ΔG_{mixing} will be ideal and therefore the same for all species involved. So for the overall amide synthesis reaction, where we need to allow for 2 starting materials and 1 product, we get:

$$\Delta\Delta G_{\text{mixing}} = RT1\text{n } V_{\text{oct}} = -4.6 \text{ kJ mol}^{-1} \text{ at } 25\ ^{\circ}\text{C} \quad (6)$$

Because of the similarity of the starting materials and products, effects due to non-ideality will usually partly cancel, so that $\Delta\Delta G_{\text{mixing}}$ will be better predicted than individual values of ΔG_{mixing}.

Step 3: partitioning. Octanol–water partition coefficients have been measured for very many compounds, and there are also rather good systems for their prediction from molecular structures.[6]

$$\Delta G_{\text{partitioning}} = RT1\text{n } P = 2.303RT\log P \quad (7)$$

where log P is for the partitioning of a molecule from water into octanol. Log P values are usually very well described as a sum of contributions from groups within the molecular structure. Due to molecular similarities between substrates and product (they are identical except for a carboxylic acid and primary amine that are converted into an amide) most contributions will cancel in calculating $\Delta\Delta G_{\text{partitioning}}$, leaving only those groups actually transformed in the reaction. In the case of amide synthesis, the contributions of the amino and carboxyl groups, minus that of the amide, give:

$$\Delta\Delta G_{\text{partitioning}} = 2.303RT\Delta\log P = +0.5 \text{ kJ mol}^{-1} \text{ at } 25\ ^{\circ}\text{C} \quad (8)$$

For most ionisable compounds, the log P values will be based on the neutral form in water, which is what is required to combine with the $\Delta G_{\text{reaction}}$. For zwitterions the situation is more complicated since some zwitterions partly ionise in octanol. Water–octanol partitioning of zwitterions was reviewed by Pagliara *et al.*[12] and it appears that a generally valid quantitative description of water–octanol partitioning of zwitterions does not exist. In the next section some semi-quantitative observations illustrate the effects of zwitterions on the thermodynamic equilibrium position.

Step 4: reaction. For the synthesis/hydrolysis reaction of amide bonds, it was found that a single equilibrium constant may be used to describe most reactions accurately. When the aqueous equilibrium constant is expressed in terms of concentrations of the unionised forms of the reactants, a constant value was observed regardless of the molecular form of the reactants. Using the

convention of taking the water thermodynamic activity as 1, the reference equilibrium constant is found to be $10^{3.6}$ M^{-1}.[7–9] This corresponds to a $\Delta G_{reaction}$ given by:

$$\Delta G_{reaction} = -RT1n\ K_{eq} = -20.5\ kJ\ mol^{-1}\ at\ 25\ °C \quad (9)$$

This ΔG corresponds to the reaction with all reactants present at 1 M concentration in water (strictly the hypothetical state where they are all still showing infinite dilution behaviour).

Steps 2 to 4 overall. As explained above, constant free energy contributions are expected for steps 2–4 for all amide synthesis reactions, giving a total $\Delta\Delta G$ contribution for mixing, partitioning and reaction of $-20.5 - 4.6 + 0.5 = -24.6$ kJ mol^{-1} at 25 °C. This must be added to $\Delta\Delta G_{fusion}$ to give an overall $\Delta G_{solid\text{-}to\text{-}solid}$:

$$\Delta G_{solid\text{-}to\text{-}solid} = -24.6 + \Delta\Delta G_{fusion}\ kJ\ mol^{-1}\ at\ 25\ °C \quad (10)$$

A negative value $\Delta G_{solid\text{-}to\text{-}solid}$ naturally indicates a favourable solid-to-solid conversion. As seen, crystal energies are the determining factor in any particular synthesis, given the fairly constant contribution from reaction (step 4). Steps 2 and 3 are needed only because the best data for reaction energetics are available for aqueous solution.

Fig. 3 shows the $\Delta G_{solid\text{-}to\text{-}solid}$ values calculated for 28 different amide forming reactions taken from the literature. It is immediately evident that reactions 9, 10, 27, 28 were not thermodynamically feasible ($\Delta G_{solid\text{-}to\text{-}solid} > 0$) while all others are. Reported experiments confirmed this outcome (see reference[10] for references to experiments). Most $\Delta G_{solid\text{-}to\text{-}solid}$ values were between −15 and −45 kJ mol^{-1}; highly favourable. Solid-to-solid reactions 25 and 26 (the synthesis reactions of acidic beta lactam antibiotics) were predicted to be thermodynamically favoured by values of around −10 kJ mol^{-1}. For these, product precipitation was not observed experimentally. It is not clear whether this was related to the accuracy of the prediction or to the experimental conditions used. One of the substrates was not present at its solubility limit in these experiments, which may have affected the outcome.[10] Of course it is always possible that a conversion that is thermodynamically favoured will not be observed because of kinetic limitations. A clear demonstration of unfavourable thermodynamics would be the observation of the reverse solid-to-solid conversion, here amide hydrolysis. In summary, the free energy change of solid-to-solid synthesis of amides can be accurately predicted from reactant's melting points.

It should be noted that if melting points are not available, theoretical predictions of crystal energies for organic molecules remain difficult. Where melting points cannot be measured because of decomposition, solubility measurements would be a basis for predictions by a method analogous to that presented here.

Solid-to-solid reactions involving salts

The organic molecules that are used as reactants in solid-to-solid conversions commonly have acidic or basic groups. Hence, in the solid form they may form salts with suitable counter-ions. Two types of salts have been used: those of charged reactants with inorganic counterions (*e.g.* eqn. 11) and those where oppositely charged reactants such as acids and bases form an 'inter-substrate' salt by proton exchange (eqn. 12).

$$R\text{–}COOH\ (s) + H_2N\text{–}R'\cdot HCl\ (s) \leftrightarrows R\text{–}CONH\text{–}R'.HCl\ (s) + H_2O \quad (11)$$

$$(R\text{–}COO^-)(^+H_3N\text{–}R')\ (s) \leftrightarrows R\text{–}CONH\text{–}R'\ (s) + H_2O \quad (12)$$

As an example of the first type of salt in peptide synthesis, it is particularly common to find hydrochlorides of basic groups in the reactants such as the side chains of Arg or Lys. In synthesis reactions, it is convenient to supply such reactants directly as solid hydrochloride salts. There are some reports of successful solid-to-solid enzymatic reactions using hydrochlorides of arginine derivatives.[14] Erbeldinger *et al* used solid Phe–OMe·HCl together with a solid base (for example $NaHCO_3$) and the free di-acid Z–Asp. The solid reaction product of the enzyme catalysed synthesis reaction was thought to be the Na salt of Z–Asp–Phe–OMe.[15]

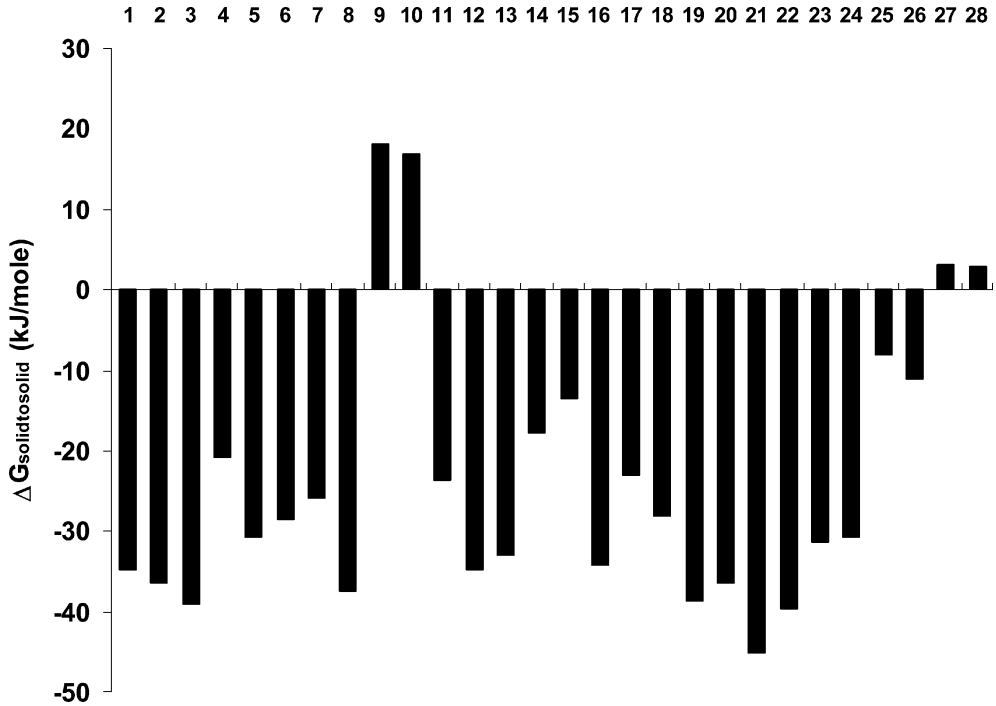

Fig. 3 Free energy change of solid-to-solid reactions calculated using eqns. 1–10. Values for variables were taken from Ulijn *et al*.[10] Reactions are synthesis of peptide bond in (1) Z–Ala–Leu–NH_2, (2) Z–Ala–Phe–NH_2, (3) Z–Ala–Phe–Leu–NH_2, from Z–Ala, (4) Fmoc–Asp–Phe–OMe, (5) Z–Asp–Leu–NH_2, (6) Z–Asp–Phe–NH_2, (7) Z–Asp–Phe–OMe, (8) Z–Gln–Leu–NH_2, (9) Bz–Gly–Tyr, (10) Bz–Gly–Phe, (11) Z–Gly–Leu–NH_2, (12) Z–Gly–Phe–Leu–NH_2, from Z–Gly (13) Z–Gly–Phe–OMe, (14) Z–His–Leu–NH_2, (15) Z–His–Phe–NH_2, (16) Z–Phe–Leu–NH_2, (17) Z–Phe–Val–NH_2, (18) For–Phe–Leu–NH_2, (19) Z–Phe–Ala–NH_2, (20) Z–Phe–Leu–NH_2, (21) Z–Phe–Met–NH_2, (22) Z–Phe–Phe–NH_2, (23) Z–Ser–Leu–NH_2, (24) Z–Ser–Phe–NH_2, (25) mandelic acid coupled to 7-ADCA, (26) phenyl acetic acid coupled to 7-ADCA, synthesis of amide bond in (27) Cephalexin, (28) Amoxicillin. All reactions are referenced elsewhere.[10]

The second type of solid salt starting material in solid-to-solid peptide synthesis is produced by proton exchange between the free amino and carboxyl groups of substrates that are to be coupled in the reaction. These inter-substrate salts have been observed for the substrate couple Z–Gln/Leu–NH_2 by Erbeldinger *et al*[16] and were also thought to be formed between the couple Z–Phe/Leu–NH_2[17] and may feature more commonly.

We were interested to find out whether the approach to $\Delta G_{\text{solid-to-solid}}$ prediction described above can be extended to the case of salt reactants and/or products. As explained above the key data that would be required for such a prediction are the melting points of the solid starting materials and products. Many hydrochlorides and ammonium carboxylates are reported to have well defined and reversible melting points at moderate temperatures (many examples appear in the Beilstein database). The melts of these salts are most likely ionic in nature, so they are examples of ionic liquids and require a different treatment from the uncharged melts that are expected for non-salt starting materials. Appendix A describes the extended thermodynamic cycle that can be used for the feasibility predictions of solid-to-solid reactions involving a single salt starting material and product with inorganic counterions (eqn. 11). Appendix B describes the equations for the reaction involving an inter-substrate salt (eqn. 12).

It was concluded that the original thermodynamic cycle (Fig. 2) can still be used when salts are involved in the reaction. For reactions where both one of the substrates and the product are in the form of salts with inorganic counterions, eqn. 10 still holds. ΔS_{fusion} values are somewhat lower for salts than for neutral molecules by 20–30 J mol^{-1} K^{-1}, which means that the overall $\Delta G_{\text{solid-to-solid}}$ may be slightly more negative (more favourable).

Reactions that start from inter-substrate salts may be less favourable than reactions starting from separate solid substrates. With the addition of an extra free energy contribution for the transfer of the molten salt to the two pure uncharged liquids, the thermodynamic cycle described above can still be used, provided that a melting point for the inter-substrate salt is known. For the reaction that produces Z–Gln–Leu–NH_2 it was found that the solid-to-solid conversion remained favourable when the starting materials form an inter-substrate salt, as observed by Erbeldinger *et al.*,[16] although less so than starting from the separate solids by 13 kJ mol^{-1}. The magnitude of this difference is in line with free energy changes found for several solid-to-solid reactions (Fig. 3). There is therefore a clear indication that in cases where the solid-to-solid reaction that starts from separate substrates is energetically less favourable, salt formation may be enough to prevent a favourable solid-to-solid conversion.

3. Using charged protecting groups to tune reactant solubilities: the special case of zwitterions

Zwitterions form a special case of reactants, because their ionisation behaviour is significantly different from that of simple weak acids, bases, or uncharged compounds. While the latter three are predominantly present as the uncharged species when dissolved in pure water, many zwitterions will be predominantly present in the zwitterionic form, and the concentration of the neutral species will only be a minor fraction. Log *P* partitioning of zwitterions was reviewed by Pagliara *et al.*[12] Zwitterions with pK_a values that are only 1–2 units apart, such as nicotinic and isonicotinic acids with ΔpK values of 2.1 and 1, exist exclusively in the uncharged form in octanol. By contrast, for zwitterions with much larger differences in p*K* values, such as alpha amino acids and peptides with ΔpK values between 4 and 6, it has been observed that the zwitterionic form is also significantly present in octanol. This is probably related to the possibility of forming intramolecular ion pairs, thus bringing opposite charges closer together and shielding them from the octanol environment. As discussed before,[10] the formation of zwitterionic products in solid-to-solid reactions is thermodynamically favoured, while the removal of zwitterionic substrates is unfavoured. This can be understood as follows. In terms of the thermodynamic cycle presented above (Fig. 2), the ionised form of zwitterions in the melt and octanol means that an additional $\Delta G_{\text{ionisation}}$ needs to be introduced to change from the ionised to the uncharged zwitterionic species. Neutralisation of zwitterions is thermodynamically unfavoured and a positive $\Delta G_{\text{ionisation}}$ contribution can therefore be expected for neutralisation of zwitterionic compounds while a negative contribution results from the ionisation of uncharged zwitterions. We have recently attempted to generate a generally valid quantitative treatment of the water–octanol partitioning of zwitterions but have not managed to obtain a satisfactory model yet (data and model are available upon request from the authors). However, a general qualitative picture can help in experimental design of successful solid-to-solid reactions.

Due to the unfavourable effects of zwitterions as substrates and the favourable effects of a zwitterionic reaction product, the most favourable situation would be to couple a di-acid and a di-base to form a zwitterion. By choosing appropriate charged protecting groups these favourable conditions can be met.

A number of protease catalysed peptide syntheses reactions with charged protecting groups have been carried out previously by the Wandrey group.[18] The aim of their work was to improve solution yields of enzyme reactions in aqueous media by using solubility enhancing charged protecting groups. In some cases, it was observed that the reaction products precipitated from the reaction mixture. We analysed these reactions in more detail and found that zwitterions were produced in each case. Although we cannot be absolutely certain that the precipitation of the reaction products reflected the equilibrium position, because the reactions were carried out under kinetic rather than thermodynamic control, it is remarkable that precipitation was observed in cases where the overall reaction involved formation of a zwitterion (Table 1, entries 1, 6). For entry 3 the expected zwitterionic di-peptide was not formed which appears to be related to the enzyme specificity rather than the thermodynamics of the reaction. In cases where no zwitterion was formed in the overall reaction, precipitation was not observed (entries 2, 4, 5, 7). Clearly, charged protecting groups can be used to create conditions where solid-to-solid reactions are favoured.

Table 1 Overall formation of zwitterions leads to product precipitation in enzymatic synthesis of di-peptides from di-acids or di-bases[18]

Entry	R–COOH[a]	H_2N–R′	Precipitation?	Overall zwitterion formed?	Yield
1	Mal–Tyr	Lys–OEt·2HCl	Yes	Yes	81%
2	Mal–Tyr	Ala–Gln	No	No	71.5%
3	Mal–Tyr	Orn–OEt·2HCl	No[c]	Yes	45–50% mixture
4	Mal–Tyr	Cit–OEt	No	No	Not stated
5	Mal–Tyr–Lys	Ala–OMe	Not stated	No	Not stated
6	Mal–Tyr	Arg–OEt	Yes	Yes	75%[b]
7	Mal–Tyr	Ala–$O(CH_2)_2$–OSO_3^-	No	No	Not stated

[a] Ethyl esters of these acids were used as acyl donors. [b] Filtration of precipitate and washing with water gave 75% yield. [c] Both Mal–Tyr–Orn and Orn delta –lactam cyclization product were observed.

4. Enthalpy changes and atom economy in solid-to-solid transformations

One reason why reactions are mostly carried out in solution rather than suspension is to help transfer heat formed during exothermic reactions. In the unstirred, mainly solid reacting mixture that is typical for solid-to-solid reactions, heat transfer may become a limitation. Because only small pockets of liquid are present throughout the mixture, natural convection may not make a substantial contribution. Conduction is then the main heat transfer mechanism, which is expected to be slow.

Furthermore, the high reactant concentrations typical for the (low volume) liquid phase present in solid-to-solid reactions can be expected to lead to higher rates of heat production (or consumption) per unit volume. Most reactions will be exothermic, and a particular concern in the use of mainly solid reaction systems would be the possibility of thermal runaway. Heat evolved in the reaction cannot be dissipated fast enough and the system temperature rises as a result. Increased temperature can in turn lead to increased reaction rates with the eventual risk of explosion (*e.g.* from vaporisation of one component). This possibility can usually be dismissed as a hazard in enzymatic reactions, because excessive temperature rise will lead to rapid inactivation of the catalyst and hence termination of the reaction—essentially the system is fail safe. Of course, even such a controlled thermal runaway is not wanted—valuable enzyme will have been lost, and probably the batch will become waste because of undesirable side reactions promoted by high temperature.

However, another feature of enzymatic reactions makes any problem typically less likely than with normal chemical conversions. Many of the reactions for which enzymatic catalysis is used have relatively low enthalpy changes, so there is less requirement for heat transfer. For example, the solid-to-solid condensation of a range of amide coupling reactions (Fig. 4) have estimated $\Delta H_{\text{solid-to-solid}}$ values between −20 and −40 kJ mol^{-1} (for methods of estimation see Appendix C). The corresponding chemical coupling reaction, using a carbodiimide, is estimated to have a ΔH value of about −140 kJ mol^{-1}; over three times as large. The relatively modest heat evolution in the enzymatic reaction should not be too difficult to remove. Indeed, Erbeldinger *et al.*[19] studied solid-to-solid synthesis of Z–Asp–Phe–OMe in a 1 kg unstirred reacting mass, and found no evidence of temperature rise in the centre of the reaction mixture.

This comparison also illustrates a rather general green feature of enzymatic processes. The enzymatic peptide synthesis operates with high atom economy—only elements of water are lost—in a direct condensation that is quite near equilibrium (although proceeds to very high yield under solid-to-solid conditions). In the chemical alternative a substantial amount of co-reagent is consumed stoichiometrically in order to give a conversion of much larger ΔH and ΔG. At best the side product will be recycled with considerable energy consumption, at worst it will be waste. Note that the direct solid-to-solid condensation would be equally thermodynamically favourable if performed as a non-enzymatic reaction. Hence the reason for employing a co-reagent carbodiimide is essentially kinetic, even though the synthetic chemist would tend to describe the purpose as driving the reaction towards synthesis.

Conclusions

In solid-to-solid reactions enzymes are used for condensation reactions in highly concentrated aqueous substrate suspensions. Reaction products precipitate from the reaction mixture and very high conversion yields can be obtained. The Gibbs free energies and reaction enthalpies of these reactions can be predicted from melting points for reactions where starting materials are simple acids or bases or salts such as hydrochlorides or inter-substrate salts. It should be noted that the methods presented here allow for the prediction of the thermodynamic direction of these reactions, but they don't necessarily indicate the practical feasibility, which also depends on factors such as the identification of a suitable catalyst and reaction kinetics.

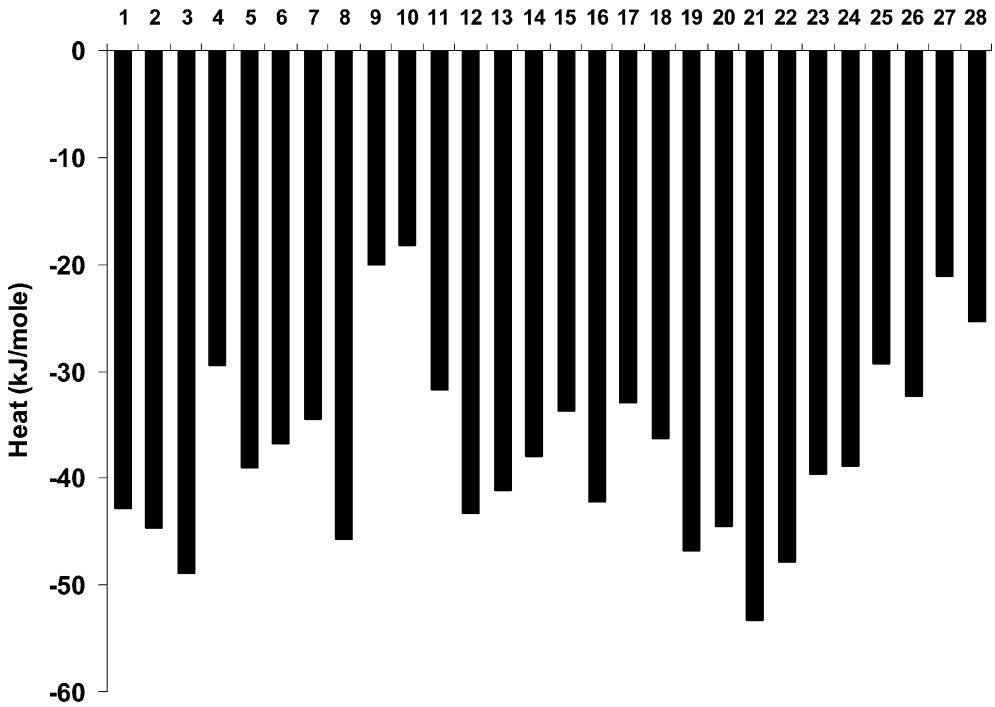

Fig. 4 Enthalpy change of solid-to-solid reactions. Values for variables taken from Ulijn *et al.*[10] Reactions are synthesis of peptide bonds in (1) Z–Ala–Leu–NH_2, (2) Z–Ala–Phe–NH_2, (3) Z–Ala–Phe–Leu–NH_2, from Z–Ala, (4) Fmoc–Asp–Phe–OMe, (5) Z–Asp–Leu–NH_2, (6) Z–Asp–Phe–NH_2, (7) Z–Asp–Phe–OMe, (8) Z–Gln–Leu–NH_2, (9) Bz–Gly–Tyr, (10) Bz–Gly–Phe, (11) Z–Gly–Leu–NH_2, (12) Z–Gly–Phe–Leu–NH_2, from Z–Gly, (13) Z–Gly–Phe–OMe, (14) Z–His–Leu–NH_2, (15) Z–His–Phe–NH_2, (16) Z–Phe–Leu–NH_2, (17) Z–Phe–Val–NH_2, (18) For–Phe–Leu–NH_2, (19) Z–Phe–Ala–NH_2, (20) Z–Phe–Leu–NH_2, (21) Z–Phe–Met–NH_2, (22) Z–Phe–Phe–NH_2, (23) Z–Ser–Leu–NH_2, (24) Z–Ser–Phe–NH_2, (25) mandelic acid coupled to 7-ADCA, (26) phenyl acetic acid coupled to 7-ADCA, synthesis of amide bonds in (27) Cephalexin, (28) Amoxicillin. All reactions are referenced elsewhere.[10]

Apart from the advantage of using enzymes as 'green catalysts' in synthesis reactions without the need for organic solvents, it was demonstrated that solid-to-solid reactions also produce significantly less heat and have better atom efficiency than conventional methods.

Appendix A: solid-to-solid reaction with inorganic counterion

Predicting the entropy of fusion to give an ionic supercooled liquid

A first question is whether the method used previously will give a good estimate of ΔG_{fusion} for production of a supercooled ionic melt at reaction temperature, or its solidification in the case of the product. This requires values for entropies of fusion, which can be calculated from measured enthalpies and melting points.

Table 2 shows all useful data found in the Beilstein and Gmelin databases for fusion enthalpies for organic salts, and the corresponding experimental estimates of entropies. Flexibility parameters have also been calculated from the structure of the organic ion (none of the inorganic ions have internal flexibility).[10,11] From these, eqns. 3 and 4 give a predicted entropy of fusion. Comparison between predicted and experimental values shows that entropies are without exception smaller than the predicted values, although there is a general correlation between the two. It is likely that ionic liquid melts are more ordered than non-ionic molecular liquids, so that there is less gain of entropy on melting. Excluding the first two small molecules (Table 2, entries 1 and 2), which seem to be outliers, the predicted values for the amine hydrochlorides (entries 3–10) average 12 J mol^{-1} K^{-1} more than the experimental values. For carboxylate salts (entries 11–17) the corresponding difference is 22 J mol^{-1} K^{-1}. The significant scatter and the limited amount of data mean that these values will remain very approximate as corrections to the prediction method.

This table includes all organic salts for which enthalpy of fusion values can be found in the Beilstein or Gmelin databases, except for a few cases: (a) where values reported are uncertain or cover a wide range; (b) some dihydrochlorides or very large molecules where unusual behaviour may be expected; (c) hydrates that will melt to a solution; (d) racemates, all related to single enantiomers that are in the table. For the last, experimental values for ΔS are usually not too different from the corresponding enantiomers, but are not used here because it is not certain whether the solid is made up of mixed crystals or a single racemic lattice.

Predicting the free energy change of solid-to-solid reactions involving salts

First, we will consider the most common case where one of each of the starting materials and products forms a solid hydrochloride salt. This situation can be expected when the salt formation involves a side chain group that is not involved in the enzymatic reaction, such as an amino acid side chain in peptide synthesis. An example could be:

$$Z\text{–Arg (s)} + Leu\text{–}NH_2\cdot HCl\ (s) \leftrightarrows Z\text{–Arg–Leu–}NH_2.HCl\ (s) + H_2O \quad (13)$$

In completing the thermodynamic cycle (Fig. 2), we suggest two possible sequences (A and B) to move on from the ionic supercooled liquid of a salt to an aqueous solution. Both involve extra steps that convert the ionic supercooled liquid melt of the salt into the 1 M solution of the uncharged molecules in octanol.

Approach A.

A1. Exchange H^+ between organic and inorganic ion. For eqn. 13 this produces a hypothetical liquid mixture of HCl and a free base.

A2. Unmix this to give pure liquid base and either gaseous or liquid HCl.

A3. Transfer the base *via* octanol to water and carry out the reaction just as in the original cycle (Fig. 2).

A4. Transfer the basic product back from water, *via* octanol to its pure liquid phase.

A5. Mix the HCl back in to give the hypothetical liquid mixture of uncharged species.

A6. Exchange H^+ to produce the ionic liquid corresponding to the product hydrochloride.

In this sequence it seems reasonable that A1 and A6 make equal and opposite contributions to the overall cycle and therefore cancel out. If the basic group is in a side chain in both starting materials and product, both A1 and A6 involve essentially the same acid–base exchange. Similarly A2 and A5 may cancel. This leaves just the steps of the original cycle in A3 and A4 to be considered, so that the equations derived above (2–10) and thermodynamic cycle Fig. 2 may still hold.

Approach B.

B1. Mix the ionic melt with octanol to give a solution of ion pairs. (This may be hypothetical if the real mixture would have significant levels of dissociated ions or neutral molecules).

B2. Carry out an exchange (in octanol solution) of H^+ and Cl^- between these ion pairs and the free base of the reaction product.

Table 2 Experimental data and predictions for ΔS_{fusion} of organic salts

Entry		T_m/°C	ΔH_{fusion}/ kJ mol^{-1}	ΔS_{fusion} (exp)/ J mol^{-1} K^{-1}	τ	ΔS_{fusion} (pred)/ J mol^{-1} K^{-1}
1	Ethylamine·HCl	110	5.5	14.4	0	50.0
2	Diethylamine·HCl	226	15.0	30.1	2	67.4
3	Ephedrine·HCl	219	31.7	64.4	2.5	71.8
4	Pseudoephedrine·HCl	182	28.0	61.5	2.5	71.8
5	Norephidrine·HCl	173	20.3	45.5	1.5	63.1
6	*N*-Methyl ephedrine·HCl	192	32.7	70.3	2.5	71.8
7	[2-Hydroxy-3-(naphthaloxy)propyl] isopropylamine·HCl	200	36.0	76.1	5.5	97.9
8	Levamisole·HCl	232	25.2	49.9	0	50.0
9	Dimethylaminoethyl methacrylate·HCl	120	30.6	77.9	4	84.8
10	Fenfluoramine·HCl	164	25.6	58.6	4.5	89.2
11	Ammonium formate	118	9.9	25.3	−0.5	45.6
12	Li formate	272	16.1	29.6	−0.5	45.6
13	Li acetate	285	11.9	21.3	−0.5	45.6
14	Na formate	257	17.7	33.4	−0.5	45.6
15	Na propionate	284	13.3	23.9	0.5	54.4
16	Na naproxen	238	26.4	51.7	2	67.4
17	Na ibuprofen	235	19.4	38.2	3	76.1

B3. Transfer the resulting starting material free base to water and carry out the reaction as in the original cycle.

B4. Transfer the basic product back from water to octanol solution.

B5. The free base product undergoes the exchange in B2.

B6. Remove the product ion pairs from octanol back to the pure liquid melt.

In this case, cancellation of B1 and B6 seems reasonable. The behaviour of ion pairs is often rather similar to that of polar molecules without formal charges, so they may mix and demix with octanol close to ideally.[13] Since B2/B5 involves protonation and ion pair formation with the same side chain group in both reactant and product, it seems reasonable that its ΔG is close to zero. Hence we again come to the conclusion that only the steps in B3 and B4 used before need be considered and the original treatment is valid for reactions involving salts. It should be noted that the current work on the physical chemistry of ionic liquids may shed further light on the likely energetics of A1/A6 and B1/B6.

A search of the Beilstein database revealed two examples where melting point data are available for hydrochlorides of a starting material and the corresponding peptide product (Table 3, entries 1 and 2). These melting points were used to predict the feasibility of the solid-to-solid synthesis of the hydrochloride salts of peptides Z–Arg–Leu–OMe and Z–Arg–Ala–OMe.

Application of eqn. 10 to these syntheses (essentially neglecting the fact that two reactants are hydrochlorides) gives overall estimates for ΔG at 40 °C of −16 and −15 kJ mol^{-1}. Given the values for ΔS_{fusion} found in Table 3 that were somewhat lower than for neutral molecules by 20–30 J mol^{-1} K^{-1} (Table 2) these estimates become slightly more negative at −17 and −16 kJ mol^{-1}. Because *both* the product and one starting material are salts, any errors in ΔS_{fusion} estimates are expected to cancel at least partly. Overall, we have a clear prediction that these solid-to-solid syntheses will be thermodynamically favourable.

Appendix B: solid-to-solid reaction with inter-substrate salt

Here we look at the influence on the overall solid-to-solid equilibrium of forming an inter-substrate salt (ammonium carboxylate) between the starting materials. Let us first look at a simple example of an ammonium carboxylate salt: ammonium acetate. The Gmelin database gives a value of 9.33 mm Hg for the vapour pressure over the solid salt at 20 °C. This value can be used to estimate the ΔG of forming the two separate neutral liquids from the solid salt, which can then be taken through the rest of the thermodynamic cycle as before (Fig. 2). The following steps are all considered at constant temperature (20 °C) and pressure (9.33 mm Hg):

C1. Transfer from solid NH_4Ac to an equimolar mixture of NH_3 and HAc vapours.

C2. Unmix the vapours to obtain pure component vapours NH_3 and HAc.

C3. Transfer from pure component vapours to liquids.

Equilibrium step C1 will have $\Delta G = 0$. For step C2 ideal gas behaviour will be a good approximation, so we can use the standard expression for mixing. We need here the negative of that, +3.4 kJ mol^{-1} (per mol of original NH_4Ac). ΔG for step C3 is estimated as $RT\ln(P_{sat}/P)$, where P_{sat} is the saturated vapour pressure of the liquid at 20 °C. P_{sat} for NH_3 is estimated as 6418 mm Hg from the correlation derived by Daubert *et al.*[20] P_{sat} for HAc is estimated as 11.62 mm Hg by interpolation ($\ln P_{sat} = A + B/T$) from 6 values in the range 6 to 40 °C from Beilstein/Gmelin database. The estimated ΔG values for transfer from the vapour to the liquid phase are then +15.9 kJ mol^{-1} for NH_3 and +0.5 kJ mol^{-1} for HAc. Summing steps C1 to C3 then gives $\Delta G_{salt\ to\ liquids}$ = +19.8 kJ mol^{-1} for the overall process of forming pure liquids from the solid salt.

ΔG for transfer from the solid NH_4Ac to its supercooled liquid at 20 °C can also be estimated. First, ΔS_{fusion} is estimated as 25 J mol^{-1} K^{-1} based on ammonium formate (Table 2, entry 11). Using a melting point of 114 °C (Gmelin database) and eqn. (2) we estimate ΔG_{fusion} = +2.8 kJ mol^{-1}.

The difference $\Delta G_{fusion} - \Delta G_{salt\ to\ liquids}$ is an estimate for the mixing of liquid NH_3 and HAc to produce the ionic liquid $NH_4^+Ac^-$ and gives: 2.8–19.8 = −17 kJ mol^{-1}. Ideal mixing would again give −3.4 kJ mol^{-1} as above. The −13.6 kJ mol^{-1} difference must therefore reflect the favourable interaction of the two species in the melt, including the proton transfer between them.

If this were a proton transfer between the carboxylic acid group and ammonia in dilute aqueous solution, the value of −13.6 kJ mol^{-1} would correspond to a ΔpK (difference between the pK_a values of both species) of around 2.4 pH units. The actual aqueous ΔpK for NH_4^+ and HAc is 4.5. This observation implies that ionisation by H^+ transfer is less favoured in the melt than in water, which is not unreasonable.

For use in predicting solid-to-solid equilibria, the key value is the +17 kJ mol^{-1} for transfer from the ionic melt to the two separate molecular liquids. To a first approximation this value may be used as an estimate for the extra unfavourable contribution in any case where the two starting materials form a solid salt. In most cases the ionic melt and both molecular liquids will be hypothetical supercooled phases, unlike NH_4Ac. However, there is no reason why the melting points of the various solids should affect the energetics of these liquid-to-liquid transfers.

The literature provides one example where solid-to-solid conversion has been reported starting from a salt of measured melting point. Erbeldinger *et al.*[16] demonstrated that Z–Gln and Leu–NH_2 formed a solid salt of melting point 150 °C. The solid-to-solid condensation reaction to form Z–Gln–Leu–NH_2 was found to be favourable at 40 °C.

Using eqns. (2–4) we estimate $-\Delta G_{fusion}$ values of −27.2 kJ mol^{-1} for solidification of the supercooled liquid reaction product, and −25.3 kJ mol^{-1} (at 40 °C) for the remaining steps starting from separate supercooled liquid starting materials (mixing with octanol, partitioning and reaction).

Assuming separate solid starting materials instead of an inter-substrate salt, as done previously,[10] the ΔG contributions from fusion of the separate starting materials were estimated using their melting points as +10.2 and +4.7 kJ mol^{-1}. This gives an overall highly favourable ΔG of −37.6 kJ mol^{-1} for the solid-to-solid conversion. Starting instead from the inter-substrate salt, ΔS_{fusion} is expected to be about 100 J mol^{-1} K^{-1}, some 22 J mol^{-1} K^{-1} less (Appendix A, Table 2) than for a neutral molecule with the same flexibility (using eqns. 3 and 4). Hence ΔG_{fusion} follows from eqn. 2 at a value of +11 kJ mol^{-1} at 40 °C. The hypothetical step of transfer from the molten salt to the two pure neutral liquids then contributes +17 kJ mol^{-1} as explained above. So the overall ΔG is estimated as −27.2 − 25.3 + 11 + 17 = −24 kJ mol^{-1}. Thus we conclude that the solid-to-solid conversion for the formation of Z–Gln–Leu–NH_2 remains favourable when the starting materials form an inter-substrate salt, as observed, although less so than starting

Table 3 Experimental data and predictions for $\Delta G_{solid\text{-}to\text{-}solid}$ for reactions involving solid hydrochloride salts

R–COOH	T_m/°C	τ	H_2N–R′	T_m/°C	τ	R–CONH–R′	T_m/°C	$\Delta G_{solid\text{-}to\text{-}solid}$/ kJ mol^{-1}
Z–Arg·HCl	155	8.5	Leu–OMe	150	3.5	Z–Arg–Leu–OMe.HCl	124	−16
Z–Arg·HCl	155	8.5	Ala–OMe	110	1.5	Z–Arg–Ala–OMe.HCl	93	−15

from the separate solids by 13 kJ mol^{-1}. In cases where the solid-to-solid reaction that starts from separate substrates is energetically less favourable, salt formation may be enough to prevent a favourable solid-to-solid conversion.

Appendix C: estimation of enthalpy changes in solid-to-solid transformations

The enthalpy of the solid-to-solid conversion can be estimated by the same thermodynamic cycle as used to estimate ΔG (Fig. 2). For a condensation reaction the overall $\Delta H_{\text{solid-to-solid}}$ is given by the sum of values for the condensation reaction in aqueous solution, dissolution (melting, mixing with octanol, partitioning into water) of solid substrates R–COOH and H_2N–R′ in water, and the precipitation (partitioning into octanol, unmixing, solidification) of R–CONH–R′ from solution.

A number of values of $\Delta H_{\text{reaction}}$ for peptide bond hydrolysis are collected in the NIST database.[21] They vary between −5 and −11 kJ mol^{-1}, for a reference reaction in which the product amino and carboxyl groups are ionised. To estimate a value for the reference reaction in which these groups are in the uncharged form, enthalpies of ionisation must be used. ΔH values for ionisation of the carboxyl group are typically small, while dissociation of the protonated amino group has ΔH of +45 to +55 kJ mol^{-1}.[22,23] Thus an average value for peptide hydrolysis will be around +45 kJ mol^{-1} for ΔH of peptide hydrolysis to the neutral products, and so −45 kJ mol^{-1} for synthesis.

Using again the thermodynamic cycle (Fig. 2), ΔH values for dissolution (and its reverse, precipitation) can be estimated as the sum of values for fusion of the solid to the supercooled liquid, its mixing with octanol, and the partitioning of the uncharged reactant into water.

Using the same approximation as for ΔG calculations we find:

$$\Delta H_{\text{fusion}} = T_{\text{m}} \Delta S_{\text{fusion}} \quad (14)$$

where T_{m} is the melting temperature (in K). The ΔS_{fusion} is estimated as before from eqns. 3 and 4. Assuming ideal mixing between the supercooled liquid and octanol means that ΔH_{mix} is zero. For octanol–water partitioning, a relatively small $\Delta H_{\text{partitioning}}$ of less than 15 kJ mol^{-1} in absolute magnitude is expected[24] so this value is also approximated as zero. Because of molecular similarities between substrates and product, errors will tend to cancel when the difference of $\Delta H_{\text{partitioning}}$ values is used in the overall calculation (as in the ΔG estimations). Hence only contributions from the fusion (and product solidification) steps are required:

$$\Delta H_{\text{solid-to-solid}} = \Delta\Delta H_{\text{fusion}} + \Delta H_{\text{reaction}} \quad (15)$$

Using the melting points and flexibility parameters from Table 4 of our previous publication,[10] we can now calculate the following enthalpies of fusion. For example, $\Delta H_{\text{solid-to-solid}}$ for peptide Z–Ser–Leu–NH_2 can be calculated from ΔH_{fusion} values (eqn. 14) of all reactants: Z–Ser, 36.6 kJ mol^{-1}; Leu–NH_2, 27.2 kJ mol^{-1}; Z–Ser–Leu–NH2, 58.4 kJ mol^{-1}. Hence the contribution to the overall solid-to-solid conversion is calculated as +36.6 + 27.2 − 58.4 = +5.4 kJ mol^{-1}. Combined with the reaction contribution, we thus estimate +5 − 45 = −40 kJ mol^{-1} for the overall conversion (entry 23 in Fig. 4). Fig. 4 summarises all $\Delta H_{\text{solid-to-solid}}$ values found for 28 different solid-to-solid reactions (for references see[10]).

Now we have values for $\Delta H_{\text{solid-to-solid}}$ we can compare the value to the non-enzymatic method to produce amide bonds. Conventional methods to bring about the same condensation usually involve reagents such as a carbodiimide. The overall reaction would be the sum of peptide condensation and hydration of the carbodiimide. There do not seem to be any measurements of ΔH for reactions of carbodiimides, but we would naturally expect a rather exothermic hydration. An estimate was made using the MOPAC2002 calculation with PM3 parameters, in the CAChe suite of programs. This gave enthalpies of formation of +77 kJ mol^{-1} for diisopropylcarbodiimide and −308 kJ mol^{-1} for *N*,*N*′ diisopropylurea. Taken with the literature value of −287 kJ mol^{-1} for the formation of liquid water, we get −308 − 77 + 287 = −98 kJ mol^{-1} for the carbodiimide hydration reaction. Hence the peptide synthesis promoted by the carbodiimide is expected to have ΔH of around −140 kJ mol^{-1}.

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