Biocatalysis in Supercritical Fluids, in Fluorous Solvents, and under Solvent-Free Conditions

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1. Introduction to Green Chemistry

In recent years, green chemistry has become an area of significant research interest. It is best defined as "the utilization of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture, and applications of chemical products".[1] Key elements of any chemical reaction are the solvents used and the employment of catalysts; therefore, the search for new environmentally benign solvents and catalysts that operate efficiently in them and can be easily recycled is of significant academic and industrial interest. Currently, there are five main "green" solvent systems: supercritical fluids (SCFs), fluorinated solvents, ionic liquids (ILs), water, and solvent-free reactions. In this review, we will focus on the research reported to date that combines enzymes (nature's catalysts) with SCFs, fluorous solvents, and under solvent-free conditions. One of the other reviews in this volume describes biocatalysis in ILs (van Rantwijk, F.; Sheldon, R. A. Biocatalysis in Ionic Liquids. *Chem. Rev.* **2007**, *107*, http://dx.doi.org/10.1021/cr050946x.) and, hence, complements this paper. There are several examples of ILs and SCFs being used together in a biphasic enzyme transformation. In this case, the role of the SCF is generally to extract and separate the products from the IL or poly(ethylene glycol) (PEG). We have included a specific section on these reactions.

1.1. SCFs

A SCF is defined as the state of a compound or element above its critical temperature (T_c) and critical pressure (p_c) but below the pressure required to condense it into a solid.[2] The phase behavior of substances at various temperatures and pressures can be represented most clearly on a phase diagram, as seen in Figure 1.

As both temperature and pressure increase, the gas−liquid coexistence curve moves upward. As the temperature increases, the liquid becomes less dense, due to thermal expansion, and as the pressure increases, the gas becomes more dense. Once the densities become equal, the phase distinction between liquid and gas disappears and the critical point has been reached. The substance is now said to be supercritical (sc), and this blurring of phases can be observed visually in a view cell (Figure 2). For instance, carbon dioxide (CO_2) at 25 °C and 50 bar can be seen as both liquid and gas with a distinct meniscus between the two phases.

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Helen Hobbs studied biochemistry and biological chemistry at the University of Nottingham where she obtained her degree in 2002. In September 2002, she started her Ph.D. in the Clean Technology Group at the University of Nottingham under the supervision of Prof. Martyn Poliakoff and Dr. Neil Thomas. Her research was focused on solubilizing biomolecules in fluorous solvents and supercritical carbon dioxide, including an in-depth characterization and activity study on these modified enzymes. She also studied the activity of cross-linked enzyme aggregates in supercritical carbon dioxide in collaboration with Prof. Roger Sheldon. After completion of her Ph.D. in November 2006, she started a postdoc at the School of Chemical, Environmental and Mining Engineering in collaboration with the Institute of Cell Signaling at the University of Nottingham. She is currently researching the hydrothermal synthesis of fluorescent nanoparticles for use in cell imaging, under the supervision of Dr. Ed Lester and Dr. Steve Briddon.

Neil R. Thomas was awarded a first-class B.Sc. (Honors) degree from the University of Southampton in 1987 and continued working there, under the supervision of Prof. David Gani, for his Ph.D. on the mechanisms of ammonia-lyases and pyridoxal 5′-phosphate-dependent enzymes. In 1990, during the final months of his Ph.D., the research group moved to the University of St. Andrews, where he completed his doctorate degree overlooking the Old Course. For the next 2 years, he worked under the supervision of Prof. Stephen J. Benkovic at Pennsylvania State University, State College, on the generation of catalytic antibodies, funded by a NATO/SERC postdoctoral research fellowship. In 1992, he returned to take up a Royal Society University Research Fellowship initially in the School of Chemistry at Bath and then in Chemistry at the University of Nottingham in 1995. He is currently an Associate Professor at the University of Nottingham, and his research interests include biocatalysis in unusual solvents, developing inhibitors of *Mycobacterium tuberculosis* and *Staphylococcus aureus* cell wall biosynthesis, constructing small protein scaffolds as antibody replacements and minienzymes, developing uses for near-infrared quantum dots in biology, and late 19th/early 20th century architecture.

On simultaneously increasing the temperature and pressure, the meniscus slowly becomes indistinct until it is no longer visible as the densities of the two phases merge. At this point, the CO_2 is said to have become sc.

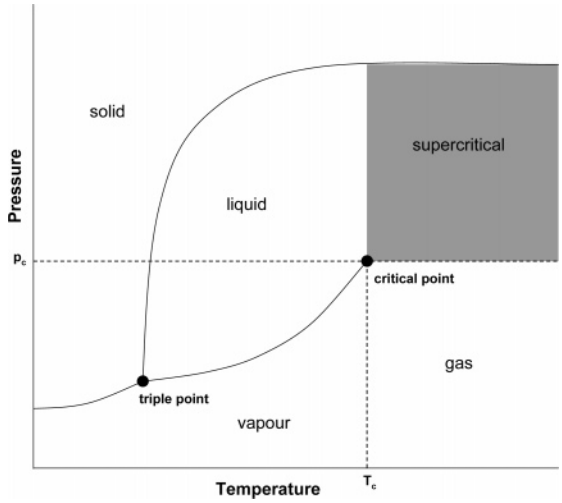


Figure 1. Phase diagram for a SCF. The critical point is the point at which the densities of the liquid and gas become identical and the fluid is said to be sc. Note that in this diagram the pressure scale is nonlinear. Adapted from ref 3. Copyright 2004 Royal Society of Chemistry.

In the sc region of the phase diagram, the fluid possesses both gaseous and liquid properties. For example, gaslike diffusivities and low viscosity coefficients allow the fluid to permeate through porous solids more quickly than a pure liquid could, hence overcoming mass transfer limitations. Liquidlike densities and dissolving powers allow the SCF to function as an effective reaction solvent. In addition, SCFs demonstrate tunable parameters such as dielectric constant, partition coefficient, and solubility. Small changes in temperature or pressure, particularly near the critical point, can result in up to 100-fold changes in solubility, and this can be useful in simplifying separations.[4]

The critical parameters for SCFs vary depending on the particular substance. A selection of the SCFs most often used for biocatalysis and their critical parameters are shown in Table 1.

The range of SCFs investigated for use as a solvent for enzyme-catalyzed reactions is relatively small due to the inherent nature of the proteins to unfold and become biologically inactive at elevated temperatures. Of those SCFs with suitable critical parameters for use with enzymes, the vast majority of reactions have employed $scCO_2$. This is because CO_2 is cheap, chemically inert, nontoxic, and readily available and because its relatively low critical parameters facilitate the use of biocatalysts. Substances such as ethane, ethene, and propane are less attractive because of their higher cost and flammability, and the use of sulfur hexafluoride or xenon is limited due to their cost and poor solvent power. However, there are some benefits for using these alternative SCFs over $scCO_2$, and these will be described below. The phase behavior of the fluid can facilitate a reaction through the use of controlled depressurization, which can allow the separation of substrates and products, without leaving harmful solvent residues. Diffusion is typically faster in SCFs as compared to liquids, which can speed up both homogeneous and heterogeneous reactions.[2] It should also be noted that water in the sc state cannot be used with enzymes as its critical parameters are well above those tolerated by proteins (Table 1).

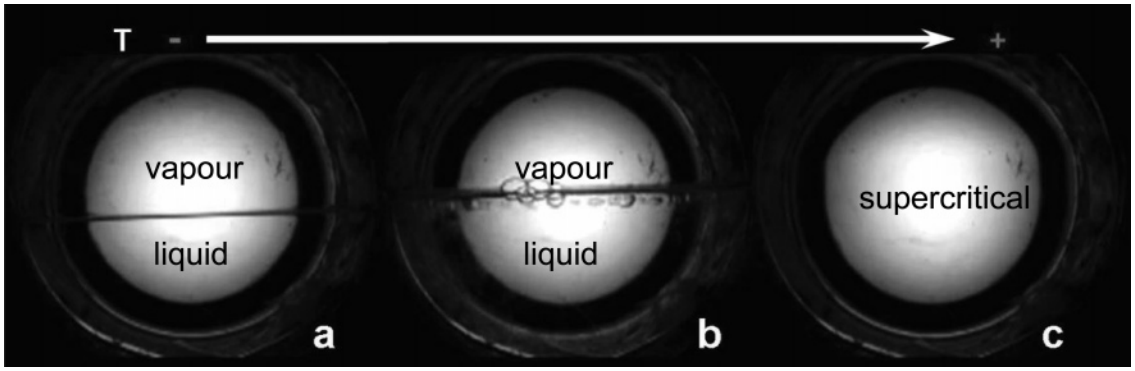


Figure 2. View cell showing the phase behavior as a substance becomes sc. (a) Biphasic system is observed at lower temperatures with a distinct meniscus between liquid and gas phases. (b) On increasing the temperature, the meniscus between the two phases starts to become blurred. (c) At a higher temperature, a homogeneous SCF is observed. The process is reversed on decreasing the temperature. Reprinted with permission from ref 3. Copyright 2004 Royal Society of Chemistry.

Table 1. SCFs Used for Biocatalysis and Their Critical Parameters[2]

substance	T_c (°C)	p_c (bar)
carbon dioxide (CO_2)	31.0	73.8
chlorodifluoromethane (ClF_2CH)	−3.7	49.7
ethane (C_2H_6)	32.3	48.8
ethene (C_2H_4)	9.2	50.5
fluoroform (CHF_3)	26.2	48.5
propane (C_3H_8)	96.7	42.5
sulfur hexafluoride (SF_6)	45.5	37.7
water (H_2O)	374.0	221.0
xenon (Xe)	17.0	5.8

1.2. Supercritical Carbon Dioxide ($scCO_2$)

$scCO_2$ can be described as a "green" solvent due to its nontoxicity and nonflammability. It is the only readily available solvent that is sc under conditions amenable to biocatalysis that is both cheap and not a volatile organic compound (VOC).

1.3. Fluorous Solvents

Fluorinated or fluorous solvents can also be described as "green" solvents since they are nontoxic and generally benign in the environment provided that they have low volatility. A monograph on all aspects of fluorous chemistry has recently been published.[5] One of the almost unique properties of fluorous solvents is their temperature-dependent miscibility with other organic solvents. This property has been elegantly exploited in fluorous biphasic systems (FBS) (see section 9). These were first described by Horváth and Rábai[6] and consist of a fluorinated solvent containing a fluorous soluble catalyst and a second product phase, which may be any organic solvent with limited, temperature-dependent, solubility in the fluorous phase.[5] This enables facile separation of products from catalysts (which can then be easily reused), which would otherwise be time-consuming and costly. Despite this advantage, there is a cloud of doubt over the "greenness" of the use of fluorous solvents due to their persistence in the environment, and this is still a matter of debate. Industrial interest in fluorous solvents is currently limited due to the high cost of these solvents.

1.4. Enzymes as "Green" Catalysts

Finally, the use of enzymes to catalyze reactions is also considered "green". For example, they have exceptionally high selectivity, which minimizes waste; they can operate on a single compound in a complex mixture, which can reduce the requirement for chromatographic or other types of separations; and they require only mild reaction conditions in comparison to some standard chemical routes, which can lower cost and energy input. If treated appropriately, enzymes are sufficiently robust to be reused with numerous batches of substrate while the byproducts of enzyme production and the enzymes themselves are readily biodegradable. The area of biocatalysis in nonaqueous media has grown immensely over the last few decades since the realization that most enzymes can function extremely well under (near) anhydrous conditions, displaying a number of useful properties such as enhanced stability and different substrate selectivity.[7] Hence, the combination of biocatalysis and SCFs or FBSs is attractive for the development of green chemistry.

2. Biocatalysis in SCFs

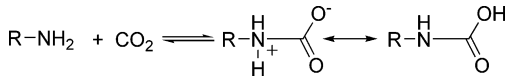
It has been over two decades since the first reports of enzyme-catalyzed reactions in SCFs were published. Randolph et al.[4] and Hammond et al.[8] both used enzymes as simple suspensions in SCFs. Since then, a number of new methods for stabilizing enzymes in SCFs have been investigated. Examples include the use of immobilized enzymes,[9] lipid-coated enzymes,[10] sol gels,[11] cross-linked enzyme crystals (CLECs),[12] cross-linked enzyme aggregates (CLEAs),[13] or enzymes combined with suitable surfactants to form reverse micelles/microemulsions.[14]

A number of reviews regarding biocatalysis in SCFs have been published since 1985 documenting the progress of the whole field[15−25] or of specific research groups.[26−28] A well-balanced and comprehensive review of the use of $scCO_2$ in the broader context of green chemical synthesis and processing has recently been produced by Eric Beckman,[29] while the earlier Chemical Reviews article on biocatalysis in SCFs by Messiano et al.[15] provides a more in-depth analysis of the physical chemistry and other parameters that affect enzymatic catalysis in SCFs and is therefore complementary to this review. This review brings together details of all of the enzyme-catalyzed reactions investigated in SCFs, fluorous solvents, and solid-to-solid/eutectic mixtures in one comprehensive summary, documenting all readily accessible literature reports in these fields of research excluding patents to the end of 2006.

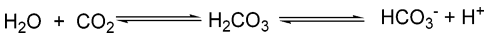
2.1. Which Solvent?

$scCO_2$ tends to be the SCF of choice for biocatalysis because it is cheap, readily available, and considered the most

Scheme 1. Reversible Carbamate Formation between CO_2 and Lysine Residues on the Surface of an Enzyme[31]



Scheme 2. Formation of Carbonic Acid and Its Dissociation to the Bicarbonate Anion in $scCO_2$



"green" of the SCFs with suitable critical parameters that are compatible with conditions required for enzymatic reactions; hence, it is the sc solvent most widely used in industry. However, carbon dioxide is involved in two chemical processes that have the potential to reduce or destroy the catalytic activity of an enzyme. These are (i) the formation of carbamates between CO_2 and lysine residues on the surface of the enzyme and (ii) the formation of carbonic acid by reaction between CO_2 and any water present in the system.

2.2. Carbamate Formation

Carbamate formation with amine groups in carbon dioxide was first reported in 1948.[30] In the presence of large amounts of an amine, both the unstable carbamoylate and the relatively stable urea can be formed. In the case of enzymes and other proteins, the ϵ-amino group of lysine and potentially the imidazole side chain of histidine can react reversibly with carbon dioxide to form an unstable carbamate that reverts to the free amine on depressurization of the system, as shown in Scheme 1.[31] In the case of enzymes, the formation of ureas is unlikely unless the proteins are highly aggregated.

Some reports have suggested that carbamate formation is advantageous as this can result in enhanced stereoselectivity of a reaction.[32−34] On the other hand, some publications claim that carbamates are the cause of enzyme inactivation in $scCO_2$[31,35,36] either through blocking the active site or causing a detrimental conformational change in the enzyme, and so, an alternative reaction medium, sc or otherwise, may provide a better solution.

2.3. Carbonic Acid Formation

The second property of CO_2 that can be detrimental to enzyme activity is the lowering in pH of water present in $scCO_2$.[18] In nonaqueous media, enzymes can change their catalytic activity if the pH of the microaqueous environment around them is altered. CO_2 can dissolve in the hydration layer associated with the enzyme, thereby altering the local pH by formation of carbonic acid by reaction between CO_2 and any water present (Scheme 2) and, hence, affecting enzyme activity.

In 1995, Toews et al. reported the first example of measuring the pH of water in the presence of CO_2.[37] They found that the pH of water in the presence of $scCO_2$ varies from pH 2.84 to pH 2.80 at 40 °C and 70−200 bar. Furthermore, Niemeyer and Bright[38] used a pH sensitive probe to examine the pH of the water core of water-in-CO_2 (w/c) reverse micelles generated with a perfluoropolyether (PFPE) surfactant and demonstrated that the pH was between 3.1 and 3.6. Comparison of this value with calculations assuming complete CO_2 saturation of the PFPE reverse micelle water pool shows that the micelle provides a barrier of 0.5 pH units to the CO_2 partitioning into the water pool.

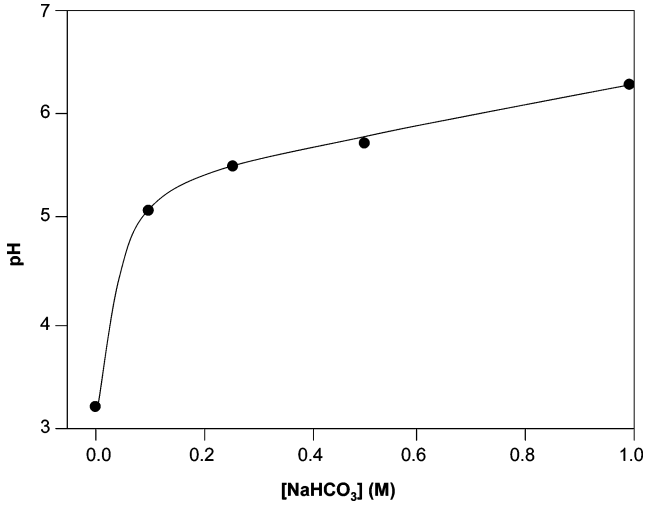


Figure 3. Effect of sodium bicarbonate concentration on the measured pH of water in contact with CO_2 ($T = 20$ °C, $p = 450$ bar). Reprinted with permission from ref 39. Copyright 1998 American Chemical Society.

2.4. Methods of Controlling the pH of Water in $scCO_2$

The control of the pH of water in CO_2 has been described by Holmes et al.[39] The proton concentration in water is primarily determined by the dissociation of carbonic acid into protons and the bicarbonate anion (Scheme 2). A simple method to control the pH would therefore be to suppress this dissociation by the addition of sodium bicarbonate, which shifts the equilibrium position of Scheme 2 to the left, reducing the proton concentration. This approach was found to be true, especially at HCO_3^- concentrations >1 M for which a pH of between 6 and 7 could be achieved (Figure 3). The pH of the buffered water was measured by ultraviolet/visible (UV/vis) spectroscopy using the indicators methyl orange, methyl red, and *p*-nitrophenolsulfonate.[39]

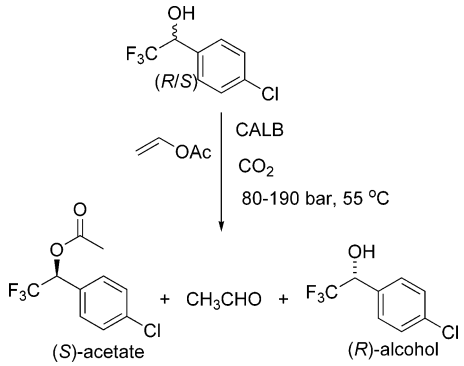
The same research group also reported that the addition of organic and inorganic buffers to the w/c microemulsion droplets stabilized by ammonium PFPE results in an increase in pH from 3 to values of 5−7.[40]

Ziegler et al.[41] reported that they were able to modulate the pH of water in $scCO_2$ by more than 1.5 pH units by adding NaOH and simply varying the CO_2 pressure over a range of 400 bar. Also reported for the first time was an aqueous phase pH within a w/c microemulsion system above neutrality.

The inhibition of enzymes in $scCO_2$ has, on several occasions, been attributed to the formation of carbonic acid resulting in a drop in the pH of the medium.[42,43] In particular, Fontes et al. studied the activity of subtilisin Carlsberg (Sub) CLECs in $scCO_2$ and suggested that the protonation of residues in the catalytic triad caused by a drop in pH results in a loss of activity as certain residues of the triad must be deprotonated for optimal catalytic function.[43]

Kamat et al.[18] calculated that the pH of water in CO_2, in the absence of buffer, is approximately 3.0 at 101 bar. On buffering the CO_2/H_2O system with, for example, phosphate buffer of pH 7.8, they demonstrated that the final pH of the buffer following CO_2 dissolution was 7.75, a negligible change. In addition, experiments with *Muchor miehei* lipase (MML) and *Candida cylindracae* lipase (CCL) show a lower activity in $scCO_2$ as compared with aqueous media yet have no pH sensitivity over a wide range in aqueous solution (pH

Scheme 3. Acetylation of *rac*-1-(*p*-Chlorophenyl)-2,2,2-trifluoroethanol with Vinyl Acetate in $scCO_2$ at 55 °C[a]



[a] Matsuda et al. demonstrate that the *E* value changes continuously on increasing pressure.[47,48]

2−10).[18,31] Chulalasananukul et al.[44] studied the effect of pH on the transesterification of propyl acetate with geraniol catalyzed by MML. Different pH buffers (pH 2−10) were added to $scCO_2$ at 40 °C, and no significant change in catalytic activity was observed over the pH range studied. In all cases, the apparent maximum velocity (V_{max}) was ca. five times higher in hexane than in $scCO_2$, suggesting that pH was *not* the main reason for the lower activity observed in $scCO_2$.[44] Care has to be taken in the interpretation of the above results as the addition of buffer salts can have a significantly different effect in systems where they dissolve in a distinct aqueous phase from those systems where the water present is limited to that directly bound to the enzyme, as discussed further in section 2.8.

Finally, Erickson et al.[45] studied the reaction between trilaurin and palmitic acid catalyzed by *Rhizopus arrhizus* lipase (RAL), in both $scCO_2$ and sc-ethane. The same drop in rate was observed on increasing pressure in both solvents; therefore, it was concluded that the drop in rate was not due to a lowering in pH.

2.5. Effects of Changing Pressure

A number of studies on the effect of changes in pressure on enzyme-catalyzed reactions have been reported, and this area has very recently been reviewed.[46] For example, Matsuda et al.[47,48] reported the enantioselective acetylation of *rac*-1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol with vinyl acetate (VA) (Scheme 3) catalyzed by Novozym SP435 (NZ 435) or *Pseudomonas cepacia* lipase (PCL) in $scCO_2$ at 55 °C.

For the reaction catalyzed by NZ 435, on increasing the pressure from 91 to 145 bar, the *E* value (a measure of enantioselectivity; $E > 100$ indicates a highly enantioselective reaction[49]) decreased from 38 to 23 while conversions remained very similar (25 and 24%, respectively). For the reaction catalyzed by PCL, the *E* value increased from 8 to 17 on increasing the pressure from 91 to 145 bar; yet, conversion was considerably reduced at the higher pressure (43% at 91 bar cf. 17% at 145 bar).

A wider range of pressures was explored for the reaction catalyzed by NZ 435, and the changes in *E* value can be seen in Figure 4. The results depicted in Figure 4 show that the *E* value changed continuously from 50 to 10 when the pressure was changed from 80 to 190 bar, regardless of the reaction time. The reasons for this tuneable enantioselectivity are currently unclear but must be the result of a change in the way the enzyme interacts with the two substrate enantiomers. Several suggestions have been made in the literature; for example, Kamat et al.[50] attributed the change in enantioselectivity of proteases from *Bacillus licheniformis* (subtilisin) and *Aspergillus* by manipulation of the pressure of sc-fluoroform to the change in the polarity of the fluoroform. Indeed, the dielectric constant for sc-fluoroform changes from 1 to 8 with a relatively small change in pressure (59−280 bar); yet, the authors were quick to state that it was not necessarily solely the solvent dielectric constant that controls activity.[51] However, in Matsuda's work, conducted in $scCO_2$ rather than in sc-fluoroform,[47,48] there is not such a marked difference in dielectric constant over the studied pressure range, but in contrast, the density of $scCO_2$ significantly changes from 0.20 to 0.42 kg/L on increasing the pressure from 80 to 110 bar at 55 °C; this could be a reason for the change in enantioselectivity. In fact, it has been suggested by Ikushima that the changes in enantioselectivity with pressure are due to the interaction of CO_2 and enzyme molecules.[32,33,52] Specifically, Ikushima studied the catalytic activity of CCL and found that the enzyme was activated near the critical point of CO_2. He suggested that CO_2, in the near critical (nc) region, triggered this activation by causing movement of the α-helical lid from a closed conformation (enzyme inactive) to an open conformation (enzyme active).[32,33] In addition, Mase et al.[34] reported enhanced enantioselectivity for the lipase-catalyzed desymmetrization of 1,3-propanediacetate in $scCO_2$ as compared to that in organic solvents. This is also attributed to the transformation of lysine groups by reaction with CO_2 to form carbamates.

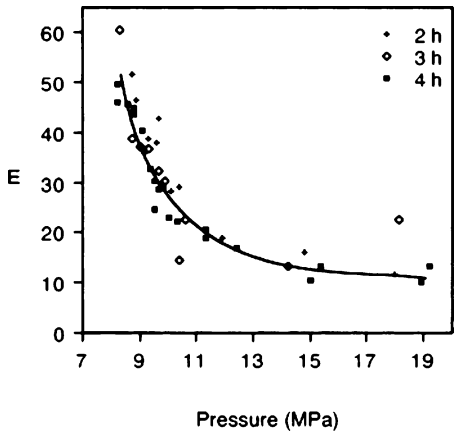


Figure 4. Effect of pressure on the enantiomeric ratio (*E*) for the reaction catalyzed by NZ 435. Reprinted from ref 47, Copyright 2001, with permission from Elsevier.

However, there is a lack of consistency in the literature regarding enzyme activity in $scCO_2$, and this makes the prediction of how to optimize new biocatalytic systems in $scCO_2$ difficult. Hartmann et al.[53] describe the peak in *E* value at 103 bar for the hydrolysis of 3-hydroxy-5-phenyl-4-pentenoic acid ethyl ester catalyzed by PCL in a biphasic buffer/$scCO_2$ system. Above and below 103 bar, the *E* value decreases, and this effect is attributed to a direct inactivation of the biocatalyst by formation of carbamates, in direct contrast to the findings of Ikushima, who reports that these interactions are the very cause for enzyme activation.

Albrycht et al.[54] demonstrate that the reactivity and selectivity of the kinetic resolution (KR) of *P*-chiral hydroxymethanephosphinates, catalyzed by NZ 435, can be controlled by tuning the pressure at values high above the

critical point. The reaction was reported to be fastest at 130 bar, way above the p_c.

Some reports have suggested that reactions are enhanced near the critical point. For example, Nakaya et al.[55] describe the transesterification of triolein and stearic acid [catalyzed by lipozyme TL IM (LZ)] and classified the reaction into three regions according to the pressure. Below 50 bar, the reaction rate was very slow and limited in the liquid triolein phase; in the nc region (50−100 bar), the rate was maximal at 59 bar possibly due to stabilization of the enzyme substrate complex; in the sc region (>100 bar), the reaction rate increased with increasing pressure, reflecting the increase in substrate solubility. In addition, Miller et al.[56] have demonstrated that an increase in pressure increased the selectivity of the reaction for the transesterification as compared with the hydrolysis of triglycerides (TGs). Erickson et al.[45] report a drop in reaction rate as the pressure increases, especially as the p_c is approached, for the reaction between trilaurin and palmitic acid catalyzed by RAL.

Conversely, Matsuda et al.[9] demonstrated that changes in pressure for the continuous KR of *rac*-1-phenylethanol catalyzed by NZ 435 in $scCO_2$ did not greatly affect either conversion or *E* values. Steytler et al.[57] stated that on increasing the pressure of $scCO_2$ to 500 bar, the synthesis of butyl laurate catalyzed by *Candida antarctica* lipase B (CALB) was not significantly affected. Also, nonyl acetate synthesis catalyzed by MML was not affected significantly by changes in pressure,[58] and the effect of changes in pressure (100−250 bar) was small on the hydrolysis of blackcurrant oil catalyzed by LZ.[59]

In fact, there seems to be no "rule of thumb" for predicting enzyme activity and enantioselectivity in $scCO_2$. Some authors say that working near the critical point is advantageous for good selectivity,[32,33,55] some say that it is sufficient that the conditions are at or above the critical point,[56] and yet still others report that it does not make any difference.[9,57−59] It may be that the effect of CO_2 on enzyme activity is very dependent on the specific enzyme, substrates, and reaction studied.

2.6. Effects of Pressurization and Depressurization

For green reactions, it is important that the enzyme can be easily recycled and that it will retain its activity over many reaction cycles; hence, the enzyme needs to be stable to many pressurization and depressurization cycles. Kasche et al.[60] provided evidence that a rapid depressurization of reactions in $scCO_2$ caused the enzymes chymotrypsin, trypsin, and penicillin amidase to become inactive, possibly through irreversible conformational changes occurring during depressurization. More recently, Bertoloni et al. have observed similar inactivation of acid and alkaline phosphatase, ATPase, and pectinase.[61]

In contrast, Habulin et al.[35,36] exposed the crude enzymes *Pseudomonas fluorescens* lipase (PFL), *Rhizopus javanicus* lipase (RJvL), *Rhizopus niveus* lipase (RNL), and porcine pancreatic lipase (PPL) to $scCO_2$, and also to nc-propane, and reported no activity change for the esterification of *n*-butyric acid following the depressurization step. The ability to perform the reaction, catalyzed by PPL in nc-propane, numerous times with the same batch of enzyme was also demonstrated. The conversion level only decreased to half the initial value after 10 reaction cycles (Figure 5), and the decrease was shown to be due to the increase in water

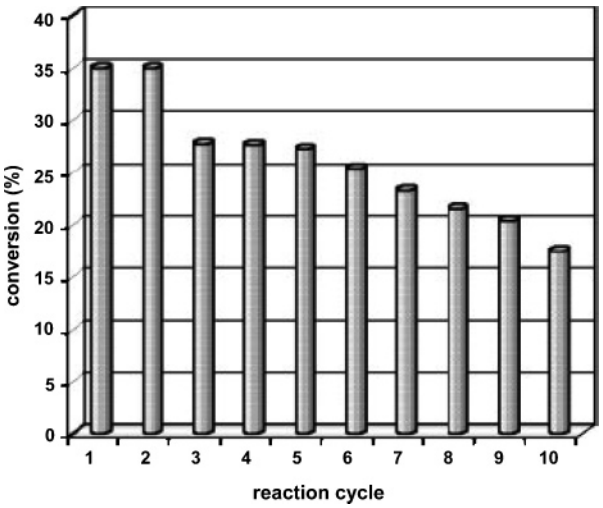


Figure 5. Half-life of PPL in nc-propane at 40 °C and 100 bar for the esterification of *n*-butyric acid with ethanol. Reprinted with permission from ref 36. Copyright 2001 Wiley Interscience.

released during the esterification reaction at the enzyme surface[36] and not inactivation due to the pressurization and depressurization steps.

Bauer et al.[62] examined the activity of both crude and purified preparations of esterase EP10 from *Burkholderia gladioli*. They found that after 30 pressurization and depressurization cycles of $scCO_2$ at 35 °C and 150 bar, the catalytic activity of the crude solution increased, possibly due to the removal of lipids, triglycerides (TGs), and fatty acids from the preparation, while there was no effect on the purified enzyme. One option available for those enzymes that are found to be highly sensitive to pressurization/depressurization is to employ a continuous reaction system that would significantly reduce the number of pressure changes to which the enzyme is subjected. A number of examples of enzymes being used in continuous SCF reactors are described in Tables 3−13. The effect of pressurization/depressurization on whole cell systems is discussed in section 4.7.

2.7. Effects of Changing Temperature

It is well-known that many enzymes are able to retain their catalytic activity in nonaqueous, hydrophobic solvents at higher temperatures in comparison to water. One reason for this may be that the enzyme is kinetically trapped in its active conformation in the hydrophobic solvent due to the lack of water that would normally lubricate its conformational flexibility;[63] this factor may also hold true in SCFs.

The thermal stability of enzymes in $scCO_2$ has also been demonstrated. For instance, Nakaoki et al. have shown that NZ 435 is still active even after heating to 140 °C in $scCO_2$.[64] Overmeyer et al.[65] also observe good NZ 435 activity and enantioselectivity at temperatures above 95 °C for the KR of ibuprofen with *rac*-1-phenylethanol, and this is supported by the work of Turner et al. for the hydrolysis of retinyl palmitate acetate by the same enzyme.[66] It is suggested that the dry compressed CO_2 stabilizes the protein structure of NZ 435[65] or that there is a faster mass transfer of the substrate to the active site of the enzyme plus higher reaction rates at elevated temperatures.[66] In contrast, Primozic et al. demonstrated the deactivation of lipolase 100T (L 100T) above 50 °C.[67] They suggest that this is due to the denaturation of the enzyme. For the esterification of oleic acid, LZ gains activity

from 40 to 60 °C but is thermally denatured at 80 °C,[66] and this is in agreement with the observations of Habulin et al.[68]

Other reports suggest an increase in enzyme thermal stability in nc-propane as compared to that in water; for example, the optimum reaction temperature for PPL in water is 40 °C, but in nc-propane, the optimum temperature is 50 °C.[35] It is suggested that this is probably a consequence of protein structural and conformational rigidity in propane,[35] and this may give better substrate specificity for the reaction studied.[36]

An optimum temperature of 40 °C in $scCO_2$ is reported for the synthesis of butyl laurate catalyzed by crude CALB,[57] for the synthesis of geranyl acetate catalyzed by LZ,[44] and for the resolution of 3-hydroxyoctanoic acid methyl esters catalyzed by PCL.[69] However, 62 °C has been reported as the optimum temperature for the hydrolysis of 3-hydroxy-5-phenyl-4-pentenoic acid ethyl ester catalyzed by PCL.[53]

Conversely, Peres et al.[70] report that changes in temperatures between 40 and 60 °C have little effect on geranyl acetate synthesis by NZ 435, and Sovova et al.[59] demonstrated that changes in temperature between 30 and 40 °C also have little effect on the catalytic activity of LZ.

The temperature dependence of the enantioselectivity of enzyme-catalyzed reactions and the importance of both entropic and enthalpic factors were first systematically studied in the late 1980s.[71] More recently, Hult et al. have conducted a number of detailed studies on the temperature dependence of the enantioselectivity of lipase-catalyzed reactions in organic solvents.[72,73] One of the first reports exploiting temperature to improve the enantioselectivity of a lipase-catalyzed reaction in an organic solvent came from Sakai.[74] An initial experiment demonstrated that 1-azirine methanols could be esterified with BCL in diethyl ether with an *E* value of 99 at −40 °C in diethyl ether, but an *E* value of only 17 was observed at room temperature. In $scCO_2$, Matsuda et al. looked at the NZ 435 catalyzed enantioselective acetylation of *rac*-1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol with VA at 31, 40, 55, and 60 °C.[48] A rapid change in *E* value was observed between 31 and 40 °C, and a more gradual change was observed at the higher temperatures. The authors note that these changes correlate well with the changes in CO_2 density and go on to evaluate *E* values at various temperatures and pressures but at the same density. They reported that the *E* values were affected by temperature with higher temperatures resulting in lower enantioselectivity in line with the observations of enzyme-catalyzed reactions in either aqueous or organic solvents.

2.8. Effects of Changing Water Content (w_o)

In the complete absence of water, enzymes are catalytically inactive. The most common explanation for this is that a minimum of a single layer of water molecules is required at critical points on the enzyme surface to maintain the native protein structure.[75] Zaks and Klibanov were the first to note that enzymes are more active in hydrophobic rather than in hydrophilic organic solvents, and they suggested that this was due to differences in water partitioning between the enzyme and the bulk solvent.[76] In essentially nonaqueous systems, any water present will partition between the enzyme and the solvent. On considering hydrophilic solvents, water will partition preferably into the solvent, and this will tend to strip the essential water off the enzyme, hence destroying the native structure and any enzyme activity. In contrast, hydrophobic solvents will not strip the essential layer of water from the enzyme, as these solvents become saturated with water at much lower concentrations; hence, the activity of the enzyme is maintained.

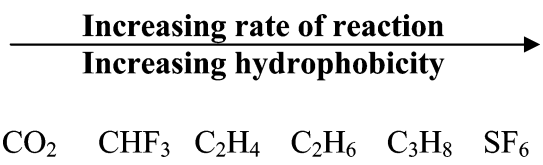


Figure 6. Comparison of reaction rates and hydrophobicity of the SCFs tested by Kamat et al.[79] Reaction rates increase on increasing hydrophobicity of the SCF due to reduced stripping of essential water molecules surrounding the enzyme.

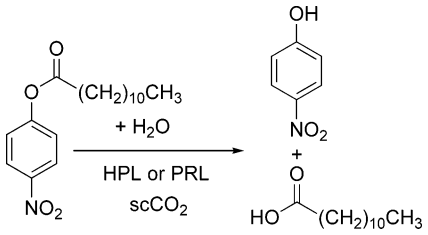
Early experiments in SCFs demonstrated that $scCO_2$ could strip the water off enzymes, reducing their activity.[77,78] In addition, Kamat et al.[79] reported the lipase (CCL)-catalyzed transesterification of methylmethacrylate in several SCFs and observed a marked decrease in enzyme activity in $scCO_2$. Reaction rate increases were found to correlate with increasing hydrophobicity of the SCFs (Figure 6). Hence, it appears that the loss of activity was the result of the enzyme losing essential water. This is surprising since CO_2 is generally considered to be a hydrophobic solvent (its w_o has been determined at 0.31 wt % at 50 °C and 344.8 bar[80]); yet, it is more hydrophilic than fluoroform or hexane and is therefore capable of stripping essential water from an enzyme thereby inactivating it. This is supported by the findings of Habulin et al.,[36] who demonstrate increased enzyme activity in nc-propane as compared with $scCO_2$ for the lipase-catalyzed esterification of *n*-butyric acid with ethanol, and they suggest that this is due to the stripping of water from the enzyme into CO_2.

In addition, Steytler et al.[57] studied the synthesis of butyl laurate using crude CALB in $scCO_2$ and demonstrated that the reaction was enhanced on addition of water. Three experiments were reported as follows: (i) dry enzyme—in the absence of water, the performance of the enzyme in $scCO_2$ was comparable with that in toluene under equivalent conditions of temperature and pressure; (ii) water-saturated enzyme—the reaction was severely retarded and hydrolysis was forced; and (iii) water-saturated $scCO_2$ was added above the enzyme contained in the water phase. In this case, the transfer of water between the two phases was minimized since both enzyme and solvent were hydrated; therefore, the reaction rate was enhanced.

Dijkstra et al.[12] have recently demonstrated a similar phenomenon for the enantioselective esterification of *rac*-1-phenylethanol by VA catalyzed by CLECs of *Candida antarctica* lipase B (ChiroCLEC-CALB). This reaction is very sensitive to the amount of water present, a concentration of 0.05 g/L resulted in optimum CLEC activity, while the enzyme is (reversibly) deactivated at lower water concentrations. This was attributed to the stripping of catalytically important water molecules from the surface of the enzyme. However, in contrast, Kmecz et al.[81] report that the use of dry or humid CO_2 makes little difference to the activity of the Amano lipase AK (AK) from *Pseudomonas fluorescens* for the acylation of 3-benzyloxypropane-1,2-diol.

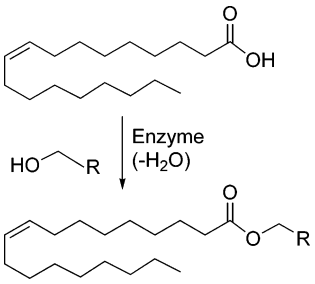
Alternative studies have looked at the effect of varying w_o in the system. For example, Vermue et al.[58] describe the decrease in transesterification of nonanol and ethyl acetate by LZ in $ncCO_2$ on increasing w_o from 0.05 to 0.2% (volume per volume, v/v). Srivasta et al.[82] studied the hydrolysis of *p*-nitrophenyl laurate to *p*-nitrophenyl catalyzed by hog pancreas lipase (HPL) or *Penicillium roqueforti* lipase (PRL)

Scheme 4. HPL- or PRL-Catalyzed Hydrolysis of *p*-Nitrophenyl Laurate to *p*-Nitrophenyl in $scCO_2$[a]



[a] Increasing w_o hinders enzyme activity.[82]

Scheme 5. Esterification of Oleic Acid with an Alcohol[a]



[a] Faster synthesis[89] and improved conversions[90,91,93] for alkyl oleate are observed in $scCO_2$.

in $scCO_2$ (Scheme 5) and reported that both enzymes were hindered on increasing the w_o due to either the inactivation of the enzyme or the formation of an aqueous layer around the enzyme that contributes to mass transfer resistance.

Still others report that changes in w_o do not affect the intrinsic activity of the enzyme[56,59] although it is generally agreed that the higher the w_o is, the greater the degree of unwanted substrate/product hydrolysis observed.

2.9. Water Activity (a_w)

Halling has suggested that the thermodynamic activity of water rather than water concentration is the key parameter in understanding the effect of water on enzymatic reactions.[83] The term water activity (a_w) describes the amount of water available for hydration of materials. A value of one indicates pure water while zero indicates the total absence of "free" water molecules; the addition of solutes always lowers a_w. a_w is defined as the product of the activity coefficient of water in the solvent (a method for estimating this value in SCFs has been described[84]) and the mole fraction of water in the solvent (eq 1).

$$a_w = \gamma x_w \tag{1}$$

Equation 1 demonstrates the calculation of a_w from the activity coefficeint (γ) and the water concentration (x_w) of the solvent.

A low a_w can be achieved and fine-tuned in $scCO_2$ using zeolite molecular sieves, such as NaA,[85] or salt hydrates, such as $Na_2CO_3 \cdot H_2O/Na_2CO_3 \cdot 1OH_2O$.[86] The effect of these solid state buffers has been extensively studied, and it was found that an acid−base effect was actually occurring.[85] A transesterification reaction, catalyzed by subtilisin CLECs, was noted to increase up to 10-fold with increasing amounts of zeolite, and therefore corresponding a_w, in $scCO_2$. The initial hypothesis was that a_w was low enough to decrease carbonic acid formation (hence minimize changes in pH) but still adequate for the function of subtilisin; the same observations were made in sc-ethane. However, it was also observed that the increase in reaction rate corresponded with the increase in the amount of zeolite present, and it could be possible that an acid−base exchange between the zeolite and the acid residue of the enzyme (eq 2) could be occurring, resulting in enhanced activity.

$$\text{protein}-\text{COO}^-\text{H}^+ + \text{Na}^+ \rightleftharpoons \text{protein}-\text{COO}^-\text{Na}^+ + \text{H}^+ \tag{2}$$

Equation 2 demonstrates equilibria to describe changes in the ionization state of a protein.[7]

It was also noted that subtilisin requires a formal negative charge on the catalytic triad for full activity. This would require removal of a proton and replacement by a counterion such as Na^+ for electroneutrality. This was tested by performing the reaction under three conditions in sc-ethane: (i) with zeolite only, (ii) with both zeolite and CAPSO [3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (sodium salt), a sodium/proton acid−base buffer], and (iii) with CAPSO only. The initial rate in the presence of buffer, regardless of the presence of zeolite, was reasonably similar; therefore, it was concluded that the zeolite effect must be of an acid−base nature.[85,87] Other such reports have been made, including the investigation into the best solid state acid−base buffer to use in SCFs. The buffer $Na_2CO_3/NaHCO_3$ was shown to increase enzyme activity up to 54-fold, probably due to its high basicity and capacity to counteract the deleterious effect of carbonic acid.[86] Six zwitterionic proton/sodium buffers were tested, and it was concluded that the higher the basicity (pK_a) of the buffer is, the higher the catalytic activity obtained.[88] Hence, this work highlights the need for the evaluation of the acid−base behavior of an extensive set of salt hydrates to identify one that is able to optimize the activity of an enzyme in $scCO_2$. Overall, Fontes et al. strongly recommend the use of acid−base buffers in enzymatic reactions in nonaqueous solvents, especially in SCFs where the use of salt hydrates still remains the most practical technique for setting and controlling a_w.[87]

3. Comparison of Activity of Lipase Enzymes in SCFs with Organic Solvents

There are plenty of papers suggesting that an enzyme-catalyzed reaction in $scCO_2$ provides superior results to those obtained in conventional organic solvents or solvent-free systems. For example, Yu et al.[89] report the faster synthesis of ethyl oleate catalyzed by CCL in $scCO_2$ as compared to that in organic solvents, Knez et al.[90,91] describe improved conversions for the synthesis of oleyl oleate (Scheme 5) catalyzed by LZ in $scCO_2$ as compared with solvent-free conditions, and Tewari et al.[92] demonstrate that the reaction rate for the transesterification of benzyl alcohol and butyl acetate by lyophilized CAL was higher in $scCO_2$ than in hexane or toluene or under solvent-free conditions.

In addition to improved reaction rates and conversions, enhanced enantioselectivity in $scCO_2$ has been reported on several occasions,[32−34,52,94,95] and this has been attributed to the specific properties of $scCO_2$ such as low viscosity and higher diffusivity of the substrates[94] as well as favorable carbamate formation on the surface of the enzyme.[32,33]

In comparison with other SCFs and gases, $scCO_2$ has been shown to be a superior reaction medium for the esterification of oleic acid with oleyl alcohol, catalyzed by LZ, when

similar enzyme concentrations were used in *n*-butane, *n*-propane, and a mixture of *n*-butane and *n*-propane.[93] Yet other reports suggest that in fact alternative SCFs are better media than $scCO_2$. For instance, Habulin et al.[35,36] report that the enzymes PFL, RJvL, RNL, PPL, and *Candida rugosa* lipase (CRL) are more stable in nc-propane than in $scCO_2$. The large loss in enzyme activity in $scCO_2$ was attributed to the interactions between CO_2 and enzyme molecules since this loss was not observed in nc-propane. Others report similar findings: PPL immobilized as a sol gel demonstrates much improved conversions for the esterification of butyric acid with isoamyl alcohol in nc-propane as compared to $scCO_2$;[11] Peres et al.[70] reported that NZ 435 is more active in sc-ethane compared with $scCO_2$ for the esterification of geraniol with acetic acid and that NZ 435 demonstrates higher catalytic activity in sc-ethane and compressed propane than in $scCO_2$ for the transesterification of butyl acetate with *n*-hexanol;[96] and Madras et al.[97] suggest that sc-methane is the SCF of choice for the synthesis of octyl palmitate catalyzed by NZ 435, possibly due to high solubility of substrates or a more favorable enzyme conformation in this medium.

Conversely, there have been a handful of reports suggesting that some enzyme-catalyzed reactions perform better in organic solvents when compared to $scCO_2$.[31,44,58,98,99] A few workers have suggested possible reasons for this phenomenon, the most favored argument for enzyme inactivation being the formation of carbamates on the surface of the enzyme as discussed earlier (section 2.2).[31]

Alternatively, several publications report comparable enzyme activities in $scCO_2$ and in organic solvents. Reaction rates for the esterification of ibuprofen with *n*-propanol catalyzed by LZ were similar in both $scCO_2$ and *n*-hexane,[100] and two reactions (a lipase-catalyzed hydrolysis of *p*-nitrophenol butyrate and lipoxygenase-catalyzed peroxidation of linoleic acid) showed essentially equivalent activity in both w/c microemulsions and water-in-heptane microemulsions.[39]

4. Stabilizing Enzymes in SCFs

Because of the relatively low activity of crude preparations of enzymes in SCFs, many attempts have been made to stabilize the enzyme by modifying the form in which it is used, such as the use of immobilized enzymes, lipid-coated enzymes, sol gels, CLECs, CLEAs, the use of reverse micelles or microemulsions, and the use of whole cells.

4.1. Immobilized Enzymes

There are a large number of enzymes immobilized on a solid support that are currently commercially available such as NZ 435 (CALB), Lipozyme (RML), Chirazymes [*Candida antarctica* lipase A (CALA), CALB, PCL, *Burkholderia cepacia* lipase (BCL) etc.], and Lipolases [*Aspergillus oryzae* lipase (AOL) and *Humicola lanuginosa* lipase (HLL)]. Other research groups have immobilized enzymes on glass beads, ACR silica gel, Celite, polypropylene granules, etc.[101−103] All of these show varying degrees of enzymatic catalysis in SCFs (see Tables 3−13 for specific details). The solid support generally makes the enzyme more robust under mechanical stress and easier to remove from the reaction and recycle as well as increases the accessibility of individual enzyme active sites.

4.2. Lipid-Coated Enzymes

The research group of Mori has pioneered the work of coating enzymes with lipids so that they are dissolved/dispersed in the solvent and catalyze homogeneous reactions[104] in $scCO_2$[105,106] and sc-fluoroform.[107,108] Two enzymes have been lipid coated with the nonionic didodecyl *N*-D-glucono-L-glutamate (Figure 7): lipase D from *Rhizopus delemar* (RDL)[105] and β-D-galactosidase from *Bacillus circulans*.[106] In these cases, the ratio of surfactant molecule to enzyme is ∼200:1. The lipid−enzyme complexes were found to catalyze the esterification of di- and triglycerides and the transgalactosylation of 1-*O*-*p*-nitrophenyl-β-D-galactoside, respectively, in $scCO_2$ with rate increases of 15-fold compared with those in conventional organic solvents. In particular, lipid-coated β-D-galactosidase was reported to be soluble (ca. 0.1 mg/mL) in $scCO_2$ in the range 32−60 °C and 74−200 bar. Furthermore, the same enzyme complex was found to be soluble in sc-fluoroform in the range 30−60 °C and 50−250 bar (0.1−0.5 mg/mL).[107] Again, a transgalactosylation reaction was successfully catalyzed with a 95% yield after 5 h. The rate of reaction was 20-fold faster than in diisopropyl ether and marginally faster than in $scCO_2$ in this case.

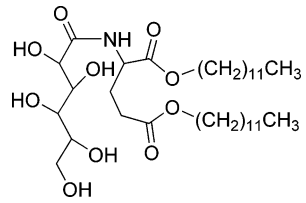


Figure 7. Structure of didodecyl *N*-D-glucono-L-glutamate, the surfactant used to coat enzymes such as RDL and β-D-galactosidase to solubilize them in $scCO_2$ and sc-fluoroform.[105,106]

Finally, a lipid-coated lipase B from *Pseudomonas fragi* (PFrL) was prepared and found to be soluble in sc-fluoroform at 0.1 mg/mL. An enantioselective acetylation was performed, and the rate was found to be very dependent on the pressure and hence dielectric constant of the solvent. Maximum conversions were a disappointing 60% within 40−100 bar.[108]

4.3. Sol Gels

The entrapment of PPL in a sol gel and its enzyme activity in both $scCO_2$ and nc-propane (40 °C and 100 bar) have been described by the research group of Habulin.[11,35] PPL in a sol gel form was shown to be much more active in a range of SCFs than the non-immobilized lipase for the esterification between butyric acid and isoamyl alcohol. It was suggested that in $scCO_2$, the sol gel protects the lipase from the adverse effects of CO_2 (carbamate formation, stress during depressurization) and that in propane the sol gel support prevents the lipase molecules from aggregating, thereby making the majority of their active sites available for catalysis. This contrasts with the nonimmobilized (native) lipase, which forms aggregates in propane.

4.4. CLECs

CLECs were developed in the 1960s, with carboxypeptidase A the first enzyme to be crystallized and then cross-linked and reported to show substantial enzyme activity.[109] CLECs are robust and maintain high activity and stability in both $scCO_2$[12] and sc-ethane.[86,88] However, the main disadvantage is that the crystallization of the enzyme is often a lengthy procedure and requires high enzyme purity, which is not possible in many cases, and results in CLECs being expensive.

Table 2. Proteins and Biocatalysts in Reverse Micelles/Microemulsions in SCFs

Proteins

entry	protein	surfactant	solvent	conditions (T, p, time, [water])	remarks	ref
1	BSA (labeled with acylodan fluorescent dye)	PFPE (mw 740) $[CF_3(CF_2CF(CF_3)O)_3-(CF_2O)_3CO_2^-NH_4^+]$	$scCO_2$	31 °C, 162 bar	w/c microemulsion	129
2	cyclosporin A BPTI (pancreatic trypsin inhibitor)	perfluoroheptanoic acid ammonium salt	7:1 v/v CO_2/MeOH	20 °C, <100 bar	w/c microemulsion	118
3	lysozyme	LS-54 (0.02 M)	water/$scCO_2$	35 °C, 220 bar $w_o$total = 12.3 $w_o$corr = 8.0	w/c microemulsion	133
4	lysozyme and Cc	AOT F-pentanol	water/$scCO_2$	38.0 °C, 345 bar		142
5	lysozyme	dynol-604	water/$scCO_2$	35 °C, 180−220 bar		143, 144
6	Cc	AOT	water/R22 (R22 = ClF_2CH)	29.3 °C, 200 bar, w_o = 9	AOT system able to solubilize >50 mM Cc	145

Biocatalysts

entry	biocatalyst	surfactant	solvent	conditions (T, p, time, [water])	reaction	yield (%) or initial rate	remarks	
7	RDL	AOT [sodium bis(2-ethylhexyl)sulfosuccinate]	ethane (C_2H_6)	32 °C, 200−320 bar, w_o = 2.78 30 min	triolein + water → oleic acid	19%	batch	115
8	horseradish peroxidase soyabean peroxidase	PFPE	$scCO_2$ ethane sc-CF_3H		oxidation of sulfides, thioanisoles, and DBTs to sulfoxides/sulfones		hemoglobin and Cc also investigated	136
9	cholesterol oxidase (*P. fluorescens*)/ catalase (*A. niger*)	1.4% PFPE ammonium salt (from Fomblin Y Aussimont)	$scCO_2$	35 °C, 200 bar	cholesterol to 4-cholestenone		active for 5 h but activity lost after 8 h	14
10	lipase from *Chromobacterium viscosum* (CVL)	diHCF4 di(1*H*,1*H*,5*H*-octafluoro-*n*-pentyl) sodium sulfosuccinate	liquid CO_2	20 °C, 450 bar w_o total = 10	*p*-nitrophenol butyrate + water → *p*-nitrophenol + butyric acid		batch; comparable result to that obtained in AOT w/o microemulsions in heptane; first example of enzyme reaction in w/c microemulsions	39
11	lipoxygenase	diHCF4 w/c microemulsion	liquid CO_2	20 °C, 450 bar, w_o = 10.	linoleic acid + O_2 → 13-hydroxyperoxy-octadecadienoic acid	K_m^{app} = 1.4 mM V_{max} = 8.0 M s^{-1} g^{-1} mL	batch; result comparable to that obtained in AOT w/o microemulsions in heptane; first example of enzyme reaction in w/c microemulsions	39

4.5. CLEAs

More recently, CLEAs have been prepared by Sheldon et al.[110] and have the advantage over CLECs since the need for a laborious crystallization of the protein is removed; yet, one obtains an immobilized enzyme, which is composed of almost entirely protein and just a small amount of cross-linking agent. To date, only three examples of the enzymatic activity of CLEAs in $scCO_2$ have been reported,[13,110,224] in all cases using CALB. In the first report by Matsuda et al., the activity of the CALB CLEA is compared with that of other forms of the CALB enzyme in $scCO_2$. In the study reported by Hobbs et al.,[110] the KR of tetralol and of *rac*-1-phenylethanol was demonstrated in a continuous reactor yielding the resolved product in excellent conversion and enantioselectivity. In addition, it was shown that a two stage reaction involving the lead-catalyzed reduction of acetophenone to *rac*-1-phenylethanol could be performed with subsequent KR of this alcohol with VA catalyzed by the CALB CLEA, avoiding the need for depressurization/repressurization between reactions. In the final example, Dijkstra et al. have shown that the CALB CLEA can be used to catalyze the formation of isoamyl acetate.[224]

Chen et al.[111] have shown that CLEAs can be made from trypsin in a AOT/water/isooctane reverse micellular solution on addition of glutaraldehyde (5% v/v). The CLEA was then precipitated by applying compressed carbon dioxide at 25.2 °C and 40 bar for 2 h. A transmission electron microscopy (TEM) study of the precipitated CLEAs showed that they had a dendritic morphology, and the size of individual CLEAs was much smaller than those produced using conventional CLEA manufacture. The size was found to be dependent on the w_o of the system: When w_o = 20, the CLEAs produced were 7−14 nm in diameter, but when w_o = 40, they were 13−23 nm diameter. The catalytic activity of the CLEAs produced by this method could be optimized to 0.133 U/mg, similar to the activity of the native enzyme.

4.6. Reverse Micelles and Microemulsions

Reverse (or inverted) micelles are small, dynamic aggregates of surfactant molecules surrounding a polar (typically aqueous) core dispersed in a nonpolar continuous (oil) phase. Reverse micelle solutions are clear and thermodynamically stable. As water is added to a reverse micelle solution, a microemulsion is formed that contains nanosized water droplets dispersed in a continuous oil phase. These are known as water-in-oil (w/o) microemulsions. The term microemulsion was coined by Jack H. Shulman from Columbia University in 1959.[112] In contrast to ordinary emulsions, microemulsions form upon simple mixing of the components and do not require high shear conditions.

Following from the pioneering work of Luisi et al. on the use of reverse micelles as hosts for proteins in organic solvents,[113] a number of groups have examined the potential of both dissolving proteins in reverse micelles/microemulsions (Table 2, first section) and conducting chemical reactions in them (Table 2, second section). It was demonstrated by Smith et al. in 1990 that the surfactant commonly used to form reverse micelles in organic solvents, aerosol

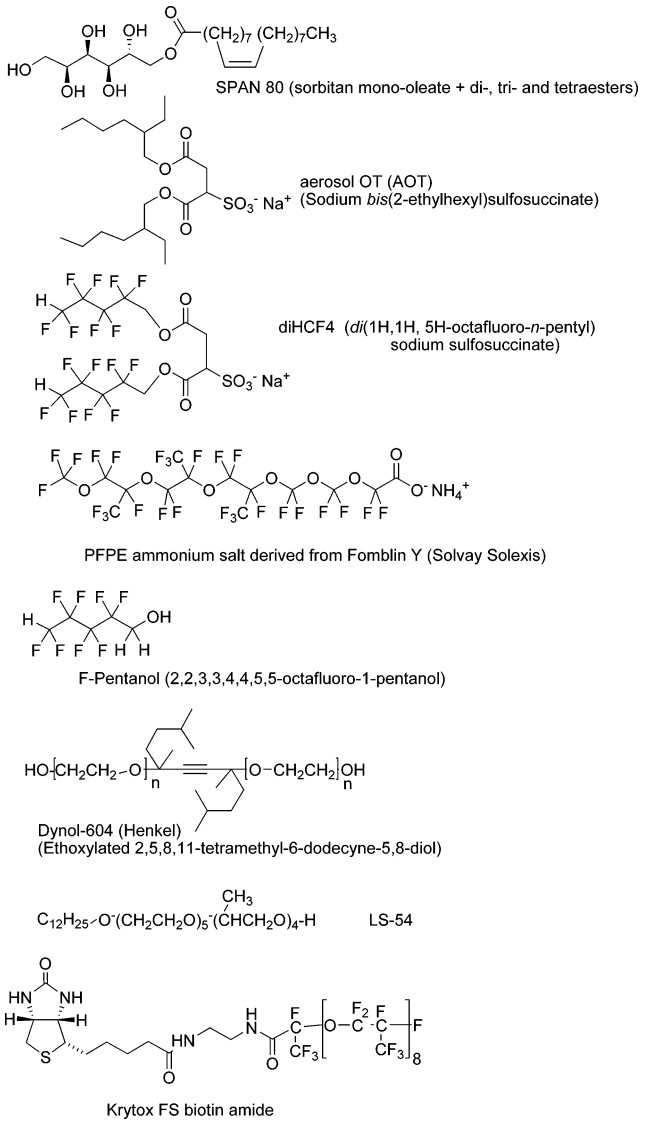


Figure 8. Structures of the surfactants used to solubilize proteins.

OT (AOT) (Figure 8), could be used to extract a small amount of the protein cytochrome c (Cc) from a sodium phosphate buffer solution into sc-propane.[114] Using dynamic light scattering (DLS), they demonstrated that the hydrodynamic diameter of the reverse micelles formed in the sc-propane layer was ~18 nm and that the size was constant at pressures between 150 and 250 bar. This study was repeated with hemoglobin, which was found not to be extracted as efficiently. In a later study, Hakoda et al. demonstrated that the lipase from *Rhizopus delemari* could be solubilized in AOT reverse micelles in sc-ethane at 32 °C and 48.8 bar.[115] The hydrodynamic diameter of the reverse micelles at 37 °C and 325 bar was found to change little with changes in the pressure or temperature of the system or the addition of the lipase. However, the diameter was significantly affected by the w_o of the system and the addition of triolein, which was hydrolyzed by the lipase to oleic acid. The use of AOT to solubilize proteins in $scCO_2$ was briefly investigated by Franco et al.[116] However, their paper reports that, while there was some visual evidence for the protein being solubilized in $scCO_2$, no protein was recovered from this phase, primarily because of the very low solubility of AOT in $scCO_2$.

Meier et al. have shown that lysozyme (14.3 kDa) and trypsin (23.8 kDa) could be solubilized in AOT reverse micelles in sc-xenon at 25 °C and 350−600 bar.[117] These solutions were examined by NMR, but no significant information is provided in regards to the structure of the protein that is solubilized in the reverse micelles. Previously, Gaemers et al. had used NMR to examine the structure of the smaller peptides cyclosporin A and pancreatic trypsin inhibitor (BPTI) in liquid CO_2.[118] In these cases, perfluoroheptanoic acid ammonium salt and trifluoroethanol were added to the liquid CO_2 to help dissolve the peptides.

During this period, there was significant research into the development of "CO_2-philic" surfactants that would be soluble in $scCO_2$ (Figure 8).[119] The main driving force for this research was the potential use of $scCO_2$ and suitable surfactants for dry cleaning as a more environmentally friendly replacement for perchloroethylene.[120] Most of the early surfactants were highly fluorinated, making them expensive and difficult to produce and dispose. More recently, it has been shown that polyoxygenated surfactant molecules can also be used to form water in CO_2 (w/c) microemulsions. The structural and electronic requirements for a good $scCO_2$ soluble surfactant have recently been reviewed by Beckman[121] and Eastoe et al.,[122] and these have culminated in the identification of a number of good nonfluorinated surfactants.[123−126] Some research has also been performed on the use of dendritic surfactants to solubilize hydrophilic molecules in $scCO_2$,[127] but the use of these molecules to solubilize proteins has not yet been reported.

The formation of thermodynamically stable reverse micelles and w/c microemulsions formed by fluorinated surfactants, water, and $scCO_2$ was achieved for the first time in 1991.[128] The potential advantage of these systems for biocatalysis is that the enzyme is maintained in a water pool and, hence, is less likely to undergo the structural changes that are sometimes observed when a protein is exposed to near anhydrous conditions, causing it to be inactivated. However, there are some significant disadvantages in employing reverse micelles or w/c microemulsions in $scCO_2$, particularly reduced pH (~3.5) for the unbuffered water pool,[38] promotion of undesirable hydrolysis reactions due to the higher w_o of the system, and difficulty in separating the products from the surfactant.

Johnston et al. were the first to demonstrate unequivocally that proteins could be dissolved within the water pools of w/c microemulsions using a PFPE surfactant through their study on bovine serum albumin (BSA) in 1996.[129] Since then, Webb et al.[130] have described a methodology for solubilizing ionic and biological species within w/c microemulsions prepared using fluorinated surfactants. Feng et al.[131] studied the effect of compressed CO_2 on the solubilization of BSA in water/AOT/isooctane reverse micelles. They determined the pH values within the water cores of reverse micelles at different CO_2 pressures and demonstrated that protein solubility increased on increasing CO_2 pressure within the low-pressure range but decreased at higher CO_2 pressures, so that the micelles eventually lost their ability to solubilize the protein. Liu et al.[132,133] formed w/c microemulsions using the surfactant Ls-54, despite it being a nonfluorinated and nonsiloxane, nonionic surfactant, and demonstrated the solubility of lysozyme within the water domain of the microemulsion. These examples all report the solubilization of proteins in w/c microemulsions while Ghenciu et al. have investigated the solubilization of subtilisin in macro- or biphasic emulsions formed in the presence of a distinct aqueous layer. They explored the use of both ionic and

nonionic perfluoroether surfactants (Twin 7500, PEG900, and Twin 7500 sodium sulfate) and demonstrated that the anionic perfluoroether surfactant was able to extract between 6.8 and 27.2% of the subtilisin from aqueous buffer into $scCO_2$ at 22 °C and 125 bar as the ratio of surfactant to protein increased from 28:1 to 75:1.[134]

The authors suggest that the increase in the amount of protein extracted at higher surfactant ratios may be due to an ion-pairing effect. The long-term stability of these systems is uncertain. Using an alternative strategy, Ghenciu and Beckman were able to extract avidin into carbon dioxide via inverse and three-phase emulsions by using a fluoroether to give [Krytox FS(H)]-tagged biotin molecule (Figure 8).[135] This approach utilizes the extremely high binding affinity that avidin has for biotin ($K_d \sim 10^{-14}$ M) and the fact that avidin is a tetrameric protein and hence is able to bind four of the fluoroether−biotin ligands. From an inverse emulsion formed from a buffer to CO_2 ratio of 4:1 with a surfactant concentration of 0.4 mmol L^{-1}, up to 40% of the avidin could be extracted. Because this process relies on the high affinity of biotin for avidin, it cannot be directly applied to the extraction of other proteins except those with a high affinity for the ligand such as streptavidin.

There have also been a handful of publications regarding the enzymatic activity in these systems as discussed below. The first published example of an enzymatic reaction conducted in pH-controlled w/c reverse micelles was reported by Holmes et al. in 1998.[39] They described the successful lipase-catalyzed hydrolysis of *p*-nitrophenol butyrate and lipoxygenase-catalyzed peroxidation of linoleic acid in reverse micelles formed by di-HCF4 (a fluorinated AOT analogue) (Figure 8) at 20 °C in liquid CO_2. This was followed by a paper from Kane et al. in 2000 describing the use of a similar solubilizing system with cholesterol oxidase from *P. fluorescens* and catalase from *Aspergillus niger*.[14]

Stanescu et al.[136] used horseradish peroxidase, hemoglobin, Cc, and soybean peroxidase in emulsions formed using PFPE as the surfactant in $scCO_2$, sc-methane, sc-ethane, and sc-trifluoromethane. They report the "bio-oxidation" of dibenzothiophenes (DBT) in $scCO_2$ with the reaction resulting in higher product yields as compared to that in aqueous systems. Research in a similar vein has also been described in an abstract from Hauck et al.[137] The systems used to solubilize proteins in SCFs are summarized in Table 2.

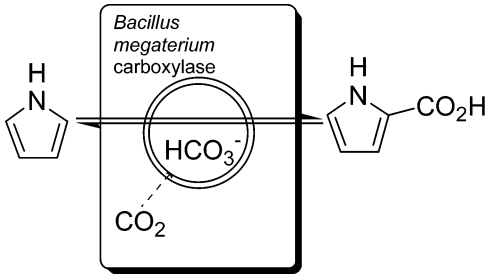
Finally, in 2006, Blattner et al.[138] encapsulated CALB and MML in lecithin w/o microemulsion-based organogels (MBG). These modified enzymes were shown to catalyze the esterification of lauric acid and 1-propanol in $scCO_2$ at 35 °C and 110 bar, and initial rates observed were higher than that in isooctane. It is also possible to make CO_2 in water emulsions that are stabilized by the presence of proteins,[139] but no catalytic studies have been performed on these recently reported systems.

$scCO_2$ has also been used as an antisolvent to precipitate both native BSA, lysozyme and trypsin, and CLEAs from reverse micelles formed by AOT in isooctane as described earlier,[111,140,141] while compressed CO_2 has also been shown to modulate the catalytic action of chloroperoxidase for the halogenation of 1,3-dihydroxybenzene in cetyltrimethylammonium chloride (CTAC)/H_2O/octane/pentanol reverse micelles.[146]

4.7. Reactions Using Whole Cells

High-pressure CO_2 has been known to have a sterilizing effect on bacteria since the 1950s[147] with $scCO_2$ having been investigated more recently.[61,148,149] There has been considerable debate over the mechanism of the bacteriocidal activity of SCFs. It was initially supposed that a rapid release of pressure in a SCF caused the rupture of the cell envelope of the bacteria and hence results in their death in a similar manner to depressurization causing protein denaturation as discussed before in section 2.6. However, Dillow et al.[148] have recently reported a scanning electron microscope study of the morphology of *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) cells before and after exposure to nc-CO_2 for 1 h at 25 °C and 205 bar. This demonstrated that most of the cells were intact after several pressurization−depressurization cycles, suggesting that neither cell rupture nor the extraction of lipids from the cell membrane was the cause of death. The authors suggest that cell death occurs because CO_2 is able to diffuse rapidly into the cell reducing the pH in the cytoplasm to ~3.0 on reacting with the water present there. More recently, the lifetime of mammalian cells in $scCO_2$ has been investigated and certain cell types have been shown to survive for extended periods in this solvent.[150] It is therefore interesting to see that there are several examples of whole cells being used as biocatalysts in $scCO_2$. In some ways, these experiments can be considered as being similar to the reactions with enzymes in reverse micelles where the cytoplasm of the cell is equivalent to the water pool of the reverse micelle, albeit with a highly complex enzyme content. Whole cells offer the significant advantage of providing any additional coenzymes and cofactors that may be required, although since the cells used in these studies are in the resting state, the supply of coenzyme may become quickly exhausted. The best studied example is the use of *Bacillus megaterium* for the carboxylation of pyrrole to pyrrole-2-carboxylate reported by Matsuda et al. (Scheme 6).[28,151]

Scheme 6. Whole Cell Reaction in $scCO_2$:Carboxylation of Pyrrole



This "carbon fixation" reaction does not require an atmosphere of CO_2 to occur.[152−154] After 1 h, the yield at just above the p_c of CO_2 (76 bar) was found to be ca. 12 times that at atmospheric pressure (1 bar). More recently, phenylphosphate carboxylase contained in *Thauera aromatica* cells has been shown to catalyze the conversion of phenol via phenylphosphate to *p*-hydroxybenzoic acid in the presence of $scCO_2$. Unlike the same reaction in aqueous buffer, the addition of sodium bicarbonate is unnecessary as the CO_2 can be used directly as a reactant.[155] Another example of the use of whole cells is that of the xylanase activity of *Aureobasidium pullulans* KK415 cells (ATCC 201145). These cells have been used to prepare *N*-octyl β-D-xylotrioside, xylobioside, and xyloside in a one-step reaction of xylan and *n*-octanol using the acetone-dried cells of *Aureobasidium pullulans* in both $scCO_2$ and sc-fluoroform.[156,157] In these cases, the cells in their acetone powder form were found to be more effective as catalysts than the

resting cells. This may be due to the resting cells having both higher β-xylosidase activity and higher w_o leading to an increase in the hydrolysis of β-D-xylotriose and xylobiose before they can react with the *n*-octanol.

A final example is the use of resting cells from the fungus *Geotrichum candidum* IFO5767 immobilized on polymer BL-100 (Osaka Yuki Kagaku Kogyo Co. Ltd.) in $scCO_2$ at 35 °C and 100 bar for 12 h for their alcohol dehydrogenase activity. These cells were used to reduce a range of prochiral ketones in yields of 11−96% with enantiomeric excess (*ee*) values in the range 96−99% being achieved.[158] This example has the advantage of not requiring additional expensive nicotinamide reducing agents or another enzyme such as formate dehydrogenase to recycle the coenzyme in situ.

5. Carboxylation: CO_2 as both Reagent and Solvent

A number of examples exist in which $scCO_2$ is utilized not only as a solvent but also as a reactant (see also Sakakura et al. *Chem. Rev.* **2007**, *107*, http://dx.doi.org/10.1021/cr068357u). Until recently, such reactions did not involve enzymes, for example, the fixation of CO_2 as propylene carbonate using an immobilized zinc pyridine bromide catalyst with propylene oxide[159] or the photolysis of anthracene leading to the production of 9,10-dihydroanthracene-9-carboxylic acid.[160]

One example of an enzyme capable of using CO_2 as a substrate under sc conditions is given by Matsuda et al.[151] They reported biocatalysis of the reversible carboxylation of pyrrole to pyrrole-2-carboxylate by whole cells of *Bacillus megaterium* (Scheme 6). Wieser et al. had previously shown that these cells can utilize bicarbonate as the carboxylate source and, by conducting the reaction under a CO_2 atmosphere (1.38 bar), the equilibrium position could be shifted to favor the production of pyrrole-2-carboxylate.[152,153] Under an atmosphere of $scCO_2$ (100 bar, 40 °C) with the cells in a potassium phosphate buffer at pH 5.5 containing ammonium acetate and potassium hydrogen carbonate, the yield of pyrrole-2-carboxylate was found to be 59%, around 12-fold higher than that at atmospheric pressure (1 bar).[151]

In a second very recently reported example, whole cells of *Thauera aromatica* containing the enzyme phenylphosphate carboxylase were shown to catalyze the conversion of phenol to 4-hydroxybenzoic acid.[155,161] This enzyme displayed excellent regioselectivity, as none of the ortho isomer was observed. The authors report that these cells did not require bicarbonate to catalyze the carboxylation but were able to use the CO_2 directly. However, given the reaction described in Scheme 2, it is obvious that some bicarbonate would be present in the reaction.

6. Enzyme-Catalyzed Polymerizations

The solubilizing properties of SCFs can be modulated using pressure as well as temperature; therefore, these solvents offer the possibility of controlling polymerization processes and the size and composition of the polymers generated. Hence, there has been considerable research on polymerization reactions in sc media.[162,163] In parallel with developments in polymerization in SCFs, there has been a rise in the use of enzymes in the preparation of synthetic polymers.[164−167] Below, we discuss the examples where enzymes and SCFs have been combined.

In 1995, Chaudhary et al.[168] formed a polyester polymer from 1,4-butanediol and *bis*-(2,2,2-trichloroethyl)adipate in a lipase-catalyzed reaction in sc-fluoroform at 50 °C. It was demonstrated that low-dispersity polyesters could be generated and that, as the pressure of the system increased from 62 to 207 bar, the average molecular mass increased from an average of 700 to 1338 Da, and the polydispersity from 1.07 to 1.23. Later, the same group reported the polymerization of 2,2,3,3-tetrafluoro-1,4-butanediol and divinyl adipate with NZ 435 in $scCO_2$ at 50 °C for 24 h at 100−200 bar. The resulting polyester had an average molecular mass of 8232 Da and a polydispersity of 1.76.[169]

In 1996, Ryu and Kim reported the horseradish peroxidase-catalyzed polymerization of *p*-cresol to a phenolic resin in $scCO_2$ at 40 °C and 76 bar in the presence of 2 mM *p*-cresol and 1 mM hydrogen peroxide. The conversion, however, was poor, with less than 50% of the *p*-cresol being converted after 5 h.[170]

Much of the recent research has focused on the ring-opening polymerization (ROP) of ϵ-caprolactone that avoids the need for a metallic Lewis acid catalyst. This was initially demonstrated with a surfactant-coated enzyme in $scCO_2$.[171] The polymerization of ϵ-caprolactone (as well as 11-undecanolide and 15-pentadecanolactone) was shown to be accelerated in $scCO_2$ as compared with microemulsions using organic solvents with the lipase from *Pseudomonas cepacia* coated in glutamic acid dioleyl ester ribitol amide. Further studies on ϵ-caprolactone polymerization have primarily utilized lipase B from *Candida antartica* supported on macroporous beads (NZ 435).[64,172−174] The poly(ϵ-caprolactone) (pCL) produced possessed M_n values in the range 12−37 kDa and polydispersities in the range of 1.4−1.6, in overall reaction yields of 95−98%. Because the enzyme was immobilized in this case, it could be readily separated from high mw polymers and recycled, and in addition, the $scCO_2$ could also be used to extract any remaining monomers and low mw polymers from the product.

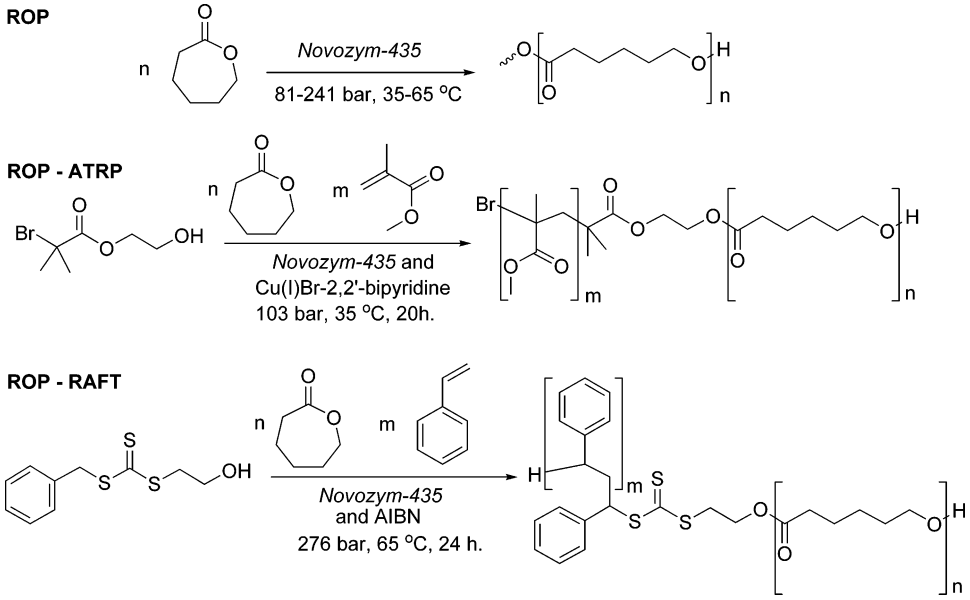
More recently, it has been demonstrated that the use of NZ 435 to catalyze ROP of lactones could be combined with either atom transfer radical polymerization (ATRP) (see Hutchinson et al. *Chem. Rev.* **2007**, *107*, http://dx.doi.org/10.1021/cr060943k) of methyl methacrylate to produce block copolymers [pCL-b-poly(methylmethacrylate) (pMMA) and pCL-b-p(MMA co-2-hydroxyethyl methacrylate (HEMA)][175−178] or with reversible addition−fragmentation chain transfer (RAFT)-mediated radical polymerization of styrene (Scheme 7).[179]

Matsumura et al. have shown that NZ 435 can be used to depolymerize poly(ϵ-caprolactone) (M_n = 100K) to form the cyclic dicaprolactone dimer (1,8-dioxacyclotetradecane-2,9-dione), which can then be repolymerized to give poly(ϵ-caprolactone) (M_n = 33K) in $scCO_2$ at 40 °C and 80 bar for 24 h.[180,181] This process has recently been modified into a continuous flow system with toluene as a cosolvent, using NZ 435. In this case, poly(*R*,*S*-3-hydroxybutanoate), poly(butylene adipate), and poly(ϵ-caprolactone) were depolymerized into cyclic oligomers.[182]

7. Biocatalysis Involving $scCO_2$ and a Second Neoteric Solvent

As described in this issue of *Chemical Reviews* (van Rantwijk, F.; Sheldon, R. A. Biocatalysis in Ionic Liquids. *Chem. Rev.* **2007**, *107*, http://dx.doi.org/10.1021/cr050946x) and elsewhere,[183,184] ILs are another type of neoteric (mean-

Scheme 7. Enzyme-Catalyzed Ring-Opening Polymerization Reactions in $scCO_2$



ing: "modern", "recent in origin" derived from Greek *neoterikos* meaning "younger") solvent that offers the possibility of eliminating the use of VOCs/volatile organic solvents (VOSs) and hence can be used in cleaner chemical processes. ILs have negligible vapor pressure and hence are not readily lost to the atmosphere, which is the fundamental property that causes them to be considered as "green solvents". However, this also means that a second phase is required to separate products generated in the IL away from the IL and any catalyst it may contain. In early studies, this second phase was normally a VOC, which therefore reduced the clean aspect of the system. In 1999, however, Blancard et al. demonstrated that CO_2 was highly soluble in 1-*n*-butyl-3-methylimidazolium [$BMIM^+$]-derived ILs but that the IL, being highly polar, was virtually insoluble in $scCO_2$.[185] Having observed this phenomenon, they were able to very efficiently extract naphthalene from [$BMIM^+$][PF_6^-] into $scCO_2$, which could then be separated, and the naphthalene was recovered after depressurization, leaving the pure IL. Since that study, it has been shown that many different ILs can dissolve considerable quantities of CO_2,[186] and more recently, the group has demonstrated that it is possible to use CO_2 as a "switch" to separate ILs from either organic solvents[187] or water.[188] The exact reasons that determine the solubility of $scCO_2$ in an IL have also been investigated.[189,190] On the basis of the different miscibilities of $scCO_2$ and ILs, a two-phase system combining these solvents has been used for a range of reactions including hydrogenation, hydroformylation, and asymmetric hydrovinylation, employing organometallic catalysts. This area is beyond the scope of this article and has recently been reviewed by Jessop, Heldebrant, and other authors.[191−193]

In 2001, Laszlo and Compton reported the activity of α-chymotrypsin (CMT) for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with 1-propanol in the ILs BMIM hexafluorophosphate [$BMIM^+$][PF_6^-] and its 1-*n*-octyl analogue [1-*n*-octyl-3-methylimidazolium $(OMIM)^+$]-[PF_6^-], both with and without $scCO_2$. The w_o of the system was found to be crucial for the reactions in the absence of $scCO_2$ with a minimum of 0.25% v/v being required. In the presence of $scCO_2$ (138 bar/45 °C), additional water was not required for the reaction to occur, but with water at 1% v/v, a doubling of the yield of the propyl ester, *N*-acetyl-L-phenylalanine propyl ester (APPE), was observed with α-chymotrypsin (CMT) freeze-dried in the presence of PEG.[194,195] In this paper, the possibility of using the $scCO_2$ phase to separate the product from the IL was suggested, and recovery of 66% of the APPE was demonstrated, but this process was not pursued further. It should be noted that in all of the studies of biocatalysis in a combination of ILs−SCFs reported to date, the enzyme does not dissolve in the IL. In early examples where the protein was soluble in the IL, it was generally found to be catalytically inactive.[196−198] More recently, a number of studies have identified ILs that dissolve proteins and retain their structure (Cc in [$BMIM^+$]-[$H_2PO_4^-$])[199] and catalytic activity (CALB in [Et_3NMe^+]-[$MeSO_4^-$])[200] while Goto et al. have demonstrated that Cc can be extracted into ILs using dicyclohexano-18-crown-6,[201] and PEG-modified subtilisin is able to dissolve in [$EMIM^+$]-[BTA^-] [EMIM = 1-ethyl-3-methylimidazolium; BTA = *bis*-(trifluoromethanesulfonamide)] and retain their structure and activity.[202,203]

Two other groups described the use of IL/$scCO_2$ systems with biocatalysts in papers that appeared almost simultaneously in 2002.[204,205] In these cases, the $scCO_2$ was also shown to have a crucial role in separating the reactants and products from the catalyst and IL. Lozano and co-workers[204] demonstrated that an aqueous solution of CALB in either [$BMIM^+$][BTA^-] or [$EMIM^+$][BTA^-] immobilized on glass wool was able to catalyze the irreversible transesterification of vinyl butyrate with 1-butanol to form *n*-butyl butyrate and acetaldehyde. In this case, the vinyl butyrate had to be introduced into the $scCO_2$ in hexane reducing the "greenness" of the reaction. The reaction was examined at 150 bar and at a range of temperatures (40−100 °C). It was shown that, at 40 °C, the enzyme only lost ca. 15% catalytic activity after the reactor had been used 11 times.[204] In the same paper, the authors describe the use of CALB immobilized on Celite in the presence of [$BMIM^+$][BTA^-] or [$EMIM^+$][BTA^-] for the KR of *rac*-1-phenylethanol with vinyl propionate. This reaction demonstrated very good enantioselectivity (99.9% *ee*), and the enzyme was found to be eight times more active when immobilized on Celite in the presence of the ILs. The authors have reported a second study on this system in which

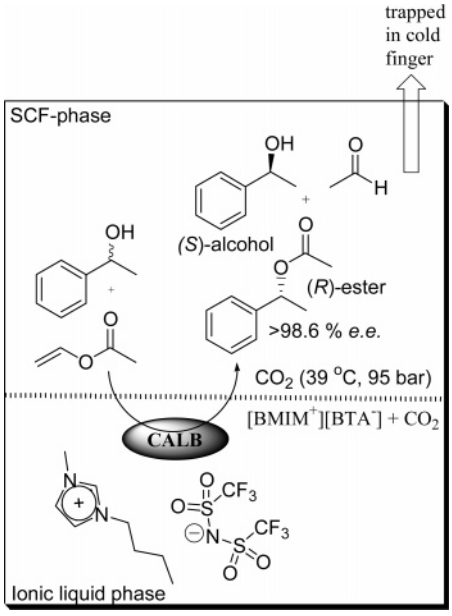
both free and immobilized CALB were subjected to temperatures of up to 150 °C and 100 bar and exhibited excellent thermal stability.[206] More recently, they have explored the effects of a range of five other ILs on CALB-catalyzed ester synthesis.[207,208] As part of this study, the stability of CALB in the ILs at 50 °C was compared with that in hexane over 50 days and it was found that there was a 2000-fold improvement in the half-life of the enzyme in the ILs. In a further study, some members of this group examined the activity of CALB immobilized on an α-alumina microporous dynamic membrane[209,210] for the synthesis of *n*-butyl propionate from 1-butanol and vinyl propionate both in $scCO_2$ on its own and also in IL/$scCO_2$ biphasic system with three different room temperature ILs {RTILs: $[BMIM^+][PF_6^-]$, [1-*n*-butyl-2,3-dimethylimidazolium $(BDiMIM)^+][PF_6^-]$, and $[OMIM^+]$-$[PF_6^-]$} at 50 °C and 80 bar. It was found that the rate of transesterification with the three different IL/$scCO_2$ biphasic systems was lower than with $scCO_2$ alone, and this was related to possible limitations in the mass transfer of substrates/products across the IL surrounding the enzyme rather than enzyme deactivation. The research of the Lozano group on combining $scCO_2$ with ILs has recently been reviewed.[211]

Reetz et al.[205] examined biocatalysis in biphasic $scCO_2$/IL systems and initially utilized the same IL $[BMIM^+][BTA^-]$, but with unmodified CALB, and examined the irreversible acylation of 1-octanol with VA. There was one significant difference in their reactor setup in that the reaction was carried out initially as a batch process with the IL functioning as a solvent rather than as a protective coating around the enzyme. This had the advantage that the substrates could be added to the IL/suspended CALB directly rather than employing a carrier solvent such as hexane. In this case, the $scCO_2$ was employed as an extraction solvent since it was only added after 30 min (at 39 °C and 95 bar) to extract the *n*-octyl acetate in 92% yield together with acetaldehyde and unreacted VA from the system for collection in a cold trap once the CO_2 had evaporated (Scheme 8).[205] The IL phase containing the CALB was recycled giving yields of 97, 98, and 98% on subsequent batches, demonstrating that the enzyme retains good activity. This system was then modified into a continuous process with $scCO_2$, allowing the production of 0.1 kg of *n*-octyl acetate per liter of reactor volume per hour, which was maintained over a 24 h period, giving an overall yield of 94%. Reetz et al. also investigated the KR of *rac*-1-phenylethanol with VA in batch where the enantioselectivity of the reaction remained very high [>98.6% of the (*R*)-acetate over four cycles].[205]

In a follow-up paper, the KR of secondary alcohols was transformed from a batch to a continuous process, and the choice of IL, ester, and the preparation of enzyme was examined. It was found that vinyl laureate, a cheaper acylating agent than VA, gave an ester that had a much lower solubility in $scCO_2$ than the unreacted *rac*-1-phenylethanol and so facilitated its downstream separation by controlled density reduction of the $scCO_2$ via a change in temperature and/or pressure. The use of NZ 435 or a sol gel immobilized CALB in place of the suspended lyophilized CALB was explored, but these catalysts gave considerably lower yields with VA as the acylating species.[212]

In a related study, Reetz and Wiesenhöfer have shown that polyethylene glycol (PEG, mw 1500), which is liquid at 50 °C and 150 bar, can be used in place of the IL for the KR of *rac*-1-phenylethanol with VA.[213] Heldebrant and

Scheme 8. Kinetic Resolution of *rac*-1-Phenylethanol with VA in a CO_2-Expanded IL with Products Extracted into the CO_2 Phase[205]



Jessop had previously demonstrated that PEG and $scCO_2$ formed a similar biphasic system to that of ILs/$scCO_2$ with CO_2 being soluble in liquid PEG but PEG having very low solubility in $scCO_2$.[214] The enantioselectivities in the PEG/$scCO_2$ system were marginally lower than those observed with the ILs (*ee* > 98.1%). After 5 h, all of the (*R*)-1-phenylethanol was converted into the desired ester. It should be noted that PEG contains two free terminal alcohol groups, but these were only acylated after extended periods (85−92% after 48 h depending on the vinyl acylating agent used). Again, changing the density of the $scCO_2$ allowed control over the relative amounts of the ester and alcohol extracted. At 50 °C/80 bar, considerably more of the (*R*)-ester (73%) was extracted from the PEG system than the IL (56%) under similar conditions. It was also demonstrated that the enzyme retained its activity and selectivity through 11 batches.

PEG offers significant advantages over current ILs in clean synthesis as it is considerably less expensive and is nonhalogenated, simplifying disposal. It has already been approved for use as a food additive, having had its toxicity fully evaluated.

Garcia et al.[25] have examined the catalytic activity of NZ 435 and *Fusarium solani pisi* cutinase immobilized on zeolite NaY in $scCO_2$/$[BMIM^+][PF_6^-]$ in a continuous system (35 °C/100 bar, $a_w = 0.12$) for the reaction of 2-phenyl-1-propanol and vinyl butyrate. In these cases, the enantioselectivity of both enzymes for this substrate was found to be low ($E = 1.5-3$); more positively, the IL appears to have protected the cutinase from inactivation by the $scCO_2$. For both enzymes, the rate observed in the $scCO_2$/$[BMIM^+]$-$[PF_6^-]$ system was higher than for the IL on its own. This enhancement was attributed to the CO_2 dissolved in the IL, decreasing its viscosity and hence improving the mass transfer of substrates to the enzyme active site.

In one final example, which is not strictly a "clean" synthesis, Broering et al. used CO_2 as a reversible switch to modulate the miscibility of aqueous and organic phases in an organic−aqueous tunable solvent (OATS) system.[215] Unmodified CALB was used to catalyze the hydrolysis of 2-phenethyl acetate in a 40:60 dioxane:buffer (150 mM

sodium phosphate) mixture to give a yield of 55% after 2 h. Addition of carbon dioxide at 50 bar caused the dioxane and aqueous layers to separate, and the organic layer was separated off and the amount of 2-phenylethanol was determined by gas liquid chromatography (GC).

8. Enzyme-Catalyzed Reactions in SCFs

The tables in this section summarize all of the enzyme reactions in SCFs reported since this area of research was first reported from 1985[4,8] to the end of 2006, excluding the

Table 3. Lipase-Catalyzed Reactions Involving Short Chain Acids and Esters (C_1-C_8) in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water])	yield (%) or initial rate	remarks	ref
1	CALB	NZ 435	a. $scCO_2$ b. scC_2H_6	acetic acid + geraniol → geranyl acetate	40 °C, 100 bar, 10 h, $a_w = 0.25$	a. 73% b. 98%	batch; temperature (40−60 °C) has no major effect	70
2	CALB	NZ 435	$scCO_2$	acetic acid + *rac*-lavandulol → (*R*)-lavandulyl acetate	60 °C, 100 bar, 1−24 h	44.3% (1.5 h)	batch; ee_s 24% ee_p 41% $E = 3.0$ yield and enantioselectivity lower than in hexane	218
3	MML	LZ	$ncCO_2$	nonanol + ethyl acetate → nonyl acetate + ethanol	60 °C, 125 bar, 0.05% v/v water	8 mmol/s/kg enzyme	continuous stirred-tank reactor; effect of pressure, polarity, and w_o studied; rate of reaction lower in CO_2 than in hexane	58
4	MML	LZ	$scCO_2$	geraniol + propyl acetate → geranyl acetate + propanol	40 °C, 140 bar, 72 h, 8−10% water w/w	30%	batch; thermostability also tested (40 °C optimum); 85% yield obtained in hexane	44
5	CALB HLL	a. NZ 435 b. lipolase	$scCO_2$	isoamyl alcohol + ammonium acetate → isoamyl acetate	a. 60 °C, 200 bar b. 40 °C, 200 bar	a. 90% b. 96%	esterification rate higher in hexane	98
6	a. CALB b. MML	a. NZ 435 b. LZ	$scCO_2$	isoamyl alcohol + acetic anhydride → isoamyl acetate	40 °C, 100 bar, 2 h	a. 100% b. 20%	batch; continuous flow was used to study stability and CO_2/substrate ratio	219
7	CALB	NZ 435	scC_2H_6	acetic acid + geraniol → geranyl acetate	40 °C, 100 bar, $a_w = 0.2$	625 mmol/ min/mg	batch	220
8	RML	LZ 77	$scCO_2$	triacetin + hexanol → hexyl acetate	46.7 °C, 182 bar, 69 min	77.3%	batch	221
9	CALB	crude	$scCO_2$	benzyl alcohol + butyl acetate → benzyl acetate + 1-butanol	20.15 °C, 100 bar	equilibrium constant $K = 0.238 \pm 0.020$	reaction proceeds more rapidly in $scCO_2$ than in hexane, toluene, or solvent free	92
10	a. PFL b. RJvL c. RNL d. PPL e. CRL	crude	$scCO_2$	a−d. *n*-butyric acid + ethanol → ethyl butyrate e. *n*-butyric acid + cytronellol → cytronellyl butyrate	40 °C, 100 bar	a. 2 b. 2 c. 2 d. 2 e. <0.5 mmol/ kg min	batch; low rates in $scCO_2$ attributed to carbamate formation and stripping of essential water from enzyme	36
11	a. PFL b. RJL c. RNL d. PPL e. CRL	crude	scC_3H_8	a−d. *n*-butyric acid + ethanol → ethyl butyrate e. *n*-butyric acid + citronellol → citronellyl butyrate	40 °C, 100 bar	a. 28 b. 17 c. 36 d. 12 e. 7.8 mmol/ kg min	batch; higher rates than above ($scCO_2$) attributed to the low dielectric constant of propane (1.7) that enables lipase lid to remain open; also, propane not able to strip essential water from enzyme surface	36
12	PPL	a. crude b. sol gel	$scCO_2$	*n*-butyric acid + oleyl alcohol → oleyl buturate	40 °C, 100 bar, 5 h	a. <3% b. 25%	batch; crude PPL deactivated in CO_2 but protected by sol gel	11
13	PPL	a. crude b. sol gel	$scCO_2$	*n*-butyric acid + oleyl alcohol → oleyl buturate	40 °C, 100 bar	a. 32% b. 70%	batch; more active sites accessible in sol gel, the enzyme agglomerates in nc-propane	11
14	PPL	a. crude b. silica aerogel	$liqC_3H_8$	*n*-butyric acid + isoamyl alcohol FAR → isoamyl butyrate	a. 50 °C, 100 bar b. 40 °C, 100 bar	a. 43% b. 70%	batch; PPL more stable in propane than in water or CO_2; immobilized PPL more efficient than crude	35
15	CVL	w/c microemulsion	$scCO_2/H_2O$	*p*-nitrophenol butyrate + water → *p*-nitrophenol + butyric acid	20 °C, 450 bar, $a_w = 10$	12.8 mL/g/s	batch; comparable result to that obtained in AOT w/o microemulsions in heptane; first example of enzyme reaction in w/c microemulsions	39
16	CCL	crude	a. $scCO_2$ b. $scSF_6$ c. scC_3H_8 d. scC_2H_6 e. scC_2H_4 f. $scCHF_3$	methylmethacrylate + 2-ethylhexanol → 2-ethyl hexylmethacrylate	45 °C, 110 bar	a. 0.05 b. 5.5 (50 °C) c. 0.5 d. 0.3 e. 0.3 f. 0.2 mM/h/ mg enz	batch	31, 79
17	CCL	crude	a. $scSF_6$ b. scC_3H_8 c. scC_2H_6 d. $scCHF_3$	methylmethacrylate + 2-ethylhexanol → 2-ethyl hexylmethacrylate	50 °C, 55 bar	a. 1.1 b. 0.2 c. 0.55 d. 0.4 mM/h	batch	51
18	HPL	crude	$scCO_2$	isoamyl alcohol + a. acetic acid → isoamyl acetate b. propionic acid → isoamyl propionate c. butyric acid → isoamyl butyrate d. octanoic acid → isoamyl octanoate	45 °C, 90 bar, 12 h, no added water	a. 4% (24 h) b. 60% c. 38% d. 77%	batch	222
19	HPL	a. crude b. L 100T c. NZ 435	$scCO_2$	isoamyl alcohol + a. acetic acid → isoamyl acetate b. octanoic acid → isoamyl octanoate c. propionic acid → isoamyl propionate	a and b. 50 °C, 150 bar c. 55 °C, 168 bar 12 h, <0.1% water	a. 30% b. 40% c. 47%	batch; comparison with solvent-free system; NZ 435 activity was nearly independent of chain length; HPL and L 100T better for short and long alkyl chain acids, respectively	223
20	CALB	CLEA	$scCO_2$	isoamyl alcohol + acetic acid → isoamyl acetate	40 °C, 110, 140, or 180 bar	0.41 mol mol^{-1} s^{-1}	batch and continuous; addition of salt hydrates had little effect on reaction rate and yield; level of conversion is highly dependent on the residence time	224

Table 4. Lipase-Catalyzed Reactions Involving Long Chain Fatty Acids or Esters ($C_{12}-C_{18}$) in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water])	yield (%) or initial rate	remarks	ref
1	CALB	immobilized on glass beads	$ncCO_2$	lauric acid + butanol → butyl laurate	40 °C, 300 bar, 21 wt % water	98%	batch; effect of temp, pressure, and humidity studied	57
2	a. CALB b. CRL	a. NZ 435 b. crude	$scCO_2$	lauric acid + D-glucose → d-glucose laurate	154 bar, 2 days a. 70 °C, $a_w = 0.75$ b. 60 °C, $a_w = 0.53$	a. 60% b. 60%	batch; LZ also studied giving much lower yields	225
3	RDL	lipid coated	$scCO_2$	lauric acid + glycerol → di- and triglycerides	40 °C, 200 bar, 3 h	>90%	batch; no catalysis by crude enzyme under comparable sc conditions; enzyme activity switches on/off by changing temp and pressure	105
4	a. CALB b. MML	MBG of: a. lecithin and HPMC b. AOT and gelatine	$scCO_2$	lauric acid + 1-propanol → 1-propyl laurate	a. 35 °C, 110 bar, 3 h b. 25.2 °C, 130 bar, 7 h	a. 36.5% b. 36.8%	batch; effect of pressure, substrate chain length, gel composition, kinetic analysis, and biocatalyst reuse studied	138
5	a. HPL b. PRL	crude	$scCO_2$	*p*-nitrophenyl laurate → *p*-nitrophenol	a. 65 °C, 83 bar, 3 h b. 50 °C, 83 bar, 3 h	a. 22.5% b. 15.3%	batch; increasing [water] decreases conversion	82
6	MML	LZ	$scCO_2$	myristic acid + ethanol → ethyl myristate	40 °C, 125 bar, 3 h, 44 mM water	100%	batch; extensive kinetics studied	226
7	MML	LZ	$scCO_2$	myristic acid + ethanol → ethyl myristate	59 °C, 125 bar, 6 h, 0.22% water	89%	continuous process	78
8	HPL	crude	$scCO_2$	myrisric acid + ethanol → ethyl myristate	45 °C, 75 bar, 3 h	37%	batch; highest conversion in $scCO_2$ as compared with MeCN or solvent-free conditions	227
9	RAL	immobilized on porous aminopropyl glass beads	$scCO_2$	myristic acid + trilaurin → 1,2(2,3)-dilauroyl-3(1)-myristoyl-*rac*-glycerol + 1,3-dimyristoyl-2-laurin	35 °C, 83−110 bar, dry CO_2	19×10^{-7} mol myristic acid incorporated/L/s	continuous; flow rate and w_o have minimal effect; increase in pressure causes an increase selectivity (transesterification vs hydrolysis)	56, 228
10	CALB	NZ 435	a. $scCO_2$ b. $scCH_4$ c. scC_2H_6	palmitic acid + octanol → octyl palmitate	a. 55 °C, 0.7 g/mL, 3 h, 7.9 mM b. 55 °C, 0.1 g/mL, 3 h, 15.8 mM c. 55 °C, 0.33 g/mL, 2 h, 31.6 mM	a. 76% b. 85% c. 80%	batch	97
11	AOL	L 100T	a. $scCO_2$ b. $scCH_4$ c. scC_2H_6	palmitic acid + octanol → octyl palmitate	a. 55 °C, 0.7 g/mL, 6 h, 15.8 mM b. 55 °C, 0.1 g/mL, 6 h, 15.8 mM c. 55 °C, 0.33 g/mL, 2 h, 39.7 mM	a. 49% b. 75% c. 65%	batch	97
12	HPL	crude	a. $scCO_2$ b. $scCH_4$ c. scC2H$_6$	palmitic acid + octanol → octyl palmitate	a. 55 °C, 0.7 g/mL, 6 h, 7.9 mM b. 45 °C, 0.1 g/mL, 6 h, 7.9 mM c. 55 °C, 0.33 g/mL, 2 h, 15.8 mM	a. 50% b. 76% c. 60%	batch	97
13	a. CALB b. AOL c. HPL	a. NZ 435 b. L100T c. crude	$scCO_2$	palmitic acid + ethanol → ethyl palmitate	55 °C, 80 bar, 6 h a. 23.6 mM b and c. 7.9 mM	a. 74% b. 44% c. 40%	batch; maximum tolerated [EtOH] = 97.3 mM; above this enzyme is deactivated	229
14	CALB	NZ 435	$scCO_2$	palmitic acid + fructose → fructose palmitate	60 °C, 100 bar, 24 h	60%	batch	230
15	RAL	crude	$scCO_2$	palmitic acid + trilaurin → 1,2-dilauryl-3-palmitoyl-*rac*-glycerol + 1,3-dipalmitoyl-2-lauryl-*rac*-glycerol (PLL)	40 °C, 90 bar, 10% v/w water	rate of PLL appearance = 50	batch; scC_2H_6 and effect of pressure also studied	45
16	CALB	NZ 435	$scCO_2$	tripalmitin + water → palmitic acid + glycerol	60 °C, 275 bar, 23.5% water	87.8%	continuous	231
17	a. CALB b. RML c. PCL	a. NZ 435 b. LZ c. immobileized on Accurel EP-100	$scCO_2$	retinyl palmitate + ethanol → retinol + ethyl palmate	60 °C, 260 bar, 75 min, 0.15 vol % water	a. 79% b. 58% c. 41%	SFE; w_o studied: NZ 435 and PCL show highest activity at low a_w (0.03%), RML at 0.09%; temp and extraction time also studied	66
18	RML	LZ	$scCO_2$	stearic acid + ethanol → ethyl stearate	50 °C, 150 bar, 30 min, 9.17 mM	0.38 M/h/g LZ	batch; increased rate on increase of pressure due to increased solubility of stearic acid	232
19	a. RDL b. RJpL c. AS d. MML	a and b. immobilized on celite c. crude d. LZ	$scCO_2$	stearic acid + triolein → fatty acids + acylglycerols	50 °C, 294 bar, 6 h, 10−12% water	a. 30% b. 32% c. 18% (3% water) d. 35%	batch; time course, w_o and reaction medium studied for both interesterification and hydrolysis; initial velocities also reported	233, 234
20	MML	LZ	$scCO_2$	stearic acid + triolein → fatty acids + acylglycerols	50 °C, 59 bar, 4.57% water	15×10^{-6} mM/min/g of LZ	batch; range of pressures tested, best reaction rate obtained near critical point	55
21	RML	LZ	$scCO_2$	ethyl stearate → stearic acid + ethanol	50 °C, 100 bar, 30 min, 31 mM water	0.58 M/h/g LZ	batch	232
22	MML	LZ	$scCO_2$	oleic acid + ethanol → ethyl oleate	40 °C, 130 bar, 80 min	100%	batch; kinetics studied and compared with those in hexane; activity was in same range in both solvents	42, 235
23	MML	LZ	$scCO_2$	oleic acid + ethanol → ethyl oleate	40 °C, 150 bar, 12 s (residence time), 10% water	100%	continuous process	236

Table 4. (Continued)

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water])	yield (%) or initial rate	remarks	ref
24	MML	LZ	$scCO_2$	oleic acid + ethanol → ethyl oleate	40 °C, 150 bar, 15 h	95%	continuous process	237
25	CCL	immobilized on Celite 545	$scCO_2$	oleic acid + 1-octanol → 1-octyl oleate	40 °C, 136 bar, 2 h, 1% v/w water	75%	batch; initial rate of reaction faster in $scCO_2$ than in organic solvents	89
26	RML	LZ	$scCO_2$	oleic acid + 1-octanol → 1-octyl oleate	50 °C, 100 bar	88%	batch and continuous processes; conditions investigated: 35−110 °C; 80−100 bar	238, 239
27	MML	LZ PT 1000L	$scCO_2$	oleic acid + oleyl alcohol → oleic acid ester	31 °C, 84.5 bar	1.428 mmol/g/h/g of enzyme	batch; higher reaction rates obtained in $scCO_2$ as compared with solvent free system	90, 91
28	MML	LZ	$scCO_2$	oleic acid + oleyl alcohol → oleic acid ester	40 °C, 150 bar, 1 L/min (CO_2 flow rate), $a_w = 0.46\%$	55%	continuous process; PT 1000L displayed lower activity than LZ	240
29	RML	a. LZ b. PT 1000L	$scCO_2$	oleic acid + oleyl alcohol → oleic acid ester	31 °C, 84.5 bar	a. 1.428 mmol/g/h b. 0.454 mmol/g/h	batch and continuous compared; various alcohol chain lengths investigated; reaction in *n*-butane studied but enzyme is deactivated	17, 68
30	RML	LZ	a. $scCO_2$ b. scC_3H_8	oleic acid + oleyl alcohol → oleic acid ester	a. 50 °C, 100 bar, 1 h b. 20 °C, 20 bar, 5 h	a. 86% b. 87.1%	batch and continuous flow; highest reaction rates observed in $scCO_2$	93
31	RML	LZ 60	$scCO_2$	oleic acid + ethanol → ethyl oleate	40 °C, 130 bar	K_m 5 mM V_{max} 650 μmol/min/g of catalyst	continuous; kinetics evaluated and competitive inhibition by ethanol observed	241
32	RML	immobilized on Accurel EP100	$scCO_2$	oleic acid + ethanol → ethyl oleate	60 °C, 180 bar, 0.9 g/L water	1600 mmol/min/g	continuous; comparison with LZ is made	103
33	RML	LZ	$scCO_2$	oleic acid + fructose (adsorbed onto silica gel) → mono-, di-, triesters	45 °C, 154 bar, $a_w = 0.48$	47%	batch; glucose and fructose also studied with lauric and palmic acid; monoesters are the dominant product	242
34	CCL	crude	scC_3H_8	oleyl oleate ester + water → oleic acid and oleyl alcohol	30 °C, 300 bar, 1.5 h	24%	high-pressure continuous flat-shape membrane reactor used	243
35	PPL	immobilized on Celite 545	$scCO_2$	triolein + water → oleic acid	40 °C, 151 bar, 120 min, 5%	28.3%	batch; various w_o and hydrolysis of partial glycerides (*rac*-1,2-diolein, 1,3-diolein, *rac*-1-monolein) also studied	244
36	RDL	AOT reverse micelles	scC_2H_6	triolein + water → oleic acid	32 °C, 200−320 bar, 30 min, $a_w = 2.78$	19%	batch	115
37	CRL	crude	$liqC_3H_8$	oleyl oleate + water → oleic acid + oleyl alcohol	35 °C, 300 bar	18%	continuous stirred tank membrane reactor	35
38	BCL	CZ L-3	$scCO_2$	various fatty acids + cholesterol or sitostanol → sterol esters	50 °C, 276 bar, 2.0 mL/min (CO_2 flow rate)	>90%	continuous; other lipases (NZ 435, CZ L-1, and LZ) screened for activity using semicontinuous process	245

Table 5. Lipase Catalysis of Reactions Involving Oils in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%)	remarks	ref
1	CALB	NZ 435	$scCO_2$	beeswax (40% long chain esters) + methanol → fatty acid methyl esters	70 °C, 275 bar	various fatty acid methyl esters: 3−51%	continuous; methanolysis also performed on jojoba oil, a mixture of TGs and waxes isolated from corn bran	246
2	MML	LZ	$scCO_2$	blackcurrant oil + water → many products, specific toward linoleic acids	30−40 °C, 104−250 bar, 55−100% saturation of CO_2 with water	100%	continuous; reaction rate decreases on increase of glycerol in enzyme bed; temp, pressure, w_o, and enzyme loading had negligible effect on hydrolysis rate	59
3	MML	LZ	$scCO_2$	canola oil + water → FFA + MG, DG, TG	35 and 55 °C, 100, 240, and 380 bar, 4 h	63−67% TG conversion	continuous	247
4	MML	LZ	$scCO_2$	canola oil + water → FFA + MG, DG, TG	35 °C, 240 bar, 0.002 mL/min water flow rate	97% TG conversion	continuous	248, 249
5	MML	LZ	$scCO_2$	canola oil + ethanol → fatty acid ethyl esters (FAE) + FFA + MG, DG, TG	55 °C, 350 bar, 360 min, 1.0% w/w EtOH	70% (FAE)	CO_2 is an extraction and reaction medium	250
6	a. PCL b. CALB c. PCL d. MML	a. sol gel b. NZ 435 c. CZ L1 d. LZ	$scCO_2$	cholesteryl stearate or phosphatidyl choline + methanol → fatty acids	50 °C, 172 bar, 80 min, 0.5% v/v MeOH	a. 45, 80% b. 98, 99% c. 98, 90% d. 96, 60%	SFE; range of commercially available lipases tested; most active examples shown	245
7	CALB	NZ 435	$scCO_2$	cod liver oil + ethanol → ethyl esters + glycerides + FFA	40 °C, 90 bar	64% ethyl esters	batch/semicontinuous; various pressures investigated and composition of product analyzed	251
8	a. HLL b. CALB	adsorbed onto methylated controlled-glass beads	$scCO_2$	cod liver oil + ethanol → fatty acid methyl esters	40 °C, 90 bar, 150 min	a. 5.84 ± 0.65 b. 2.71 ± 0.1 $\mu mol\ min^{-1}\ mg^{-1}$	batch; varying degrees of hydrophobicity for glass beads investigated; glyceride synthesis catalyzed by HLL also studied	252
9	CALB	NZ 435	$scCO_2$	corn oil + methanol → fatty acid methyl esters	50 °C, 241 bar	>98%	continuous	253
10	RML	LZ	$scCO_2$	corn oil + caprylic acid → structured lipids	55 °C, 241.3 bar, 6 h, 1% water	62.2%	batch	254

Table 5. (Continued)

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%)	remarks	ref
11	a. RML b. CALB	a. LZ b. NZ 435	$scCO_2$	palm kern oil + ethanol → fatty acid methyl esters	a. 55 °C, 136 bar, 4 h, 5% water b. 40 °C, 73 bar, 4 h, 10% water	a. 26.4% b. 63.2%	batch	255, 256
12	CALB	NZ 435	$scCO_2$	soybean oil + glycerol, 1,2-propanediol, methanol → MG, DG, TG + fatty acid methyl esters	70 °C, 276 bar, 0.7% water	MG 84% DG 15.4% TG 0.6%	continuous	257
13	CRL	crude	a. $scCO_2$ b. ncC_3H_8	soybean oil + water → fatty acid methyl esters	40 °C, 100 bar, 6 min, 1:10 w/w (lipase:water) 50 °C, 100 bar	a. very low b. 7.5%	batch	258
14	CALB	NZ 435	a. $scCO_2$ b. ncC_3H_8	soybean oil + water → fatty acid methyl esters		a. 7 mg/g min b. 9 mg/g min	batch	258
15	MML	immobilized (Novozymes)	$scCO_2$	FFA of hydrolyzed soy deodorized distillate (DOD) + butanol → fatty acid butyl esters (FABE)	36 °C, 122 bar, 3 h, 15% [enzyme] w/w	95%	batch	259
16	CRL	immobilized on zeolite	$scCO_2$	TG (soy deodorized distillate) + water	43 °C, 180 bar, 90 min, 40 wt %	94%	batch; TGs in soy deodorized distillate, removal process	260
17	ANL	L 100T	$scCO_2$	sunflower oil + water → oleic acid + linoleic acid	50 °C, 200 bar, 48 h, oil:buffer ratio 1:1	0.193 g oleic acid/g oil phase 0.586 g linoleic acid/g oil phase	batch and high-pressure continuous flat-shape membrane reactor used; thermodynamic and kinetic properties of lipase established	67, 261
18	ANL	L 100T	$scCO_2$	sunflower oil + water → FFA	50 °C, 200 bar, 1 h	50%	high-pressure continuous flat-shape membrane reactor	243
19	MML	LZ	$scCO_2$	triolein + ethylbehenate → 1,3-dibehenoyl-2-oleoyl glycerol (BOB)	50 °C, 150 bar	37 mol % BOB	batch	262, 263
20	CALB	NZ 435	$scCO_2$	triolein + ethyl 4-hydroxy-3-methoxy cinnamate → monoferuloyl-monooleoyl-glycerol + feruloyldioleoyl-glycerol	80 °C, 241 bar, 48 h	74%	batch; higher yields and shorter reaction times than in toluene	264
21	CALB	NZ 435	$scCO_2$	1-octanol + ethyl 4-hydroxy-3-methoxy cinnamate → octyl ferulate	80 °C, 138 bar, 24 h	53%	batch	264

Table 6. Lipase-Catalyzed Stereospecific Reactions Involving *rac*-1-Phenylethanol in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%) or initial rate	stereoselectivity (ee_p unless otherwise stated)	remarks	ref
1	CALB	crude	$scCO_2$ + $[BMIM^+][BTA^-]$	*rac*-1-phenylethanol + vinyl propionate → (*R*)-1-phenylethyl propionate	50 °C, 150 bar, <4% v/v water	1.7 ± 0.2 U/mg enzyme	>99.9%	continuous	204
2	CALB	a. crude (NZ 525L) b. NZ 435	$scCO_2$ + $[EMIM^+][BTA^-]$	*rac*-1-phenylethanol + vinyl propionate → (*R*)-1-phenylethyl propionate	120 °C, 100 bar, 4 h	a. 0.6 ± 0.02 U/mg b. 9.1 ± 0.3 U/g	a. >99.9% b. >99.9%	continuous biphasic CO_2 flow over IL/lipase phase	206
3	CALB	NZ 435	$scCO_2$	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	95 °C, 150 bar, 3 h	48%	>99%	batch; esterification of ibuprofen also studied, lower *ee* observed; excellent thermostability of enzyme	65
4	CALB	NZ 435	$scCO_2$	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	40 °C, 90 bar, 7 h	48%	ee_p 99.8% ee_s 90.6% E > 100	batch	9
5	CALB	NZ 435	$scCO_2$	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	42−43 °C, 130 bar, 7 h	47%	ee_p 99.7% ee_s 89.6% E = 1850	continuous process; pressure change has little effect	9
6	CALB	ChiroCLECTM	$scCO_2$	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	40 °C, 90 bar, 60 min, 0.05 g/L	100%	>99%	batch and continuous; catalytic turnover number (k_{cat}) = 0.95 s^{-1}	12
7	PCL	immobilized on functionalized silica gel	a. $scCO_2$ b. $scSF_6$	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	a. 50 °C, 200 bar, 350 min b. 50 °C, 200 bar, 130 min	a. 46% b. 50%	a. >99% b. >99%	batch	265
8	PFrL	lipid coated	$scCHF_3$	*rac*-1-phenylethanol + lauric acid → (*R*)-1-phenyl ethyl laurate	40 °C, 60 bar, 40 h	60%		batch; activity controlled by changing temp and pressure	108

Table 7. Other Sterospecific Esterification Reactions Catalyzed by Lipases in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%) or initial rate	stereoselectivity (ee_p unless otherwise stated)	remarks	ref
1	CCL	immobilized on aminopropyl glass beads	$scCO_2$	(±)-citronellol + oleic acid → (3)-(−)-oleic acid 3,7-dimethyl-6-octenyl ester	31.1 °C, 84.1 bar	3.6%	98.9%	continuous flow; stereoselectivity not observed in cyclohexane, e.e. highest nc point	52
2	PPL	immobilized	$scCO_2$	*rac*-glycidol + butyric acid → (*S*) glycidyl butyrate	35 °C, 140 bar, 10−15 h, 20−25% water	25−30%	83 ± 2%	batch; a series of supports for enzymes were screened	266, 267
3	MML	LZ	$scCO_2$	*rac*-ibuprofen + *n*-propanol → (*S*)-*n*-propyl ester of ibuprofen	50 °C, 100 bar, 23 h, 0.54 mL/L	75%	70% (at 15% conversion; 150 bar)	batch; reaction rates similar in hexane and $scCO_2$	100

Table 7. (Continued)

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%) or initial rate	stereoselectivity (ee_p unless otherwise stated)	remarks	ref
4	CCL	immobilized on glass beads	$scCO_2$	(±)-citronellol + *n*-valeric acid → (*S*)-(−) *n*-valeric acid 3,7-dimethyl-6-octenyl ester	35 °C, 75.5 bar	282.1 μmol/h/g enzyme	98.9% (at 84 bar)	continuous process; first report of pressure dependence of enzyme activity	268, 269
5	PCL	crude	$scCO_2$	*rac*-3-hydroxyoctanoic acid methyl ester + VA → (*S*)-3-hydroxyoctanoic acid methyl ester acetate	50 °C, 110 bar, 71 h	62.8%	ee_s 77.8% ee_p 46.1%	batch; on-line analysis	270
6	PCL	immobilized on celite or VA epoxy	$scCO_2$	*rac*-3-hydroxyoctanoic acid methyl ester + VA → (*S*)-3-hydroxyoctanoic acid methyl ester acetate	40 °C, 120 bar, 120 h	85%	ee_s 98% ee_p 18%	batch; effect of cosolvent, water, pressure, and temp investigated	69
7	PS	immobilized on ACR-silica gel	$scCO_2$	*rac*-1-(4-bromophenyl) ethanol + acetic anhydride → 1-(4-bromophenyl)ethyl acetate	40 °C, 200 bar, 360 min	55%	96%	batch; conversion and *ee* higher in $scCO_2$ as compared with organic solvents; other substrates also studied	94
8	CRL	lipase AY30	$scCO_2$	(±)-menthol + isopropenyl acetate → menthol acetate + acetone	50 °C, 100 bar	4.3 μmol h^{-1} g^{-1} enzyme	$E = 70$	batch; four lipases and esterase EP10 tested; menthol and citronellol tested for transesterification with various acylating reagents; poor stereoselectivity with (±)-citronellol	271
9	PCL	immobilized on ACR-silica gel	$scCO_2$	*rac*-1-(4-*tert*-butylphenyl) ethanol + acetic anhydride → (*S*)-1-(4-*tert*-butylphenyl) ethyl acetate	40 °C, 200 bar, 6 h	50%	99%	batch; many other substrates tested with excellent conversions and *ee*, all higher than in organic solvents	272
10	CRL	crude	$scCO_2$	*rac*-naproxen + *n*-hexanol → (*S*)-naproxen ester	44 °C, 100 bar	none given	$E = 8$	continuous; other alcohols studied: ethanol deactivates CRL	273
11	CRL	immobilized on silica gel	$scCO_2$	*rac*-*trans*-2-phenyl-1-cyclohexanol + VA → (1*R*,2*S*)-*trans*-2-phenyl-1-cyclohexyl acetate	40 °C, 400 bar, 24 h	48%	100%	batch; rate of reaction higher than in hexane	274
12	CALB	NZ 435	$scCO_2$	*rac*-1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol + VA → (*S*)-1-(*p*-chlorophenyl)-2,2,2-trifluoro-ethyl acetate	55 °C, 100 bar, 5 h	50%	$E = 50$ (80 bar, 2 h)	batch; decreasing pressure increased E value from 10 to 50	47
13	CALB	NZ 435	$scCO_2$	*rac*-1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol + VA → *rac*-1-(*p*-chlorophenyl)-2,2,2-trifluoro-ethylacetate	55 °C, 100 bar, 6 h	55%	$E = 60$ (90 bar)	batch	48
14	CALB	NZ 435	$scCO_2$	*rac*-3-methyl-2-butanol + vinyl octanoate → (*R*)-3-methyl-2-butyl octanoate	40 °C, 214 bar	-	$E = 325$	batch; lower degree of enantio-selectivity observed in $scCO_2$ as compared with organic solvents	275
15	MML	LZ	$scCO_2$	*rac*-1-acetoxy-1-(benzofuran-2-yl)-ethane + ethanol → (*R*)-1-acetoxy-1-(benzofuran-2-yl)-ethanol	38 °C, 120 bar, 4 h	11%	ee_p 1.6% ee_s < 1% $E = 1.3$	batch; other nucleophiles investigated; reaction occurs more efficiently under solventless conditions or in hexane	99
16	CALB	CZ L-2	$scCO_2$	2-benzyl-1,3-propanediace-tate + methanol → 2-benzyl-1,3-propane monoacetate	40 °C, 100 bar, 3 h	16	49%	batch; enantioselectivity not observed in organic media; suggested that a conformational change in enzyme due to carbamate formation gives good *ee*	34
17	CALB	crude	$scCO_2$ + [BMIM] [NTf_2]	1-butanol + vinyl butyrate → butyl butyrate	100 °C, 150 bar, <4% v/v water	71 ± 3.9 U/mg enzyme	99 ± 0.9%	continuous	204
18	CALB	NZ 435	$scCO_2$	1-butanol + butyl vinyl ester → butyl butyrate	a. 50 °C, 90 bar, 3 h b. 50 °C, 80 bar, 6 h	a. 100%, 2.55 U/mg b. 100%, 1.38 U/mg	a. >99% b. 99%	a. batch b. continuous membrane reactor	276
19	a and b. CALB c and d. MML	a. crude (NZ 525L) b. NZ 435 c. crude (NZ 388L) d. LZ	$scCO_2$ + [EMIM] [NTf_2]	*rac*-glycidol + a. VA b. vinyl butyrate c. VA d. vinyl butyrate → glycidyl esters	a. 40 °C, 100 bar b. 50 °C, 150 bar c. 40 °C, 100 bar d. 50 °C, 150 bar	a. 153.7 U/mg b. 50.1 U/g c. 13.5 U/mg d. 17.5 U/g	*S*/*R* ratios a. (*S*) 3.0 b. (*S*) 2.9 c. (*R*) 2.6 d. (*R*) 2.6	continuous biphasic CO_2 flow over IL/lipase phase	277
20	CALB	NZ 435	$scCO_2$	*p*-chiral hydroxy-methane-phosphinate + VA → *p*-chiral hydroxymethanephosphinate acetate	40 °C, 130 bar, 2 h	46%	27% $E = 3$	batch; no reaction below 80 bar; range of enzymes tested (AK, LPL LZ, PS-C), effect of *p*, reaction time, and substrate specificity studied	
21	PFL	lipase AK	$scCO_2$	3-benzyloxypropane-1,2-diol + VA → mono acetate, diacetate, cyclic acetals	38 °C, 120 bar, 270 min, humid $scCO_2$	84.7%	71.6% (diacetate)	batch; water does not significantly influence productivity or selectivity of reaction; other lipases also screened	
22	CALB	a. NZ 435 b. CLEA	$scCO_2$	*rac*-*p*-bromo-1-phenylethanol + VA → (*R*)-*p*-bromo-1-phenyl-ethyl acetate	40 °C, 90 bar, 2 h	a. 48% b. 48%	a. 99.8% E > 1000 b. >99.9% E > 1000	batch; first use of CLEAs in $scCO_2$; various 1-arylethanols also studied	
23	CALB	NZ 435	$scCO_2$	ethylene glycol + ethyl acetate → ethylene glycol mono- and diacetate (EGMA and ethylene glycol diactetate, EGDA)	50 °C, 100 bar	65%	89.7% (ethylene glycol monoacetate, EGMA)10.3% (EGDA)	batch; conversion higher than in absence of CO_2	

Table 8. Sterospecific Hydrolysis Reactions Performed in SCFs and Catalyzed by Lipases

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%) or initial rate	stereoselectivity (ee_p unless otherwise stated)	remarks	ref
1	MML	LZ 20	$scCO_2$	*rac*-3-(4-methoxyphenyl)-glycidic acid methyl ester + water → (2*R*,3*S*)-3-(4-methoxyphenyl)glycidic acid + methanol	60 °C, 130 bar, 5 h, 3 mL/L	53%	87%	batch; faster rate in CO_2 than toluene/water mixture	95
2	HLL	lipolase	$scCO_2$	*bicyclo*[3.2.0]heptanol esters + water → hydroxyesters	35 °C, 80 bar, 20 h, 0.5% water	35−40%	ee_p 68−92% ee_s 50−67%	batch	279
3	a. CVL b. PPL c. PCL	crude	$scCO_2$/ buffer biphasic system	*rac*-3-hydroxy-5-phenyl-4-pentanoicacidethylester + buffer → (*R*)-3-hydroxy-5-phenyl-4-pentanoicacid	40 °C, 110 bar a. 22.4 h b. 165 h c. 15.5 h	a. 50% b. 13% c. 50%	a. 65%; $E = 11$ b. 90%; $E = 21$ c. 83%, $E = 40$	batch; 15 lipases and one esterase screened for highest enantioselectivity; PCL was found to be the best enzyme	53

Table 9. Lipase-Catalyzed Polymerizations and Depolymerizations in SCFs

entry	species	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%) or mw	remarks	ref
1	PPL	crude	$scCHF_3$	*bis*-(2,2,2-trichloroethyl) adipate + 1,4-butanediol → poly(1,4-butylene adipate)	50 °C, 372 bar	average mw 1340	SFE system; range of pressures studied (70−372 bar)	168
2	CALB	NZ 435	$scCO_2$	divinyl adipate + octafluorooctandiol → fluorinated polyester	50 °C, 200 bar, 24 h	mw 8232	batch	169
3	CALB	crude	$scCO_2$	polyanhydride + glycol → polyester	60 °C, 80−140 bar, 24 h	80%	batch; various substrates tested	280
4	CALB	NZ 435	$scCO_2$	dicaprolactone → polycaprolactone	70 °C, 80 bar, 6 h, no additional water	82%	batch	180
5	CALB	NZ 435	$scCO_2$	ϵ-caprolactone → polycaprolactone	35−65 °C, 81−241 bar, 6−72 h	38−98% mw 23000−37000	batch	172
6	CALB	NZ 435	$scCO_2$	block copolymer formation: ROP of ϵ-caprolactone + ATRP of methyl methacrylate	35 °C, 103 bar, 20 h	60% mw 41000	batch; chemoenzymatic catalysis; ϵ-caprolactone used as cosolvent and monomer, allows control of ATRP	175
7	CALB	NZ 435	$scCO_2$	ϵ-caprolactone + MMA + HEMA → p(MMA-*co*-HEMA)-β-pCL (graft copolymer)	35 °C, 103 bar	47−76% yield depending on MMA/HEMA/CL feed ratio	batch; combination of enzyme initiated ROP + ATRP; two step, one pot synthesis	178
	CALB	NZ 435	$scCO_2$	ϵ-caprolactone + → pFOMA-β-pCL (diblock copolymer)	45 °C, 110 bar, 45/55 pFOMA/pCL	57%	batch; combination of enzyme initiated ROP + ATRP; two step, one pot synthesis	281
8	CALB	NZ 435	$scCO_2$	polycaprolactone → cyclic and linear oligomers	40 °C, 80 bar, 16 h, 200% water w.r.t. polycaprolactone	100% mw <500	adjust water concentration to control cyclic or linear oligomers	181
9	CALB	Crude	$scCO_2$	polycaprolactone → ϵ-caprolactone	60 °C, 80 bar, 24 h	mw 1900 (lowest)	addition of acetone (0.5 mL) gives oligomer mw 710; increase degradation by increasing [enzyme]	282
10	CALB	NZ 435	$scCO_2$	polycaprolactone → ϵ-caprolactone	40 °C, 180 bar, 6 h, water 50 wt % w.r.t. polycaprolactone	90%	batch	180
11	CALB	NZ 435	$scCO_2$/ subcritical CO_2 + toluene	degradation of: poly(*R*,*S*-3-hydroxybutanoate, polycaprolactone, and poly(butylene adipate) to cyclic oligomers	40 °C, 150 bar, 80% CO_2 20% toluene	>99.9%	chemical recycling; flow rates and $scCO_2$ content studied; pressure and [polymer] not important	182
12	a. MML b. PCL	unknown	$scCO_2$	polycaprolactone → ϵ-caprolactone	60 °C, 80 bar, 24 h, 50 mg polycaprolactone, 0.5 mL acetone	mw 1700−2000	addition of a very small amount of water is also effective	282

Table 10. Esterification and Glycosylation Reactions Catalyzed by Nonlipase Enzymes in SCFs

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%), intial rate, kinetic parameter and/ or selectivity	remarks	ref
1	β-D-galactosidase (*Bacillus circulans*)	lipid coated	$scCO_2$	1-*O*-*p*-nitrophenyl-β-D-galactopyranoside + 5-phenylpentan-1-ol → 5-phenylpentyl-β-D-galactosidase + *p*-nitrophenol	40 °C, 100−150 bar, 3 h	72%	reaction is 15-fold faster than in *iso*-propanol; also reaction with cholesterol β-galactoside (15%); suspended enzyme is inactive	106
2	β-D-galactosidase (*B. circulans*)	lipid coated	$scCHF_3$	1-*O*-*p*-nitrophenyl-β-D-galactopyranoside + 5-phenylpentan-1-ol → 5-phenylpentyl-β-D-galactosidase + *p*-nitrophenol	37 °C, 60 bar, 5 h	>90%	slow reaction below 48 bar or above 100 bar	107
3	CMT (bovine pancreas)	immobilized	$scCO_2$			first-order kinetic behavior	fluid density a key parameter for enzyme stability; pressurization/depressurization cycles and w_o increase loss of activity	283

Table 10. (Continued)

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%), intial rate, kinetic parameter and/or selectivity	remarks	ref
4	CMT (bovine pancreas)		$scCO_2$/MeCN	*N*-acetyl-L-tyrosine ethyl ester + AA amides	5 h	91%		284
5	CMT (bovine pancreas)	PEGylated	$scCO_2$/[BMIM][PF_6]	APEE + 1-propanol → APPE + ethanol	0.5% v/v water		the combination of $scCO_2$ and an IL was more efficient than the IL on its own	195, 285
6	CMT (bovine pancreas)	lipid coated (L-gluamic acid dialkyl ribitol amide)	a. $liqCO_2$ b. $scCO_2$	APEE + hydrochlorides of L-glycinamide and L-leucinamide → dipeptides	a. 35.2 °C, 61 bar b. 60.2 °C, 101 bar, 12 h, 4% water	a. 50% b. 80%	surfactants AOT and span 60 also investigated; reaction faster in $scCO_2$	285
7	cutinase (*Fusarium solani pisi*)	immobilized on Accurel EP100	$scCO_2$	hexanoic acid + hexanol → hexyl hexanoate	45 °C, 130 bar, 5 days, $a_w = 0.76$	29%	enzyme lost 10% activity over 6 days; salt hydrates used to control a_w	286
8	cutinase (*F. solani pisi*)	immobilized on zeolite	a. scC_2H_4 b. $scCO_2$	*rac*-1-phenylethanol + vinyl butyrate → (*R*)-1-phenylethyl butyrate	a. 15 °C, 80 bar, a_w 1.0 b. 35 °C, 80−250 bar, $a_w = 0.6$	V_{max}/K_m a. 0.02 b. 0.03 M/min	salt hydrate used to control a_w ee_p ~100% for both solvents	287
9	esterase	EP10	$scCO_2$	(±)-menthol + isopropenyl acetate → menthol acetate + acetone	50 °C, 100 bar	14 μmol h^{-1} g^{-1} enzyme $E = 180$	batch; four lipases and esterase EP10 tested; menthol and citronellol tested for transesterification with various acylating reagents	288, 289
10	Sub (*Bacillus licheniformis*)	alcalase	$scCO_2$	resolution of *N*-protected AA derivatives	35 °C, 80 bar, 20 h, 0.5% water	98% (L-isomer) 47% conversion L-selective >99%		290
11	Sub (*B. licheniformis*)	crude (lyophilized from pH 7.8)	$scCO_2$	*N*-acetyl-L-phenylalanine chloroethyl ester + ethanol → APEE + chloroethanol	45 °C, 150 bar, 15 min, [ethanol] 0.5−5% (v/v)	10−54%	T and CO_2/ethanol ratios investigated; faster in $scCO_2$ than anhydrous organic solvents	291
12	a. Sub (*B. licheniformis*) b. protease (*Aspergillus*)	crude	$scCHF_3$	*N*-acetyl-D/L-phenylalanine ethyl ester + methanol → *N*-acetyl-D/L-phenylalanine methyl ester + ethanol	50 °C, 69 bar	k_{cat}/K_m (mM^{-1} s^{-1}) a. 0.06 b. 0.032	decrease in activity with increasing pressure in fluoroform, no change in propane; subtilisin selective for L-ester, little selectivity for protease	50
13	Sub (*B. licheniformis*)	crude	a. $scCHF_3$ b. ncC_3H_8	APEE + methanol → *N*-acetyl-L-phenylalanine methyl ester + ethanol	50 °C a. 124 bar b. 69−345 bar constant a_w	a. 1.5 mM/h b. 4 mM/h	increasing pressure decreases initial rate in $scCHF_3$, but no effect observed in ncC_3H_8; pH and w_o controlled using salt hydrate	292
14	Sub (*B. licheniformis*)	CLEC	a. $scCO_2$ b. scC_2H_6	APEE + 1-propanol → APPE + ethanol	40 °C, 100 bar a. a_w 0.01 b. a_w 0.3	a. 50 b. 580 nmol/min/mg	$scCO_2$ inhibits activity of subtilisin CLECs probably due to carbonic acid formation; CLECs superior to crude enzyme	85, 293
15	Sub (*B. licheniformis*)	crude (lyophilized from pH 7.8)	$scCO_2$	*N*-acetyl-D/L-phenylalanine methyl ester + ethanol → APEE + methanol	47 °C, 114 bar, 1 h, 0.74 M water	90% ee_p > 99%	other substrates, alcohol concentration and w_o also investigated	294
16	xylanase (*Aureobasidium pullulans*)	acetone-dried cells	$scCO_2$	xylan + *n*-octanol → 1-octyl β-D-xylotrioside and xylobioside	65 °C, 147 bar, 16 h, 0.1 M acetate buffer (pH 4.0)	52%	without CO_2 1-octyl-β-D-xylotrioside hardly produced conversion is better than using resting cells	156, 157

Table 11. Hydrolysis Reactions Catalyzed by Nonlipase Enzymes in SCFs

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (T, p, time, [water]	yield (%)	remarks	ref
1	α-amylase (*B. licheniformis*)	crude	$scCO_2$	corn starch + water → glucose	35 °C, 101 bar		reaction rate enhanced at higher temp and pressure	295
2	alkaline phosphatase	crude	$scCO_2$	disodium *p*-nitrophenyl phosphase + water → *p*-nitrophenyl-	35 °C, 101 bar, 0.1%	71%	first reaction in CO_2 (batch); reaction rate limited by rate of dissolution of substrate in CO_2	296
3	cellulase (Logan Corp., Ottawa)	crude	$scCO_2$	avicel (cellulose material) + water → glucose	46 °C, 137 bar, 2% w/w water	70%	>90% active after 5 days; reaction rate increased in CO_2	297
4	cellulase		$scCO_2$	cellulose + water → glucose	50 °C, <160 bar, 90 min	100%		298
5	cellulases (various sources)		$scCO_2$	boll fibers of cotton + water → glucose	50 °C, 160 bar, 48 h		yield is 1.2 times that of reaction conducted at atmospheric pressure	299
6	cellulase (*Humicola insolens*)	immobilized on ceramic membrane	$scCO_2$	carboxymethyl cellulose + water	45 °C, 100 bar, up to 9 h		high-pressure continuous enzymatic tubular membrane reactor (HP CETMR)	243
7	cellulase		scH_2O pretreatment	cellulose + water → glucose + cellbiose	380 °C, 250−330 bar, 0.1−0.4 s	74−84%	substrate is processed 13 times faster than non-scH_2O treated cellulose	300

Table 11. (Continued)

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (*T*, *p*, time, [water]	yield (%)	remarks	ref
8	isoamylase	free	$scCO_2$, buffer biphasic system	amylopectin + water	40 °C, 103 bar, 60 min, initial pH 4.25	absorbance (represents amount of product formation) 3.6	batch; initial pH, pressure, agitation rate and % starch investigated	301
9	papain	latex (Promod 144P) (Biocatalysts, U.K.)	$scCO_2$/ phosphate buffer pH 7.0	casein hydrolysis	35 °C, 200 bar	96%	reaction monitored by UV; lower reaction at 50 °C and 300 bar	302

Table 12. Oxidation and Carboxylation Reactions Catalyzed by Nonlipase Enzymes in SCFs

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (*T*, *p*, time, [water]	yield (%) or intial rate or kinetic parameter	remarks	ref
1	carboxylase (*Bacillus megaterium*)	whole cells	$scCO_2$	pyrrole + CO_2 → pyrrole-2-carboxylate	40 °C, 100 bar, 1 h, $KHCO_3$, NH_4OAc in phosphate buffer	59%	yield is 12 times higher than at atmospheric pressure; effect of pressure investigated	151
2	cholesterol oxidase (*Gloeocysticum chrysocreas*)	immobilized on glass beads	$scCO_2$ + 2% v/v *t*BuOH	cholesterol + O_2 → cholest-4-ene-3-one + H_2O_2	35 °C, 100 bar trace amount of water	k_{cat} 75−274 s^{-1}	cholesterol oxidase (*Streptomyces* sp., *Nocardia* sp., *Pseudomonas* sp.) also tested and found not to be stable	77, 303
3	cholesterol oxidase (*Pseudomonas fluorescens*)	reverse micelles of PFPE	$scCO_2$	cholesterol + O_2 → cholest-4-ene-3-one + H_2O_2	35 °C, 150 bar, 1 M buffer (pH 7)	$k_{cat,app}$ 3.1 s^{-1}	optimum activity when H_2O:PFPE >12; enzyme inactivated after 5 h	14
4	L-AA oxidase (snake venom *Crotalus adamanteus*)		$scCO_2$	3,4-dihydroxyphenyl-L-alanine (L-DOPA) + O_2 f 3,4-dihydroxyphenyl pyruvate + NH_3 + H_2O_2	40 °C, 110 bar, 3 h	1.47 mmol U^{-1}	L-AAO more stable in $scCO_2$ than in phosphate buffer; reaction includes catalase to destroy peroxide	304
5	lipoxygenase	w/c microemulsion	CO_2	linoleic acid + O_2 → 13-hydroxyperoxyocta-decadienoic acid	20 °C, 450 bar, $a_w = 10$	$K_{m,app}$ 1.4 mM $V_{max,app}$ 8.0 M s^{-1} g^{-1} mL	batch; result comparable to that obtained in AOT w/o microemulsions in heptane; first example of enzyme reaction in w/c microemulsions	39
6	lipoxygenase-1 (soybean)	immobilized	$scCO_2$	linoleic acid + O_2 → 13*S*-hydroperoxyocta-decadienoic acid (13*S*-HPODE)	33 °C, 250 bar	83%	only 67% yield in aqueous media	305
7	polyphenol oxidase (mushroom)	immobilized on glass beads	a. $scCO_2$ b. $scCHF_3$	*p*-cresol or *p*-chlorophenyl + O_2 f *o*-benzoquinone	a. 36 °C b. 34 °C 340 bar, 40 min or 1 L/min flow rate	a. 15−20% b. 70%	batch and continuous	8
8	phenylphosphate carboxylase (*Thauera aromatica*)	whole cells	$scCO_2$	phenol + CO_2 → 4-hydroxybenzoic acid	30 °C, 200 bar, 4.5 h, water solution	4%	avoids the need for sodium bicarbonate that is required when water is used as a solvent	155
9	alcohol dehydrogenase (*Geotrichum candidum*)	immobilized cells	$scCO_2$	*o*-fluoroacetophenone + 2-propanol f (*S*)-1-(*o*-fluorophenyl)-ethanol + propanone	35 °C, 100 bar, 12 h	81% ee_p > 99%	the stereoselective reduction of six acetophenones, benzyl acetone and cyclohexanone is reported	158,306

Table 13. Biocatalysis in Carbon Dioxide and a Second Neoteric Solvent

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (*T*, time, other)	yield (%)	remarks	ref
1	CMT	lyophilized from phosphate buffer in the presence of PEG	$scCO_2$/ $[BMIM^+]$ $[PF_6^-]$ or $[OMIM^+]$ $[PF_6^-]$	APEE + 1-propanol → *N*-acetyl-L-phenyl-alanine propyl ester + ethanol	45 °C, 138 bar, 24 h	40.7% (/$[BMIM^+][PF_6^-]$) 20.3% ($[OMIM^+][PF^{6-}]$)	$scCO_2$ was not used to extract	194, 307
2	CALB	on glass wool	$scCO_2$/ $[EMIM^+]$ $[BTA^-]$ or $[BMIM^+]$ $[BTA^-]$	vinyl butyrate + 1-butanol → *n*-butyl butyrate + acetaldehyde	40−100 °C, 150 bar			204, 206
3	CALB	immobilized on Celite	$scCO_2$/ $[EMIM^+]$ $[BTA^-]$ or $[BMIM^+]$ $[BTA^-]$	*rac*-1-phenylethanol + vinyl propionate → (*R*)-1-phenyl ethyl propionate	up to 150 °C and 100 bar	99.9% ee_p	2000 times more stable than the enzyme in hexane under the same conditions; a further 5 ILs have been investigated	204, 206−208
4	CALB	α-alumina with PEI and glutaraldehyde	$scCO_2$/ $[BMIM^+]$ $[PF_6]$ or $[BDiM^+]$ $[PF_6]$ or $[OMIM^+]$ $[PF_6^-]$	1-butanol + vinyl propionate → *n*-butyl propionate + acetaldehyde	50 °C, 20 bar		rate of tranesterification reaction was lower with $scCO_2$/IL systems than $scCO_2$ alone	209, 210
5	CALB		$scCO_2$/ $[BMIM^+]$ $[BTA^-]$	1-octanol + VA → *n*-octyl acetate + acetaldehyde	39 °C, 95 bar, 0.5 h	92%	batch and continuous reaction; the $scCO_2$ was added AFTER the reaction to extract the ester product from the IL	205

Table 13. (Continued)

entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (T, time, other)	yield (%)	remarks	ref
6	CALB		$scCO_2$/[BMIM$^+$][BTA$^-$]	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	15 min then add $scCO_2$ at 40 °C and 110 bar	quantitative conversion of the (*R*)-alcohol, 98.7% *ee*	use of NZ 435 or a sol gel immobilized enzyme less effective	212
7	CALB		$scCO_2$/PEG (mw 1500)	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	50 °C, 150 bar, 5 h	quantitative conversion of the (*R*)-alcohol, *ee* > 98.1	at 50 °C/80 bar considerably more (73%) of the (*R*)-ester was extracted from the PEG system than the IL (56%)	213
8	CALB		CO_2/40:60 dioxane: phosphate buffer	2-phenethyl acetate → 2-phenethanol + acetic acid	25 °C, 50 bar, 2 h	55%	CO_2 at 50 bar used to separate the miscible aqueous and dioxane phases; hydrolysis occurs ifreaction is left longer	215
9	cutinase from *F. solani pisi*	immobilized on zeolite NaY	$scCO_2$/[BMIM$^+$][PF_6^-]	2-phenethyl-1-propanol + vinyl butyrate → 2-phenethyl-1-propanol butyrate + acetaldehyde	35 °C, 100 bar, $a_w = 0.12$	130 nmol^{-1} min^{-1} mg^{-1}	continuous reaction $E = 1.5-2.5$	25
10	CALB	NZ 435	$scCO_2$/[BMIM$^+$][PF_6^-]	2-phenethyl-1-propanol + vinyl butyrate → 2-phenethyl-1-propanol butyrate + acetaldehyde	35 °C, 100 bar, $a_w = 0.2$	412 nmol min^{-1} mg^{-1}	continuous reaction $E = 2-3$	25
11	CALB	NZ-525L immobilized on butyl silica	[BTMA$^+$][BTA]	*rac*-1-phenylethanol + vinyl propionate → (*R*)-1-phenylethyl propionate	50 °C, 100 bar, $a_w = 0.90$	>99.9% ee_p 6× higher rate cf. hexane/IL	11 other alkyl modified silicas investigated as well as [TOMA$^+$][BTA$^-$]	308

patent literature. Lipases are the most popular choice for enzymatic reactions studied in SCFs; therefore, these are grouped together at the beginning of section 8. Tables 3−5 are focused on esterifications. Table 3 lists reactions involving short chain acids and esters, with reactions sorted by acid substrate chain length, starting with the shortest chain first (acetic acid) and ending with the longest chain molecule (octanoic acid). Table 4 lists reactions involving longer chain fatty acids, again sorted with the shortest fatty acid first (lauric acid) and ending with the longest fatty acids (stearic and oleic acid). Table 5 focuses on the esterification of oils with the reactions listed alphabetically by type of oil.

Tables 6−8 summarize all stereospecific reactions. The substrate *rac*-1-phenylethanol is one of the most popular substrates for study, so these reactions have been grouped in Table 6 and all other stereospecific esterifications are listed in Table 7. Table 8 completes the set with the remaining hydrolysis reactions performed in SCFs. Finally, Table 9 lists all of the polymerization and depolymerization reactions performed in SCFs with lipases.

Tables 10−12 list all of the other non-lipase enzyme reactions carried out in SCFs. These reactions are grouped as follows: Table 10 lists esterification and glycosylation reactions, Table 11 lists hydrolysis reactions, and Table 12 lists oxidation, reduction, and carboxylation reactions. The reaction conditions, rates, and percentage conversions are given where they have been provided in the paper cited. If a wide range of substrates is tested with an enzyme, typically the data for the "best" substrate are presented and other substrates are listed in the remarks section. Earlier versions of this tabulation first appeared in the theses of Helen M. Kirke[216] and Helen R. Hobbs[217] at the University of Nottingham, United Kingdom.

9. Biocatalysis in Fluorous Solvents

The number of reports regarding biocatalysis involving fluorous solvents is limited to just a handful of publications, partly due to the insolubility of enzymes in fluorous solvents and also to a lack of examples of enzyme substrates that are soluble in fluorous solvents. Recently, however, there have been a variety of attempts to exploit some of the properties of fluorous solvents in combination with the benefits of using enzymes for catalysis to develop cleaner synthetic processes, and these are discussed below.

9.1. Basic Properties of Fluorous Solvents

Fluorous solvents have been produced for commercial use in a range of roles: low boiling point compounds as alternatives to chlorofluorohydrocarbons (CFCs) as refrigerants (R-134, R-227ea); greases and lubricants based on polyfluoropolyethers that operate at >300 °C; and compounds such as 1-bromoperfluorooctane that are used as a component of artificial blood. Because the intermolecular reactions in a fluorous solvent are very weak, the *n*-perfluoroalkanes have lower boiling points than their corresponding *n*-alkanes and exhibit extremely low polarities. The solubility of water in fluorous solvents is, therefore, very low due to the lack of suitable H-bonding interactions, while gases such as carbon dioxide, oxygen, and hydrogen are much more soluble in fluorous solvents than in water and exhibit several fold better solubility than in hydrophobic organic solvents.[309] Table 14 summarizes the physical properties of the fluorous solvents that have been used in processes involving enzymes.

The cheapest commonly available perfluorinated solvent is perfluorohexane, also known as FC-72. This is composed of an undefined mixture of isomers in a similar manner to the "hexanes" used in conventional synthesis. Therefore, many chemical reactions reported to date make use of perfluoromethylcyclohexane (PFMC), a more expensive alternative, but one that ensures improved reproducibility.

From the synthetic chemistry viewpoint, perhaps the most useful general property of fluorous solvents is their temperature-dependent miscibility with organic solvents. This behavior is an example of a thermomorphic effect, which manifests itself in many different forms. In the context of synthesis, this property can be used to switch a reaction that is heterogeneous into homogeneous with the mass transfer benefits that this can accrue. This property has been exploited widely in synthetic chemistry in the form of fluorous biphasic catalysis.[311,312] The miscibilities of fluorous and nonfluorous solvents as a function of temperature is an area that has not been extensively reported.[310] Table 15 summarizes the upper "critical" or consolute temperatures for a range of solvents mixed in a 1:1 ratio. Above this temperature, the two solvents

Table 14. Basic Data on the Fluorous Solvents Used in Biocatalysis[a]

solvent	formula	bp (°C)	mp (°C)	density (g/cm^3)	code name	ClogP
perfluorohexane	C_6H_{14}	57.1	−90	1.669	FC-72	5.653
perfluoroheptane	C_7F_{16}	82.4	−78	1.745		6.542
perfluorooctane	C_8F_{18}	97−104	−25	1.766	FC-77 FC-3255	7.432
PFMC	$CF_3C_6F_{11}$	76.1	−37	1.784	PFMC	5.166
PFPE	$F(CF_2O)CF_3$ mw ∼350	57	-	1.65	HT55	
1,1,1,2-tetrafluoroethane	F_3CCH_2F	−26.1	−103.3	0.0425 (gas phase)	R-134a	0.44
1,1,1,2,3,3,3-heptafluoropropane	$F_3CCHFCF_3$	−16.4	−131	1.46 at −16 °C	R-227ea	1.742
difluoromethane	H_2CF_2	−51.6	−136	0.5265 at −52 °C	R-32 Freon 32	0.304
1-HFE (1,1,1,2,2,3,3,4,4-nonafluoro-4-methoxybutane)	$MeO(CF_2)_3CF_3$	61	−135	1.520	CF 61 HFC 7100 (NOVEC)	4.669

[a] Modified from ref 310.

Table 15. Summary of the Consolute Temperatures of Different FBSs (Except the Three Component Final Entry)[310]

solvent system (1:1)	two phases at (°C)	one phase above (°C)	ref
$CF_3C_6F_{11}/CCl_4$	RT	26.7	313
$CF_3C_6F_{11}/CHCl_3$	RT	50.1	313
$CF_3C_6F_{11}$/hexane	0	RT	310
$CF_3C_6F_{11}/Et_2O$	0	RT	310
C_6F_{14}/C_6H_{14}	0	∼24	314
perfluorodecalin/$PhCH_3$	RT	64	315
$CF_3C_6F_{11}$/hexane/$PhCH_3$ (ratio 3:3:1)	RT	36.6	6

involved cannot be separated into two distinct phases. Those chosen can be partitioned on cooling below 60 °C and hence would be amenable to use with enzymes. It should be borne in mind that the ratio of the two solvents and the presence of dissolved species can both have an affect on its phase behavior.

The following sections describe the reports of the use of biocatalysis in conjunction with fluorous solvents ending with the only example to date of a biocatalyst being used in conjunction with a flurous biphasic system.

9.2. Biocatalysis in Fluorous Solvents

A number of enzyme-catalyzed reactions have been conducted in sc fluoroform, and these are collected together in the first section of Table 16. Fluoroform offers some advantages over $scCO_2$ as a reaction medium as discussed in section 1.1. In this section, we focus on the reports of biocatalysis in perfluorinated solvents that have been conducted under non-sc conditions.

In 2002, Panza et al.[316−318] reported the attachment of a PFPE tail to nicotinamide adenine dinucleotide (NAD), forming a fluorinated NAD molecule (FNAD) soluble in fluorous solvents. They demonstrated that FNAD forms micelles when dissolved in a fluorous solvent (methoxynonafluorobutane, HFE) and these micelles were able to extract the enzyme horse liver alcohol dehydrogenase (HLADH) from an aqueous phase into the fluorous phase. The addition of butyraldehyde and ethanol as cosolvents enhanced the efficiency of HLADH extraction into the reverse micelles. The catalytic activity of the enzyme was reported for the reduction of butyraldehyde to butanol in HFE. The enzyme in FNAD reverse micelles was also shown to be both soluble and active in liquid CO_2.[317]

In 2004, Saul et al. reported heterogeneous biocatalysis in low boiling point fluorous solvents used as refrigerants (R-134a, R-227ea).[319] The activity of NZ 435 for the KR of *rac*-1-phenylethanol was shown to be increased both in rate and in yield in hydrofluorocarbons (HFCs) as compared to those in hexane and methyl *tert*-butyl ether (MTBE). This increase was attributed to the low viscosity and consequently increased solute diffusivity in the HFC. Improved activity of subtilisin for the transesterification of *rac*-*N*-phenylalanine propyl esters in HFCs was also demonstrated in 1,1,1,2-tetrafluoroethanol (R-134a) when compared to that in conventional organic solvents.

9.3. Fluorous Tagging and Facile Separations

Theil and co-workers have reported the use of chirazyme L-2 (CZ L-2) for the KR of racemic alcohols, including *rac*-1-phenylethanol using a highly fluorinated carboxylic acid in acetonitrile.[320−323] The corresponding fluorinated (*R*)-ester and nonfluorinated (*S*)-alcohol are then partitioned into a fluorous solvent (perfluorohexane) and methanol, respectively, hence removing the need for a costly chromatographic separation. In addition, the reverse reaction was also performed such that the racemic fluorinated ester was enantioselectively hydrolyzed by CALB in acetonitrile to the nonfluorinated (*R*)-alcohol, leaving the fluorinated (*S*)-ester unreacted. These are separated by partitioning into methanol and perfluorohexane, respectively.[324]

In addition to this research, the resolution of *rac*-1-(2-napthyl)ethanol in combination with a fluorous triphasic separative reaction has been reported.[325] Alcoholysis of the corresponding fluorinated ester was performed by CZ L-2 in acetonitrile, and the resulting products and a fluorinated acid (catalyst) were transferred to the source phase (methanol) of a U-tube. The nonfluorinated (*R*)-alcohol remained in the source phase while both fluorinated (*S*)-ester and catalytic fluorinated acid passed to the fluorous phase (FC-72). The (*S*)-ester undergoes alcoholysis whereby its fluorous tag is removed and the resulting (*S*)-alcohol passes to the receiving phase (methanol) on the opposite side of the U-tube to the source phase. Hence, both enantiomers of 1-(2-napthyl)ethanol are obtained and separated in high yields and enantioselectivities as shown in Scheme 9. Teo et al. have also described the use of fluorous tagging for the KR of *rac*-1-phenylethanol by NZ 435 catalysis and partitioning using a fluorous solvent.[326] These techniques are not strictly termed FBS, but it seems that there is great potential to combine the KR and partitioning in one step.

9.4. Biocatalysis in a FBS

The concept of a FBS was first described by Horváth and Rábai in 1994.[6] The idea is elegant in its simplicity: A catalyst is dissolved in a fluorous solvent and combined with substrates dissolved in an organic solvent to form a biphasic system. On warming the system, the two phases become

Table 16. Biocatalysis in Supercritical Fluoroform and Involving Fluorous Solvents

Supercritical Fluoroform

entry	enzyme (species)	preparation	substrates + products	reaction conditions (*T*, time, other)	yield (%)	remarks	ref
1	CCL	crude	methylmethacrylate + 2-ethylhexanol → 2-ethyl hexylmethacrylate	45 °C, 110 bar	0.2 mM/ h/mg enz	batch	31, 79
2	CCL	crude	methylmethacrylate + 2-ethylhexanol → 2-ethyl hexylmethacrylate	50 °C, 55 bar	0.4 mM/h	batch	51
3	PFrL	lipid coated	*rac*-1-phenylethanol + lauric acid → (*R*)-1-phenyl ethyl laurate	40 °C, 60 bar, 40 h	60%	batch; activity controlled by changing temp and pressure	108
4	PPL	crude	*bis*-(2,2,2-trichloroethyl) adipate + 1,4-butanediol → poly(1,4-butylene adipate)	50 °C, 372 bar	average mw 1340	SFE system; range of pressures studied (70−372 bar)	168
5	β-D-galactosidase (*B. circulans*)	lipid coated	1-*O*-*p*-nitrophenyl-β-D-galactopyranoside + 5-phenylpentan-1-ol → 5-phenylpentyl-β-D-galactosidase + *p*-nitrophenol	37 °C, 60 bar, 5 h	>90%	slow reaction below 48 bar or above 100 bar	107
6	a. Sub (*B. licheniformis*) b. protease (*Aspergillus*)	crude	*N*-acetyl-D/L-phenylalanine ethyl ester + methanol → *N*-acetyl-D/L-phenylalanine methyl ester + ethanol	50 °C, 69 bar	k_{cat}/K_m ($mM^{-1}\,s^{-1}$) a. 0.06 b. 0.032	decrease in activity with increasing pressure in fluoroform, no change in propane; subtilisin selective for L-ester, little selectivity for protease	50
7	Sub (*B. licheniformis*)	crude	APEE + methanol → *N*-acetyl-L-phenylalanine methyl ester + ethanol	50 °C, 124 bar constant a_w	a. 1.5 mM/h	increasing pressure decreases initial rate in $scCHF_3$, but no effect observed in ncC_3H_8; pH and w_o controlled using salt hydrate	292
8	polyphenol oxidase (mushroom)	immobilized on glass beads	*p*-cresol or *p*-chlorophenyl + O_2 → *o*-benzoquinone	34 °C, 340 bar, 40 min or 1 L/min flow rate	70%	batch and continuous	8

Fluorous Solvents

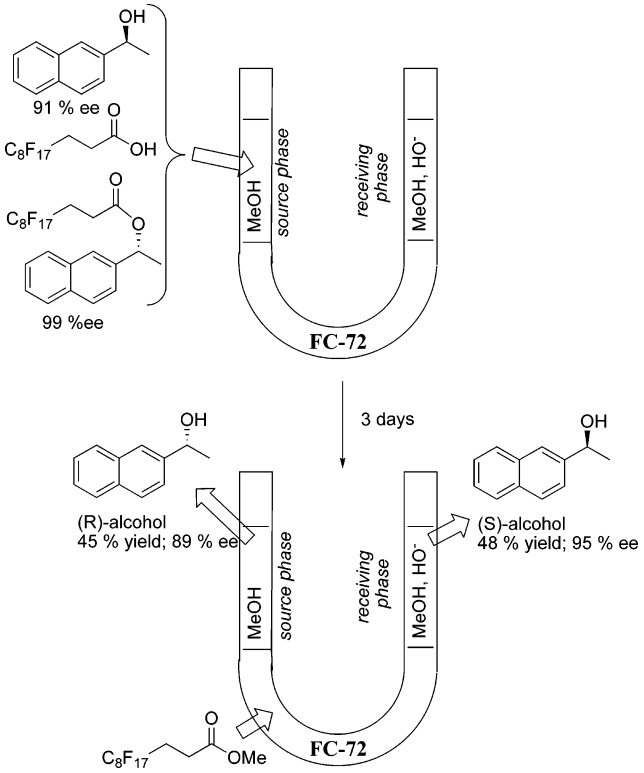
entry	enzyme (species)	preparation	solvent	substrates + products	reaction conditions (*T*, time, other)	yield (%)	remarks	ref
1	alcohol dehydrogenase (horse liver)	FNAD micelles	methoxy-nonafluoro-butane	butaraldehyde + ethanol → butanol	30 °C, 5 h, shaking 250 rpm	9 mM butanol	enzyme activity of HLADH in FNAD micelles in CO_2 at 2600 psi and RT shown to be greater than without solubilization	316−318
2	CALB	NZ 435	R-32, R-227ea, and R-134a	*rac*-1-phenylethanol + VA → (*R*)-1-phenyl ethyl acetate	RT, 5 h	49−50% ee_p > 99%		319, 323
3	CALB PCL	NZ 435 crude	R-32, R-227ea, and R-134a	*meso*-2-cyclopentene-1,4-diol + VA → monoacetate	RT, 3−5.5 h	55−61% ee_p > 99% 42−58% ee_p > 99%		319
4	Sub	crude	R-32 and R-134a	*N*-protected phenylalanine propyl esters + MeOH → *N*-protected phenylalanine methyl esters	RT, 19 or 72 h	10−33% ee_p > 99%		319
5	CALB	CZ L-2	MeCN	*rac*-1-phenylethanol + highly fluorinated ester → highly fluorinated (*R*)-product + (*S*)-1-phenylethanol	RT, 19 h	46% ee_p > 99%	*n*-perfluorohexane was used to extract the fluorinated product from unreacted (*S*)-1-phenylethanol	320−322
6	CALB	CZ L-2	MeCN/FC-72	*rac*-1-(2-napthyl)ethanol + highly fluorinated ester → highly fluorinated (*S*)-product + remaining (*R*)-substrate	RT, 7 days	50% ee_p > 99%	fluorous triphasic separative reaction	325
7	CRL	crude	perfluoro-hexane/ hexane	(*rac*)-organic acid or ester + fluorinated alcohol → (*R*)-organic acid or ester + (*S*)-fluorous ester product	40 °C, 44−149 h	48−53% ee_p 72−95%		329, 330
8	AS	PEG complex	perfluoro-octane	vinyl cinnamate + benzyl alcohol → benzyl cinnamate + acetaldehyde	55 °C	5.5 mmol/ h/g enzyme	reaction proceeds 10× as fast in perfluorooctane cf. isooctane	331

miscible to form a single phase, and a homogeneous reaction can occur. The catalyst and products can then be easily separated from each other by simply recooling the reaction mixture, and the two phases separate; the catalyst remains in the fluorous solvent, and the product is retained in the organic solvent, as shown in Scheme 10.

Catalysts that are soluble in fluorous solvents need to be highly fluorinated themselves since "like dissolves like".[327] There are two possibilities for the design of such catalysts: New catalysts can be specifically designed to dissolve in the fluorous environment, or many "conventional" catalysts can be converted to "fluorous-like" ones by incorporation of fluorous ponytails.[6]

The temperature at which a single phase or two phases are observed will vary depending on the composition of the system. For example, Horváth and Rábai describe the phase

Scheme 9. Fluorous Triphasic Reaction/Separation[325a]



[a] Nonfluorinated (*R*)-2 alcohol remains in the source phase. The fluorinated carboxylic acid and (*S*)-alcohol pass into the fluorous phase (FC-72) where the fluorous tag of the (*S*)-ester is hydrolyzed by base in the receiving phase, yielding nonfluorinated (*S*)-alcohol, which subsequently passes into the receiving methanol phase, leaving the methyl ester of the fluorous tag in the fluorous phase.

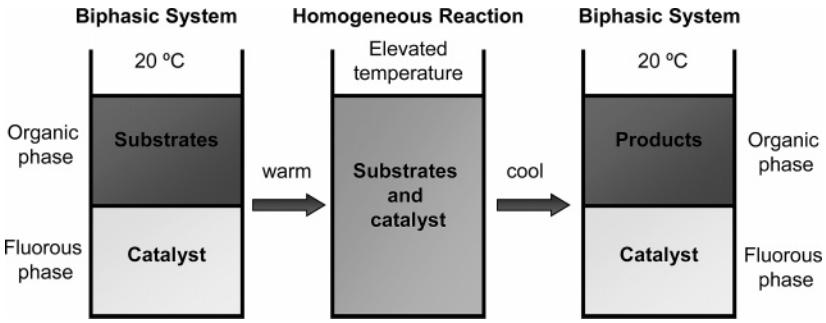
coalescence of *n*-hexane:toluene:PFMC in the ratio 3:1:3 on hand warming (36.5 °C), which reverts to a two phase solution after cooling to room temperature.[6] The temperatures at which a variety of solvent systems become one phase and separate to form two phases have been reported elsewhere (Table 15).[310,328]

Only one FBS incorporating heterogeneous biocatalysis has been reported. In 2002, Beier and O'Hagan reported the high activity of crude CRL for the enantioselective esterification reaction of *rac*-methylpentanoic acid with a fluorinated alcohol in a FBS (perfluorohexane and hexane).[329,330] The acid was solubilized in hexane, and the alcohol and corresponding fluorinated ester product were both solubilized in perfluorohexane. In this case, the catalyst was not soluble in either solvent but was used as a suspension and was easily filtered off at the end of the reaction. CRL selectively catalyzed the esterification of (*S*)-methylpentanoic acid to the corresponding (*S*)-fluorinated ester product, which was retained in the fluorous solvent, and the remaining (*R*)-methylpentanoic acid could be easily removed following phase separation on cooling of the system (Scheme 11).

In 2004, Maruyama et al. reported the use of PEG-lipase complexes to catalyze the alcoholysis of vinyl cinnamate with benzyl alcohol in perfluoro-octane.[331] On screening five PEG−lipase complexes, the authors found that PEG−lipase from *Alcaligence* sp. (PEG-AS) demonstrated the highest alcoholysis activity, >16-fold that of the native AS (lipase from *Alcaligences* sp.) powder. The lipase activity in fluorous solvents was remarkably high as compared with that in conventional organic solvents, such as isooctane and *n*-hexane, possibly due to the hydrophobicity of the fluorous solvents. A small volume of isooctane was required to dissolve the nonfluorinated substrates, but on increasing this volume, the activity of the PEG-AS complex was reduced.

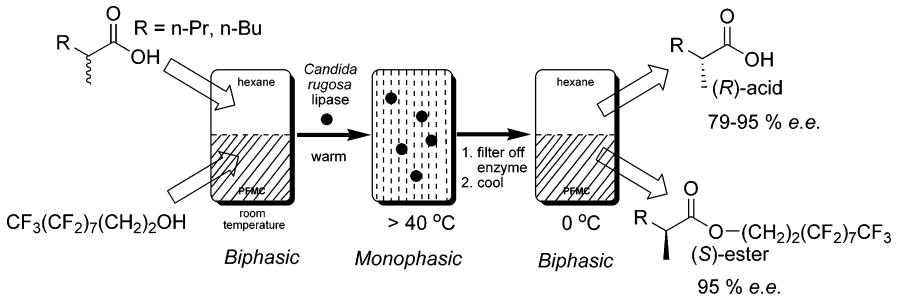
True fluorous biphasic catalysis as defined by Rábai and Horváth has yet to be demonstrated, as it would require the enzyme to be modified to be highly soluble in both the fluorous solvent on its own and the single phase formed when the organic and fluorous solvents are warmed above their consolute temperature. Beier and O'Hagan have, however, elegantly demonstrated that a FBS involving a suspended

Scheme 10. Fluorous Biphasic System (FBS)[a]



[a] At ambient temperature, the fluorous and organic phases are immiscible. On warming, the two phases become miscible and a homogeneous reaction occurs. On cooling, the phases separate, thereby removing the catalyst from the product without the need for costly separation techniques.

Scheme 11. Kinetic Resolution of α-Methyl Acids with in Situ Enantioselective Separation Using a FBS[329,330]



enzyme that can be filtered off and potentially recycled can be used to separate a fluorinated ester product generated from a racemic carboxylic acid in a KR catalyzed by a suspension of the lipase from *Candida rugosa*.

Biocatalysis in FBS is a relatively new area of research, and it seems that its full potential has yet to be realized. Most reports within this area have discussed the use of fluorous tagging of a single enantiomer by enzyme catalysis and then demonstrated facile separation by partitioning the two enantiomers into a fluorous and organic solvent. It is disappointing that only one project has made the crucial next step of combining enzyme catalysis and fluorous tagging in a one pot FBS.[329]

A different thread to this field of research was tackled by Panza, Russell, and Beckman,[316,317] whereby an enzyme was solubilized in the fluorous solvent by means of forming fluorinated reverse micelles. The enzyme was successfully extracted from aqueous solution into the fluorous phase and above all retained excellent enzyme activity. However, separation of enzyme from products is surely made complex in such a system. Would it be possible to produce a system whereby the enzyme is solubilized in the fluorous phase and substrates are solubilized in the organic phase? This would provide a homogeneous reaction mixture at elevated temperatures and straightforward separation of the products from the biocatalyst on simply cooling the solution, thereby facilitating phase separation.

The combination of biocatalysis and FBSs, although relatively new, is an attractive one and further research would certainly be of interest especially in the area of green chemistry. Facile separations of biocatalyst from product provide cost- and time-effective processes, plus the biocatalyst is easily reused and recycled. It is just unfortunate that there is a cloud of doubt over the persistence in the environment of the fluorinated solvents and their byproducts; however, this is still a topic of debate in this field.

10. Solvent-Free and Solid-to-Solid Biocatalysis

An alternative strategy to the use of $scCO_2$, ILs, or fluorous solvents is to avoid the use of solvents completely with the reaction involving only reagents and any associated catalysts—so-called solvent-free or solventless reactions (see Walsh et al. *Chem. Rev.* **2007**, *107*, http://dx.doi.org/10.1021/cr0509556).[332−335] This approach is relatively simple if one or more of the reagents is a liquid, or as described in section 5, a SCF or gas, but if both substrates and catalysts are solids, then the accessibility of the catalyst to the substrates will limit the speed and yield of the reaction. In the case of biocatalysis, a further concern is the stability of the enzyme in the substrate when this is also used as the solvent, especially as it is known that polar organic compounds such as alcohols that are widely used in (trans)esterification reactions can denature enzymes when employed as solvents.[75] There are numerous examples of "solvent-free" biocatalysis with liquid substrates involving either free enzymes or immobilized enzymes.[336−339] These reactions generally proceed in a similar manner to those involving enzymes in nonaqueous solvents, except that the initial rates of reaction and levels of conversion at equilibrium may be higher. Solvent-free polymerization reactions may exhibit unusual kinetics due to the lack of monomer availability and entanglement of the catalyst in the polymer during the late stages of the reactions. Of more relevance to this review are the studies on heterogeneous systems involving "mainly undissolved" substrates. The research in this area has followed two approaches that have been termed "heterogeneous eutectic" and "solid-to-solid" reactions. In many ways, the composition of the reaction systems used in either of these approaches shares many common features. The essential requirement of both solid-to-solid and heterogeneous reactions is that they require a small amount of a liquid phase in which the enzyme can conduct the reaction, generally to give a product that then precipitates out of solution.

Solid state enzyme reactions date back to the early 1970s with the work of Roslyakov and co-workers on the hydrolysis of cinnamoyl-modified CMT[340] and *N*-succinyl-L-phenylalanine-*p*-nitroaniline by the same enzyme[341−343] together with work on subtilisin.[344] This research was primarily concerned with investigating the mechanism of action of the enzyme rather than its use in biotransformations. Later studies on papain and proteinase K[345] demonstrated that the rates of reaction were highly dependent on the w_o of the system, the physicomechanical properties of the reactants and products, and the presence of buffer salts and lyoprotectants.

The potential advantages of solid state biocatalysis are the requirement for smaller reactor volumes, and the associated cost savings from not needing to purchase or dispose of a solvent. Solid state biocatalysis does have limitations in that most industrial scale chemical plants are designed with the handling and transportation of liquid intermediates in mind. The solvent can also provide a useful role in dissipating heat in an exothermic reaction, and the biocatalyst and substrates must be well-mixed during the reaction to ensure that the substrate is brought in close proximity to the active site of the enzyme. For most of the solid state reactions described to date, the products would need to be separated from the reactants through chromatography or other means.

10.1. Heterogeneous Eutectic Reactions

The heterogeneous eutectic method requires that the mixture of substrates either on their own or in combination with a very small amount of an adjuvant (water or an organic compound) possesses a sufficiently low melting (eutectic) point (generally <60 °C) to make it compatible with the thermal stability of a biocatalyst.[346−354] In their initial investigations of the eutectic method, Gill and Vulfson demonstrated in 1993 that it was possible to perform peptide coupling reactions on substrate mixtures that formed heterogeneous mixtures or eutectics (low melting point mixtures made up of two or more components).[348] Both subtilisin and CMT were used in their initial study. Immobilized subtilisin was shown to catalyze the formation of the dipeptide from solid L-Leu-NH_2, L-Met-NH_2, and GlyNH_2 dispersed in liquid L-Phe-OEt in 83, 75, and 36% yields, respectively.[346−354] In parallel,[346,355−357] a range of proteases were investigated together with the use of an adjuvant, a small quantity of an organic solvent added to ensure eutetic formation. A range of commercially important peptides were produced using this methodology including aspartame, sweet lysine peptide, kyotorphin amide, angiotensin converting enzyme (ACE)-inhibiting and immunoactive tripeptides, Leu-enkephalin amide, and fragments of the so-called "delicious octapeptide", with overall yields of 21−84% (Table 17). Through the use of Fourier transform infrared (IR) microscopy and polarizing microscopy, López-Fandiño et al. demonstrated that the reaction was occurring in the liquid phase formed just above the eutectic point.[356]

Table 17. Selected Solventless/Solid-to-Solid/Eutectic Reactions Involving Biocatalysts

entry	enzyme (species)	preparation	"adjuvant"	substrates + products	reaction conditions (*T*, time, other)	yield (%)	remarks	ref
1	Sub	immobilized on Celite	none	Leu-NH_2 + Phe-OEt → Phe-Leu-NH_2 + EtOH	preheated to 60 °C and then 37 °C after enzyme is added, 48 h	74%	0.19 mmol min^{-1} mg^{-1}	356
2	Sub	immobilized on Celite	10% w/w water	Leu-NH_2 + Phe-OEt → Phe-Leu-NH_2 + EtOH	preheated to 60 °C and then 37 °C after enzyme is added, 48 h	70%	0.65 mmol min^{-1} mg^{-1}	356
3	Sub	immobilized on Celite	10% w/w EtOH	Leu-NH_2 + Phe-OEt → Phe-Leu-NH_2 + EtOH	preheated to 60 °C and then 37 °C after enzyme is added, 48 h	81%	0.26 mmol min^{-1} mg^{-1}; similar overall yields with 2-methoxyethyl acetate, 2-methoxyethyl ether, or triethyleneglycol dimethyl ether	356
4	thermolysin	enzyme dissolved in HEPES (Na^+) buffer, 0.5 M, pH 7.0	11% water	Leu-NH_2 + *Z*-Gln → *Z*-Gln-Leu-NH_2 + EtOH	40 °C	80%	rates up to 20 mmol s^{-1} kg^{-1}	378, 379
5	thermolysin		0.5M Na-HEPES buffer, pH 7	Phe-OEt + Leu-NH_2 → Phe-Leu-NH_2 + EtOH	20 h	95%		380
6	CALB	NZ 435	NaOH, NaOH/DMSO or NaOH/DMSO/2M2B	sorbitol + fatty acid → sorbitol-fatty acid ester + H_2O (capric acid; lauric acid; myristic acid)	25 h, 60 °C, 100 rpm	94−98%	eutectic medium reaction; molecular sieves added to remove water generated/ 0.086−0.098 mmol g^{-1} h^{-1}	352
7	CALB	immobilized on polypropylene (EP 100)	*t*-butanol	*β*-D-glucose + vinyl palmitate → 6-*O*-palmitoyl-*β*-D-glucose + acetaldehyde	24 h, 60 °C, 250 rpm	84%	CALB immobilized on other media also investigated	381, 382
8	CMT		1−15% water (v/w), 5% DMF (v/w)	*Z*-Tyr-OEt + Arg-NH_2 → *Z*-Tyr-Arg-NH_2 + EtOH	9 h, 30 °C	90%	substrate content >80% (w/w)	358
9	CMT	immobilized on Celite	water 20% w/w	*Z*-Asp(OAll)OAll + L-Glu (OAll)OAll → *Z*-Asp(OAll)-Glu(OAll)OAll	17 h, 37 °C	56%	also examined activity of chymopapain, subtilisin, papain, and thermolysin to produce a range of peptides	357
10	thermolysin		18% (v/v) MEA, 9% (v/v) DMSO	*Z*-Asp(OEt)OEt + Ala-NH_2 → *Z*-Asp(OEt)-Ala-NH_2EtOH	8 h	70%	alitame derivative	350
11	thermolysin		12.5 M NaOH(aq)	*Z*-Asp + Phe-OMe → *Z*-Asp-Phe-OMe	50 °C	60%	2.2 μmol g^{-1} s^{-1} initial rate	383
12	CRL			(*R*/*S*)-naproxen → (*S*)-naproxen	50 °C, 72 h	40%		384
13	penicillin acylase	immobilized on Eupergit C	0.01 M phosphate buffer, pH 6.5	6-APA + D-PGM → ampicillin + MeOH		up to 70%	w_o 3−30% w/w; use of salt hydrates also examined	373
14	glycyl endopeptidase (from latex of *Carica papaya*)		water 10% w/w L-Cys	*Z*-Gly-OH + H-Phe-NH_2 → *Z*-Gly-Phe-NH_2	40 °C, 30 h	54%	23 nmol min^{-1} mg $enzyme^{-1}$ initial rate	385
15	CALB	NZ 435	DMSO (20−40%)	naringin + palmitic acid → naringin palmitate + H_2O	80 °C, 10 h	33%	addition of activated molecular sieves increased yield by 7%; termed a "highly concentrated homogeneous reaction" by the authors as palmitic acid is a liquid	386

More recently, Gill and Valivety achieved the multigram scale stepwise synthesis of a tetrapeptide under eutectic conditions using Celite-deposited chymopapain and subtilisin and the presence of 16−20% w/w water and ethanol as an adjuvant.[347] The peptide bonds were formed in yields of 73, 74, and 76%. Other groups have examined alternative enzymes and different adjuvants in the synthesis of the precursor of aspartame (*N*-CBZ-L-Asp-L-PheOMe)[349,351] and the kyotorphin precursor, *N*-carbobenzoxyl-L-tyrosine-L-arginine amide (*N*-CBZ-L-Tyr-L-$ArgNH_2$),[358] using the enzymes thermolysin, *Bacillus amyloliquefaciens* KCCM 12091 protease, and CMT, respectively. A synthesis of a protected precursor of the artificial sweetner alitame (L-Asp-D-Ala) was achieved from *N*-Cbz-L-Asp(OEt)OEt and D-alanine amide using CMT.[350] These form a semiliquid mixture with a eutectic point of 27 °C in the presence of an adjuvant composed of dimethyl sulfoxide (DMSO), 2-methoxy ethyl acetate, and water.

10.2. Solid-to-Solid Reactions

The alternative approach began from the work of Kuhl et al. looking at biocatalysis of suspensions of substrates in organic solvents with salt hydrates being added to provide the only source of water[359−361] or with small amounts of water.[362] This system was then developed further by Halling et al. who coined the term "solid-to-solid" reaction.[363] In this approach, most of the reactants remain in the solid state with a small amount progressively dissolving in the added solvent whereupon they are converted into products that then precipitate. The system is not at its eutectic point but requires an added liquid phase to be present. It is the precipitation of the products that drives the reaction to give high yields. This area has been reviewed previously.[353,354,364,365] Using the solid-to-solid approach, a range of amides, esters, and glycosides have been produced. Halling et al. have investigated the underlying kinetics[366] and thermodynamics[363,367−370]

of these systems focusing on peptide bond formation. Michielsen et al. have modeled the conversion of maleate to malate by *Pseudomonas pseudoalcaligenes*.[371] Ulijn et al. have also investigated the synthesis of β-lactam antibiotics such as amoxicillin by the so-called "direct condensation" approach using penicillin acylase and 6-aminopenicillanic acid (6-APA).[372]

In 2002, Youshko and Švedas described the use of Eupergit C-immobilized penicillin acylase for the synthesis of ampicillin.[373,374] In this reaction, equimolar amounts of lyophilized 6-APA and D-phenylglycine methyl ester (D-PGM) lyophilized at pH 6.5 were combined and mixed with the immobilized penicillin acylase and a salt hydrate ($Na_2SO_4 \cdot H_2O$, $MgSO_4 \cdot 7H_2O$, or $AcONa \cdot 3H_2O$) to provide an overall w_o of between 5 and 30% w/w. This system was shown to catalyze the synthesis of ampicillin with a rate comparable to that of a homogeneous system saturated with 6-APA. A similar approach has recently also been used for the preparation of the related β-lactams amoxicillin[375] and cephalexin[376] with penicillin acylase and in the use of immobilized glutaryl acylase for the hydrolysis of glutaryl-7-aminocephalosporanic acid, respectively.[377]

One major concern with solid-to-solid reactions is how these can be scaled up to be useful on an industrial scale. The production of *Z*-aspartame has been conducted at the 3 mol level using a stirred tank reactor to give the product in ca. 90% yield. Erbeldinger et al. have examined methods of scaling up the thermoase-catalyzed synthesis of *Z*-Asp-Phe-OMe using a jacketed reactor with an anchor stirrer and also suggested a method to isolate and recycle the enzyme.[387,388] Computer modeling and experimental studies on the formation of eutectic mixtures of protected amino acids (AAs) have been conducted by Kim et al.[389,390] to investigate the fact that *N*-carbobenzoxy (*Z*)-protected AA/second amino-acid/methanol mixtures preferentially form eutectic melts while mixtures in which both AAs have free amines do not. The area of reactor design for solid-to-solid reactions has been discussed in detail by Michielsen et al.[391] However, the perceived problems with scale-up of these types of reactions have so far prevented them from being more widely investigated.

11. The Future

This review brings together a number of strands of research in the field of biocatalysis under "green" conditions that have taken place over the past 21 years. Much of the initial research focused on examining the activity of enzymes in SCFs, especially carbon dioxide. Major hurdles to this research have been carbamate formation on the enzyme and the control of the pH of the reaction due to carbonic acid formation. Therefore, it has proved difficult to obtain conditions in which enzyme turnover was sufficiently better than in other solvents to justify the establishment of a commercial process based on biocatalysis in $scCO_2$. This problem appears to have been circumvented recently with the use of $scCO_2$ in combination with an IL, where the IL appears to protect the enzyme from the detrimental effects of direct contact with $scCO_2$. There is plenty of scope for the further study of biocatalysis in $scCO_2$ or other SCFs in combination with ILs and other neoteric solvents such as liquid PEG.

The study of enzyme reactions in fluorous solvents is much more recent with only a handful of examples where the enzyme is actually catalyzing the reaction in the fluorous phase. Beier and O'Hagan's work has elegantly demonstrated that biocatalysis and fluorous biphasic separation can be combined.[329,330] To be completely true to the original fluorous biphasic catalysis system described by Horváth and Rábai,[6] the enzyme should be soluble in the fluorous and homogeneous mixed phases and retained in the fluorous phase at the end of the reaction for reuse. Maruyama's study on the PEG-modified enzyme that shows good activity in perfluorooctane is a step toward this goal.[392] The combination of fluorous phases with SCFs, ILs, and other neoteric solvents in both chemical and biocatalysis is also currently under explored.

The study of solvent-free biocatalysis on solid substrates was an area of significant activity in the period 1992−2002, but publications in this area have become more infrequent. This is due primarily to the perceived problems with poor mass transfer in the solid-to-solid reaction systems, and the absence of large amounts of solvent in these systems is currently not a paradigm with which many synthetic chemists are comfortable. However, Halling and co-workers and others have demonstrated that it is possible to get high yields in these reactions, especially in the preparation of commercially useful peptides; therefore, a broader exploration of the potential of this approach is certainly warranted.

12. Abbreviations

6-APA	6-aminopenicillanic acid
AA	amino acid
AK	amano lipase AK (PFL)
ANL	*Aspergillus niger* lipase
AOL	*Aspergillus oryzae* lipase
AOT	aerosol OT
APEE	*N*-acetyl-L-phenylalanine ethyl ester
APPE	*N*-acetyl-L-phenylalanine propyl ester
AS	lipase from *Alcaligences* sp.
ATRP	atom transfer radical polymerization
a_w	water activity
BCL	*Burkholderia cepacia* lipase
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CALA	*Candida antarctica* lipase A
CALB	*Candida antarctica* lipase B
CAPSO	3-(cyclohexylamino)-2-hydroxy-1-propane sulfonic acid
Cc	cytochrome c
CCL	*Candida cylindracae* lipase
CLEA	cross-linked enzyme aggregate
CLEC	cross-linked enzyme crystal
CLogP	calculated logarithm (1-octanol/water partition coefficient)
CMT	α-chymotrypsin
CRL	*Candida rugosa* lipase
CVL	*Chromobacterium viscosum* lipase
CZ L-1	Chirazyme L-1 (PCL)
CZ L-2	Chirazyme L-2 (CALB)
CZ L-3	Chirazyme L-3 (BCL)
CZ L-5	Chirazyme L-5 (CALA)
DBT	dibenzothiophene
DG	diglyceride
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
D-PGM	D-phenylglycine methyl ester
E	enantiomeric ratio
ee	enantiomeric excess
EGDA	ethylene glycol diactetate
EGMA	ethylene glycol monoacetate
FAE	fatty acid ester
FBS	fluorous biphasic system
FFA	free fatty acid

FNAD	fluorinated nicotinamide adenine dinucleotide
FOMA	1*H*,1*H*,2*H*,2*H*-perfluorooctylmethacrylate
GC	gas−liquid chromatography
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid
HFC	hydrofluorocarbon
HFE	methoxynonafluorobutane
HLADH	horse liver alcohol dehydrogenase
HLL	*Humicola lanuginosa* lipase
HPL	hog pancreas lipase
IL	ionic liquid
IR	infrared
KR	kinetic resolution
Krytox	Krytox 157 FSL
L 100T	Lipolase 100T (AOL)
Lipolase	Lipolase (HLL expressed in recombinant *Aspergillus* sp.)
LPL	lipoprotein lipase
LZ	Lipozyme TL IM (MML/RML)
MBG	microemulsion-based organogel
MG	monoglyceride
MML	*Mucor miehei* lipase
MTBE	methyl *tert*-butyl ether
mw	molecular weight
NAD	nicotinamide adenine dinucleotide
nc	near critical
NZ	novozym
OATS	organic−aqueous tunable solvent
p_c	critical pressure
pCL	poly(caprolactone)
PCL	*Pseudomonas cepacia* lipase
PEG	poly(ethylene glycol)
PEI	poly(ethyleneimine)
PFL	*Pseudomonas fluorescens* lipase
PFMC	perfluoromethylcyclohexane
PFPE	perfluoropolyether
PFrL	*Pseudomonas fragi* lipase
pMMA	poly(methylmethacrylate)
PPL	porcine pancreas lipase
PRL	*Penicillium roqueforti* lipase
PS	lipase from *Pseudomonas* sp.
PT 1000L	palatase 1000L (water-soluble form of RML)
RAFT	reversible addition−fragmentation chain transfer
RAL	*Rhizopus arrhizus* lipase
RDL	*Rhizopus delemar* lipase
RJpL	*Rhizopus japonicus* lipase
RJvL	*Rhizopus javanicus* lipase
RML	*Rhizopus miehei* lipase
RNL	*Rhizopus niveus* lipase
ROP	ring-opening polymerization
sc	supercritical
SCF	supercritical fluid
SFE	supercritical fluid extraction
Sub	subtilisin Carlsberg
T_c	critical temperature
TEM	transmission electron microscopy
TG	triglyceride
U	unit enzyme activity
UV/vis	ultraviolet/visible
V_{max}	maximum velocity
v/v	volume per volume
v/w	volume per weight
VA	vinyl acetate
VOC	volatile organic compound
VOS	volatile organic solvent
w_o	water content
w/c	water-in-CO_2
w/o	water-in-oil

Pressure Conversion: 1 bar = 14.504 psi = 0.1 MPa

Ionic Liquids:

BDiMIM	1-*n*-butyl-2,3-dimethylimidazolium
BMIM	1-*n*-butyl-3-methylimidazolium
BTA	*bis*-(trifluoromethylimidazolium)
EMIM	1-*n*-ethyl-3-methylimidazolium
OMIM	1-*n*-octyl-3-methylimidazolium
TOMA	trioctylmethylammonium

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