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Applied Biotransformations in Green Solvents

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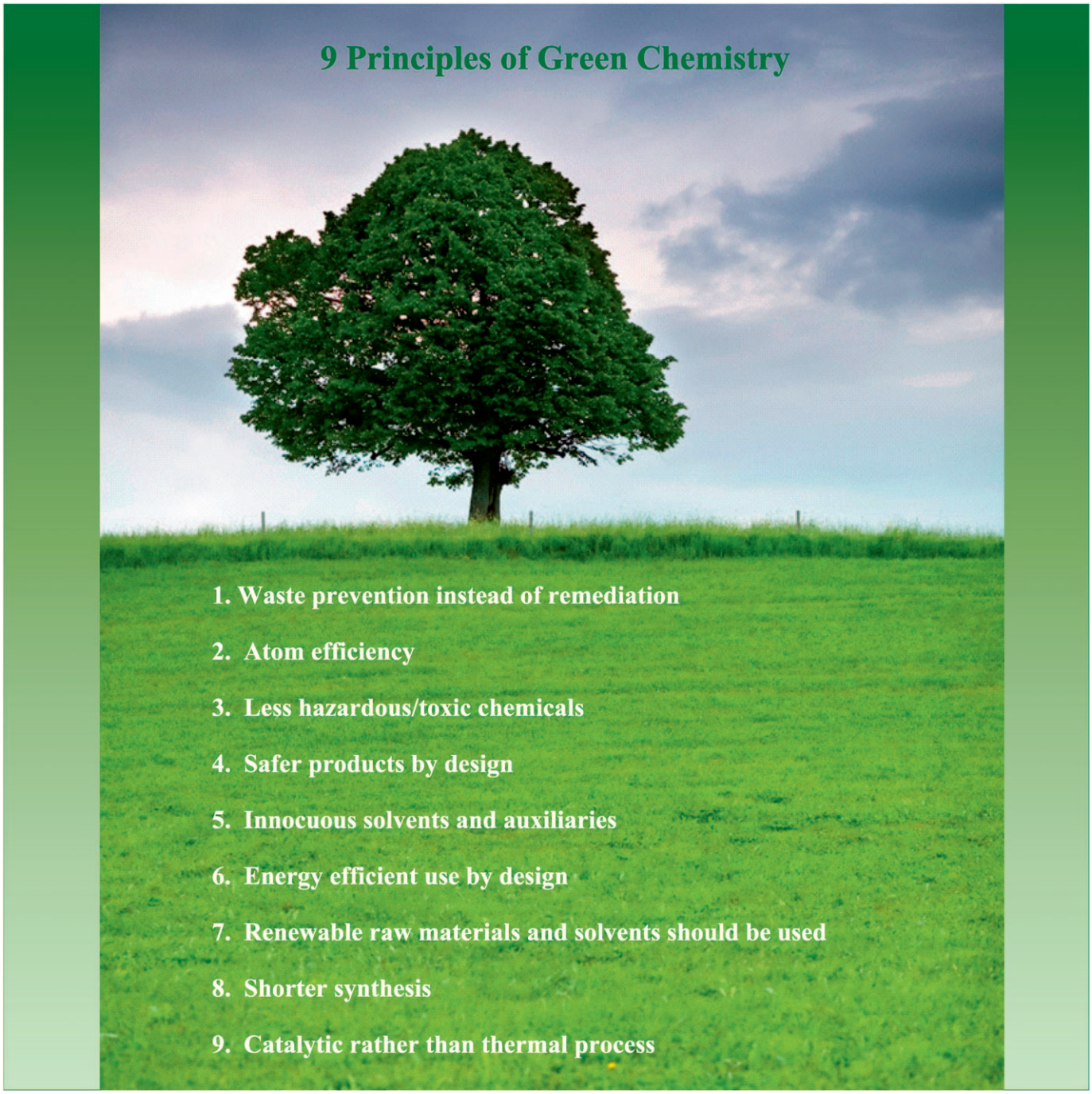

Abstract: The definite interest in implementing sustainable industrial technologies has impelled the use of biocatalysts (enzymes or cells), leading to high chemo-, regio- and stereoselectivities under mild conditions. As usual substrates are not soluble in water, the employ of organic solvents is mandatory. We will focus on different attempts to combine the valuable properties of green solvents with the advantages of using biocatalysts for developing cleaner synthetic processes.

Keywords: biocatalysts · biotransformations · green chemistry · sustainable chemistry · white biotechnology

Introduction

There is growing interest in developing more environmentally acceptable processes in chemical or biotechnological industries. This trend towards what has become known as sustainable technologies can be defined as green chemistry and white biotechnology, when the emphasis is focussed on chemical or biotechnological processes, respectively. The definition and concept of green chemistry was first formulated at the beginning of the 1990s,[1] and adopted by the "US Green Chemistry Program" from the US Environmental Protection Agency (EPA) in 1993. On the other hand, the European Association for Bioindustries (Europabio) defined white biotechnology as an emerging field within modern biotechnology that serves industry, in fact, the concept white biotechnology is commonly accepted in the industrial world as the application of biotechnological tools (genetically modified organisms (GMO), new enzymes from extremophiles, etc.) to produce substances of interest, taking into the account the principles of green chemistry. Therefore, white biotechnology can help realise substantial gains for the environment, consumers and industry.

As reported by Anastas and Horvath, the guidelines for these new technological philosophies are the well-known 12 principles of green chemistry[2,3] that can be conceptually summarised as the following:

1) Waste prevention instead of remediation
2) Atom efficiency
3) Less hazardous/toxic chemicals
4) Safer products by design
5) Innocuous solvents and auxiliaries
6) Energy efficient use by design
7) Renewable raw materials and solvents should be used
8) Shorter synthesis (reducing protection–deprotection steps)
9) Biocatalytic or catalytic rather than thermal processes
10) Design the products for degradation without pollutant problems
11) Efficient analytical methodologies for pollution detection and prevention
12) Inherently safer processes

As we can deduce from principles 2, 8 and 9 of green chemistry,[1–3] biocatalysis plays an important role in green processes, since it provides an alternative to classic organic chemistry.[4] Whole cells and enzymes offer suitable tools for industrial reactions, which can be carried out under mild conditions, without the use of heavy metals and with a great control over chemo-, regio- and stereoselectivity by using appropriate enzymes. However, enzymes and whole cells also have disadvantages: high cost, easy deactivation under incorrect conditions and inhibition by substrate and/or reaction products. Fortunately, developments in large-scale DNA sequencing, structural biology, protein expression, high-throughput screening (HTS) from different microorganism collections or other natural sources, clone banks and metagenomic gene discovery[5,6] or even in enzyme promiscuity,[6–9] direct enzyme evolution and metabolic engineering[10–12] have contributed to reduce the cost of biocatalysts and improve the yields; on the other hand, efficient immobilisation techniques can assist the stabilisation of the enzymes.[13] In this way, Reetz et al. have developed a useful single-cell HTS system to identify enantioselective hydrolytic enzymes.[14] Another remarkable option is to focus on extremophiles for biocatalyst discovery. Enzymes synthesised by thermophiles or hyperthermophiles are thermostable, working between 60 and 125 °C, and being highly stable in the presence of high concentrations of organic solvents. Therefore, these microorganisms offer useful enzymes to expand the range of reaction conditions suitable for biocatalysis, and the enzymes obtained from them represent superior starting points for further stability optimisation.

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There are various thermostability mechanisms depending on the enzyme. Nevertheless some common features can be identified: high number of hydrogen bonds, electrostatic interactions, hydrophobic interactions, high number of disulfide bonds, etc.[15] In this way, some interesting enzymes (such as esterases, lipases, proteases, alcohol dehydrogenases, etc.[16–18] or nucleoside phosphorylases) from *Thermus thermophilus*[19] have been described.

Today, in the fine-chemical or pharmaceutical industries, solvents are used in large quantities relative to the product (the solvent/product ratio varies between 100 and 1000). Therefore, solvents are considered as the major cause of the environmental damage attributed to an industrial process.[20] The idea of a "green solvent" expresses the aim to minimise the environmental impact resulting from the use of solvent in one chemical process. Therefore, the term green solvent should be associated with low toxicity, low vapour pressure, good biodegradability or non-environmentally damaging. Nevertheless, the introduction of a new solvent in an industrial process must be critically analysed from technological, chemical, toxicological and environmental points of view, because it is not easy to have a goal in all of the aforementioned topics. Nowadays, a new generation of solvents has been developed within the SOLVSAFE Network (http://www.solvsafe.org/), whose search has become an area of interest, although in some cases their greener properties are still in doubt. Currently, water, supercritical fluids (SCFs), fluorous solvents and solvents from renewable sources are considered green solvents. On the other hand, the sustainability of ionic liquids (ILs), initially considered to be green solvents, is now under debate, as we will comment in the next section.

Finally, the key to success in developing green, sustainable industrial processes is the effective integration of catalytic

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technologies—chemical or biocatalytic—into organic synthesis processes. It is also necessary to look at the whole picture; not only the reaction steps, but also to downstream processing. Therefore, it is necessary to integrate separation, removal, recovery and recycling of solvents, products, by-products and concomitant products. Herein, we will focus on the use of green solvents combined with biocatalysed reactions and their current applications in sustainable process.

Green Solvents

Solvents are almost ubiquitous in chemical transformations. Although in many green chemistry books and monographs one can find that "the greenest solvent is no solvent at all"[20] there are definitely clear advantages in using solvents as part of chemical processes. We can highlight the fact that reactions proceed faster and more smoothly when the reactants are dissolved, because mass transfer restrictions are reduced. In addition, solvents may have a positive effect on the rate and/or selectivity of the reaction, due to the differential solvation of reagents, intermediates or transition states. Solvents act as heat transfer media, removing heat liberated in an exothermic reaction, reducing thermal gradients in a reaction vessel and allowing a smooth and safe reaction. Finally, solvents often facilitate separation and purification of reaction products.

In the EU, solvent use is regulated by increasingly demanding environmental legislation. The removal of residual solvent from products is usually achieved by evaporation or distillation, therefore, most organic solvents used are highly volatile. This results in atmospheric pollution, which is a major environmental issue of global proportions. Moreover, workers' exposure to volatile organic compounds (VOCs) is a serious health issue. Recent environmental legislation is aimed at strict control of VOC emissions and the eventual phasing out of greenhouse gases and ozone-depleting compounds.

The concept of a green solvent started with green chemistry, and the term has been increasingly used in the first decade of this century. Solvents are explicitly mentioned in the 5th principle of green chemistry (safer solvents and auxiliaries),[2,3] and is implied in others, such as the 3rd and 4th principles. The qualitative and quantitative importance of solvents in chemical processes, together with the increasing legal restrictions concerning the use of organic solvents derived from petrol, has resulted in a remarkable growth in interest for green solvents for industry. Traditionally, green solvents are classified in five main categories, although, as we will discuss shortly below, there is still controversy concerning the labelling of some of these categories as truly "green": 1) water, 2) ILs, 3) fluorous solvents, 4) SCFs and 5) organic solvents from renewable sources.

Water is at the top of all green solvent lists, and for very good reasons. Water is non-toxic, non-flammable, colour- and odourless (making contamination easy to spot), widely available and inexpensive. Furthermore, its polar nature facilitates, in many cases, product separation, and even reaction selectivity, by differential solvation. As water is immiscible with many organic solvents, as well as with other green solvents, such as ILs, fluorous solvents and $scCO_2$, it is well suited for biphasic catalysis. On the negative side, many organic compounds do not dissolve in water, whereas reactants must have some solubility to have a chemical reaction proceeding at a reasonable rate. Furthermore, reactants and or catalysts may not be stable or may be deactivated in water, which limits the scope of application. From the toxicity and eco-toxicity side, although water is non-toxic, it can be easily contaminated by organic and metallic impurities. It is also difficult to eliminate, which require expensive end-of-pipe solutions to return clean water to the environment.

ILs constitute probably the largest group of those solvents labelled as neoteric solvents, traditionally considered as being environmentally benign. It must be emphasised that when referring to an IL it is usually understood that the ionic compound is liquid below 100 °C, and normally at room temperature, so very often they as referred to as room-temperature ionic liquids (RTILs). Although the use of ILs shows several advantages, we must also mention that they show many inconveniences, such as high cost, difficulties in purification, and, above all, toxicity issues. There is already enough evidence to conclude that many ILs are potentially as harmful as the conventional organic solvents that they are supposed to substitute. Antibacterial activity, as well as cytotoxicity and toxicity towards multicellular organisms have been determined in different studies.[21] Ecotoxicity has also been studied in the case of aquatic organisms (algae, crustacea, fishes)[21] and terrestrial plants (soil toxicity).[22] Therefore, currently used ILs are not intrinsically green solvents, therefore, careful design in the synthesis of ILs is required to reduce their toxicity.[23]

Fluorous solvents, a term coined by Horvath,[24] describe highly fluorinated alkanes, ethers and tertiary amines that present some special features that made them suitable for biphasic catalysis. They are simultaneously hydro- and lipophobic, so they form biphasic systems with water, but also with hydrocarbons. The immiscibility with the hydrocarbons depends on temperature, so one can have thermoregulated phase separation.[4,24,25] Usually, the catalytic reaction takes place in a homogeneous phase at high temperature; then, phase separation is obtained by cooling to room temperature, allowing easy separation of the catalytic (fluorous) phase from reaction products (hydrocarbon phase). As for ILs, fluorous solvents display good gas solubility properties, so they are particularly well suited for being used in combination with $scCO_2$ as the extraction phase. There are relatively few works dealing with biocatalysis in fluorous solvents,[26] but in many cases, they are connected with supercritical extraction of reaction products using $scCO_2$. The main shortcomings of fluorous solvents are their very high prices, and the debatable greenness of their production and fate. In this regard, it has been found that nearly all humans, and a large proportion of wildlife, are contaminated with en-

vironmentally persistent long-chain perfluoroalkyl compounds.

Supercritical fluids (SCFs) have been broadly studied and several reviews have been reported devoted to this field[4,27–32] since 1985, when the first publication appeared.[32] A SCF is defined as the state of a compound above their critical temperature and critical pressure, but below the pressure required to condense it into a solid.[6,27] SCFs have properties between liquids and gases, since their density is comparable to a liquid, whereas their viscosity is comparable to that of gases.[27] Each substance has particular critical properties and they should be selected according to the properties that best fit the purpose.

Properties of SCFs depend on temperature and pressure. Because of this, we can tune the reaction conditions to adapt them to the specific requirement for biocatalysts. Parameters such as dielectric constant, partition coefficient or solubility are pressure sensitive.[33] For this reason, small changes in pressure and temperature near to the critical point can cause important alterations in solubility and produce high diffusivity and low viscosity, which allow high rates of mass transfer. In addition to these advantages, changes in the solubility also provide benefits to separate the components by extraction using controlled depressurisation.[4] The solubility of the compounds in SCFs depends on vapour pressure, polarity and molecular weight, and the best conditions for a compound to be solubilised in SCFs are high vapour pressure, low polarity and low molecular weight.[34]

Several features of $scCO_2$ make it an interesting solvent in the context of green chemistry and catalysis. For carbon dioxide, the critical pressure and temperature are moderate: 74 bar and 31 °C, respectively. Hence the amount of energy required to generate supercritical carbon dioxide is relatively small. In addition, carbon dioxide is non-toxic, chemically inert towards many substances, non-flammable, and simple depressurisation results in its removal, allowing easy separation from reaction products. Its main uses are as a replacement for VOCs in extraction and cleaning processes, but applications in biocatalysis[35] are also well known; above all it is used in multiphase catalysis. However, the use of $scCO_2$ requires special equipment, which makes its use more difficult and expensive when applied to industrial or semi-preparative systems.

Another important issue in SCF research is to determine the precise nature of the enzymes that are going to be used, since enzymes need a certain percentage of water to retain their activity. When the physical properties change, the enzyme can be affected. For example, some lipase[36] and protease[37] activities depend on the dielectric constant of the solvent. However, changes in pressure do not affect the stability of most enzymes.[38] To improve the activities and stabilities of enzymes, many techniques have been developed that use various SCFs: immobilised enzymes,[39,40] lipid-coated enzymes,[41,42] cross-linked enzyme crystals (CLECs),[43–45] cross-linked enzyme aggregate (CLEAs)[46,47] or reverse micelles.[48]

The last category of green solvents is composed those organic solvents obtained from renewable sources, also called "bio-solvents". Although they share some common properties with organic solvents derived from petrol (such as flammability, for instance), they also present clear advantages, such as sustainable production, better biodegradability and lower toxicity. Some commercially available bio-solvents are lactic acid esters, fatty acid esters, glycerol derivatives (triacetin, solketal, glycerol formal), 2-methyltetrahydrofuran (MeTHF, obtained from agricultural by-products), *N,N*-dimethylamides from fatty acids or ethanol, for example.

A key issue when dealing with green solvents is how to find the best solvent substitution for a given application. For instance, the oil seed extraction field is largely dominated by hexane, a highly flammable hydrocarbon with known neurotoxic effects. Pharmaceutical synthesis, in turn, is very dependent on the use of dichloromethane, despite the increasingly strong recommendations to avoid its use. It is clear that an integral approach is necessary to optimise the efforts directed to substitute harmful solvents by other more benign options. As we mentioned before, SOLVSAFE (Advanced Safer Solvents for Innovative Industrial Eco-processing) is a European initiative joining industry and academia, initially as part of an integrated EU project, whose main goals are the reduction in the number and quantity of hazardous solvents utilised in industrial applications, the reduction of VOC emissions to the atmosphere, the decrease of CO_2 emissions and the increase in the quantity of renewable materials utilised. To fulfil these objectives, fundamental research and development have been carried out in the following areas: 1) non-conventional, non-toxic solvents; 2) solvents from renewable feedstocks; 3) water-based processes and 4) innovative synthetic strategies.

As an example of the kind of developments achieved, we will briefly mention glycerol-derived solvents. In recent years, the increase in the production of biodiesel has leaded to a surplus in the production of glycerol. If valuable solvents are synthesised from glycerol, then not only will it have new applications substituting other more toxic solvents, but also the large surplus of glycerol will be given additional value. Within the SOLVSAFE context, several tens of glycerol derivatives (many of them not previously known) have been synthesised, and their physicochemical and solvation properties determined.[48–50] Applications in the biocatalysis field have also been found.[50,51]

Toxicity of Organic Solvents versus Enzymes and Whole Cells

The natural solvent of whole cells and enzymes is water, which has serious drawbacks for organic synthesis, due to the poor solubility of most of the organic compounds. Furthermore, sometimes side reactions, such as hydrolysis, polymerisation or racemisation, can take place in the presence of water, leading to secondary products. Until the mid-1980s, enzyme-catalysed reactions in organic solvents were

considered impossible, because it was assumed that enzymes need water to maintain their natural structure and thus their catalytic activity.[52] However, over the last 20 years, it has been demonstrated that many enzymes can catalyse reactions in organic solvents,[53,54] which has brought new possibilities to light.

The solvent systems that have commonly been used for enzyme-catalysed reactions containing organic media can be classified into three different categories: monophasic aqueous–organic solutions (solvents miscible with water), biphasic aqueous–organic solutions (solvents inmiscible with water), and enzymes suspended in pure organic solvent. Although the activity of enzymes in organic solvents is in general lower than that observed in water,[55] there are several advantages to them, since regio- and chemoselectivity can be controlled by using the appropriate solvents.[54] Indeed, for many nucleases, lipases or proteases studied in our group, the acylation is solvent dependent and therefore can be modulated by the medium.[56,57]

In the case of whole cells, the toxicity of the organic solvents is well known. The solvents alter the cell membrane avoiding and/or interfering with mass transfer into and outside the whole cell, and can even affect the cell viability.[58] To maintain the integrity of the cell membrane, biphasic conditions have been recommended[59,60] by using solvents with $\log P > 4$. Another alternative is whole-cell trapping in different matrixes, such as porous polyacrylamide or polyacrylonitrile,[61] calcium[58] or barium alginate,[62] or polyurethane foam,[48] which act as a barrier against the toxic solvent. Finally, the use of hyperthermophiles (growing at $T > 80\,^{\circ}C$) allows the possibility of employing useful enzymes that expand the range of the reaction conditions suitable for biocatalysis,[19] especially in the presence of organic solvents.

Biotransformations in Sustainable Conditions

Solvent-free enzymatic reactions: The most sustainable strategy is to avoid the use of solvents completely.[15] This approach is relatively simple if one or more of the reagents is a liquid, 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.[20] This methodology has been used in different biotransformations especially in lipase-catalysed transesterifications.[63,64] Evidently, this strategy is not useful in the case of whole cells.

Biotransformations in water: As we have mentioned before, it is generally established that, inside the green chemistry philosophy, the best solvent is no solvent;[28] nevertheless, if a solvent needs to be used, then water is preferred.[65] In fact, water is the greener solvent par excellence, although its use leads to some problems in biotransformations, especially related to the low solubility of organic compounds in water, as discussed above. Of course, a plethora of biotransformations have been described in water both in the laboratory[66,67] and at an industrial scale.[68] As examples, we will mention the stereoselective reduction of prochiral 4*R*-carvone (**1**), which is sequentially reduced to 1*R*,4*R*-dihydrocarvone (**2**) and then to 1*R*,2*S*-4*R*-dihydrocarveol (**3**) (Figure 1),[66] or the synthesis of different nucleosides catalysed by nucleoside phosphorylases or adenosine deaminase with excellent yields, stereo- and regioselectivity.[67] The use

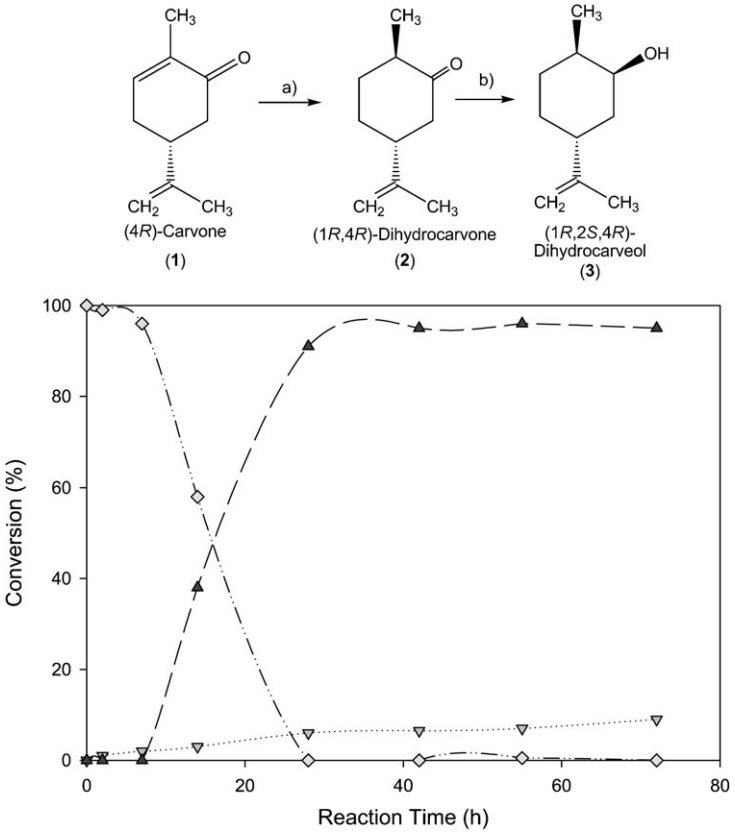


Figure 1. Stereoselective reduction of **1** by using *Diplogelasinospora grovesii* whole cells in water.[67] ◆: **1**, ▼: **2**, ▲: **3**.

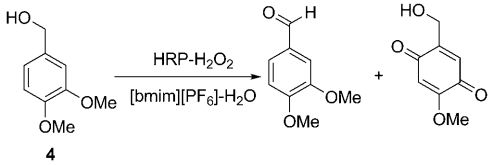
of water is extremely important in redox processes, using either isolated enzymes or whole cells, because of the deleterious effect of most organic solvents on these biocatalysts, as we have mentioned in the previous section. Another complexity arises from the fact that redox enzymes require precise co-factors for their activity, and these molecules are relatively unstable and expensive if used in stoichiometric amounts; thus, they should only be used in catalytic amounts and must be regenerated in situ by using a second redox reaction to allow them to re-enter the reaction cycle, therefore, leading to a drastic economic reduction.[69–72]

Co-factor recycling can be avoided if whole microbial cells are used as biocatalysts for redox reactions;[70] in this case, inexpensive sources of redox equivalents, such as carbohydrates, can be used, because all of the enzymes and co-factors required for the metabolism are indeed present inside the cell. However, from the perspective of green chemistry, the use of isolated enzymes (or non-growing whole cells) is generally recommended, due to the fact that

less biomass is produced, therefore facilitating product recovery and productivity (usually not very high, since non-natural substrates are only tolerated at concentrations as low as 0.1–0.3 %[73]). Therefore, the E factors (defined as kg waste per kg of product[74,75]) for whole-cell processes are generally very high.

We have already mentioned the different ways in which water can be present in the reaction medium of biotransformations. For redox processes, it is quite common to use some co-solvents to improve the solubility of the reactants and facilitate enzyme–substrate interactions. In this sense, the use of glycerol as a green bio-co-solvent in whole-cell-catalysed bioreductions of ketones has been recently described,[76,77] leading to excellent yields and stereoselectivity values. Although this procedure has not been implemented so far at an industrial scale, the indisputable advantages of employing polar, non-toxic, biodegradable and recyclable glycerol, manufactured from renewable sources, led us to foresee its unquestionable potential.

ILs have been also used as water co-solvents for oxidoreductase-catalysed reactions.[78,79] Thus, the use of [BMP][NTf_2] (BMP = butylmethylpyrrolidinium, NTf_2 = bis(trifluoromethylsulfonyl)imide), a water immiscible IL, resulted in a dramatic improvement in the enzymatic reduction of ketones with co-factor recycling catalysed by alcohol dehydrogenase (ADH) from *Rhodococcus erythropolys*[80] opening the possibility of an industrial application, due to the increase in the maximum product concentration, the half-life of the enzyme (more than 250 h in the presence of 10 % (v/v) [BMP][NTf_2]), the initial rate of the reaction (more than 4 times), as well as the facilitation of product isolation. Kumar and co-workers have described the oxidation of veratryl alcohol (**4**, Scheme 1), a model compound for lignin



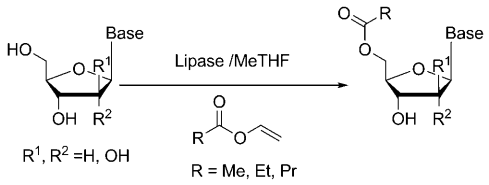
Scheme 1. Biomimetic biooxidation of veratryl alcohol (**4**).[81] HRP = horseradish peroxidase, bmim = 1-butyl-3-methylimidazolium.

sub-structures, using hydrogen peroxide and horseradish peroxidase (HRP) in ILs,[81] describing similar yields to those in aqueous solution, but reporting a considerable increase in the stability of HRP, which could be reused with appreciable activity for up to five cycles. Thus, the poor stability of HRP in the presence of an excess of hydrogen peroxide (limiting factor for its industrial use in delignification) can be overcome.

Biotransformations in solvents obtained from renewal sources: A possible solution to eliminate the petroleum-based solvents is the use of a new generation of solvents derived from biomass. Nowadays, several groups are involved in the research of these bio-solvents and their applications. Some of them, such as glycerol, ethanol, 2-methyl-tetrahydro (MeTHF) or cyclopentylmethyl ether, are also available from commercial sources, although their use in biocatalysis is scarce.

Candida antartica lipase B (CALB) is a well-known enzyme that efficiently catalyses kinetic resolution of representative secondary alcohols, esters, and amines, by using glycerol as the solvent and acyl donor/acceptor. High conversions and enantioselectivities were achieved and the product was easily separated by simple extraction with diethyl ether.[82] The resolution of a racemic mixture of 2-methyl heptanoate has been described with immobilised CALB in glycerol.[83] High alcohol yields and high enantioselectivities of the ester and of the corresponding alcohol were achieved. Enantiopure compounds were also prepared by enantioselective transformation of prochiral compounds. This method increases the theoretical product yield to 100 % and avoids complicated separation of the two enantiomers.

High regioselective esterification of nucleosides has been described in our group using immobilised CALB and MeTHF as the solvent,[51] leading to high yields and regioselectivity in the acylation of 1-β-arabinofuranosyluracil; this is now is being used in industrial processes (Scheme 2).

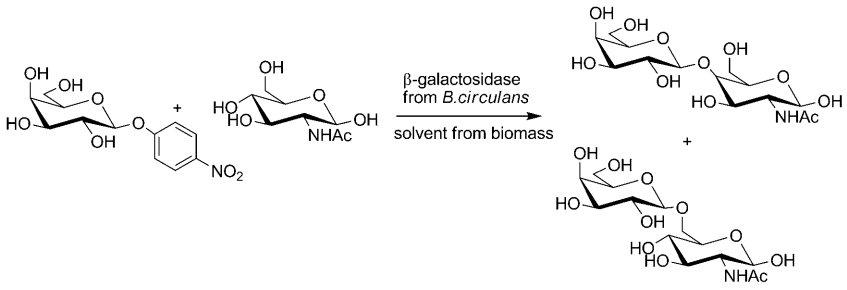


Scheme 2. Regioselective acylation of nucleosides in MeTHF.[51]

Using fatty acids from biomass, COGNIS S.A. has developed *N*,*N*-dimethylamides that have been used in biotransformations. Thus, Hernáiz et al.[84] have described a change in the regioselective transglycosylations catalysed by β-galactosidase from *Bacillus circulans*, changing from β-1,4- to β-1,6-linkages by using these bio-solvents, as shown in Scheme 3.

Organic carbonates could theoretically be considered as a solvent family obtained from alcohols and CO_2.[85] Indeed, dimethylcarbonate can be obtained from $scCO_2$ and MeOH.[86] The addition of CO_2 to epoxides is another industrial methodology to prepare organic carbonates. These compounds offer several advantages when used as solvents in biotransformations: 1) they are commercially available, 2) they are biodegradable, 3) they are polar aprotic solvents, 4) they show low eco- and cytotoxicity and 5) they can be considered as non-VOC-producing solvents.[88]

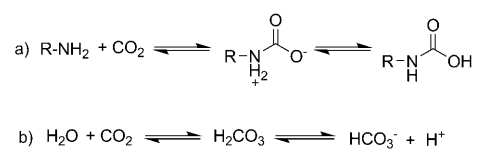
Biotransformations in organic carbonates have not been extensively developed. This could be because many enzyme-catalysed hydrolysis reactions require biphasic conditions and pH control, which is not trivial in these biphasic condi-



Scheme 3. Change in regioselectivity in transglycosylation by using solvents derived from biomass.[84]

tions. Furthermore, lipase-catalysed hydrolysis of dialkylcarbonates or cyclic carbonates has been described under these conditions.[89] As a consequence, these compounds cannot be used in many lipase-catalysed hydrolysis reactions. Alternatively, enzyme-catalysed reactions in monophasic conditions have given promising results, such as in the lipase-catalysed resolution of racemic alcohols by using vinyl acetate in propylene carbonate, yielding conversion values between 40–50% and *ee* values higher than 99%.[89] Chiral carbonates can be used as chiral solvents. These carbonates can be prepared by lipase-catalysed kinetic resolution of racemic carbonates. The most developed process is the large-scale synthesis of homochiral (*R*)-or (*S*)-glycerolcabonate.[90] These compounds can be interesting as solvents for stereocontrolled chemical or enzymatic processes.

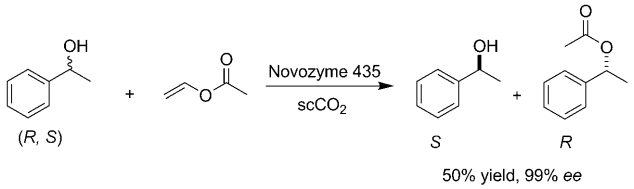
Biotransformations in SCFs: The most popular SCF employed as a green solvent in biocatalysis is CO_2.[91] Supercritical CO_2 ($scCO_2$) is chemically inert, has unique transport properties, it is cheap, not flammable, non-toxic, readily available and its critic parameters (31 °C and 73.8 bar) are compatible with the conditions required for enzyme reactions. Supercritical water cannot be used in biotransformations due to its critic parameters (374 °C and 221 bars) being too high for the enzymes. Other substances, such as ethane or propane, are less commonly employed because of their high cost and higher flammability than CO_2. Although CO_2 is the most usual supercritical solvent, some side reactions can take place when $scCO_2$ is employed, which can affect the enzymatic activity. The first one, carbamate formation, may occur between lysine residues present in the enzymatic surface and the medium CO_2 (Scheme 4a). However, the influence of this carbamate formation in enzymes is still being debated. Many groups believe that carbamate formation can contribute to increasing the selectivity of the reaction.[92,93] For example, in the asymmetrisation of 1,3-propanediacetate, when the pressure increases, the *ee* value improves to 50%.[94] On the other hand, Habulin and Knez[94,95] reported that carbamates may have an effect on enzyme conformation, leading to enzyme deactivation. This deactivation has also been described by Kamat et al.[96]



Scheme 4. Side reaction produced by $scCO_2$.

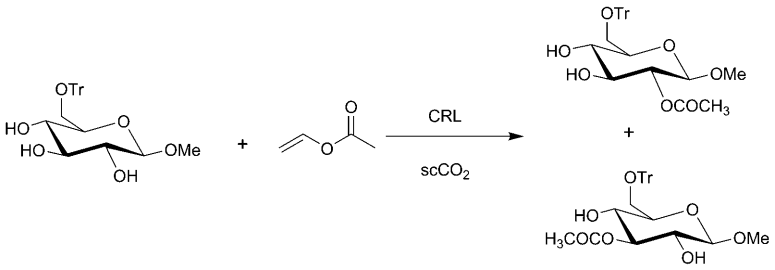
The second side reaction that can take place when $scCO_2$ is employed is the formation of carbonic acid between the CO_2 and the water present in the reaction medium (Scheme 4b). This formation increases the pH, which can cause damage to the enzyme.[97] A study on how pH changes in presence of supercritical water–CO_2 was reported by Toews et al.[98] However, these alterations in pH can be controlled by addition of salts, such as sodium bicarbonate or sodium hydroxide.[99]

Different hydrolases[5,100,101] have been employed in SCFs, especially lipases, proteases and galactosidases. Lipases have shown good catalytic activity in SCFs.[7,100,101] There are several papers showing that lipases can operate in SCFs better than in organic solvents,[102–104] and describing how the use of $scCO_2$ has produced an improvement in enantioselectivity.[105–107] Many of these reported effects on lipases take place if the enzyme is immobilised on different surfaces, such as Celite, silica gel or polypropylene.[39,40,108] The employment of immobilisation techniques for industrial purposes is highly advantageous, due to the fact that this solid state confers robustness to the biocatalysts, and facilitates its recyclability. For instance, Matsuda et al.[29] have reported a kinetic resolution of 1-phenylethanol with a Novozyme 435 (immobilised CALB). The process was carried out by using $scCO_2$ to give the product in 99% *ee* and 50% yield (Scheme 5).



Scheme 5. Enantioselective acylation of 1-phenylethanol in $scCO_2$.[29]

Palocci et al.[109] have described how $scCO_2$ can modulate regioselectivity in the acylation of 6-*O*-trityl-β-D-glucopyranoside by using lipase from *Candida rugosa*. The regioselectivity of the reaction was shifted towards the synthesis of 3-*O*-acetyl-6-*O*-trityl-β-D-glucopyranoside, and this is a function of the physicochemical parameters of the SFC employed (Scheme 6) with a conversion of 91% as the only regioisomer.



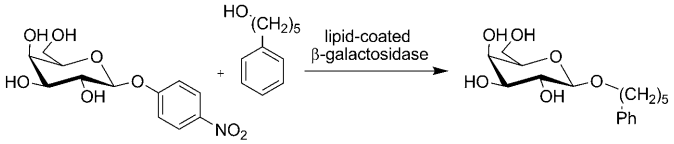
Scheme 6. Regioselective acylation of 6-*O*-trityl-β-D-glucopyranoside in $scCO_2$ (modified from ref. [109]). Tr = trityl, CRL = *Candida rugosa* lipase.

Proteases have seldom been studied in the presence of SCFs (Table 1). Subtilisin has been used in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APPE) and methanol in the presence of supercritical CHF_3,[52] demonstrating that changes in the physical properties of the SCF can affect the catalytic efficiency of the enzyme. Fontes et al.[113] proved that the same enzyme in $scCO_2$ worked with a rate of 97% at 114 bar; however, the higher the pressure, the lower the enzymatic activity, up to 300 bar, at which pressure the enzyme is completely deactiviated.

Finally, glycosidases are enzymes scarcely used in the presence of SCFs because the reaction conditions that preserve their activities are very restrictive.[52] They are commonly used in aqueous medium, while the employment in organic medium is still being studied.[105,106] However, Mori et al.[41,42] have described the use of $scCO_2$ or $scCHF_3$ in the transglycosylation of 1-*p*-nitrophenyl-β-D-galactopyranoside and 5-phenylpentan-1-ol, catalysed by lipid-coated β-galactosidase from *B. circulans* (Table 2).

ILs have been used as co-solvents in downstream processes when $scCO_2$ has been used as the reaction medium. Due to the low solubility of ILs in $scCO_2$, mixtures of both must be used. In this sense, Lozano et al. described several examples of using CALB in the dynamic kinetic resolution of *rac*-1-phenylethanol by transesterification with vinyl propionate in different biphasic IL/$scCO_2$ systems, reporting excellent yields (76%) and enantioselectivities (*ee* values between 91–98%).[114]

Table 2. Synthesis of galactosides using *B. circulans* β-galactosidase and different SCFs as reaction medium.



Product	SCF	*t* [h]	Yield [%]	Ref.
1-*O*-(5-phenylpentyl)-β-D-galactopyranoside	CO_2	3	72	[41]
1-*O*-(5-phenylpentyl)-β-D-galactopyranoside	CHF_3	5	90–95	[42]

Table 1. Protease-catalysed transesterification reactions using subtilisin from *B. licheniformis* in different SCFs.

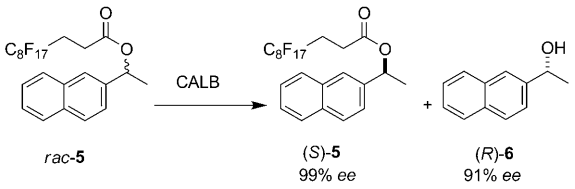
Product[a]	SCF	Reaction conditions			Yield	Initial rate	Ref.
		T [°C]	*p* [bar]	*t* [min]	[%]		
APEE + chloroethanol	CO_2	45	150	15	10–54		[110]
APEE + methanol	CHF_3	50	69			0.06 $mM^{-1}s^{-1}$	[111]
APEE + methanol	CHF_3	50	124			1.5 $mM h^{-1}$	[112]
APEE + methanol	CO_2	47	114	60	97		[113]

[a] APEE = *N*-acetyl-L-phenylalanine ethyl ester.

Biotransformations in fluorous solvents: As previously described, the main characteristic of fluorous solvents is their temperature-dependent miscibility with conventional organic solvents, which allows the biocatalyst and the substrate to be dissolved in different phases at room temperature and a monophase to be obtained by warming the reaction. Once the reaction is completed and the mixture is cooled, a biphase is produced again, so that the product can be easily isolated.[5,24,25]

The use of these solvents in biocatalysis is limited by the low solubility of the enzymes in such media. Saul et al.[115] used subtilisin Carlsberg in the transesterification of *N*-acetyl-phenylalanine propyl esters using tetrafluoroethane as solvent, reporting higher yields in tetrafluoroethane than in conventional solvents, maintaining an excellent enantioselectivity (99% *ee*).

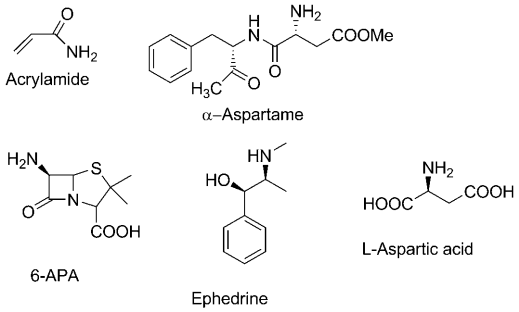
On the other hand, Luo et al.[116] described the kinetic resolution of 1-phenylethanol catalysed by Novozyme 435 (immobilised CALB), through a transesterification process using difluoromethane, 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoropropane as solvents. In all cases, the maximum yields were achieved at shorter reaction times than those reported for a conventional organic solvent (hexane), and the *ee* value obtained was 99%. These same authors[116–119] described the kinetic resolution (KR) of a racemic fluorous ester (*rac*-**5**) with CALB, leading to a mixture of enantioenriched alcohol (*R*)-**6** and fluorous ester (*S*)-**5** (Scheme 7). The mixture was performed in triphasic conditions with a simple fluorous–organic liquid–liquid extractive purification.



Scheme 7. Enantioselective hydrolysis of a fluorous ester with CALB.[110–113]

Applied Chemoenzymatic Processes

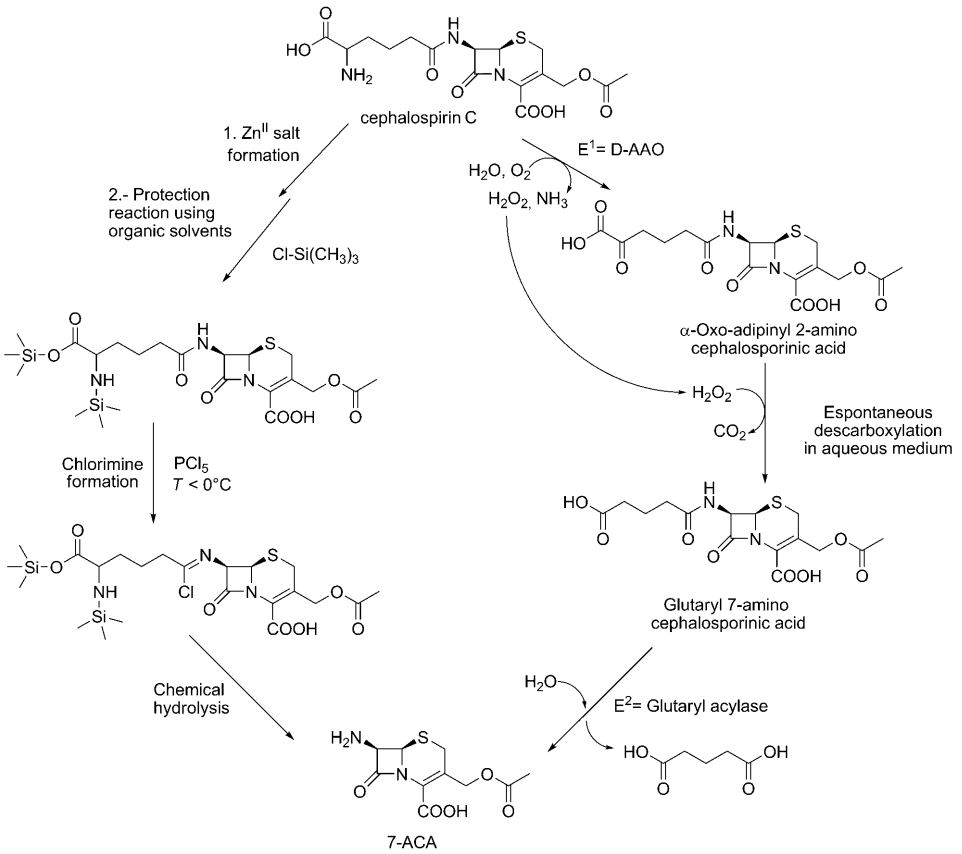
Biocatalysts (both as isolated enzymes and whole-cell systems) are increasingly being used to assist in synthetic routes, leading to complex molecules of industrial interest.[9,68,73,120] Whereas there is a particular interest in the use of biocatalysis to create new routes to lower value chemicals,[120,121] the biggest role for biocatalysis still remains in the pharmaceutical and chemical sectors,[122–124] where its regioselective and stereoselective properties enable difficult syntheses (often requiring multiple protection and deprotection steps) to be prevented. Nevertheless, the application of biocatalytic steps into a well-established synthetic procedure is complicated, due to investment costs, rebuilding of industrial plants and so forth. In general, the introduction of a biocatalytic process in cascade reactions is justified to reduce the number of synthetic steps, especially protection/deprotection steps. Even more, the total synthesis of industrially attractive molecules cannot be carried out by using exclusively biocatalytic steps. In fact, biotransformations are generally combined with traditional synthetic steps, therefore, increasing the sustainability of the overall process, especially if green solvents are also involved. Nowadays, large-scale industrial applications of biocatalysts include, for example, the thermolysin-catalysed synthesis of the low-calorie sweetener aspartame, the production of acrylamide and nicotinamide (assisted by nitrile hydratases) and, more recently, the production of biopolymers such as polylactic acid (Scheme 8).[120,125] Thus, some greener processes are critically presented and compared with the traditional approaches, taking into account the green chemistry principles.[2,3]



Scheme 8. Examples of molecules manufactured by using industrial applications of biocatalysts.

Good examples of the replacement of traditional organic processes by a greener chemoenzymatic methodologies include the industrial synthesis of semisynthetic penicillins and cephalosporins,[4] the transformation of natural and synthetic fibers,[126] the pulp kraft-bleaching and recycling of paper[127] and the multi-step synthesis of polyketide and glycopeptide antibiotics.[128]

In Scheme 9 we show a classical example, a comparison between the pure chemical synthesis of 7-aminocephalosporanic acid (7-ACA) and the chemoenzymatic strategy, start-



Scheme 9. Chemical versus chemoenzymatic synthesis of 7-ACA.

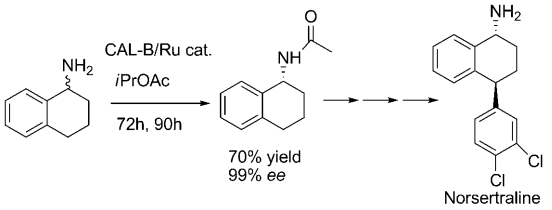
ing in both cases with the same compound, cephalosporin C obtained by conventional fermentation.

On the one hand, the conventional chemical synthesis uses two contaminants and dangerous reagents ($ZnCl_2$ and PCl_3) as well as chlorinated hydrocarbons as solvents, producing a lot of waste and concomitant sub-products, such as $Zn(NH_4)_2{\cdot}PO_4$, HCl, H_3PO_4. On the other hand, the chemoenzymatic process is carried out in aqueous solutions, generating only NH_3, CO_2 and glutaric acid as sub-products. Mother liquors requiring incineration are reduced from 29 to 0.3 tonnes, non-toxic Zn^{II} salts are produced and the VOC emissions are reduced from 7.5 to 1 kg per kg of product.[129]

Enantiomerically pure chiral amines are key intermediates in a number of pharmaceutical compounds that possess a wide range of biological activities.[130] Biocatalytic approaches to these building blocks have traditionally relied upon KR of racemic substrates by using hydrolytic enzymes[131] such as lipases, acylases or proteases, combined with different chemical steps. Some of these processes are successfully operated on a large scale.[132] In certain cases it is possible to racemise the unreactive enantiomer by addition of a racemisation catalyst leading to a dynamic kinetic resolution (DKR) process.[133] Alternative approaches based upon deracemisation have also been developed in which enantioselective amine oxidases are combined with non-selective reducing agents, resulting in yields higher than 50% and high enantioselectivities.[134] Recently transaminases have emerged as viable biocatalysts for chiral amine production.[135]

Truppo et al.[136] have described a process for the resolution of amines using a transaminase in combination with an amino acid oxidase and a catalytic amount of pyruvate as the amine acceptor. This overcomes the inherent limitation in productivity of such systems due to inhibition of ω-transaminase at higher pyruvate concentrations. A range of benzylamines were resolved with excellent enantioselectivity (99%) and conversion (50%). The amine enantiomer could be selected by using *S*- or *R*-selective transaminase[136] (Scheme 10).

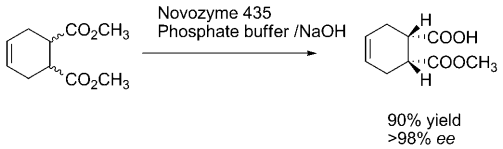
Another chemoenzymatic approach to chiral amines has been published by Bäckvall's group.[137] Racemisation with a Shvo-type Ru catalyst in conjunction with KR by acylation with donors, for example, isopropyl acetate/bibenzylcarbonate and Novozyme 435, gave the corresponding *R*-amides/carbamates in high yield and high purity. Carbamates could be deprotected under mild hydrogenolysis conditions. This DKR was applied to different structural kinds of amines, giving yields of 60–95%, and *ee* values of 90–99%. The developed methodology was applied to a synthesis of norsertraline (Scheme 11).



Scheme 10. KR of benzylamines catalysed by a coupled transaminase/oxidase system.

Scheme 11. DKR of chiral amines.[137]

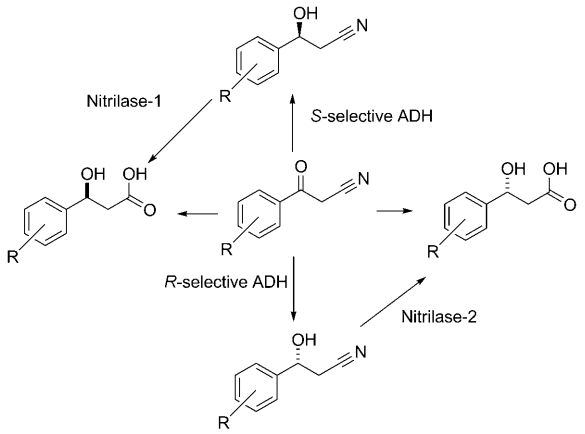
The enzymatic desymmetrisation of simple *meso* compounds can also be applied to pro-chiral compounds. The chiral monoester, (1*S*, 2*R*)-2-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid is a key chiral intermediate for the synthesis of a drug candidate for the modulation of chemokine receptor activity.[138] 1*S*,2*R*-Monoester and its enantiomer 1*R*,2*S*-monoester can be obtained by resolution[139] of the racemic acid with alkaloids, for example, cinchonidine and ephedrine. However, as the maximum theoretical yield of the resolution process cannot be more than 50%, a process that affords desymmetrisation of a *meso* compound would be greatly preferred. Goswami and Kissick,[140] have described an efficient process catalysed by *C. antarctica* B lipase to prepare the chiral monoester from the corresponding *meso* compound. The enantiomer obtained is the opposite of that obtained from pig liver esterase hydrolysis (Scheme 12).



Scheme 12. Asymmetrisation of a *meso*-diester.[140]

Optically active β-hydroxynitriles and β-hydroxycarboxylic acids are key building blocks for the synthesis of a variety of pharmaceutically important compounds. For example, many biologically active compounds, such as β-blocker drugs, contain 1,3-aminoalcohol moieties, which are often prepared by reduction of β-hydroxynitriles. These structures to be used as anti-inflammatory drugs,[141] chiral β-hydroxy carboxylic acids can be converted to β-amino acids,[142] β-lactams[143] or β-lactones.[144] The β-hydroxy carboxylic acid structure has often been found in polyketide natural products such as amphotericin B,[145] tylosin, rosaramicin[146] and

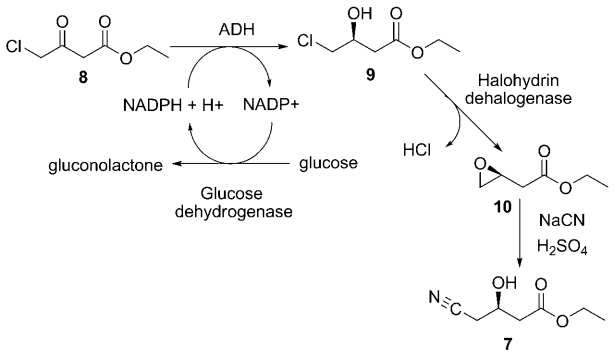
in the marine natural product hapalosin.[147] Ankati et al.[148] have shown that chiral β-hydroxycarboxylic acids or chiral β-hydroxynitriles can be efficiently prepared by alcohol dehydrogenase (ADH) catalysed reduction, followed by hydrolysis of the nitrile function with a nitrilase. By correct choice of ADH, both enantiomers are accessible. Aryl and alkyl examples are reported; with aryl compounds giving *ee* values in the range 95–99%. The application of a nitrilase to perform the hydrolysis reduces side reactions and maintains chiral integrity. The β-hydroxy acids can be prepared in a one-pot process by sequential addition of the two enzymes (Scheme 13).



Scheme 13. Production of optically active β-hydroxynitriles and β-hydroxycarboxylic acids.

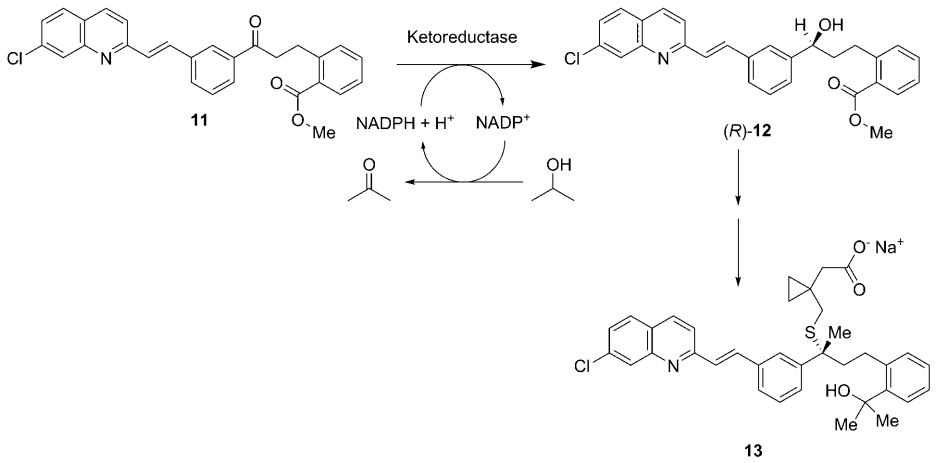
Very recently, ADHs have been also used by the company Codexis.[149,150] to prepare the hydroxynitrile (*R*)-**7** (Scheme 14), a key intermediate in the synthesis of atorvastatin, by using a coupled two enzyme process, in which the biocatalyst activity has been increased by using directed evolution.

In the first step, (*S*)-ethyl-4-chloro-3-hydroxybutyrate (**9**) is prepared by reduction of the ketoester **8**, using a mutant ketoreductase from *Geotrichum candidum* SC 5469 ($T=25\,^{\circ}C$, in aqueous buffer, pH 7) coupled to a nicotinamide adenine dinucleotide phosphate (NADPH) dependent glucose dehydrogenase for co-enzyme regeneration. The product (*S*)-**9** is obtained in 96% isolated yield with 99.5% *ee*, starting with a concentration of **8** of 160 $g\,L^{-1}$. In the second step, a halohydrin dehalogenase is used to catalysed the substitution of Cl by CN at pH 7.3 and 40 °C (yield = 99.5%, $t=24$ h, 99% *ee*). These modifications, compared with the traditional chemical synthesis, decreasing the overall E factor from >100 down to 18, also avoiding, in addition, an energy-consuming vacuum distillation. As a consequence, this enzymatic methodology clearly fits with the 1st, 6th and 9th green chemistry principles.[2,3]



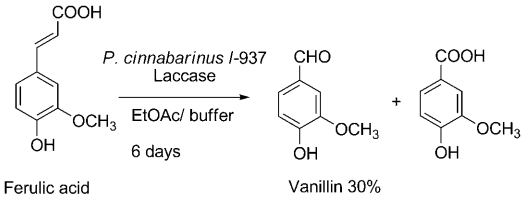
Scheme 14. Chemoenzymatic synthesis of **7**.

Liang et al.[151] have recently described the reduction of the hydrophobic ketone **11**, using genetically modified ketoreductases (Scheme 15). The *R* alcohol ((*R*)-**12**) was obtained in 95% yield with >99% *ee* for the crude product ($t=24$ h, $T=48\,^{\circ}C$), improving the chemical process (85–90% yield). The biocatalyst is less expensive than water sensitive (−)-chlorodiisopinocanphenylborane (DIP-Cl) used in the chemical reduction, which must be conducted at −20 °C.[152] The high hydrophobicity of **11** required the use of a mixture of *i*PrOH, toluene and triethanolamine buffer of pH 8.0 (5:1:3 v/v), in which *i*PrOH is used as an auxiliary substrate to regenerate the oxidised co-enzyme ($NADP^+$), avoiding the use of a second enzyme as described in the previous example. This process has been scaled up to 200 kg per batch to obtain (*R*)-**12**, the chiral intermediate for the synthesis of sodium montelukast **13**, an anti-asthma drug.



Scheme 15. Asymmetric reduction of **11** by using a ketoreductase and *i*PrOH as the second substrate.

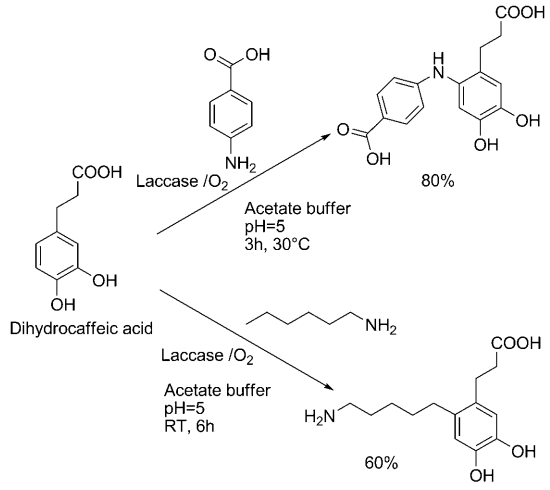
Laccases (benzenediol oxygen oxidoreductases, EC 1.103.2) are very interesting commercially available enzymes, which are able to catalyse the oxidation of low-molecular-weight phenols or anilines under very mild conditions.[153] The oxidations are performed in low ecotoxic conditions (monophasic media such as acetone/water; EtOAc/water, etc.), as in the case the oxidation of isoeugenol with laccases from fungus (*Pycnoporous coccineus*).[154] The oxidation of ferulic acid has been used to generate stable yellow colorants related to vanillin to be used in the food industry (Scheme 16). The reaction is performed in EtOAc/phosphate buffer.[155] Increasing the percentage of EtOAc, hydrophobic substances such as steroid hormones[156] can be also oxidised to form anabolic steroids.



Scheme 16. Production of vanillin from ferulic acid.

Another interesting laccase-catalysed reaction is the coupling of phenolic groups and amines. Due to the hydrophilic properties of these structures, the reaction can be performed in water, as shown in Scheme 17, starting from the industrially interesting dihydrocaffeic acid.

The reaction takes place at room temperature and can be carried out with aliphatic or aromatic amines. This methodology has been used to produce Trinuvin, a UV absorbing benzotriazole base useful as a photoprotector.[147] The key step for its synthesis is performed in water, therefore, avoiding the use of traditional reagents for chemical coupling, which also require solvents such as hexane, octane or toluene.



Scheme 17. Laccase-catalysed coupling of phenolic groups and amines.

Finally, glycerol (a typical bio-solvent) is widely used in the biotransformations of terpenois or aromatic ketones used in perfumes.[77] Glycerol solubilises the substrates and it is immiscible with traditionally used green solvents, such as EtOAc and recently $scCO_2$.

Summary and Outlook

Biocatalysis is a very useful tool when examining a chemical process from a green chemistry perspective. In fact, the mild conditions required and the high selectivities obtained definitely contribute to improve chemometrics such as the E factor or atom economy. In addition, the use of low-ecotoxic green solvents combined with the biotransformation step is undoubtedly highly beneficial from an environmental point of view. So, in a synthetic scheme leading to the production of drugs and/or fine chemicals, the substitution of one or more chemical steps for the corresponding biocatalysed alternatives increases the "green qualification" of the overall procedure, both in the lab, pilot-plant or on fully industrial scales.

As a consequence, the upmost chemical and pharmaceutical companies are increasingly modifying their synthetic procedures under this philosophy, mainly pushed by some legislative initiatives, such as REACH (Registration, Evaluation and Authorization of Chemical Substances), instigated by the EU. Thus, this is certain to increase basic and applied research in biocatalysis in coming years.

Acknowledgements

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