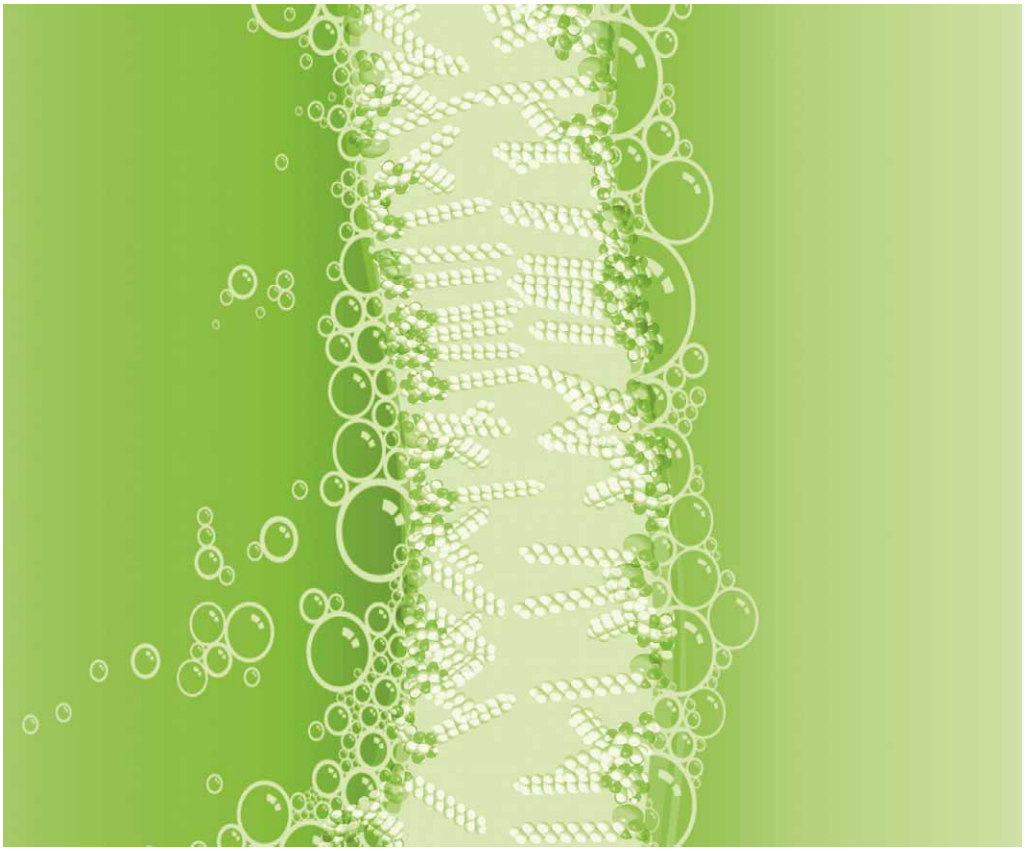
Chem Soc Rev

This article was published as part of the

Green Chemistry themed issue

Guest editors Chao-Jun Li and Paul Anastas

Please take a look at the issue 4 2012 table of contents to access other reviews in this themed issue



Chem Soc Rev

Dynamic Article Links

Cite this: *Chem. Soc. Rev.*, 2012, **41**, 1585–1605

www.rsc.org/csr

CRITICAL REVIEW

Expanding the organic toolbox: a guide to integrating biocatalysis in synthesis†

Christopher M. Clouthier‡[ab] and Joelle N. Pelletier*[abcd]

Received 20th October 2011
DOI: 10.1039/c2cs15286j

This *critical review* presents an introduction to biocatalysis for synthetic chemists. Advances in biocatalysis of the past 5 years illustrate the breadth of applications for these powerful and selective catalysts in conducting key reaction steps. Asymmetric synthesis of value-added targets and other reaction types are covered, with an emphasis on pharmaceutical intermediates and bulk chemicals. Resources of interest for the non-initiated are provided, including specialized websites and service providers to facilitate identification of suitable biocatalysts, as well as references to recent volumes and reviews for more detailed biocatalytic procedures. Challenges related to the application of biocatalysts are discussed, including how 'green' a biocatalytic reaction may be, and trends in biocatalyst improvement through enzyme engineering are presented (152 references).

[a] *Département de Chimie, Université de Montréal, Montréal, Canada. E-mail: joelle.pelletier@umontreal.ca*
[b] *PROTEO, the Québec Network for Protein Function, Structure and Engineering, Canada*
[c] *Département de Biochimie, Université de Montréal, Montréal, Canada*
[d] *CCVC, the Québec Center for Green Chemistry and Catalysis, Canada*

† Part of a themed issue covering the latest developments in green chemistry.

‡ Current address: Department of Chemistry, University of Ottawa, Ottawa, Canada.

1. Introduction

Over the past decade, an increasing number of chemists have turned to biocatalytic steps within their synthetic schemes, both in academia and industry. The synthetic advantages of enzymes have long been recognized: their potential for regio-, chemo- and stereo-control is immediately attractive, particularly when working with complex functionalized molecules or with scaffolds and functional groups that cannot withstand harsh reaction conditions. Enzymes are often used in aqueous media, and always at moderate reaction temperatures (< 100 °C)

Christopher M. Clouthier

Christopher Clouthier obtained his PhD in the area of biocatalysis in 2008 under the guidance of Prof. Margaret Kayser (University of New Brunswick). He then moved to Montréal where he joined the group of Prof. Joelle Pelletier as a postdoctoral scholar at the Université de Montréal where his research focused on probing the role of protein dynamics on enzyme function and evolvability.

Joelle N. Pelletier

Joelle Pelletier obtained a PhD in enzymology (McGill, 1995). She was a postdoctoral scholar at U. de Montréal (1998) and at U. Zürich (1999) developing enzyme-based selection methods. Now a Professor of Bio-Organic Chemistry, and Adjunct Professor in Biochemistry, at U. de Montréal, her research in biocatalyst engineering integrates physical-organic chemistry, high-throughput screening and molecular modelling. Her contributions include 50 refereed publications and chapters, and 10 patents. She is an executive board member of the Canadian Green Chemistry and Engineering Network and co-directs PROTEO, the Québec Network for Protein Function, Structure and Engineering. She will co-chair the 2014 GRC on Biocatalysis.

and pressures; because they are catalysts, are non-toxic (though may be allergenic), leave no residual heavy metal contamination and offer inherent biodegradability, biocatalysts offer a 'green' option that merits exploration.

The purpose of this critical review is to present basic considerations concerning adoption of biocatalysis (Section 2), key successes and challenges in biocatalysis over the past 5 years (Section 3), and to provide resources for the non-initiated (Section 4). While presenting an overview of key advances in biocatalysis, we point out recent volumes and reviews which provide a more comprehensive survey. We also highlight specialized websites and service providers directed to identifying and obtaining suitable biocatalysts. Our main focus will be the recent use of biocatalysts in the synthesis of value-added synthetic targets, with an emphasis on pharmaceutical intermediates while touching on bulk chemicals and polymer synthesis. Where relevant, biocatalyst improvement through the application of directed enzyme evolution will be presented, to highlight the key role that enzyme engineering is likely to hold in furthering biocatalysis in the future (Section 5). We end with a brief discussion of some remaining challenges in the area (Section 6).

2. The right context for applying biocatalysis to organic synthesis

Early developments in biocatalysis date back millennia, under the form of controlled fermentation for the preparation of ethanol, and later in bread-making and in development of fermented dairy products. These developments were based on whole cells, where bacterial or yeast cells are used as a source of biocatalysts. This science became common lore, with few fundamental advances made in the discovery, application and modification of biocatalysts for the transformation of matter.

Over the past century or so, the majority of advances in the realm of organic synthesis have concerned 'classical' organic synthesis, conducted in organic solvents and excluding biocatalysts. Classical chemical catalysts have evolved tremendously, increasing in efficiency, robustness, and offering better stereocontrol and chemoselectivities. In parallel with those advances, the nature of the catalytic power of biocatalysts has been largely unveiled since the 1960's, when enzyme structures were first revealed and biochemical characterization was undergoing rapid development. Nonetheless, we still struggle to understand how biocatalysts—or enzymes—procure rate enhancements as high as 10^{20} over the uncatalyzed reaction,[1] with turnover rates generally in the area of 1–1000 s^{-1} and 'total turnover numbers' (TTN) that match or surpass their most efficient chemical counterparts. Interestingly, increasing the efficiency of cell-based ethanol production—as a biofuel, rather than as a mind-altering substance—is one of the current drivers of biocatalyst discovery, development and improvement. Biofuel production is an example of enzyme-catalyzed bulk chemistry on the MT per year scale. In this review, some examples will be presented of well-established industrial biocatalytic conversions for production of bulk chemicals and fine chemicals; however, the majority of examples report recent advances shown to provide advantages on the laboratory-scale that have not yet been integrated into disclosed industrial processes.

An inspiring exemplification of the powerful nature of biocatalysis is at the source of the tons-per-year bioconversion processes of steroid drugs, for wound healing, athletic doping and the birth control (Fig. 1). The capacity for enzymes to display chemoselectivity, regioselectivity and stereoselectivity is all clearly illustrated in that example.

Enzyme-catalyzed reactions can offer significant advantages relative to reactions catalyzed by other means. The large surface contact established between a typical enzyme and its substrate offers high potential for regiocontrol, and for stereocontrol: only one area (regiocontrol) and face (stereocontrol) of the substrate is well positioned with respect to the catalytic

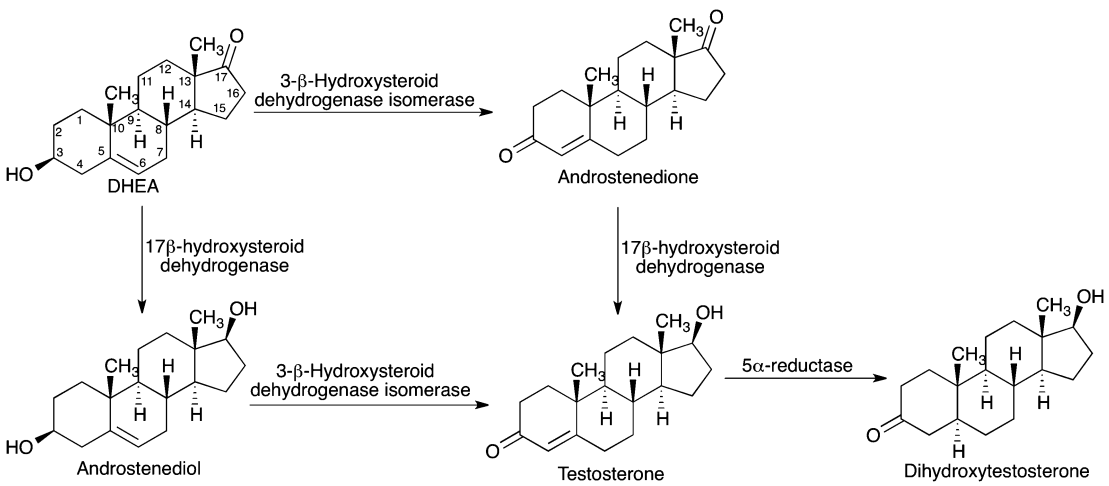


Fig. 1 Biocatalyst selectivity in steroid conversions. Myriad biocatalysts act upon the steroid ring, each one illustrating exquisite chemoselectivity and, often, stereoselectivity. Classical chemical approaches to these transformations are challenging due to steric hindrance and similar chemical reactivity of a number of atom sets. Mining microorganisms for new steroid bioconversions over the past six decades has resulted in a toolbox of biocatalysts affording diverse selective steroid transformations of industrial significance, including reduction of steroid ketones and alkenes, oxidation, isomerization, deacetylation and hydroxylation. Enzymes from *Actinobacteria* genera alone are known to selectively hydroxylate positions 1, 2, 6, 7, 9, 11, 12, 14–17 and 19 of the steroid ring.[151] Figure adapted from ref. 152.

machinery while the remainder of the substrate is excluded. As a result, a substrate molecule can often include multiple atoms of similar reactivity, only one of which will be the favoured target for enzyme-catalyzed conversion (chemoselectivity).

Such high selectivity can be advantageous, as it can eliminate tedious separation steps of undesired isomers. However, it can also prove limiting: many enzymes have a strictly defined natural substrate scope, which typically translates into a fairly narrow synthetic substrate scope. For this reason, enzymes that naturally accept a broader substrate distribution can prove advantageous as multipurpose catalysts. In the sections below, certain enzymes will be highlighted not only for their capacity to transform a breadth of substrates, but also to perform a range of chemistries. For example, one of the most commonly applied biocatalysts over the past decade is lipase B from *Candida antarctica* (CALB). While natively catalyzing the hydrolysis of long-chain fatty acid esters, it has also been applied to hydrolysis and the synthesis of a broad variety of esters and to diverse chemistries including Michael additions and ring-opening polymerization.

Certain advantages of including biocatalyzed steps in a synthetic route are illustrated in a large-scale chemoenzymatic process for the production of pregabalin, the active ingredient in the chronic pain relief block-buster drug, Lyrica.[2] In that process, the key stereocenter was chemically generated in its racemic form for enzymatic kinetic resolution using a lipase from *Thermomyces lanuginosus* (Lipolase). With recycling of the undesired enantiomer, the overall process provided an almost doubled yield, with 99.5% purity and 99.75% ee, as well as a reduced environmental footprint: 5 times less reagents and 8 times less solvent were required than the previous manufacturing route, per kg of product. This example of a large-scale industrial chemoenzymatic synthesis of an active pharmaceutical ingredient is not isolated.[3] Indeed, the past 5 years have seen an increase in the development of industrially-relevant chemoenzymatic processes, where biocatalysts are called upon to efficiently resolve problematic synthesis steps, while being environmentally and economically advantageous.[4] Comparisons are most informative when conducted *via* cradle-to-gate life cycle impact estimations comparing 'traditional' and biocatalytic syntheses, as reported for the synthesis of the important pharmaceutical intermediate 7-aminocephalosporic acid.[5]

BOX 1: How green is my biocatalysis?

It is easy to perceive biocatalysis as inherently 'green': reaction conditions are generally mild and environmentally benign, and the catalyst itself is biodegradable. Nonetheless, the full impact of biocatalysis on a process requires deeper analysis of the entire life-cycle.[5,6] The use of a biocatalyst generally reduces or eliminates requirement for protective group chemistry, thus reducing the number of reaction steps. In turn, each reaction step that is avoided reduces the requirement for reagents, solvent, energy and time. This contributes to improve reaction 'greenness' and, as a rule, economic viability. Some concepts will be introduced here; a more detailed presentation of green metrics applied to biocatalysis is available.[7]

One hurdle to biocatalysis may be the choice of reaction medium appropriate for both the biocatalyst and the reagents, particularly when the reagents are poorly soluble in aqueous media. The overall process becomes less green if one must remove water with additional extraction steps or undertake energy-intensive evaporation of water; removal of water with selective membranes may be envisaged if the physical properties of the reaction components allow. The addition of organic co-solvent to an aqueous reaction medium may provide the requisite properties allowing both adequate solubilisation of reagents, and maintenance of enzyme activity. Over the last decade, research efforts have been devoted towards the application of biocatalysts in non-aqueous reaction media. While an in-depth discussion of this area falls outside the scope of this review, the reader is directed towards excellent reviews (and references therein) of the topic, indicating important developments within this area.[8–13] The behaviour of specific enzymes in the presence of varying concentrations of different organic solvents and reaction components differs widely and merits exploration, because certain conditions may stabilize enzymes or modulate their activity in ways that can be advantageous. For example, lyophilisation in the presence of kosmotropic salts can allow for recovery of enzyme activity upon solubilization in organic solvent.[14] While the main thrust of research in this area dates back to the 1980's and 1990's, developments in this area continue to be of interest, as illustrated by specific, recent reports on the activity of a phenylalanine ammonia lyase[15] or of a feruloyl esterase for breakdown of lignocellulosic biomass.[16]

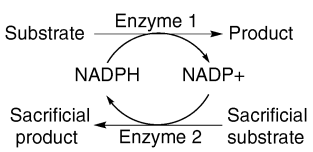
The choice of co-solvent has an important impact not only on enzyme activity and reagent solubilisation, but also on reaction 'greenness'; an excellent source of information is the 'GSK solvent selection guide', which considers the environmental sustainability of over 100 solvents, in the context of manufacturing active pharmaceutical ingredients.[17] Computational tools have also been developed to aid in the selection of solvents that are both 'green' and compatible with specific experimental requirements.[18] Further ingenious means to increase reaction 'greenness' have been reported, such as 'enzymatic transcrystallization'. Here, the key to efficient synthesis of the industrially-relevant 12-aminolauric acid from ω-laurolactam was the insoluble, crystalline form of both the reagent and the product under the aqueous biocatalytic reaction conditions.[19] Because the hydrolytic conversion was efficient ($\geq$ 95%), the product was recovered by simple filtration, in this multi-gram (0.1 mol) scale demonstration. While transcrystallization is attractive for this application, it will be challenging to transfer the process to other reactions.

Biocatalysis may reduce the number of required reaction steps relative to conventional catalysts, and eliminate harmful metals, as illustrated by the award-winning synthesis of the anti-diabetic, sitagliptin (Januvia™). High-pressure asymmetric hydrogenation using a rhodium-based chiral catalyst and a recrystallization were replaced by the direct biocatalytic synthesis of the desired product.[20] Another approach to reducing the number of steps is to perform

'one-pot', multistep cascade reactions. These eliminate the need to isolate reaction intermediates, thus increasing reaction 'greenness', and can be compatible with use of biocatalysts, including biocatalytic oxidation–reduction cascades.[21] Sheldon and colleagues have reported such a two-enzyme cascade for the synthesis of enantiopure aliphatic α-hydroxycarboxylic amides from aldehydes.[22] Ultimately, how green a process is must be assessed by a full life cycle analysis, which includes all steps of a process from the environmental impact pertaining to feedstock production to the disposal of each spent reaction component.[23] For example, industrial margarine production proceeds by interesterification of fats. Biocatalysis improved all environmental factors considered, relative to chemical catalysis, including energy consumption, global warming, acidification and smog formation.[23] As an additional benefit, chemically catalyzed interesterification of fats results in undesired darkening of the product, as opposed to the biocatalyzed process.

Biocatalysts can be applied in solution, though large-scale applications tend to use immobilized enzyme. Ideally, the immobilized enzyme is separable from the reaction components by filtration, facilitating product purification and catalyst recycling. The interested reader is directed to recent reviews on the topic.[24–27] Biocatalysts can be immobilized under the form of covalently linked or cross-linked enzyme aggregates[22] or adsorbed on an inert resin, among other approaches, often providing increased stability under various reaction conditions. A relevant example of adsorbed biocatalyst is Novo-435, the most commonly used form of CALB. Enzyme immobilization can reduce global process cost when the enzyme is sufficiently robust to maintain activity upon recycling into reaction cycles, or in continuous-feed processes. A practical and commonly used form of enzyme immobilization is the use of whole cells, which is presented in greater detail in reviews such as ref. 28 and 29. Briefly, whole microorganisms—such as the yeast *Saccharomyces* and *Pichia*, and bacteria such as *Escherichia coli* and *Streptomyces*—efficiently express the enzyme for the relevant bioconversion. It has long been known that whole cell bioconversions may be performed in aqueous media, or may include co-solvent or be performed in a 2-phase system.[30] Indeed, whole cells are surprisingly robust, being able to adapt to growth in many water-miscible solvents, and in 2-phase systems, including 2-phase aqueous–ionic liquid systems.[31]

In addition, whole cells are highly efficient at supplying expensive, essential co-factors such as NAD(P)/NAD(P)H, FAD/FADH and coenzyme A (coA), as part of their normal metabolic equilibrium. An alternative approach to cofactor regeneration is the use of 'coupled' reactions, where a second enzyme is included in the reaction along with its substrate which, upon being converted to product, recycles the cofactor back to the state required for the reaction of interest (Scheme 1). The coupled reaction should be performed under conditions ensuring that it is not rate-limiting.[32] It can be advantageous to pull the reaction equilibrium through formation of a precipitate, or a volatile product.[33] Coupled systems hold the advantage of working with purified components, with little concern about undesired side-reactions.



Scheme 1 Conceptual representation of a typical coupled enzyme co-factor recycling strategy.

However, the inclusion of additional reaction components, particularly a second enzyme, may significantly increase cost.

For a more detailed survey of biocatalytic concepts and reactions of interest, we direct the reader to a number of recent, comprehensive volumes. Of particular interest is an in-depth presentation by Sheldon of the 'green' potential of biocatalysis.[7] In their well organized and complete volume, Tao, Lin and Liese[34] focus on biocatalysis for industrial drug discovery and manufacturing, including reaction engineering, an overview of metabolic engineering and specific considerations of green chemistry. Whittall and Sutton structured a wealth of practically oriented short protocols, providing the background and the how-to for application of readily available biocatalysts, directed towards non-experts.[35] Fessner and Anthonsen have collated expert contributions on specific topics within the multidisciplinary field of biocatalysis.[36] Yeh, Yang and McCarthy edited a volume comprising an excellent introduction to powerful approaches to enzyme discovery, such as metabolic engineering, laboratory evolution and computational advances, as well as practical applications of biocatalysis to drug synthesis.[37] Most recently, Tao and Kazlauskas have garnered contributions covering emerging biocatalysts and industrial applications as diverse as biomass conversion and synthesis of polymers, pharmaceuticals, and fragrances.[38]

3. Reactions of synthetic interest

A survey of the main biocatalytic reactions of synthetic interest follows, highlighting recent advances in each of those areas (Table 1).

3.1 Hydrolytic reactions

A prominent example of mature, large-scale industrial biocatalysis is the application of penicillin G acylase to the hydrolysis of penicillin G for production of 6-aminopenicillanic acid (6-APA). This efficient conversion is fuelled by the requirement for hundreds of tons per year of semi-synthetic β-lactam drugs derived from the common 6-APA core. Further incorporation of biocatalysis in derivatization steps of 6-APA, including the synthesis of the common antibiotic ampicillin *via* the enzyme penicillin amidase, on a scale of >100 T per year, has been promoted by the strict regulatory requirements surrounding pharmaceutical compounds (recently reviewed in ref. 39). Indeed, the use of biocatalysts rather than chemical catalysts eliminates potential sources of contaminants and reduces concerns about undesired side-products.

Table 1 Main biocatalytic synthesis reactions discussed

Reaction catalyzed	Biocatalysts of interest	Main industrial applications in synthesis (*and industrial scale*)
Ester hydrolysis, esterification	Hydrolase, esterase (*i.e.*: lipase)	Chiral resolution and transesterification in fine and bulk chemistry (*g to kT per year*)
Polymerization	Hydrolase, esterase (*i.e.*: lipase)	(*not mature technology*)
Epoxide resolution, diol formation	Epoxide hydrolase	Fine chemistry (*g scale*)
Nitrile hydrolysis	Nitrilase	Fine chemistry, bulk chemistry (*g to kT per year*)
Amide bond formation	Nitrile hydrolase, peptidase, protease, transglutaminase	Fine chemistry, pharma (*g to kT per year*)
Carbonyl reduction	Ketoreductase, alcohol dehydrogenase	Fine chemistry, pharma (*g to kg per year*)
Alkene reduction	Enoate reductase	Fine chemistry (*g to kg per year*)
Oxidative insertion	Baeyer–Villiger monooxygenase, dioxygenase	Fine chemistry (*small-scale*)
Carbon–carbon and carbon–nitrogen bond formation	Aldolase, transaminase	Fine chemistry, pharma (*small to kg scale and rapidly increasing*)
Conjugate addition	Hydrolase, esterase (*i.e.*: lipase), transglutaminase	(*small-scale*)

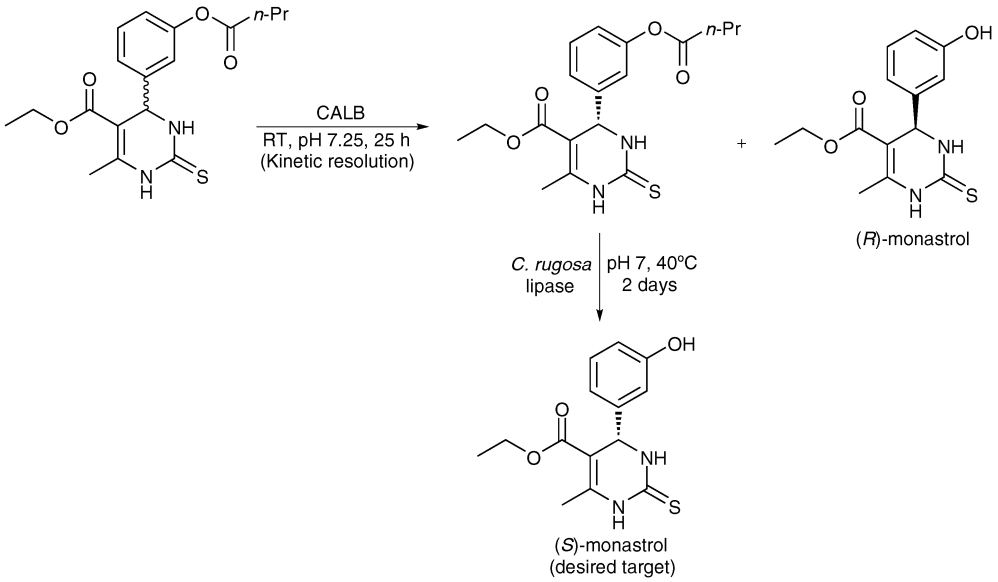
This is but one example of the biocatalyzed hydrolytic reactions geared towards product synthesis which have proliferated in academic and industrial settings over the past decades. Often showing sufficient breadth in substrate spectrum to be multi-purpose, requiring no biological co-factors, offering high turnover and volume efficiency, hydrolases are preferred industrial biocatalysts. The most broadly applied hydrolytic enzymes are the lipase class of hydrolases: as a result of their natural role in hydrolyzing lipids, they are active in more hydrophobic environments than most enzymes, and are thus compatible with formulation for use in organic solvents[40] where starting materials can readily be dissolved. Many lipases are commercially available (refer to Section 3), as they are used on a very large scale in the food and laundry detergent industries.[41] Lipases and other hydrolases perform a vast array of stereoselective hydrolytic reactions. Importantly, if used in dry media (in the absence of water), they can be made to catalyze bond formation rather than hydrolysis. This has allowed the development of a panoply of hydrolase-catalyzed syntheses of esters, amides (including peptide bonds) and peracids. Some recent, diversified synthetic applications of hydrolases will be presented below, as an indication of their broad synthetic utility and ease of application.

3.1(a) Chiral resolution *via* hydrolases. Hydrolases are routinely applied to chiral resolution of secondary alcohols and amines and, more recently, to the resolution of tertiary alcohols. These high value synthetic building blocks can be resolved by the selective enzymatic conversion of a single stereoisomer (often in a simple acetylation or deacetylation reaction), allowing facile separation and recovery of the desired isomer. The additional steric bulk of tertiary alcohols has proven refractory for reaction with many hydrolases that efficiently resolve secondary alcohols. A lipase from *Candida antarctica*—this time lipase A rather than lipase B—shows high activity and enantioselectivity towards tertiary alcohols, as do other lipases with a wider active site. Other strategies to obtain optically-pure chiral alcohols include enantioselective ring opening of epoxides using epoxide hydrolases or halohydrin dehalogenases, as well as numerous other approaches recently reviewed.[42]

The production of enantiomerically pure carboxylic acids is a further topic of interest for fine chemical production. Nitrile hydrolases (nitrilases) have been successfully applied to this task. For example, *E. coli* cells expressing a nitrilase from *Alcaligenes* sp. ECU0401 were applied to the production of (*R*)-(−)-mandelic acid from racemic mandelonitrile. This enzyme was, however, inhibited by high concentrations of substrate (>200 mM), limiting its utility. To relieve the inhibition, the whole cells were used in an aqueous–organic biphasic system constituted of 10% toluene. This allowed 96% conversion of 500 mM starting material in 4 h with product of 98% ee, in a 2 L stirred reactor.[43] It should be noted that the reaction was carried out at pH 10, to promote *in situ* racemization of the unreacted (*S*)-mandelonitrile and provide access to a theoretical yield of 100%.

In an example highlighting the chemo- and regioselectivities of lipases, a two-step biocatalyzed chiral resolution of monastrol was recently reported.[44] Among the novel microtubule targeting class of compounds with potential anticancer activity, monastrol is the first reported small molecule inhibitor. As (*S*)-monastrol shows a 15-fold increased potency relative to the (*R*)-isomer, the chiral resolution of monastrol takes on its importance. Resolution of monastrol butanoyl acetate, by CALB-catalyzed hydrolysis of the undesired isomer, was followed by *Candida rugosa* lipase-catalyzed hydrolysis of the desired isomer to provide (*S*)-monastrol (Scheme 2). While the second step afforded a high conversion, the key first step was not entirely stereoselective, and had to be halted near 60% conversion to provide an isolated yield of 31% of the desired isomer, with 97% ee. This was demonstrated on a scale of 0.3 mmol, and may offer an avenue for synthesis of this promising new drug class.

The synthesis of optically active precursors of β-substituted-γ-amino acids, *via* resolution with commercially available lipases, was recently reported.[45] The lipases efficiently resolved a variety of aliphatic and aromatic substrates with high enantioselectivity and good yields. This was followed by a thermal decarboxylation in aqueous solution to produce the desired precursor molecules, which are flexible synthetic precursors. Similarly, the lipolase-catalyzed preparation of enantiomerically enriched γ-amino acids provides the key reaction in the above-mentioned Pfizer chemoenzymatic process for production of pregabalin (Lyrica).[2] As a result of using a racemic starting material and running the reaction at



Scheme 2 Application of enzymatic hydrolytic kinetic resolution for the preparation of the small molecule inhibitor (*S*)-monastrol.

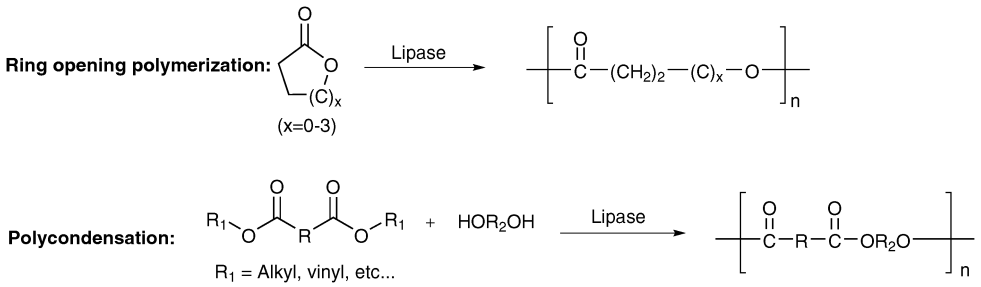
room temperature and atmospheric pressure, this process is run at approximately 50% of the cost of previous process methods that required specialized reaction conditions.[46]

In another application, a lipase resolution step was included in a total synthesis of the massively prescribed anti-depressant sertraline. Demonstrated on the 0.1 mmol scale, a key racemic diol was chemically prepared from the corresponding dione. Dynamic kinetic resolution was performed using a combined CALB- and Ru-complex-catalyzed asymmetric transformation to afford the desired enantiomerically pure diol in extremely high diastereomeric purity (*trans*/*cis* = 99.9 : 0.1, >99.9% ee).[47] Interestingly, certain conditions allowed the selective oxidation of bicyclic diols to enantioenriched ketoalcohols.

Recently, significant academic effort has been devoted towards the application of hydrolases in ionic liquid (IL)-based media. ILs show potential as greener solvents in organic synthesis due to their non-volatile, highly stable nature, along with their ability to dissolve a wide range of both polar and non-polar organic compounds.[8,48,49] ILs have found utility in reactions involving the hydrolase-mediated regioselective acylation of carbohydrates. A range of ILs was screened as alternative solvents for the small scale (0.25 mmol) enzymatic acylation of levoglucosan using an immobilized lipase from *Pseudomonas cepacea*, with a range of acyl donors.[50] 3-(2-Methoxyethyl)-1-methyl-1*H*-imidazol-3-ium tetrafluoroborate ([MOEMim][BF4]) was found to be the most useful and green organic solvent for the acylation of levoglucosan with short and long chain acyl donors with moderate yields and generally good regioselectivities. CALB-mediated kinetic resolution of racemic 1-phenylethanol, an important agrochemical and pharmaceutical building block, was also systematically screened in a number of ILs.[51] 1-Butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF6]) achieved the highest conversion (50%) within 3 hours in >99% ee for the desired product. Interestingly, [bmim][PF6] was also found to be the best IL for the hydrolase-based kinetic resolution of racemic 1-chloro-3-(3,4-difluorophenoxy)-2-propanol, a key intermediate used in the synthesis of lubeluzole.[52] In addition, efforts are being directed towards the development of "designer" ILs for enzyme-mediated reactions. Specifically, Abe *et al.* have developed a class of phosphonium-based ILs that appear to be superior to the 1-butyl-3-methylimidazolium-based ILs in retention of enzymatic activity.[53] Designer ILs with properties tuned for enzyme-catalyzed reactions represent a significant step towards integration of ILs into bioprocess development.

Despite the desirable properties of ILs as green solvents in biocatalytic processes, a limitation that has hampered their widespread use is their cost. A more cost-effective alternative being explored in the academic setting as possible next-generation green solvents for biocatalysis are deep eutectic solvents (DES). DES are mixtures of an ammonium salt and a hydrogen bond donor species such as choline chloride or urea.[54,55] Readily accessible, inexpensive and biodegradable, DES may represent a viable green replacement for conventional organic solvents. A screen of a range of DES in model reactions, including the transesterification and aminolysis of ethyl valerate by lipase as well as the hydrolysis of styrene oxide by epoxide hydrolase, revealed their potential. Reactions were carried out on the 40–100 mM scale with generally good conversions (>90%).[55]

3.1(b) Polymer synthesis. The application of enzymes in biocatalytic polymerization reactions represents an alternative to classical chemical polymerization methods that employ either acid–base or transition metal catalyzed processes. Unlike chemical methods, enzymatic polymerization reactions are generally performed under mild reaction conditions (ambient temperature, pressure, and at neutral pH) and possess a high degree of reaction control in terms of their chemo-, regio-, and stereoselectivities, generally providing polymers with highly regulated structure. Biocatalytic polymerization thus offers novel, greener routes for the preparation of polymeric materials and biocompatible products. Excellent surveys of the



Scheme 3 Major modes of lipase catalyzed polyester synthesis.

overall development and application of enzymes in polymer synthesis are found in ref. 56–58.

Biocatalytic polymerizations have successfully been applied over the last two decades in two major polymerization strategies, namely the ring-opening polymerization of lactones and polycondensation type reactions (Scheme 3) catalyzed by lipases.[57,58]

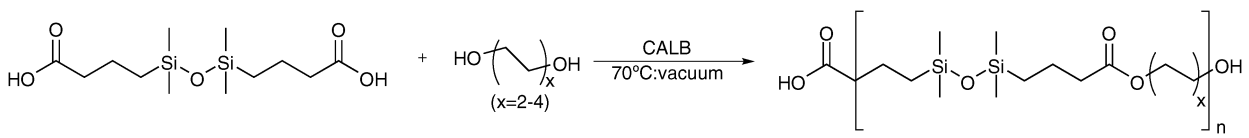
While a number of lipases have been screened for their ability to perform biocatalytic polymerizations, to date the best-studied enzyme used for polymerizations is CALB lipase.[58] Applications of CALB in biocatalytic polymerizations include the synthesis of biocompatible and biodegradable polyesters *via* lipase-catalyzed copolymerization of lactones, alkyl diester, and diol sub-units.[59] A two-stage biocatalytic copolymerization reaction has been successfully performed, for the production of aliphatic copolyesters with average molecular weights of 77 000 g mol^{-1}, possessing generally good thermal stabilities. CALB has also been used to catalyze the polycondensation reactions of a mixture of diethyl carbonate, diester, and a diol on the multi-gram scale, to produce aliphatic poly(carbonate-co-esters) which are useful building blocks for the preparation of specialty polyurethanes.[60]

An important application of biocatalytic polymerizations was recently reported involving the preparation of siloxane polymers, an important building block in the preparation of large number of value-added products including electronics, resins, and polymeric coating agents.[61] Specifically, immobilized CALB was used to catalyze the condensation of 1,3-bis-(carboxypropyl)-tetramethyldisilane and a series of alkanediol units and produce organosiloxane polymers ranging from 6100 to 11 000 g mol^{-1} in molecular weight depending on the alkanediol used (Scheme 4). The development of a lipase-based biocatalytic method for an organosilane polymer product represents an important step towards the development of a greener strategy for the production of these high value polymer targets.[61]

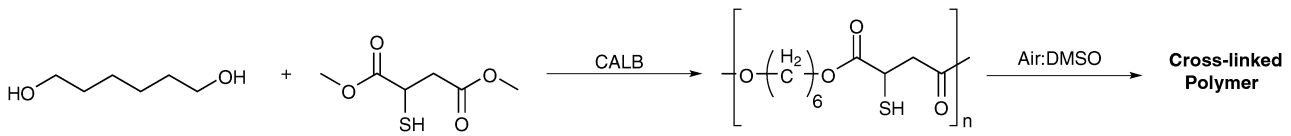
Immobilized CALB has also been used to catalyze the direct polycondensation of hexane-1,6-diol and dimethyl-2-mercapto-succinate to yield aliphatic polyesters containing free pendant mercapto groups with molecular weights of 14 000 g mol^{-1} (Scheme 5). No formation of thioester linkages was observed. The pendant thiol-containing polyesters were cross-linked to form gels upon air oxidation in DMSO. The ease of cross-linking the polyesters, coupled with the ability to reduce disulfide linkages with reducing agents, means this method could represent a green chemical route towards the synthesis and recycling of cross-linked polymers.[62] Sugars were also integrated into polymers, where formation of low molecular weight linear and cyclic ester oligomers from succinic acid and di-anhydro hexitols was catalyzed by CALB in a toluene based medium. Enantiopreference of isomannide over isosorbide and over isoidide was rationalized according to docking into the CALB structure.[63]

Despite the progress made in biocatalytic polymerization reactions, applications remain limited to those monomers and growing polymers with relatively low viscosities at modest temperatures.[56] This limitation is a barrier to broad industrial application of these methods, which will require means to thermostabilize the biocatalyst to a greater extent than the current limit of ~100 °C. Globally, lipase-catalyzed ring-opening and polycondensation-based polymerization offer excellent control of regio-, chemo-, and enantioselectivities under mild conditions towards a growing array of monomers, making this an increasingly flexible tool for a selected range of polymerization reactions.

3.1(c) Epoxidation and diol formation. Enantiopure epoxides are important synthons for the production of useful value-added



Scheme 4 CALB catalyzed polymerization of organosiloxane with alkanediols in bulk at elevated temperature and reduced pressure.



Scheme 5 CALB based biocatalytic polymerization of free mercapto containing polyesters.

chiral chemicals. Epoxides are key chemical building blocks owing to their relatively high reactivity with other nucleophilic groups such as halides, nitrogen, oxygen, and sulfur. The classical chemical route for the production of epoxides involves oxidation of olefins with strong oxidizing agents such as permanganate or peracids, however, the products of such oxidative processes are typically racemates. Excellent chemical approaches to chiral epoxide synthesis have been reported, such as the Sharpless and Jacobsen asymmetric epoxidation methods. However, they suffer either from limited substrate scope or the need for complex chiral metal catalysts.

Chiral epoxides can be generated biocatalytically, through the kinetic resolution of racemic epoxides using an epoxide hydrolase catalyzed hydrolysis, yielding an enantiopure dihydroxylation product and an enantioenriched epoxide.[64] Epoxide hydrolases are robust biocatalysts that have attracted much attention from synthetic organic chemists due to their ability to selectively hydrolyze racemic epoxides, representing a powerful asymmetric tool in organic synthesis. The biocatalytic kinetic resolution of epoxides mediated through epoxide hydrolases also represents a more environmentally compatible and greener alternative to classical chemical methods.

Recently efforts have focused on the development of epoxide hydrolases from novel microbial sources such as bacteria and yeast as well as practical methods for their application. An epoxide hydrolase from *Rhodotorula glutinis* was expressed using a recombinant *Pichia pastoris* expression system and allowed for the enantioselective resolution of high concentrations of racemic styrene oxide in good yield and excellent enantioselectivity, on a small scale (μmol).[65]

In research directed towards the development of an efficient synthesis of useful intermediates for drug synthesis, Li *et al.* utilized an expoxide hydrolase for racemic epoxide resolutions.[66] Specifically, whole bacterial cells of *Sphingomonas* sp. HXN-200 were used to catalyze the selective resolution of a series of racemic substituted chlorostyrene oxides to yield the corresponding enantiomerically enriched (*R*)-diols and (*S*)-epoxides (Scheme 6). For the chlorostyrene oxides tested, hydrolysis of the 3-chlorostyrene was best, with yields of 44% for the (*S*)-3-chlorostyrene oxide and 99% ee on the μmol scale.[66]

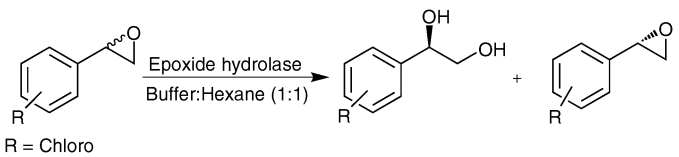
Building on their earlier work, Li *et al.* employed an epoxide hydrolase from *Sphingomonas* sp. HXN-200 in conjunction with a styrene monooxygenase overexpressed in *E. coli* to perform sequential enantioselective epoxidation and regioselective hydrolysis to yield chiral vicinal diols *via* a tandem biocatalyst approach[67] (Scheme 7). A two-phase solvent system allowed maximal production of the intermediate epoxide as well as easy separation of the desired chiral diol. The tandem biocatalysis approach successfully produced the desired chiral diols in good yields and excellent enantioselectivities and represents an interesting stepping-off point for the development of other tandem biocatalyst transformations.[67]

An epoxide hydrolase has also been used by Faber and co-workers in the chemoenzymatic synthesis of chromanemethanol, a key building block used to make α-tocopherols.[68] Using an epoxide hydrolase expressed by native *Rhodococcus ruber* CBS717.73, racemic 2-benzylmethyl-2-methyloxirane was resolved on the mg scale to yield the enantiopure (*R*)-2-benzylmethyl-2-methyloxirane in 40% isolated yield and >99% ee, as well as the corresponding (*R*)-diol (Scheme 8). The resulting (*R*)-oxirane was then used in the asymmetric synthesis of (*R*)-chromanemethanol.

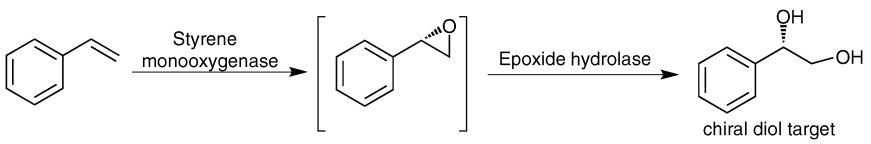
Epoxide hydrolases represent an important class of biocatalysts for the generation of enantiopure epoxides and 1,2-diol species. While the vast majority of epoxide hydrolases currently utilized are derived from microbial sources, these enzymes are ubiquitous in nature and therefore further opportunities exist in the development and application of these enzymes in asymmetric synthesis (see ref. 69 and references therein).

3.1(d) Nitrile hydrolysis. Hydrolysis of the nitrile group provides a synthetically accessible route for the formation of a wide variety of carboxylic acids, which represent important intermediates for the generation of a wide array of value-added building blocks and chemicals. Of note is the biocatalyzed synthesis of acrylamide, on the half-million T per year scale worldwide. Classical chemical methods for nitrile hydrolysis require the use of either strongly acidic or basic conditions, and high reaction temperatures, making this process incompatible with a number of hydrolysable functional groups. The harsh reaction conditions, formation of unwanted by-products, and significant amounts of inorganic waste generated *via* classical chemical methods have made the development of greener and more eco-friendly routes of nitrile hydrolysis an attractive target for synthetic chemistry.

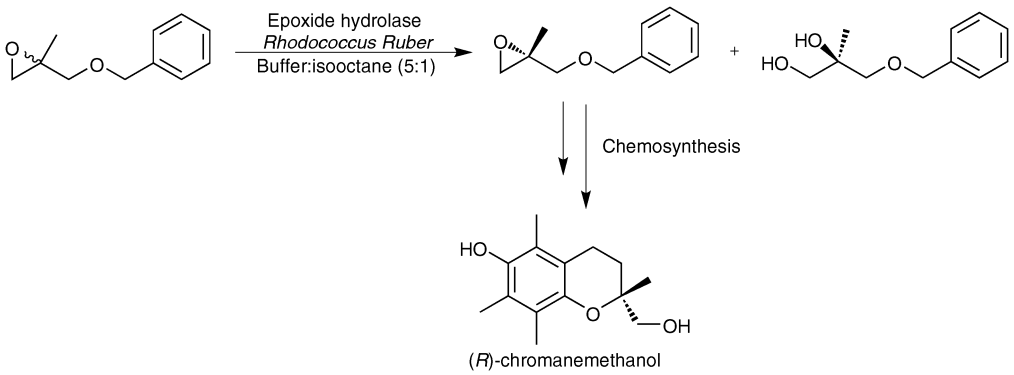
A more environmentally compatible method for the hydrolysis of nitriles to carboxylic acids involves the use of nitrilases.



Scheme 6 Biocatalytic kinetic resolution of racemic 2-, 3-, and 4-chlorostyrene oxides using epoxide hydrolase.



Scheme 7 Asymmetric dihydroxylation of an aryl olefin *via* a tandem monooxygenase and epoxide hydrolase biocatalysis approach.

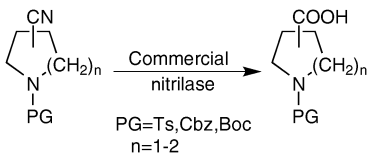


Scheme 8 Epoxide hydrolase based kinetic resolution of (*rac*)-2-benzyloxymethyl-2-methyloxiane.

Typically, nitrilase-based biotransformations are performed under physiological conditions of temperature and pH (typically using whole cells overexpressing the desired nitrilase). This limits undesired by-products and waste generated, allowing for a wide array of chemical diversity in the nitrile species. In addition to their functional group tolerance, nitrilases often possess good chemo-, regio-, and enantioselectivities. A comprehensive survey of nitrilases and their development is found in recent reviews by Martínková and co-workers.[70,71]

In an industrial context, nitrilase from *Acidovorax facilis* 72W recombinantly overexpressed in *E. coli* has been used by Dupont for the production of glycolic acid *via* a three-step chemoenzymatic kg-scale process. In this process formaldehyde is reacted with hydrogen cyanide to produce glycolonitrile, which is directly converted to the desired glycolic acid by a nitrilase that was immobilized under conditions allowing for retention of enzyme activity upon recycling.[72] In order to explore the synthetic utility of commercially available nitrilases, Winkler *et al*. screened the nitrilases NIT-101 through NIT-108 from Biocatalytics, for selective hydrolysis of a series of heterocyclic amino nitriles to their corresponding carboxylic acids[73] (Scheme 9). The most active and selective nitrilases identified were applied to demonstrate the chemoenzymatic enantioselective synthesis of *cis*- and *trans*-configured five- and six-membered ring carbocyclic γ-amino acids in good yields.[74]

Rational genome mining has been used by Hua and co-workers to identify novel nitrilases within the genome of *Bradyrhizobium japonicum* USDA 110, followed by substrate specificity profiling.[75,76] One nitrilase was found to selectively



Scheme 9 Enzymatic transformation of pyrrolidine and piperidine carbonitriles to the corresponding carboxylic acids by commercial nitrilases.

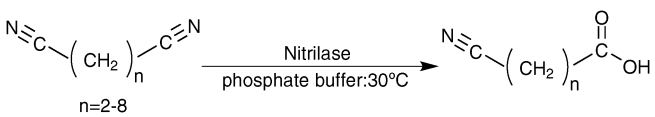
hydrolyze a range of aliphatic dinitrile species to the corresponding cyanocarboxylic acids, a task that is virtually impossible *via* chemical methods (Scheme 10).[77] The aforementioned nitrilases were found to efficiently and selectively hydrolyze dinitriles independent of aliphatic chain length, adding to their synthetic utility.

Using these newly identified nitrilases, Zhu and colleagues reported a two-step, one-pot tandem biocatalysis strategy, involving enzymatic reduction and nitrile hydrolysis on the 0.1 g scale, for the synthesis of β-hydroxy carboxylic acids.[78] The β-ketonitrile starting material was asymmetrically reduced to the corresponding (*R*)- or (*S*)-β-hydroxynitrile species using a suitable carbonyl reductase. A suitable nitrilase was then added to the reaction mixture to carry out the second step. Enantiopure β-hydroxynitriles were thus prepared in excellent yields without isolation of intermediates, reducing the cost and potential environmental footprint of the reaction (Scheme 11).

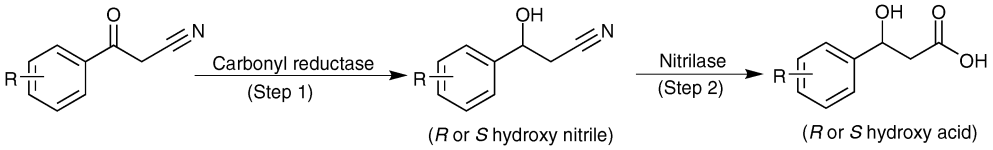
Cobalt-containing nitrile hydratases from diverse microorganisms, upon screening towards chiral nitriles, exhibited enantioselectivtities ranging from being non-selective to procuring enantiomeric ratios of >100, the latter being illustrated by successful synthesis of the pharmaceutical intermediate naproxennitrile. Rules for nitrile composition were derived, where nitriles on quaternary carbons were poor substrates, and at least one bulky group near the nitrile was necessary for enantioselectivity.[79]

3.2 Reduction reactions

3.2(a) Reductions of aldehydes and ketones. Reduction of the carbonyl functionality represents a corner-stone reaction within the synthetic organic chemist's toolbox. Owing to its importance, a great deal of effort has been devoted towards the development of green catalytic methods for the regio- and stereoselective reduction of carbonyl compounds. Within the biocatalytic toolbox of enzymes, ketoreductases (KRED) or carbonyl reductases represent the enzymatic counterpart to the traditional boron or phosphine-containing ligand-based chemical methods for carbonyl reduction. The virtues and limitations of ketoreductases in the synthesis of fine chemical



Scheme 10 Selective hydrolysis of α,γ-dinitriles catalyzed by *Bradyrhizobium japonicum* USDA110.



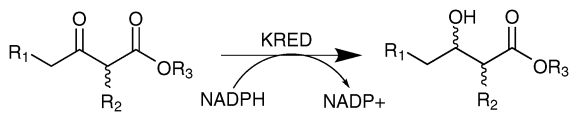
Scheme 11 One-pot tandem bioreduction/nitrile hydrolysis reaction catalyzed by isolated carbonyl reductase and nitrilase enzymes for the preparation of enantiopure hydroxy carboxylic acids.

and pharmaceutical production have been highlighted in recent review articles.[80,81]

A major practical consideration when considering the use of KRED is their requirement for the NADPH co-factor as a source of hydride ions in the reduction process. As discussed above, efficient cofactor recycling options are available, such as coupled enzyme systems based on glucose or formate dehydrogenase, or the use of whole microbial cells over-expressing the KRED of interest.[81] Nonetheless, even when using whole cells, it may be required to add additional reducing cofactor (NADPH) from an external source to drive the reaction, which significantly increases cost; the creation of 'designer cells' which provide an efficient co-factor regeneration system has overcome that problem.[82]

Sheldon and co-workers recently screened a range of KREDs derived from a number of microbial and fungal sources for their ability to selectively perform carbonyl reductions on a range of carbonyl esters and aromatic ketones (Scheme 12). A number of the KREDs screened generated a range of enantiopure β-hydroxy esters in good yields, though organic cosolvents reduce the activity of the KREDs. The choice of the KRED used controlled the enantioselectivity, generally providing access to both enantiomers of the β-hydroxy ester.[83] Another example made use of ketoacyl-[acyl carrier protein] (β-ketoacyl-ACP) reductases, which are involved in lipid biosynthesis. Among this family of enzymes, a (β-ketoacyl-ACP) reductase from a *Bacillus* has been shown to provide the asymmetric reduction of a broad spectrum of prochiral ketones and keto esters. Conducted in whole *E. coli* cells, ethyl 2-oxo-4-phenylbutyrate was converted to ethyl (*S*)-2-hydroxy-4-phenylbutyrate on the 50 g scale, with >99% conversion and ee.[84]

Recently, Ma *et al.* reported a 'green-by-design' strategy for the biocatalytic synthesis of a key intermediate of atorvastatin (Lipitor) that employs a KRED biocatalyst.[85] Their strategy



Scheme 12 Example of KRED based reductions of β-keto-esters.

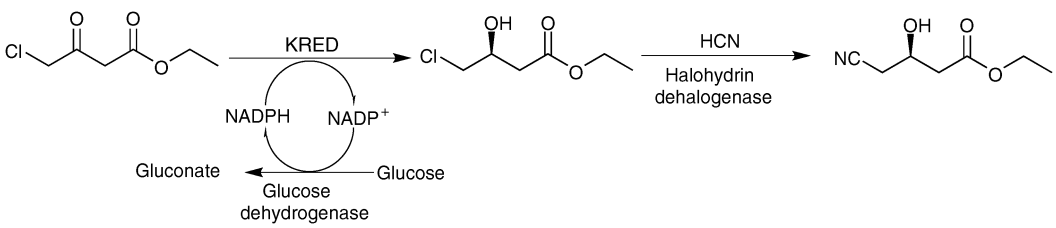
involved a two-step, three enzyme process in which the first step involved the biocatalytic reduction of ethyl-4-chloro-acetate using an isolated NADPH-dependent keto-reductase, with a NADP-dependent glucose dehydrogenase-based co-factor recycling scheme (Scheme 13). Isolation of the desired (*S*)-alcohol and subsequent reaction with a second biocatalyst, halohydrin dehalogenase, catalyzed substitution of the chlorine with a cyano group by reaction with HCN at ambient temperature and neutral pH. This tandem biocatalytic process, demonstrated on the 100 g scale, allowed for quick access to the desired atorvastatin intermediate.[85]

KREDs have also been applied towards the stereoselective synthesis of chiral, 2-substituted 1,3-diols through a two-KRED biocatalytic reduction cascade[86] (Scheme 14). Inclusion of a glucose dehydrogenase co-factor recycling system reduced the global cost and enhanced the synthetic utility of that reductive cascade process, illustrated on the mg scale.[87]

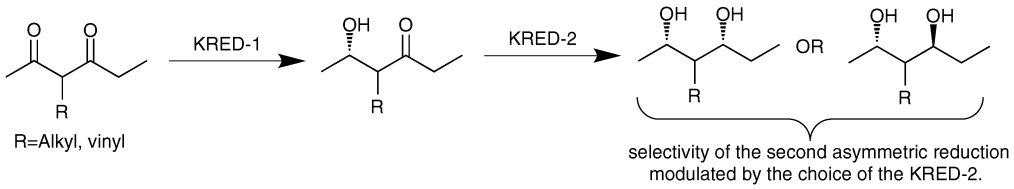
Given the growing number of KREDs available and the continuing development of practical, efficient and affordable co-factor recycling methods, KREDs are now established as highly useful reagents for organic synthesis. Due to their general utility, biocatalytic reductions are considered 'reagents of choice' for industrial process development, because of their 'green' value and the opportunity to avoid the cost of operating specialized hydrogenation equipment.

3.2(b) Alkene reductions. A further class of reduction reactions attracting much attention is the asymmetric reduction of C=C bonds, resulting in the generation of two chiral centers. Enoate reductases from the Old Yellow Enzyme family (EC 1.3.1.X) are flavin and NADPH-dependent enzymes that asymmetrically reduce activated C=C bonds. Having a moderately broad substrate scope, they reduce α,β-unsaturated carbonyls, nitroalkenes, and various carboxylic acid derivatives (such as esters, nitriles, and lactones) with a high degree of enantioselectivity.[88] For activated alkenes bearing an electron-withdrawing substituent, the degree of activation of the alkene can be directly related with the enoate reductase substrate acceptance (Scheme 15).

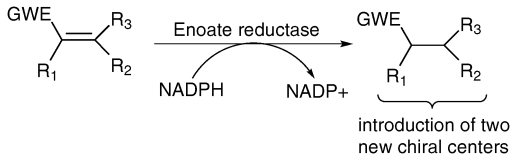
By screening the asymmetric bioreduction of a range of methyl-2-hydroxymethylacrylate derivatives, an efficient and



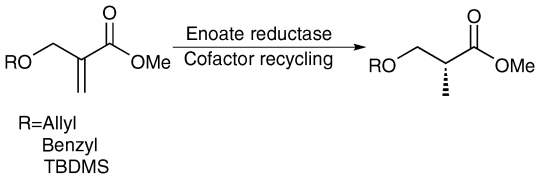
Scheme 13 Two step biocatalytic route for the synthesis of the desired hydroxynitrile intermediate for the atorvastatin synthesis.



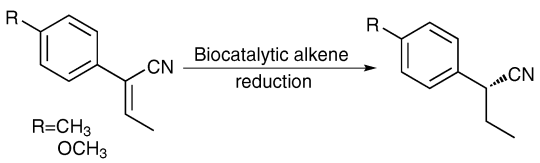
Scheme 14 Representative double reduction of substituted 1,3-diketones using isolated KRED biocatalysts.



Scheme 15 Example of the reduction of activated alkenes using enoate reductase based biocatalysis.



Scheme 16 Asymmetric bioreduction of methyl-2-hydroxymethyl acrylate derivatives using enoate reductase yielding enantiopure methyl-3-hydroxy-2-methylpropionate.



Scheme 17 Asymmetric reduction of α,β-unsaturated nitriles catalyzed by enoate reductase biocatalysis.

green route for the production of (*R*)-3-hydroxy-2-methyl-propanoate derivatives (commonly known as the 'Roche ester') was developed through the mg-scale application of an enoate reductase (Scheme 16).[89]

Enoate reductases have also been used to perform the asymmetric reduction of α,β-unsaturated nitriles and ketones. Using a series of commercially available enoate reductases, Kosjek and co-workers selectively reduced a range of unsaturated nitriles with a high degree of enantioselectivity (Scheme 17), yielding highly functionalized compounds that provide a nice stepping-off point towards the synthesis of more complex targets.[90]

The continuing discovery and characterization of enoate reductases as asymmetric reducing agents will provide broader access to stereocontrol for a variety of alkene reductions.[91]

3.3 Oxidative reactions

Oxidative reactions constitute one of the most fundamental steps for the introduction of chemical functionality/diversity into cheap or readily available starting materials such as alkanes, alkenes, or aromatic/heteroaromatic compounds. Despite their importance, traditional oxidative methodology suffers from disadvantages including the use of environmentally incompatible reagents (such as chromium, nickel, or copper-based reagents) and the potential for undesired side reactions due to harsh reaction conditions or lack of chemoselectivity. Here, we provide an overview of biocatalytic alternatives and recent applications of bio-oxidants in organic synthesis.

3.3(a) C–H activation. Cytochrome P450s (or P450s) catalyze the oxidation of non-activated carbon atoms, which is chemically demanding. Hundreds of naturally-occurring P450s have been identified, catalyzing a broad range of oxidative reactions: alkene epoxidation, aromatic hydroxylation, dehalogenation and deamination reactions have been demonstrated. In addition, engineering reaction conditions can allow P450s to catalyze such reactions as C–C bond cleavage and rearrangements including Baeyer–Villiger oxidations.[92] As a result of being enzyme-catalyzed, those reactions generally benefit from high chemo-, regio- and stereoselectivities, which makes them highly attractive for synthetic applications. Indeed, they obviate the need for specific activation of the target reaction site and require no protective groups, thus eliminating many reactions steps. P450s have been applied to the synthesis of building blocks for the synthesis of such valuable compounds as the antimalarial artemisinine[94]—demonstrated on a mg scale—as well as Pravastatin, an inhibitor of HMG CoA reductase (hyperlipidemia), the anticancer drug candidate epothilone, indinavir for the treatment of AIDS, and the β-blocker atenolol.[93]

Nonetheless, P450s have significant limitations, including instability, narrow substrate specificity, complex cofactor requirements (NADPH and FAD) and limited electron flow.[95] Self-sufficient P450s alleviate some of these problems, as they contain in a single protein all the machinery necessary to ensure the electron flow and heme-Fe regeneration required for the oxidation reaction, in contrast to the majority of P450s which depend on companion proteins. Among these, the self-sufficient P450 from *Bacillus megaterium* (BM3) is the most prominent, and it has been engineered to catalyze a host of synthetically-attractive reactions including hydroxylation of cyclic and acyclic alkanes and terpenoids, steroids, peralkylated monosaccharides, production of native and synthetic drug metabolites and ene epoxidation (see ref. 96 and references therein). Subsequent fluorination at selectively hydroxylated sites offered a broad range of compounds that were fluorinated at otherwise poorly reactive loci,[97] expanding the synthetic utility of the biocatalytic step. Engineered BM3s were also identified that perform highly regio- and diastereo-selective hydroxylation of testosterone at carbons 2 and 15, which the

native BM3 does not differentiate.[98] As illustrated, synthetically-relevant advances have been made in biocatalyzed oxidation reactions. These generally remain demonstrated only on a small scale, and there remain a vast number of valuable targets to be tested for enzymatic oxidation.

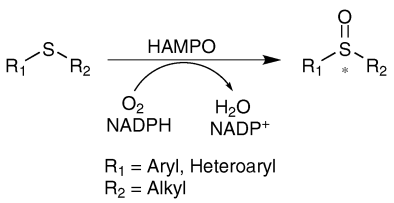
3.3(b) Oxidative insertion across C–C bonds. The regio- and stereoselective carbon–carbon cleavage and oxygen insertion, known as the Baeyer–Villiger oxidation reaction, is a key synthetic transformation. Chemical reagents utilized for this reaction, such as *m*-chloroperbenzoic acid and trifluoroacetic acid, are toxic, explosive, and lack specificity. Highly sophisticated metal-based oxidants provide good selectivities but possess a narrow substrate range, limiting their general applicability. As an alternative, the Baeyer–Villiger monooxygenase (BVMO) family of enzymes, which are flavoprotein-based biocatalysts found in a variety of bacteria and fungi, have been shown to catalyze this transformation. The most extensively studied and utilized BVMO is cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIB 9871, which has been shown to accept hundreds of different compounds (see ref. 99–101 and references therein for a comprehensive list) and offers relatively high chemo-, regio-, and enantioselectivity. It can oxidize a range of acyclic, cyclic, bicyclic, tricyclic, and heterocyclic ketones with a variety of substituents and substitution patterns, to chiral lactones. $CHMO_{Acineto}$ also oxidizes a broad selection of sulfides, dithienes, dithiolanes to chiral sulfoxides and tertiary, secondary, and hydroxylamines to *N*-oxides, hydroxyamines, and nitrones, respectively. Through protein engineering, $CHMO_{Acineto}$ has been made into a readily accessible tool for synthetic chemists.[100]

Recent applications of BVMOs have been directed towards the synthesis of β-amino acids and β-amino alcohols, which are of growing industrial interest as building blocks for β-peptides, alkaloids, and terpenoid synthesis. Bornscheuer *et al.* screened an in-house toolbox of 16 BVMOs (recombinantly expressed in *E. coli*) to identify a number which were of utility for enzymatic kinetic resolution of racemic, substituted aliphatic β-amino ketones. Interestingly, the BVMOs were promiscuous in regioselectivity for β-amino ketones, with oxidative insertions into the carbon–carbon bond yielding both the normal ester and/or abnormal Baeyer–Villiger ester products (Scheme 18),[102,103] the latter of which are not currently attainable by established chemical methods.

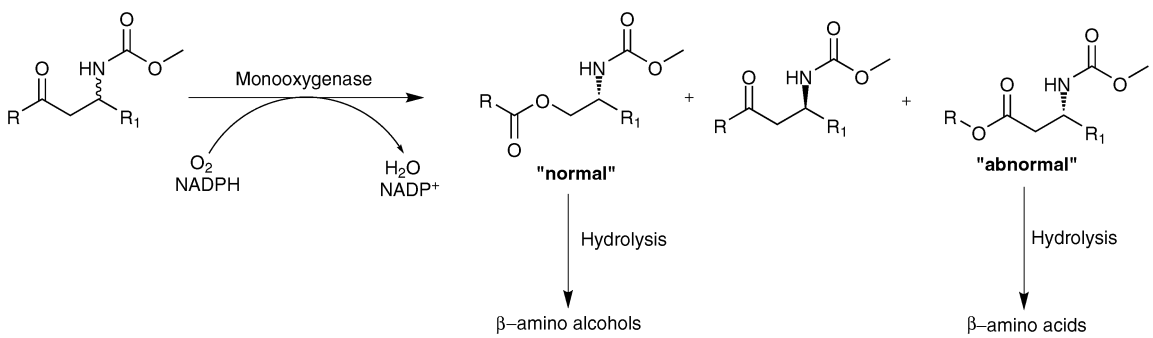
The enzyme-mediated Baeyer–Villiger oxygenation class of biocatalytic reactions is a rapidly growing area of catalysis and the number of chiral synthons accessible through biocatalytic oxidations is expanding. Despite the utility of the BVMO-mediated oxidations in chemical synthesis and their demonstration in whole cells on a pilot-plant scale (200 L),[104] further research is needed to make biocatalytic BVMO oxidations more appealing for use in both a laboratory and industrial setting.

3.3(c) Oxidation of heteroatoms. BVMOs catalyze the asymmetric oxidation of sulfides to their corresponding chiral sulfoxides, with minimal over-oxidation to the undesired sulfone. The generation of chiral sulfoxides has gained importance owing to their use as chiral ligands in asymmetric catalysis, for their potential as chiral auxiliaries, and due to the presence of sulfinyl groups within many molecules with high biological activity. Phenylacetone monooxygenase (PAMO) and 4-hydroxyacetophenone monooxygenase (HAPMO) were screened for their ability to selectively oxidize a range of alkyl, aryl, and heteroaryl sulfides to their corresponding chiral sulfoxides (Scheme 19). While HAPMO was found to oxidize a broad range of sulfides with generally high yields and moderate to high enantioselectivities, PAMO offered more limited substrate tolerance and selectivity.[105]

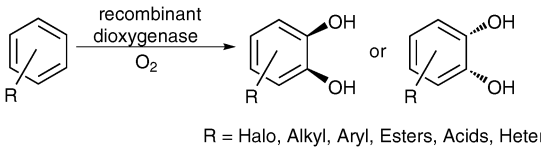
3.3(d) Dioxygen insertion across a C=C bond. The regiospecific hydroxylation of aromatic compounds through purely chemical methods remains a highly challenging and notoriously difficult synthetic transformation. The typical dioxygenase reaction involves the simultaneous incorporation of two oxygen atoms onto the target reagent. Dioxygenases are remarkable and highly-utilized biocatalysts that perform this reaction. Unlike the chemical equivalent, the enzymatic dihydroxylation of aromatic compounds offers high regio-, stereo-, and enantiospecificity, generally yielding dihydrodiols in relatively high yields. Investigations into the substrate scope and tolerance to functional groups of toluene dioxygenase (TDO) have



Scheme 19 BVMO-catalyzed oxidation of aryl and heteroarylsulfides to the corresponding chiral sulfoxide.



Scheme 18 Examples of kinetic resolution of aliphatic protected β-amino ketones catalyzed by BVMO oxidation.



Scheme 20 Possible *cis*-dihydrodiol stereoisomers resulting from dioxygenase catalyzed dihydroxylations of single arene rings.

found more than 400 arene *cis*-dihydroxydiol reaction products generated *via* TDO in whole cells, by supplying the cells with substituted benzene, benzoate acids/esters, and tri-cyclic azaarene-based substrates (Scheme 20).[106,107]

Over the last two decades, Hudlicky and co-workers, as well as others, have applied a range of *cis*-dihydrodiol products of dihydroxylation-based fermentative biocatalysis towards the efficient and exhaustive synthesis of a number of chemically and biologically relevant targets such as cyclitols, terpenes, alkaloids, and carbohydrates.[106,108,109] Notably, the application of arene metabolites of ethyl benzoate allowed introduction of complexity in a practical synthesis of oseltamivir (Tamiflu), and the efficient synthesis of (−)-idesolide was devised through enzymatic hydroxylation of benzoic acid (Scheme 21);[110,111] in both cases, the key chiral diol was produced on the gram scale.

As demonstrated, chemical transformation of readily available reagents with TDO has given rise to a wealth of diversified, enantiopure building blocks. However, the vast majority of those has not yet been utilized in synthetic ventures and thus provide a rich opportunity for further development.[106]

3.4 CC-bond forming reactions

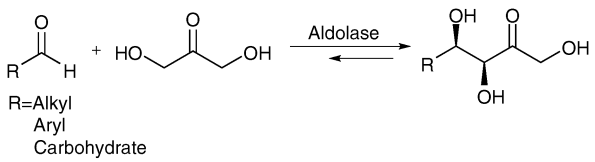
3.4(a) Aldol addition. The asymmetric formation of new carbon–carbon bonds *via* aldol addition remains a challenging transformation in organic synthesis. While significant progress has been made in this area through the use of chiral auxiliaries and metal- or organocatalyst-based catalyst complexes, limitations exist including the need for stoichiometric quantities of reagents.[112] In addition, performing aldol additions under aqueous conditions remains challenging due to the sensitivity to reaction conditions and the need to extensively protect multifunctional polar compounds to limit undesired cross-reactivity. Biological aldol catalysts, known as aldolases, are utilized under aqueous conditions at neutral pH, offering a greener alternative and powerful method for asymmetric aldol additions. Aldolases form new C–C bonds between a range of one-, two-, or three-carbon nucleophilic donor species and either an electrophilic aldehyde or ketone acceptor moiety, with a high degree of stereospecificity.[112] For an excellent review highlighting the general utility, progress, and limitations of aldolases in stereoselective synthesis, see ref. 113 and references therein.

An aldolase that has recently attracted attention is the D-fructose-6-phosphate aldolase from *E. coli*. It catalyzes the reversible aldol addition between a non-phosphorylated (unactivated) donor species, such as dihydroxyacetone, and a variety of aldehyde species (Scheme 22).

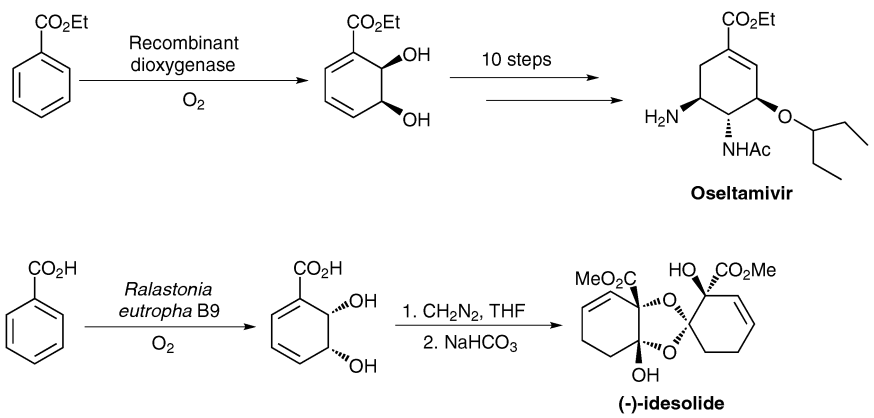
The utility of D-fructose-6-phosphate aldolase as a robust tool in organic synthesis has recently been demonstrated through its use in a cascade-type chemoenzymatic synthesis of polyhydroxylated compounds. Concia and co-workers catalyzed the stereoselective aldolase addition of hydroxyacetone and dihydroxyacetone to a number of aldehyde acceptors on the mg to 1 gram scale, in good yields and excellent enantioselectivities[114] (Scheme 23).

D-Fructose-6-phosphate aldolase was also applied to asymmetric self- and cross-aldol type reactions with glycolaldehyde, providing a highly interesting synthetic route to a variety of polyol frameworks.[115] To expand the utility of this aldolase, it was immobilized onto a layered double hydroxide inorganic support, followed by testing of the addition of hydroxyacetone to formaldehyde on the μmol scale. The solid-supported aldolase was stable for up to 3 months as a suspension in water with no loss in activity, allowing catalyst recycling.[116]

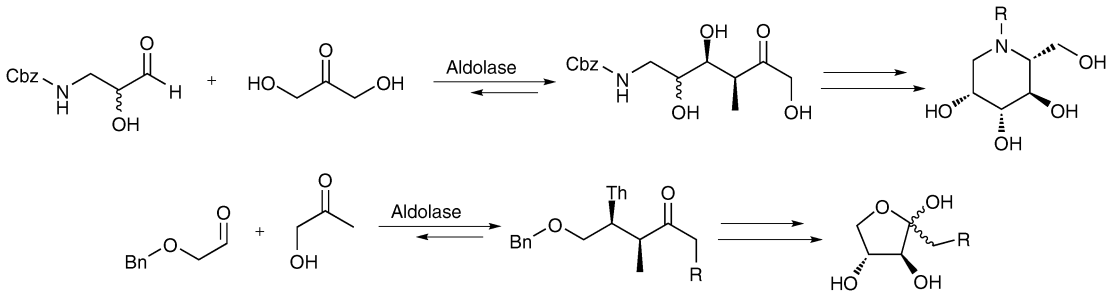
A second class of aldolases that has been the subject of much interest is L-threonine aldolase which can perform the aldol addition of glycine to aldehydes to yield a product containing



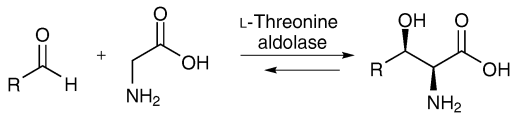
Scheme 22 Representative aldol addition between dihydroxyacetone and an aldehyde catalyzed by D-fructose-6-diphosphate aldolase from *E. coli*.



Scheme 21 Asymmetric synthesis of *cis*-dihydrodiols for the total synthesis of the natural products oseltamivir and (−)-idesolide obtained using dioxygenase biocatalysis.



Scheme 23 D-Fructose-6-diphosphate aldolase catalyzed reaction of hydroxyacetone and dihydroxyacetone with aldehydes for the synthesis of carbohydrates and imino sugars compounds.



Scheme 24 Reaction of glycine with an aldehyde catalyzed by L-threonine aldolase.

two new stereocenters (Scheme 24).[113] Interestingly, L-threonine aldolases are uniquely able to catalyze asymmetric aldol reactions without the need to protect the amino acid donor moiety.

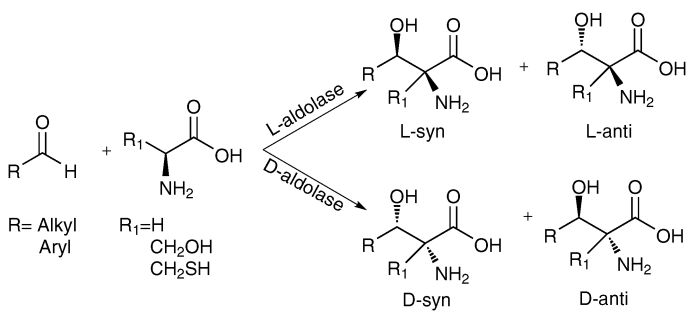
Crude extract from aldolases over-expressed in *E. coli* and *Saccharomyces cerevisiae* catalyzed the aldol addition reaction of glycine with a series of substituted benzaldehydes to yield the corresponding β-hydroxy-α-amino acids in excellent yields and high selectivity, even under process-like conditions of high substrate concentration (250 mM), as verified on the 0.1 mmol scale.[117] L-Threonine aldolases have also been used to access the highly important α,α-dialkyl-α-amino acid building block. Using a biocatalytic aldolase strategy, a series of β-hydroxy-α,α-dialkyl-α-amino acids were prepared on the scale of 0.1 mmol using either the L- or D-threonine aldolases from *Aeromonas jandaei* or *Pseudomonas* sp., respectively (Scheme 25).[118] Promiscuous donor group specificity with amino acids such as alanine, serine, and cysteine was observed. These findings expand the synthetic scope of threonine aldolases and open up simple biocatalytic routes for the synthesis of D- or L-α-alkyl amino acid derivatives.[118]

Work continues on the identification and development of novel aldolases, for their potential as emerging and powerful tools in the synthetic chemist's repertoire.

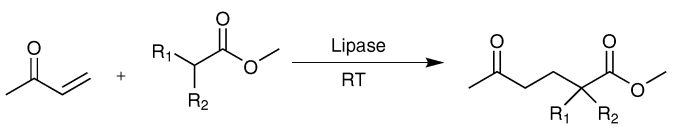
3.4(b) Conjugate additions. Carbon–carbon bond forming reactions represent a mainstay in synthetic organic chemistry. The Michael addition reaction, which is a conjugate addition type reaction, is a powerful and highly-used method for the formation of new carbon–carbon and carbon–heteroatom bonds. Michael-type reactions typically involve the use of either strongly basic or acidic conditions, leading to the generation of potentially hazardous waste by-products and/or undesired side-products. Recent efforts geared to discovery of greener, more environmentally compatible catalysts have been directed towards the development of biocatalytic versions of the Michael reaction.

Investigations into the ability of hydrolases to catalyze Michael-type reactions have identified a number of lipases that possess some latent Michael addition-type activity.[119–121] Screening lipases for reactivity with a series of acidic esters and small α,β-unsaturated ketones or nitroalkenes found four lipases, namely CALA and CALB, *Mucor miehei* lipase, and *Thermomyces lanuginosas* lipase that were able to catalyze most of the investigated conjugate addition reactions tested[121] (Scheme 26).

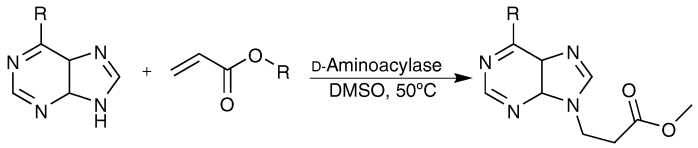
The Michael acceptor substrates required strong electron withdrawing substituents and highly nucleophilic C–H acid compounds to proceed. Unfortunately, the reactions were not enantioselective.



Scheme 25 Biocatalytic synthesis of DL-syn or DL-anti mixtures of β-hydroxy-α-quaternary amino acids using threonine aldolases.



Scheme 26 Representative example of a lipase-catalyzed Michael addition between an unsaturated ketone and an ester.

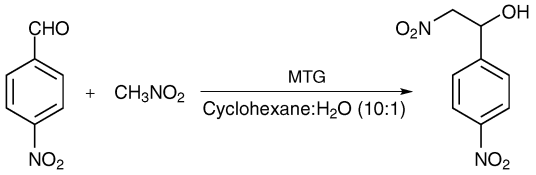


Scheme 27 Michael addition of aminopurines with acrylates catalyzed by D-aminoacylase.

More recent work by He and coworkers showed that enzymatic asymmetric Michael additions were possible using immobilized lipase from *T. lanuginosus* for the reaction of cyclohexenone with a wide range of 1,3-dicarbonyl compounds.[119] Despite the generally good yields of the reaction, only modest enantioselectivities were observed.

Screening work by the group of Lin identified a series of commercially available hydrolases that performed Michael-type additions in a range of neat organic solvents. In particular, D-aminoacylase was found to have high Michael addition-type activity for a range of 1,3-dicarbonyl donors and a series of α,β-unsaturated acceptors.[122] The Michael addition activity of D-aminoacylase has been extended to the synthesis of structurally diverse purine derivatives in good yields under relatively mild reaction conditions[123] (Scheme 27). Due to their well established nature and general ease of use, hydrolase-catalyzed Michael additions represent a viable next generation method for conjugate addition reactions.

3.4(c) Henry type (nitroaldol) reactions. The nitroaldol (a.k.a the Henry) reaction is a powerful route for the formation of new carbon–carbon bonds in organic synthesis. The reaction involves the coupling of an alkyl nitrocompound (nitroalkane) with a carbonyl (either an aldehyde or ketone) *via* a base-catalyzed process, resulting in the formation of β-nitroalcohols. β-Nitroalcohols are valuable intermediates in the synthesis of a range of 2-aminoalcohols, 2-nitroketones, and nitroalkenes. The use of strong bases such as lithium diisopropylamide (LDA), butyl lithium, and $Ba(OH)_2$ can promote formation of undesired side products (from competing aldol addition or Cannizzaro reactions). In the first reported example of a biocatalytic Henry reaction, Tang *et al*. recently reported the reaction of 4-nitrobenzaldehyde and nitromethane in a cyclohexane : water (10 : 1) solvent system in the presence of microbial transglutaminase (MTG) isolated from *Streptoverticillium griseoverticillatum* at room temperature,



Scheme 28 Henry reaction of 4-nitrobenzaldehyde and nitromethane catalyzed by MTG.

yielding the corresponding β-nitroalcohol with no unwanted side products[124] (Scheme 28).

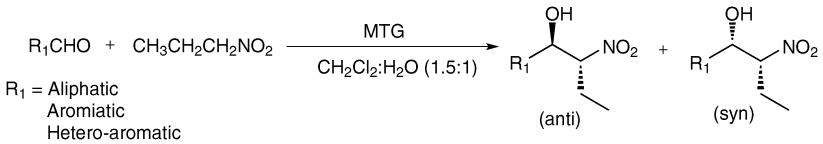
A 1 mmol-scale survey of the substrate scope for this biocatalytic reaction revealed that MTG recognizes a broad range of aliphatic, aromatic, and hetero-aromatic aldehyde substrates. Small aliphatic or aromatic aldehydes bearing electron-withdrawing substituents were found to be the best substrates for this MTG-catalyzed Henry reaction. Modulation of the nitroalkyl compound had little effect on the ability of MTG to catalyze the reaction.[124] It is interesting to note that MTG-catalyzed coupling of aldehydes with nitroalkyl groups such as nitroethane and nitropropane generated products corresponding to an asymmetric Henry reaction showing *anti*/*syn* selectivity, with production of the *syn* product being favoured (Scheme 29).

3.5 Transfer reactions

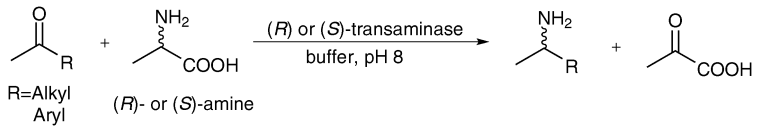
3.5(a) Transamination. The past years have been marked by an increased prevalence of biocatalytic transamination reactions for the synthesis of chiral amines from prochiral ketones, as a result of the increased availability of transaminase enzymes (also named aminotransferases). Transaminases are also applied to kinetic resolution and deracemisation (Scheme 30).

The tremendous synthetic utility of chiral amines has resulted in their widespread adoption in key industrial processes, which has been recently reviewed.[125] One factor contributing to their potential for industrial application is the possibility of using low-cost amine donor compounds, in conjunction with the prochiral ketone of interest. In the past, α-transaminases were the main targets of development, where the enzyme is specific for an amino group adjacent to the carboxylate. However, the chemical accessibility of a variety of carboxylate-containing prochiral reagents such as ketoacids and hydroxyketones has promoted the discovery of a growing array of transaminases, which provide access to an increasing diversity of amines. In particular, the looser substrate specificity of ω-transaminases is attracting attention.[126]

The increasing availability of (*R*)-specific transaminases is a further enabling factor; these are being made available through genomic mining[127] or through engineering[20] (see Section 4 for an important example of transaminase engineering). Seven such novel (*R*)-specific transaminases were recently identified and validated for their potential in synthesis of aliphatic, aromatic and arylaliphatic amines, several showing



Scheme 29 MTG-catalyzed Henry reaction of aldehydes with nitropropane.



Scheme 30 Representative example for the biocatalyst-based synthesis of chiral amines using (*R*)- or (*S*)-transaminases.

excellent properties.[127] In the preparation of chiral amines, the unfavorable thermodynamic equilibrium of the amination reaction is a challenge that must be addressed, as by removal of one of the products.[125] For example, in the case where the amine donor compound is the amino acid alanine, the corresponding product pyruvate can be removed by further enzymatic conversion, thus pulling the equilibrium to afford better yields.[128] The potential gains in driving a one-step chiral amination to completion are fuelling many efforts in this direction. This was convincingly demonstrated in a 100 mg-scale, 4-step chemoenzymatic synthesis of (*S*)-rivastigmine, a potent drug used in early-stage Alzheimer's disease, using a coupled enzyme system to drive the reaction to completion.[129] The key chiral intermediate was obtained by biocatalyzed transamination with ee > 99%. 'Substrate engineering'—in this case, masking a phenolic hydroxyl group with the aid of a protecting group—was required to improve the yield, affording >96% conversion to the desired product using (*S*)-selective *Vibrio fluvialis* ω-transaminase. Of note, the (*R*)-selective ATA-117 ω-transaminase was used to provide the opposite enantiomer with ee > 99%, although conversion was lower (76%).

Transaminases can be applied to racemisation, to improve overall yields of resolution reactions. One recent example was provided on a small scale for racemisation of α-chiral primary amines, using ω-transaminases.[130] Chemical racemisation of amines generally requires metal-based catalysis, which can be beneficially replaced by enzyme-catalyzed processes. Indeed, biocatalytic racemisation can be gentler than chemical methods, thus allowing recycling of the undesired enantiomer with higher yield. These early efforts require further fine-tuning but are very promising. Similarly, ω-transaminases were applied to the kinetic resolution of racemic organoselenium amines.[131] Chiral selenides have been shown to be useful chiral ligands in asymmetric synthesis, and in some cases present biological activity, justifying the interest they generate. By applying either (*R*)- or (*S*)-selective transaminase-catalyzed resolution, both enantiomers of various organoselenides were obtained with enantioselectivities that were often excellent; yield varied from poor to good, indicating that further development is required.

3.5(b) Transamidation. Enzymatic synthesis of amide bonds is attractive, as it obviates the need for some protection/deprotection steps. Proteases and peptidases have long been run 'backward', to synthesize peptide bonds, as performed using thermolysine in the kT per year synthesis of the dipeptide aspartame. Recently, two aminopeptidase-based syntheses of the β,α-dipeptide L-carnosine, an important food additive, were devised to replace chemical synthesis.[132]

Nonetheless, fighting equilibrium towards the native hydrolysis reaction is challenging, as clearly outlined in ref. 132. Transglutaminases natively catalyze transamidation, between a glutamine amide (acyl donor substrate) and the amine of the lysine (acyl acceptor substrate), resulting in protein cross-linking. Transglutaminases accept a broad range of amine acceptor substrates, which has enabled adding polyethylene glycol to proteins (PEGylation) for pharmaceutical purposes. Co-solvents can increase selectivity of microbial transglutaminase to a narrow subset of glutamine residues on the target protein.[133] The nature of the acyl donor group can also be varied, where a glutaminyl-glycine dipeptide N-terminally modified with fluorophores was successfully transferred onto a protein,[134] suggesting that the combination of small molecules other than peptides will be possible either with native, or engineered transglutaminases. These reactions show promise but have found little application to small-molecule synthetic chemistry to date.

4. Identifying a suitable biocatalyst for a key reaction

When a key reaction appears to be a candidate for biocatalysis—perhaps because of the presence of functional groups incompatible with standard chemistry, or for stereochemical considerations—one must identify an appropriate biocatalyst. As with chemical catalysts, the first consideration is the nature of the desired chemical transformation, and the second is the overall structure of the reagent, beyond the bond of interest. Three levels of increasing complexity are considered: first, where a similar reaction has been reported, second when a new reaction is likely to require biocatalyst library screening, and finally when screening fails and biocatalyst engineering is required. The two first cases are discussed below, and the third case in Section 4. The acquisition of a practical guide such as ref. 35 and 135 may prove valuable. Directed to the non-expert, these recent books cover theory and practice of working with biocatalysts and developing a biocatalytic reaction.

If the target biocatalytic reaction has been reported with a similar substrate (*i.e.* same key bond to be transformed, and similar functional groups especially in the vicinity of the key bond), it is worth trying the same, or a similar biocatalyst. Websites such as the 'Enzyme Directory' (www.enzymedirectory.com), Biocatalysis (www.bio-catalyst.com/enzyme-sources), the CoEBio3 site at the University of Manchester (www.coebio3.org) and the biocatalysis group pages at the University of Graz (borgc185.kfunigraz.ac.at) provide lists of biocatalyst suppliers. Similarly, if the microbial strain to be used for a bioconversion is known, one can obtain it from a collection; the World Federation for Culture Collections holds up-to-date links to culture collections worldwide.

If no similar reaction has been reported, a broader search may involve testing a few, promiscuous commercial enzymes, or screening enzyme libraries, or libraries of whole cells. Financial considerations for purchasing these, as well as material considerations for screening capacity, will be invoked.

Literature searches to direct the selection of biocatalysts can be complemented by Web-based tools. The University of Minnesota Biocatalysis/Biodegradation database provides an easily queried collection of microbial biocatalytic reactions and biodegradation pathways. Of limited coverage with respect to synthetically attractive reactions, it is of interest for *cis*-dihydroxylations of a breadth of reagents and is searchable by graphical structure search. One can query the BRENDA enzyme database, to search by substrate or product substructure, or by reaction type, either by name or with a graphical interface. This process is painstaking, may yield many putative hits without ranking them, and is restricted to known reactions, therefore of limited utility for new reactions. To overcome this limitation, Arzeda Corp offers an Enzyme Identification™ service: using a chemical descriptor of the expected transition state for the desired reaction, and a crystal structure for an enzyme belonging to a plausible biocatalyst class, a Rosetta-based algorithm searches enzyme sequence and structure databases to rank enzymes according to their potential for providing the appropriate catalytic machinery and substrate binding pocket for the target reaction. Methods providing some predictive capacity of substrate scope have also been developed, such as an experimental active site mapping of P450 variants using semisynthetic chromogenic probes that are structurally related to a desired substrate.[136]

Individual biocatalysts and biocatalyst screening kits are offered by an increasing number of companies. Some kits contain all necessary reagents and controls for the reaction. Others offer specialty products or services, such as immobilized enzymes, proprietary enzymes, customized biocatalyst improvement or development, all the way to process development. To counter the investment required to purchase or build up a versatile biocatalyst library, a number of institutions specializing in organic synthesis also act as service providers. Among the many product and service providers, we note the following.

Access to diversified biocatalyst collections is rapidly growing, as major enzyme producers such as Novozymes and Genecor make their products available through large chemical suppliers such as Sigma-Aldrich. Biocatalysts are provided individually or in small panels, in free or immobilized form. Codexis, a strong driver in biocatalyst discovery, development and application, provides biocatalyst screening panels in the 96-well plate format, including ketoreductases, acylases, ene reductases, transaminases, and halohydrin dehalogenases. As do Ingenza, they also work with industrial partners to develop new biocatalytic routes for production of chiral chemicals, biopharmaceuticals and biofuels. DSM's InnoSyn services scout for greener synthesis routes *via* enzyme assays including new lyases, transaminases and dehydrogenases. Others offer specialized products, such as Nzomics which specializes in nitrile-manipulating enzymes and carbohydrate-active enzymes, and also offers enzyme customizing services. Chiralvision offers synthetic route development comprising biocatalyzed steps using commercial enzymes, as well as enzyme immobilization. Their screening kits include a lipase kit for reactions under water-free conditions. Among its varied services, Almac offers enzyme kits, reaction screening services with off-the-shelf biocatalysts, microbial screens to identify novel biocatalysts, and process scale-up services. CoEBio3 is a UK academia-based institute offering in-house partnerships with industry and academia, for training or services in discovery and development of biocatalysts and bioprocesses ranging from early stage consulting to process scale-up.

Commercial biocatalysts may be provided as a single isomer, or as a less purified mixture of similar enzymes with somewhat differing specificities (as for many commercial glycosidases). Mixtures may prove less reproducible but may be cheaper and more flexible with respect to preferred substrates. For example, while a mixture of isoforms known as pig liver esterase (PLE) holds numerous synthetic applications, a single isoform of PLE produced in a microbe—thence termed 'alternative pig liver esterase' (APLE), was found to have attractive catalytic properties.[137] In that example, the switch from a porcine to a microbial source may facilitate regulatory matters for specific applications.

Eventually, a successful biocatalysis scheme may need to be scaled up. In that context, a recent cost analysis of biocatalyst production can be of aid in assessing that aspect of economic viability of a biocatalyzed process.[138]

5. Biocatalyst engineering: improving the shortcomings

Maximizing the benefits of biocatalysis requires optimizing reaction conditions. It may also require improving the biocatalyst, as recently discussed with an industrially-oriented mindset in ref. 139 and 140. One main route to obtaining better biocatalysts is genome mining, where large-scale DNA sequencing efforts allow identification of natural variants of a given enzyme class, offering the potential for differences in stability, substrate scope and fine selectivity. A recent example of mining for new P450s is provided in ref. 141; other efforts target genome mining from extremophilic organisms, to provide biocatalysts that tolerate high or low temperatures, high salt concentrations or extreme pH, with implications for improved process productivity. The second main route to improving enzymes is through engineering: as done in nature, when needs arose throughout evolution for improved biocatalysts or for biocatalysts catalyzing new reactions, so does enzyme engineering propose to do. Engineering existing biocatalysts has become broadly practiced over the past 5–10 years, the tools for doing so now being readily available. A clear review of biocatalyst engineering methodologies is provided in ref. 142.

Biocatalyst properties that lend themselves well to engineering include the modulation of enantioselectivity, enzyme stability under various reaction conditions (temperature, solvent, high reagent concentration), alteration of substrate scope, reduction of product inhibition and increased enzyme production. Below, some recent examples are provided, which vary in the properties that were modulated and in the extent of the engineering efforts. It should be noted that companies offering biocatalyst engineering services have sprung up around the globe. They are mostly modest-sized, offering a targeted set of specialty services, though major players such as Verenium and Codexis have deployed massive efforts in high-throughput strain and enzyme screening, and in enzyme and

process engineering, for the development of targeted industrial applications.

The power of evolving an enzyme using progressively more stringent selection parameters, to attain the target process conditions, is perfectly exemplified by the evolution of a methyl-ketone transaminase into a prositagliptin ketone transaminase, thus accommodating a much larger substrate.[20] In addition, the best evolved enzyme showed increased thermostability and increased tolerance to high co-solvent concentration (50% DMSO) and to high substrate loading (200 g L^{-1}). The evolution scheme was complex, requiring stepwise generation of libraries to transform successively larger substrate analogs under increasingly process-like conditions, making use of computational modeling and a variety of protein engineering methodologies. The final enzyme included 27 mutations with 10 in the active-site area, to provide a $>10^4$-fold increase in enzyme turnover under process-like conditions. This achieved a doubled productivity relative to the original chemically-catalyzed reaction, producing 1/5th less waste and eliminating heavy metals and specialized reaction equipment. Most known transaminases are *S*-selective; importantly, *R*-selective transaminase variants generated throughout the engineering process in that work also enrich the toolbox available for transaminase screening.

Thermostability was one of the first enzyme properties to be a target for improvement, first by mining thermophilic organisms for stable homologs (where thermostable DNA polymerases offer a brilliant case story) or by engineering. Enantioselectivity has also been improved or even inverted in a number of enzyme systems, as nicely illustrated in ref. 143. For example, the enantioselectivity of a P450 was recently both improved and inverted for stereoselective hydroxylation of a pyrrolidine. The hydroxylation of *N*-benzylpyrrolidine to both the (*R*)- and (*S*)-*N*-benzyl-3-hydroxypyrrolidines was desired but only the (*S*)-enantiomer was biocatalytically attainable, with 43% ee. Directed evolution afforded a variant with enhanced (*S*)-selectivity (65% ee), and another variant giving an ee of 83% for the (*R*)-enantiomer.[144]

Broadened substrate scope can be achieved by screening random mutations. However, recent approaches tend to favour targeting the active site for modification, to keep library sizes manageable. Examining some recent engineering efforts pertaining to P450s, combinatorial replacement of active site residues by the small-sized alanine allowed accommodation of large substrates such as steroids, alkaloids and peralkylated monosaccharides in P450 BM3.[145] In another effort, alignment-based rational library design guided the conception of a very small (2 randomized active-site positions) but productive library of P450 BM3 variants, allowing identification of variants catalyzing the yet unknown oxidization of C8–C12 cycloalkanes.[146] Finally, computational molecular dynamics simulations were applied to identify active-site 'hot-spots' for selectivity, which allowed identification of P450 CYP102A1 variants from a small library, capable of oxidizing limonene to the valuable perillyl alcohol.[147]

Most of the enzyme classes presented in this review have been subjected to engineering, or stand to be. Modification of the biocatalyzed chemistry, including a change in mechanism, can also be achieved although this area is still young: rational design followed by random mutagenesis and directed evolution allowed conversion of an esterase into an epoxide hydrolase. This change in mechanism required many mutations and provided a moderately efficient biocatalyst (turnover number of 0.01 s^{-1}) that was hampered by substrate inhibition.[148] Increased reaction rate can more readily be achieved, as illustrated by molecular modeling and redesign of CALB for the ring opening polymerization of D,D-lactide, providing a 90-fold increased activity with improved rate and degree of polymerization.[149] Also, the process productivity of the 'green by design' cascade reaction for the biocatalyzed synthesis of atorvastatin (Section 2.1) was initially too low to be economically viable. Directed evolution by DNA-shuffling of the two transforming enzymes and the cofactor recycling enzyme improved them 7-fold (KRED), 13-fold (glucose dehydrogenase) and >2500-fold (halohydrin dehalogenase), allowing for increased substrate loading, decreased biocatalyst loading and reduced costs.[85]

6. Challenges and outlook

The ever-increasing rate of discovery of new enzymes and the growing capacity to engineer enzymes are gradually filling the gaps of desired selectivity for reactions of interest. Some remaining challenges surround issues of incompatibility between the solubilities of biocatalysts and reagents; the use of alternative solvents such as supercritical fluids may offer advantages, although technological transformation would be required for their application in a biocatalytic process. Heterogenous systems may offer more ready solutions and merit exploration. Engineering hydrophobic reagents with removable solubilising groups may also be an option, though this would have to be ingeniously devised into a recyclable system to avoid generation of waste. We must continue to improve approaches to biocatalyst immobilization, stabilization and recycling, to reduce process costs; here, nanosciences may provide some solutions, by application of techniques such as nanopore encapsulation for stabilization of isolated enzymes. This has not yet been demonstrated at a large scale. Improvement of yeast and bacterial strains for whole cell applications is likely to remain a better investment for large-scale applications.

Enzyme engineering is continuing to become more streamlined, as a result of including computational steps, robotic screening and new assays. The inclusion of non-natural amino acids into protein sequences, by modified genetic encoding, has been practised for nearly 2 decades, but remains difficult; developments in cell-free expression may hold the key to making this more efficient and accessible. An area showing rapid development and promising to become much greater yet is pathway engineering: the combination of whole-cell biocatalysis and improved enzyme engineering methodologies is enabling important advances. Finally, computational methods of enzyme design, or enzyme improvement, are growing at a rapid pace. '*De novo*' biocatalyst design shows promise for creating biocatalysts to promote reactions for which no equivalent bio/catalyst is yet known, such as a 'Kemp-eliminase'.[150] The rate constants achieved to date with designed enzymes remain modest, yet can be improved by post-computational directed evolution.[150]

Acknowledgements

The authors thank Dr Robert Lortie and Prof. Nicolas Doucet for their insightful comments, and to Maximillian Ebert for assistance in creating the TOC graphic.

References

1 R. Wolfenden, *Ann. Rev. Biochem.*, 2011, **80**, 645–667.
2 C. A. Martinez, S. Hu, Y. Dumond, J. Tao, P. Kelleher and L. Tully, *Org. Process Res. Dev.*, 2008, **12**, 392–398.
3 R. N. Patel, *Coord. Chem. Rev.*, 2008, **252**, 659–701.
4 J. Tao and J.-H. Xu, *Curr. Opin. Chem. Biol.*, 2009, **13**, 43–50.
5 R. K. Henderson, C. Jimenez-Gonzalez, C. Preston, D. J. C. Constable and J. M. Woodley, *Ind. Biotechnol.*, 2008, **4**, 180–192.
6 S. Kim, C. Jiménez-González and B. Dale, *Int. J. Life Cycle Assess.*, 2009, **14**, 392–400.
7 R. A. Sheldon, in *Biocatalysis for Green Chemistry and Chemical Process Development*, ed. J. A. Tao and R. Kazlauskas, John Wiley & Sons, Inc., 2011, pp. 67–88.
8 F. van Rantwijk and R. A. Sheldon, *Chem. Rev.*, 2007, **107**, 2757–2785.
9 P. Lozano, *Green Chem.*, 2010, **12**, 555–569.
10 M. Moniruzzaman, K. Nakashima, N. Kamiya and M. Goto, *Biochem. Eng. J.*, 2010, **48**, 295–314.
11 A. M. Klibanov, *Nature*, 2001, **409**, 241–246.
12 S. H. Krishna, *Biotechnol. Adv.*, 2002, **20**, 239–267.
13 *Organic Synthesis with enzymes in non-aqueous media*, ed. G. Carrea and S. Riva, Wiley, 2008.
14 A. L. Serdakowski and J. S. Dordick, *Trends Biotechnol.*, 2008, **26**, 48–54.
15 A. J. Quinn, M. J. Pickup and G. B. D'Cunha, *Biotechnol. Prog.*, 2011, **27**, 1554–1560.
16 C. B. Faulds, M. Pérez-Boada and Á. T. Martínez, *Bioresour. Technol.*, 2011, **102**, 4962–4967.
17 R. K. Henderson, C. Jimenez-Gonzalez, D. J. C. Constable, S. R. Alston, G. G. A. Inglis, G. Fisher, J. Sherwood, S. P. Binks and A. D. Curzons, *Green Chem.*, 2011, **13**, 854–862.
18 M. Folic, R. Gani, C. Jimenez-Gonzalez and D. J. C. Constable, *Chin. J. Chem. Eng.*, 2008, **16**, 376–383.
19 Y. Fukuta, H. Komeda, Y. Yoshida and Y. Asano, *Biosci., Biotechnol., Biochem.*, 2009, **73**, 980–986.
20 C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman and G. J. Hughes, *Science*, 2010, **329**, 305–309.
21 J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti and W. Kroutil, *Curr. Opin. Chem. Biol.*, 2011, **15**, 249–256.
22 S. van Pelt, F. van Rantwijk and R. A. Sheldon, *Adv. Synth. Catal.*, 2009, **351**, 397–404.
23 P. H. Nielsen, K. M. Oxenboll and H. Wenzel, *Int. J. Life Cycle Assess.*, 2007, **12**, 432–438.
24 R. A. Sheldon, *Adv. Synth. Catal.*, 2007, **349**, 1289–1307.
25 R. Sangeetha, I. Arulpandi and A. Geetha, *Res. J. Microbiol.*, 2011, **6**, 1–24.
26 U. Hanefeld, L. Gardossi and E. Magner, *Chem. Soc. Rev.*, 2009, **38**, 453–468.
27 D. Brady and J. Jordaan, *Biotechnol. Lett.*, 2009, **31**, 1639–1650.
28 B. Pscheidt and A. Glieder, *Microb. Cell Fact.*, 2008, **7**, 25.
29 H. Fukuda, S. Hama, S. Tamalampudi and H. Noda, *Trends Biotechnol.*, 2008, **26**, 668–673.
30 P. Nikolova and O. P. Ward, *J. Ind. Microbiol. Biotechnol.*, 1993, **12**, 76–86.
31 S. A. Gangu, L. R. Weatherley and A. M. Scurto, *Curr. Org. Chem.*, 2009, **13**, 1242–1258.
32 K. Tauber, M. Hall, W. Kroutil, W. M. F. Fabian, K. Faber and S. M. Glueck, *Biotechnol. Bioeng.*, 2011, **108**, 1462–1467.
33 M. Höhne, S. Kühl, K. Robins and U. T. Bornscheuer, *ChemBioChem*, 2008, **9**, 363–365.
34 *Biocatalysis for the Pharmaceutical Industry*, ed. J. A. Tao, G.-Q. Lin and A. Liese, John Wiley & Sons, Ltd, 2009, pp. i–xi.
35 *Practical Methods for Biocatalysis and Biotransformations*, ed. J. Whittall and P. W. Sutton, John Wiley & Sons, Ltd, 2009, pp. i–xxix.
36 *Modern Biocatalysis*, ed. W.-D. Fessner and T. Anthonsen, Wiley-VCH Verlag GmbH & Co. KGaA, 2009, pp. I–XXIII.
37 *Enzyme Technologies*, ed. W.-K. Yeh, H.-C. Yang and J. R. McCarthy, John Wiley & Sons, Inc., 2010, pp. i–xi.
38 *Biocatalysis for Green Chemistry and Chemical Process Development*, ed. J. A. Tao and R. Kazlauskas, John Wiley & Sons, Inc., 2011, pp. i–xii.
39 G. Volpato, R. C. Rodrigues and R. Fernandez-Lafuente, *Curr. Med. Chem.*, **17**, 3855–3873.
40 F. Secundo and G. Carrea, *J. Mol. Catal. B: Enzym.*, 2002, **19–20**, 93–102.
41 F. Hasan, A. A. Shah and A. Hameed, *Enzyme Microb. Technol.*, 2006, **39**, 235–251.
42 R. Kourist and U. T. Bornscheuer, *Appl. Microb. Biotechnol.*, 2011, **91**, 505–517.
43 Z. J. Zhang, J. Pan, J. F. Liu, J. H. Xu, Y. C. He and Y. Y. Liu, *J. Biotechnol.*, 2011, **152**, 24–29.
44 M. A. Blasco, S. Thumann, J. Wittmann, A. Giannis and H. Groger, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 4679–4682.
45 H. Mukherjee and C. A. Martinez, *ACS Catal.*, 2011, **1**, 1010–1013.
46 S. Hu, C. A. Martinez, J. Tao, W. E. Tully, P. Kelleher and Y. Dumond, Preparation of Pregabalin and Related Compounds, *US Pat.*, 20110065168, 2011.
47 P. Krumlinde, K. Bogar and J. E. Bäckvall, *Chem.–Eur. J.*, 2010, **16**, 4031–4036.
48 M. Sureshkumar and C. K. Lee, *J. Mol. Catal. B: Enzym.*, 2009, **60**, 1–12.
49 J. Gorke, F. Srienc and R. J. Kazlauskas, *Biotechnol. Bioprocess Eng.*, 2010, **15**, 40–53.
50 P. Galletti, F. Moretti, C. Samori and E. Tagliavini, *Green Chem.*, 2007, **9**, 987–991.
51 M. Habulin and Z. Knez, *J. Mol. Catal. B: Enzym.*, 2009, **58**, 24–28.
52 M. Singh, R. S. Singh and U. C. Banerjee, *J. Mol. Catal. B: Enzym.*, 2009, **56**, 294–299.
53 Y. Abe, K. Kude, S. Hayase, M. Kawatsura, K. Tsunashima and T. Itoh, *J. Mol. Catal. B: Enzym.*, 2008, **51**, 81–85.
54 P. Domínguez de María and Z. Maugeri, *Curr. Opin. Chem. Biol.*, 2011, **15**, 220–225.
55 J. T. Gorke, F. Srienc and R. J. Kazlauskas, *Chem. Commun.*, 2008, 1235–1237.
56 R. A. Gross, M. Ganesh and W. Lu, *Trends Biotechnol.*, 2010, **28**, 435–443.
57 S. Kobayashi, *Macromol. Rapid Commun.*, 2009, **30**, 237–266.
58 S. Kobayashi and A. Makino, *Chem. Rev.*, 2009, **109**, 5288–5353.
59 Z. Jiang, *Biomacromolecules*, 2008, **9**, 3246–3251.
60 Z. Jiang, C. Liu and R. A. Gross, *Macromolecules*, 2008, **41**, 4671–4680.
61 Y. Poojari, A. S. Palsule, M. Cai, S. J. Clarson and R. A. Gross, *Eur. Polym. J.*, 2008, **44**, 4139–4145.
62 M. Kato, K. Toshima and S. Matsumura, *Biomacromolecules*, 2009, **10**, 366–373.
63 D. I. Habeych, P. B. Juhl, J. Pleiss, D. Vanegas, G. Eggink and C. G. Boeriu, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 1–9.
64 N. Bala and S. S. Chimni, *Tetrahedron: Asymmetry*, 2010, **21**, 2879–2898.
65 S. S. Yoo, S. Park and E. Y. Lee, *Biotechnol. Lett.*, 2008, **30**, 1807–1810.
66 X. Jia, Z. Wang and Z. Li, *Tetrahedron: Asymmetry*, 2008, **19**, 407–415.
67 Y. Xu, X. Jia, S. Panke and Z. Li, *Chem. Commun.*, 2009, 1481–1483.
68 M. Fuchs, Y. Simeo, B. T. Ueberbacher, B. Mautner, T. Netscher and K. Faber, *Eur. J. Org. Chem.*, 2009, **6**, 833–840.
69 M. Widersten, A. Gurell and D. Lindberg, *Biochim. Biophys. Acta*, 2010, **1800**, 316–326.
70 L. Martínková, V. Vejvoda, O. Kaplan, D. Kubac, A. Malandra, M. Cantarella, K. Bezouska and V. Kren, *Biotechnol. Adv.*, 2009, **27**, 661–670.
71 L. Martínková and V. Kren, *Curr. Opin. Chem. Biol.*, 2010, **14**, 130–137.
72 A. Ben-Bassat, A. M. Walls, M. A. Plummer, A. E. Sigmund, W. L. Spillan and R. DiCosimo, *Adv. Synth. Catal.*, 2008, **350**, 1761–1769.

73 M. Winkler, D. Meischler and N. Klempier, *Adv. Synth. Catal.*, 2007, **349**, 1475–1480.
74 M. Winkler, A. C. Knall, M. R. Kulterer and N. Klempier, *J. Org. Chem.*, 2007, **72**, 7423–7426.
75 D. Zhu, C. Mukherjee, E. R. Biehl and L. Hua, *J. Biotechnol.*, 2007, **129**, 645–650.
76 D. Zhu, C. Mukherjee, Y. Yang, B. E. Rios, D. T. Gallagher, N. N. Smith, E. R. Biehl and L. Hua, *J. Biotechnol.*, 2008, **133**, 327–333.
77 D. Zhu, C. Mukherjee, E. R. Biehl and L. Hua, *Adv. Synth. Catal.*, 2007, **349**, 1667–1670.
78 H. Ankati, D. N. Zhu, Y. Yang, E. R. Biehl and L. Hua, *J. Org. Chem.*, 2009, **74**, 1658–1662.
79 S. van Pelt, M. Zhang, L. G. Otten, J. Holt, D. Y. Sorokin, F. van Rantwijk, G. W. Black, J. J. Perry and R. A. Sheldon, *Org. Biomol. Chem.*, 2011, **9**, 3011–3019.
80 R. Wohlgemuth, *Curr. Opin. Biotechnol.*, 2010, **21**, 713–724.
81 G. W. Huisman, J. Liang and A. Krebber, *Curr. Opin. Chem. Biol.*, 2010, **14**, 122–129.
82 H. Gröger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker and O. May, *Angew. Chem., Int. Ed.*, 2006, **45**, 5677–5681.
83 M. J. Sorgedrager, F. van Rantwijk, G. W. Huisman and R. A. Sheldon, *Adv. Synth. Catal.*, 2008, **350**, 2322–2328.
84 Y. Ni, C.-X. Li, J. Zhang, N.-D. Shen, U. T. Bornscheuer and J.-H. Xu, *Adv. Synth. Catal.*, 2011, **353**, 1213–1217.
85 S. K. Ma, J. Gruber, C. Davis, L. Newman, D. Gray, A. Wang, J. Grate, G. W. Huisman and R. A. Sheldon, *Green Chem.*, 2010, **12**, 81–86.
86 D. Kalaitzakis and I. Smonou, *J. Org. Chem.*, 2010, **75**, 8658–8661.
87 D. Kalaitzakis and I. Smonou, *Tetrahedron*, 2010, **66**, 9431–9439.
88 R. Stuermer, B. Hauer, M. Hall and K. Faber, *Curr. Opin. Chem. Biol.*, 2007, **11**, 203–213.
89 C. Stueckler, C. K. Winkler, M. Bonnekessel and K. Faber, *Adv. Synth. Catal.*, 2010, **352**, 2663–2666.
90 B. Kosjek, F. J. Fleitz, P. G. Dormer, J. T. Kuethe and P. N. Devine, *Tetrahedron: Asymmetry*, 2008, **19**, 1403–1406.
91 N. J. Mueller, C. Stueckler, B. Hauer, N. Baudendistel, H. Housden, N. C. Bruce and K. Faber, *Adv. Synth. Catal.*, 2010, **352**, 387–394.
92 V. B. Urlacher and M. Girhard, *Trends Biotechnol*, 2011, DOI: 10.1016/j.tibtech.2011.06.012.
93 A. Goswami, *Enzyme Technologies*, John Wiley & Sons, Inc., 2010, pp. 125–184.
94 J. A. Dietrich, Y. Yoshikuni, K. J. Fisher, F. X. Woolard, D. Ockey, D. J. McPhee, N. S. Renninger, M. C. Y. Chang, D. Baker and J. D. Keasling, *ACS Chem. Biol.*, 2009, **4**, 261–267.
95 E. O'Reilly, V. Kohler, S. L. Flitsch and N. J. Turner, *Chem. Commun.*, 2011, **47**, 2490–2501.
96 S. T. Jung, R. Lauchli and F. H. Arnold, *Curr. Opin. Biotechnol.*, 2011, **22**, 809–817.
97 A. Rentmeister, F. H. Arnold and R. Fasan, *Nat. Chem. Biol.*, 2009, **5**, 26–28.
98 S. Kille, F. E. Zilly, J. P. Acevedo and M. T. Reetz, *Nat. Chem.*, 2011, **3**, 738–743.
99 G. de Gonzalo, M. D. Mihovilovic and M. W. Fraaije, *ChemBioChem*, 2010, **11**, 2208–2231.
100 M. M. Kayser, *Tetrahedron*, 2009, **65**, 947–974.
101 H. Leisch, K. Morley and P. C. Lau, *Chem. Rev.*, 2011, **111**, 4165–4222.
102 J. Rehdorf, M. D. Mihovilovic and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2010, **49**, 4506–4508.
103 J. Rehdorf, M. D. Mihovilovic, M. W. Fraaije and U. T. Bornscheuer, *Chemistry*, 2010, **16**, 9525–9535.
104 C. V. F. Baldwin, R. Wohlgemuth and J. M. Woodley, *Org. Process Res. Dev.*, 2008, **12**, 660–665.
105 A. Rioz-Martinez, G. de Gonzalo, D. E. T. Pazmino, M. W. Fraaije and V. Gotor, *Eur. J. Org. Chem.*, 2010, **33**, 6409–6416.
106 T. Hudlicky and J. W. Reed, *Synlett*, 2009, 685–703.
107 F. Fabris, J. Collins, B. Sullivan, H. Leisch and T. Hudlicky, *Org. Biomol. Chem.*, 2009, **7**, 2619–2627.
108 D. R. Boyd, N. D. Sharma, C. A. Acaru, J. F. Malone, C. R. O'Dowd, C. C. R. Allen and P. J. Stevenson, *Org. Lett.*, 2010, **12**, 2206–2209.
109 T. Hudlicky and J. W. Reed, *Chem. Soc. Rev.*, 2009, **38**, 3117–3132.
110 L. Werner, A. Machara and T. Hudlicky, *Adv. Synth. Catal.*, 2010, **352**, 195–200.
111 D. R. Adams, C. Aichinger, U. Rinner and T. Hudlicky, *Synlett*, 2011, 725–729.
112 K. Faber, *Biotransformations in Organic Chemistry*, Springer Publishing, 2011, pp. 211–228.
113 P. Clapes, W. D. Fessner, G. A. Sprenger and A. K. Samland, *Curr. Opin. Chem. Biol.*, 2010, **14**, 154–167.
114 A. L. Concia, C. Lozano, J. A. Castillo, T. Parella, J. Joglar and P. Clapes, *Chem.–Eur. J.*, 2009, **15**, 3808–3816.
115 X. Garrabou, J. A. Castillo, C. Guerard-Helaine, T. Parella, J. Joglar, M. Lemaire and P. Clapes, *Angew. Chem., Int. Ed.*, 2009, **48**, 5521–5525.
116 C. Guerard-Helaine, B. Legeret, C. Fernandes, V. Prevot, C. Forano and M. Lemaire, *New J. Chem.*, 2011, **35**, 776–779.
117 K. Baer, N. Duckers, T. Rosenbaum, C. Leggewie, S. Simon, M. Krausser, S. Osswald, W. Hummel and H. Groger, *Tetrahedron: Asymmetry*, 2011, **22**, 925–928.
118 K. Fesko, M. Uhl, J. Steinreiber, K. Gruber and H. Griengl, *Angew. Chem., Int. Ed.*, 2010, **49**, 121–124.
119 J.-F. Cai, Z. Guan and Y.-H. He, *J. Mol. Catal. B: Enzym.*, 2011, **68**, 240–244.
120 R. de Souza, L. M. C. Matos, K. M. Goncalves, I. C. R. Costa, I. Babics, S. G. F. Leite, E. G. Oestreicher and O. A. C. Antunes, *Tetrahedron Lett.*, 2009, **50**, 2017–2018.
121 G. A. Strohmeier, T. Sovic, G. Steinkellner, F. S. Hartner, A. Andryushkova, T. Purkarthofer, A. Glieder, K. Gruber and H. Griengl, *Tetrahedron*, 2009, **65**, 5663–5668.
122 J.-M. Xu, F. Zhang, Q. Wu, Q.-Y. Zhang and X.-F. Lin, *J. Mol. Catal. B: Enzym.*, 2007, **49**, 50–54.
123 J. L. Wang, J. M. Xu, Q. Wu, D. S. Lv and X. F. Lin, *Tetrahedron*, 2009, **65**, 2531–2536.
124 R. C. Tang, Z. Guan, Y. H. He and W. Zhu, *J. Mol. Catal. B: Enzym.*, 2010, **63**, 62–67.
125 P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto and J. M. Woodley, *Biotechnol. Bioeng.*, 2011, **108**, 1479–1493.
126 J. Ward and R. Wohlgemuth, *Curr. Org. Chem.*, 2010, **14**, 1914–1927.
127 S. Schätzle, F. Steffen-Munsberg, A. Thontowi, M. Höhne, K. Robins and U. T. Bornscheuer, *Adv. Synth. Catal.*, 2011, **353**, 2439–2445.
128 E. Ricca, B. Brucher and J. H. Schrittwieser, *Adv. Synth. Catal.*, 2011, **353**, 2239–2262.
129 M. Fuchs, D. Koszelewski, K. Tauber, W. Kroutil and K. Faber, *Chem.l Commun.*, 2010, **46**, 5500–5502.
130 D. Koszelewski, B. Grischek, S. M. Glueck, W. Kroutil and K. Faber, *Chem.– Eur. J.*, 2010, **17**, 378–383.
131 L. H. Andrade, A. V. Silva, P. Milani, D. Koszelewski and W. Kroutil, *Org. Biomol. Chem.*, 2010, **8**, 2043–2051.
132 T. Heck, V. S. Makam, J. Lutz, L. M. Blank, A. Schmid, D. Seebach, H.-P. E. Kohler and B. Geueke, *Adv. Synth. Catal.*, 2010, **352**, 407–415.
133 A. Mero, M. Schiavon, F. M. Veronese and G. Pasut, *J. Controlled Release*, 2011, **154**, 27–34.
134 N. Kamiya, H. Abe, M. Goto, Y. Tsuji and H. Jikuya, *Org. Biomol. Chem.*, 2009, **7**, 3407–3412.
135 *Practical Biotransformations: A Beginner's Guide*, ed. Gideon Grogon, Wiley-Blackwell, 2009.
136 K. Zhang, S. El Damaty and R. Fasan, *J. Am. Chem. Soc.*, 2011, **133**, 3242–3245.
137 M. Hermann, M. U. Kietzmann, M. Ivancic, C. Zenzmaier, R. G. Luiten, W. Skranc, M. Wubbolts, M. Winkler, R. Birner-Gruenberger, H. Pichler and H. Schwab, *J. Biotechnol.*, 2008, **133**, 301–310.
138 P. r. Tufvesson, J. Lima-Ramos, M. Nordblad and J. M. Woodley, *Org. Process Res. Dev.*, 2010, **15**, 266–274.
139 P. Kaul and Y. Asano, *Microb. Biotechnol.*, 2011, DOI: 10.1111/j.1751-7915.2011.00280.x [Epub ahead of print].
140 A. S. Bommarius, J. K. Blum and M. J. Abrahamson, *Curr. Opin. Chem. Biol.*, 2011, **15**, 194–200.
141 T. Furuya and K. Kino, *Appl. Microbiol. Biotechnol.*, 2010, **86**, 991–1002.

142 Y. L. Boersma, M. J. Droge and W. J. Quax, *FEBS J.*, 2007, **274**, 2181–2195.
143 M. T. Reetz, *J. Org. Chem.*, 2009, **74**, 5767–5778.
144 W. L. Tang, Z. Li and H. Zhao, *Chem. Commun.*, 2010, **46**, 5461–5463.
145 J. C. Lewis, S. M. Mantovani, Y. Fu, C. D. Snow, R. S. Komor, C. H. Wong and F. H. Arnold, *ChemBioChem*, 2010, **11**, 2502–2505.
146 E. Weber, A. Seifert, M. Antonovici, C. Geinitz, J. Pleiss and V. B. Urlacher, *Chem. Commun.*, 2011, **47**, 944–946.
147 A. Seifert, M. Antonovici, B. Hauer and J. Pleiss, *ChemBioChem*, 2011, **12**, 1346–1351.
148 H. Jochens, K. Stiba, C. Savile, R. Fujii, J. G. Yu, T. Gerassenkov, R. J. Kazlauskas and U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2009, **48**, 3532–3535.
149 M. Takwa, M. W. Larsen, K. Hult and M. Martinelle, *Chem. Commun.*, 2011, **47**, 7392–7394.
150 O. Khersonsky, D. Röthlisberger, A. M. Wollacott, P. Murphy, O. Dym, S. Albeck, G. Kiss, K. N. Houk, D. Baker and D. S. Tawfik, *J. Mol. Biol.*, 2011, **407**, 391–412.
151 M. V. Donova, *Prikladnaia biokhimiia i mikrobiologiia*, 2007, **43**, 5–18.
152 M. J. Reed, A. Purohit, L. W. L. Woo and B. V. L. Potter, *Drugs Future*, 2008, **33**, 597–606.