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Role of Biocatalysis in Sustainable Chemistry

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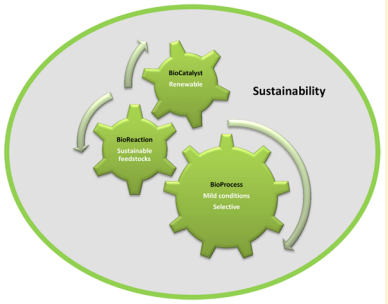
ABSTRACT: Based on the principles and metrics of green chemistry and sustainable development, biocatalysis is both a green and sustainable technology. This is largely a result of the spectacular advances in molecular biology and biotechnology achieved in the past two decades. Protein engineering has enabled the optimization of existing enzymes and the invention of entirely new biocatalytic reactions that were previously unknown in Nature. It is now eminently feasible to develop enzymatic transformations to fit predefined parameters, resulting in processes that are truly sustainable by design. This approach has successfully been applied, for example, in the industrial synthesis of active pharmaceutical ingredients. In addition to the use of protein engineering, other aspects of biocatalysis engineering, such as substrate, medium, and reactor engineering, can be utilized to improve the efficiency and cost-effectiveness and, hence, the sustainability of biocatalytic reactions. Furthermore, immobilization of an enzyme can improve its stability and enable its reuse multiple times, resulting in better performance and commercial viability. Consequently, biocatalysis is being widely applied in the production of pharmaceuticals and some commodity chemicals. Moreover, its broader application will be further stimulated in the future by the emerging biobased economy.



CONTENTS

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1. INTRODUCTION

Process chemists have long recognized the need to consider both environmental and economic metrics when developing processes that ultimately will find application in chemicals manufacture. Beyond stoichiometric reagent use, catalysis offers significant benefits, and today, increasing emphasis is being placed on biologically mediated chemical reactions, more generally termed bioconversion. The scope of bioconversion is very broad, and it is instructive to classify bioconversions into two main types.[1] The first type is growth-associated whole-cell bioconversion, which is usually defined as fermentation. In such cases, the reactant (often termed the substrate) is used for the production of the biocatalyst (and its maintenance), as well as the reaction of interest, and furthermore, the productivity is coupled to the growth rate of the cells (which can be low in aerobic fermentations). In contrast, biocatalytic processes can be defined as those in which the production of the biocatalyst (enzyme or "resting" cell) and the conversion step operate independently. Today, fermentation processes have been established for the production of a range of products (including amino acids from modified *Corynebacterium glutamicum* and *Escherichia coli*).[2] Other products include lactic acid, succinic acid, and 3-hydroxypropionic acid,[3] as well as recent attempts toward itaconic acid and adipic acid.[4] Nevertheless, in this review, we shall be concerned only with this second type, namely, bioconversions with biocatalysts that have been produced beforehand.

Figure 1 classifies such biocatalytic processes into three groups depending on the format of the biocatalyst used for the conversion: (a) resting whole-cell biocatalysis, (b) isolated-enzyme biocatalysis, and (c) immobilized-enzyme biocatalysis. The format of the biocatalyst is of utmost importance in developing a suitable process structure and, ultimately, the flowsheet.

The first case, resting whole-cell biocatalysis, is schematically shown in Figure 1a and represents those cases in which the growth of the biocatalyst and the subsequent conversion of the substrate to product can be separated while still operating in whole-cell format. Compared to fermentation, this approach brings several advantages, including the use of alternative reaction media, with benefits for downstream processing. Likewise, an extra degree of freedom is introduced through the option to change the cell concentration after the fermentation. In this way, the productivity (space-time yield) of the reaction can be increased many-fold over that of a conventional aerobic fermentation. This also implies that the two steps, growth of the biocatalyst and use of the biocatalyst, can each operate optimally.

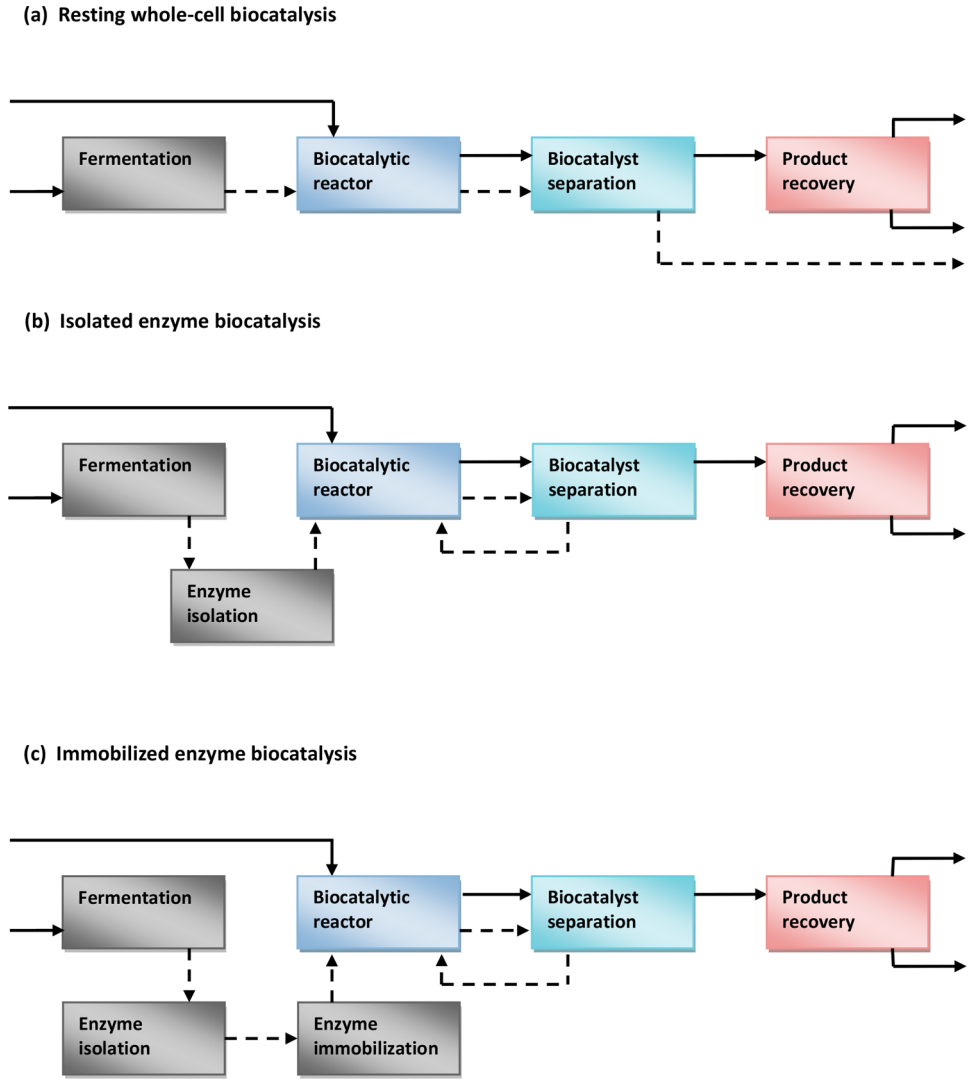

Figure 1. Flowsheets of the three classes of biocatalysis. Solid lines indicate substrate-/product-rich streams. Dotted lines indicate biocatalyst streams.

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Chem. Rev. XXXX, XXX, XXX−XXX

In principle, the cells could be recycled at the end of the conversion step to reduce the cost contribution of the fermentation to the final product, although this is rarely justified in practice.[5] Nevertheless, such whole-cell biocatalysis is fraught with the problem of cross-reactivity with cellular metabolites. Likewise, cofactors might be used suboptimally, because they might also be used elsewhere in the metabolic network. Additionally, substrates and products can be decomposed through competing cellular reactions, thereby lowering the yield. Finally, such a scheme also has the disadvantage of potential substrate diffusional limitations into cells, which can result when non-natural substrates are used, because no active transport mechanism into the cell is available.[6] Unsurprisingly, this effect is more pronounced in the conversion of bulky substrates.[7]

The second group, isolated enzyme biocatalysis, is schematically shown in Figure 1b and represents those reactions where the enzyme is used outside the cell in which it was produced. Primarily, this approach attempts to overcome the diffusional limitations of substrates into (and products out of) cells. In such cases, either the enzyme has been secreted during the fermentation or the cells need to be disrupted following fermentation (and the cell debris removed), to produce a soluble, although unpurified, enzyme. Using an unpurified protein avoids the added cost of purification. Provided that it does not contain proteases, the additional protein present in such an unpurified mixture will usually be beneficial to the desired enzyme stability.[8]

Conditions for the use of an isolated enzyme catalyst usually include the requirement that the enzyme can be easily separated from the product-rich stream. In many cases, the cost of the enzyme is such that reuse is necessary,[9] and this can most effectively be achieved according to the third scheme, using an immobilized enzyme,[10−13] as schematically shown in Figure 1c. Here, the enzyme is immobilized, usually on a spherical solid or, more frequently, within a porous support, to facilitate removal from the product stream by simple (micro)filtration and, thereby, enable subsequent recycle. It is also possible to cross-link enzymes (in various degrees of purity), which often requires the handling and separation of very small particles, which can be readily achieved, for example, by making the cross-linked enzyme particles magnetizable and separating them magnetically (see section 4.3.3). Many processes operate with multiple recycles (in some cases, more than 100) to reduce the cost contribution of the biocatalyst to the final product. An additional benefit of immobilization can be the added stability conferred on the enzyme by immobilizing it in a more stable conformation.

Because of the enormous advances that have been made in biocatalysis in the past decade, in this review, we limit ourselves mainly to the literature from 2006 onward. Earlier references are largely limited to discussions of the historical development of the field.

2. SUSTAINABLE CHEMISTRY

In the mid-1980s, there was growing concern regarding the copious amounts of waste being generated by the chemical industry. A paradigm shift was clearly needed, from traditional concepts of reaction efficiency and selectivity, which focus largely on chemical yield, to one that assigns value to the maximization of raw materials utilization, elimination of waste, and avoidance of the use of toxic and/or hazardous substances.[14,15] There was a pressing need for alternative, cleaner chemistry that was more resource-efficient and produced less waste. As a result of increasing environmental awareness, emphasis gradually switched to waste prevention at the source as opposed to waste remediation and pollution control by end-of-pipe solutions. The U.S. Pollution Prevention Act of 1990[16] focused attention on the need to reduce environmental pollution and recognized that waste prevention at the source not only eliminates the cost of waste treatment but actually strengthens economic competitiveness through a more efficient use of raw materials. This act led to a fundamental shift in the strategy for environmental protection—from "end-of-pipe" waste treatment to waste prevention—and led to the emergence of the term "green chemistry" at the U.S. Environmental Protection Agency (EPA) in the early 1990s.

A succinct definition of green chemistry is as follows: *Green chemistry efficiently utilizes (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.*[17] Green chemistry is primarily pollution prevention rather than waste remediation. The term gained formal recognition with the publication of the 12 principles of green chemistry, by Anastas and Warner in 1998 in their book *Green Chemistry: Theory and Practice*,[18] in which the overall guiding element is "benign by design".

The 12 principles of green chemistry can be summarized as follows.

(1) Waste prevention instead of remediation
(2) Atom efficiency
(3) Less hazardous materials
(4) Safer products by design
(5) Innocuous solvents and auxiliaries
(6) Energy efficient by design
(7) Preferably renewable raw materials
(8) Shorter synthesis (avoid derivatization)
(9) Catalytic rather than stoichiometric reagents
(10) Design products for degradation
(11) Analytical methodologies for pollution prevention
(12) Inherently safer processes

The term sustainable development was introduced in 1987 with the publication of the Brundtland report, *Our Common Future*, by the World Commission on Environment and Development.[19] The report recognized the necessity for industrial and societal development to provide a growing global population with a satisfactory quality of life, but that such development must also be sustainable over time. Sustainable development was defined as "development that meets the needs of the present generation without compromising the ability of future generations to meet their own needs".[19] According to Graedel,[20] to be sustainable, a technology must fulfill two conditions: (i) natural resources should be used at rates that do not unacceptably deplete supplies over the long term, and (ii) residues should be generated at rates no higher than can be assimilated readily by the natural environment. It is abundantly clear that an economy based on nonrenewable fossil resources—oil, coal, and natural gas—consumes natural resources at a much higher rate than they are produced in Nature and that carbon dioxide is being generated at a much higher rate than it can be assimilated by the natural environment, leading to undesirable climate change. Hence, a balance needs to be found among societal equity, environmental impact, and economic development, often referred to as the three Ps—people, planet, and profit—or the triple bottom line. In contrast to green chemistry, sustainable development comprises an economic component. If

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Chem. Rev. XXXX, XXX, XXX−XXX

a technology is not economically competitive, it will not be sustainable in the long term.

Increased interest in green and sustainable growth, coupled with a growing concern for climate change, has focused attention on resource efficiency and is driving the transition from a traditional linear flow of materials in a "take−make−use−dispose" economy, to a greener, more sustainable circular economy.[21]

2.1. Role of Catalysis

A major cause of waste, particularly in the manufacturing of fine chemicals and pharmaceuticals, is the use of stoichiometric, mainly inorganic, reagents in organic synthesis. Pertinent examples are reductions with metals (Na, Mg, Zn, Fe) and metal hydride reagents ($LiAlH_4$, $NaBH_4$) and oxidations with permanganate, manganese dioxide, and chromium(VI) reagents. Mineral acids (H_2SO_4, HF, H_3PO_4) and Lewis acids ($AlCl_3$, $ZnCl_2$, BF_3), employed as reagents in the reaction or in downstream processing, are another major source of waste. In addition, because of the molecular complexity of most pharmaceuticals, their synthesis involves multistep syntheses that are likely to generate more waste than those of simpler commodity chemicals. The solution to these problems is evident: substitution of these wasteful stoichiometric methodologies with more atom- and step-economical[22] catalytic alternatives. In short, the key to green and sustainable chemistry is the use of catalysis—heterogeneous catalysis, homogeneous catalysis, organocatalysis, and biocatalysis—in organic synthesis.[23,24]

2.2. Biocatalysis Is Green and Sustainable

The landmark work by Zaks and Klibanov[25] in 1984, showing that lipases are more thermally stable in organic solvents, such as toluene, than in water, led to the realization that enzymes could be broadly applied in organic synthesis. Moreover, the growing need for cost-effective methods for the industrial synthesis of pure enantiomers of chiral drugs[26] provided an opportunity for the broad application of highly enantioselective biocatalytic methods.[27] However, at that time, widespread application was limited by the paucity of commercially available enzymes, which consisted mainly of hydrolases, such as lipases and proteases, used in food and beverage processing and laundry detergents. Largely thanks to advances in biotechnology, this situation has changed dramatically in the past two decades. Taking advantage of advances in high-throughput DNA sequencing, more than 20000 bacterial and fungal genomes have been sequenced, and the data have become available in the public domain. Currently, a target gene can be identified in silico by "genome mining" of such databases. The gene can then be synthesized, within a week; subsequently cloned into a host microorganism; and thanks to recombinant DNA technology, produced on an industrial scale for acceptable prices.

Twenty-five years ago, it was necessary to modify the process to accommodate the available enzyme. In keeping with the need for a new paradigm,[28] directed (in vitro) evolution[29−34] was used to engineer enzymes to exhibit targeted process parameters, such as activity, specificity, and stability.[35,36] Today, it is eminently feasible to optimize enzymes to fit a predefined optimum process, that is, genuine benign by design. In addition, their storage and operational stabilities can be optimized by effective immobilization, thus enabling their cost-effective recovery and multiple recycling as free-flowing solids.[37]

Hence, biocatalysis has evolved into an industrially attractive technology[38] and has been integrated into mainstream organic synthesis,[39−44] particularly for the enantioselective synthesis of active pharmaceutical intermediates (APIs).[45−49] Indeed, Turner and O'Reilly[50] proposed biocatalytic retrosynthesis guidelines and rules to identify beneficial enzymatic syntheses of target molecules.

The widespread use of biocatalytic methods is also a consequence of their numerous economic and environmental benefits. The catalyst (an enzyme) is produced from readily available renewable resources and is biodegradable and essentially nonhazardous and nontoxic. Moreover, the use of scarce precious metals and the subsequent costs of removing traces of noble metals from end products are circumvented. Enzymatic reactions are generally performed under mild conditions (ambient temperature and atmospheric pressure) in water, often without the need for functional-group activation, protection, and deprotection steps. This affords routes that are more step-economical[22] and generate less waste than conventional organic syntheses. Consequently, biocatalytic methods are more environmentally attractive, more cost-effective, and therefore more sustainable. Furthermore, processes with isolated enzymes can be conducted in standard multipurpose batch reactors, thus avoiding the need for extra investments, such as in high-pressure equipment.

Because enzymatic processes are generally conducted under approximately the same conditions of temperature and pressure, it is relatively easy to integrate multiple transformations into economically and environmentally attractive cascade processes.[51] As shown in Table 1, biocatalysis conforms with 10 of the 12 principles of green chemistry, with the remaining two (principles 4 and 10) not really being relevant because they relate to the product, rather than process design. In short, biocatalysis is a truly green and sustainable technology. It is not only environmentally beneficial, but it is also, thanks to advances in biotechnology, very cost-effective.

Table 1. Green Chemistry and Biocatalysis

	green chemistry principle	biocatalysis
1	waste prevention	significantly reduced waste
2	atom economy	more atom- and step-economical
3	less hazardous syntheses	generally low toxicity
4	design for safer products	not relevant (product not process)
5	safer solvents and auxiliaries	usually performed in water
6	energy efficiency	mild conditions/energy-efficient
7	renewable feedstocks	enzymes are renewable
8	reduced derivatization	avoids protection/deprotection steps
9	catalysis	enzymes are catalysts
10	design for degradation	not relevant (product not process)
11	real-time analysis	applicability to biocatalytic processes
12	inherently safer processes	mild and safe conditions

2.3. Metrics of Green Chemistry and Sustainability

Improvements in the sustainability of all manufacturing processes, including biocatalytic ones, need to be quantitatively measured. Indeed, if it is not possible to measure the sustainability of a process, it is not possible to benchmark it and define targets for improvement. Likewise, industrial implementation necessitates quantitative measures to assess improvements to processes to justify the required investment. The two oldest green metrics are atom economy[52] and the *E* factor,[14,53] first proposed in 1991 and 1992, respectively. Atom economy (AE) is calculated by dividing the molecular weight of the product by the sum total of the molecular weights of all substances formed in the stoichiometric equation for the reaction

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Chem. Rev. XXXX, XXX, XXX−XXX

involved. AE is a theoretical number, based on the use of exact stoichiometric quantities of starting materials and a chemical yield of 100%. Substances that do not appear in the stoichiometric equation, such as solvents and auxiliary chemicals used in product recovery, are disregarded. Nonetheless, it is an extremely useful metric for the initial evaluation, prior to performing experiments, of the resource utilization and the associated waste generation in alternative routes to a targeted product.

The *E* factor, in contrast, is the actual amount of waste produced in the process. It takes the product yield into account, along with waste from all of the auxiliary operations, for example, solvent losses and chemicals used in workup. In the original publication,[14] waste was defined as "everything but the desired product", with the exception of water. The rationale for excluding water was that including it could lead to a skewing of *E* factors, making meaningful comparison of processes difficult. That is, an otherwise waste-free process using substantial amounts of water could be construed as being less green than a process producing much more undesirable waste but using little water. However, the current trend in the pharmaceutical industry is actually to include water in the *E* factor.

A high *E* factor means more waste and, consequently, a negative environmental impact. The ideal *E* factor is zero. It can be calculated for a particular product, production site, or even a whole company. Interestingly, lower *E* factors have been shown[54] to correlate well with reduced manufacturing costs of APIs, which is a reflection of the lower process materials input, reduced costs of hazardous and toxic waste disposal, improved capacity utilization, and reduced energy demand.

Various alternative metrics have also been proposed[55] for measuring the environmental footprints of processes, and attempts have been made to unify the different green metrics.[56] Constable and co-workers at GlaxoSmithKline (GSK),[57,58] for example, proposed the use of reaction mass efficiency, a refinement of atom economy that takes yield and use of excess reagents into account, and mass intensity (MI), defined as the total mass (including water) used in a process divided by the mass of product (MI = *E* factor + 1). The Green Chemistry Institute Pharmaceutical Round Table adopted this metric, renaming it Process Mass Intensity (PMI), to benchmark the environmental footprints of processes for APIs and to use these data to drive the greening of the pharmaceutical industry.[59,60] However, none of these alternative metrics offers any particular advantage over the *E* factor for describing how wasteful a process is. The ideal PMI is 1, whereas the ideal *E* Factor is 0, which perhaps more clearly reflects the ultimate goal of zero waste. The *E* factor also has the advantage that, in evaluating a multistep process, *E* factors of individual steps are additive but PMIs are not because PMI does not discount step products from the mass balance. PMI and the *E* factor represent two sides of the same coin. The PMI, which is favored by the Pharmaceutical Round Table, emphasizes resource efficiency,[59] whereas the *E* factor emphasizes waste generated. In the early 1990s, emphasis was clearly on waste elimination, and the goal of the chemical industry was "zero-waste production plants" for chemicals manufacture, which is better reflected in the *E* factor.

A major source of waste in the pharmaceutical industry is solvent losses. In early analysis, if solvent losses were not known, it was generally assumed that 90% of the solvent could be recovered and reused. In hindsight, this assumption might have been too optimistic, especially because solvent use is scale-dependent. More recently, a distinction has been made[54] between simple *E* factors (sEFs), which exclude solvents and water and are useful for early route scouting, and complete *E* factors (cEFs), which include solvents and water, assuming no recycling. The true commercial *E* factor will fall somewhere between the sEF and cEF and can be calculated when reliable data for solvent losses are available.

A shortcoming of simple mass-based metrics is that they assign the same weighting to all types of waste. Hence, the term "environmental quotient", EQ, where *Q* is an arbitrarily assigned unfriendliness multiplier, was introduced.[61] Although arbitrarily assigned *Q* values are debatable, it is clear that (monetary) values can be assigned to waste streams, and much attention has been devoted in the past two decades to developing methodologies for quantification of *Q*. Early examples are the EATOS (Environmental Assessment Tool for Organic Synthesis) software of Eissen and Metzger[62] and EcoScale,[63] a semiquantitative method for evaluating both economic and environmental impact factors of organic syntheses based on yield, cost, safety hazards, conditions, and ease of downstream processing.

Life cycle assessment (LCA)[64,65] was specifically designed to assess the environmental impact of a product, in all stages of its "life", from raw materials extraction through materials processing to distribution, use, and disposal or recycling. LCA is conducted on the basis of quantifiable environmental impact indicators, such as energy usage, global warming, ozone depletion, acidification, eutrophication, smog formation, and human and ecotoxicity, in addition to waste generated. Graedel noted, in 1999, that "adding a life-cycle perspective to green chemistry enlarges its scope and enhances its environmental benefits".[66] It should be noted, however, that conducting a full-scale cradle-to-grave or cradle-to-cradle LCA in the design or development phase is generally too difficult and time-consuming.[67]

Integration of mass-based green metrics with LCA metrics for the quantitative assessment of the environmental impact of processes has been described by several authors.[68] Jessop and co-workers,[69] for example, used a combination of nine LCA environmental impact indicators in a gate-to-gate assessment of the greenness of alternative routes to a particular product. Similarly, the EPA introduced GREENSCOPE, based on the four Es, namely, environment, energy, efficiency, and economics, for evaluating and designing more sustainable processes.[70]

Some companies have developed their own LCA methodologies. GSK, for example, developed FLASC (Fast Life Cycle Assessment of Synthetic Chemistry)[71] for the evaluation of synthetic routes to APIs and recently described the evolution of LCA in pharmaceutical and chemical applications.[72] BASF developed Eco-efficiency Analysis,[73] and L'Oreal, the cosmetic ingredients producer, introduced Eco-footprint,[74] consisting of a manufacturing footprint and an eco-design footprint. Similarly, the flavor and fragrance company Mane introduced Green Motion for evaluating environmental, health and safety (EHS) impacts of their manufacturing processes[75] and claimed that a full assessment could be made in only 30 min. Interestingly, the environmental indicators used by companies are also determined by the market segment that is being served. For example, the cosmetic ingredient/flavor and fragrance manufacturers allocate considerable importance to the renewable versus synthetic origin of raw materials, whereas this is essentially a moot point for pharmaceutical companies.

An interesting example in which a direct comparison of a synthetic route with a two-enzyme route was made concerns the synthesis[76] of 7-aminocephalosporanic acid (7-ACA) starting from the potassium salt of cephalosporin C. A full EHS and LCA

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Chem. Rev. XXXX, XXX, XXX−XXX

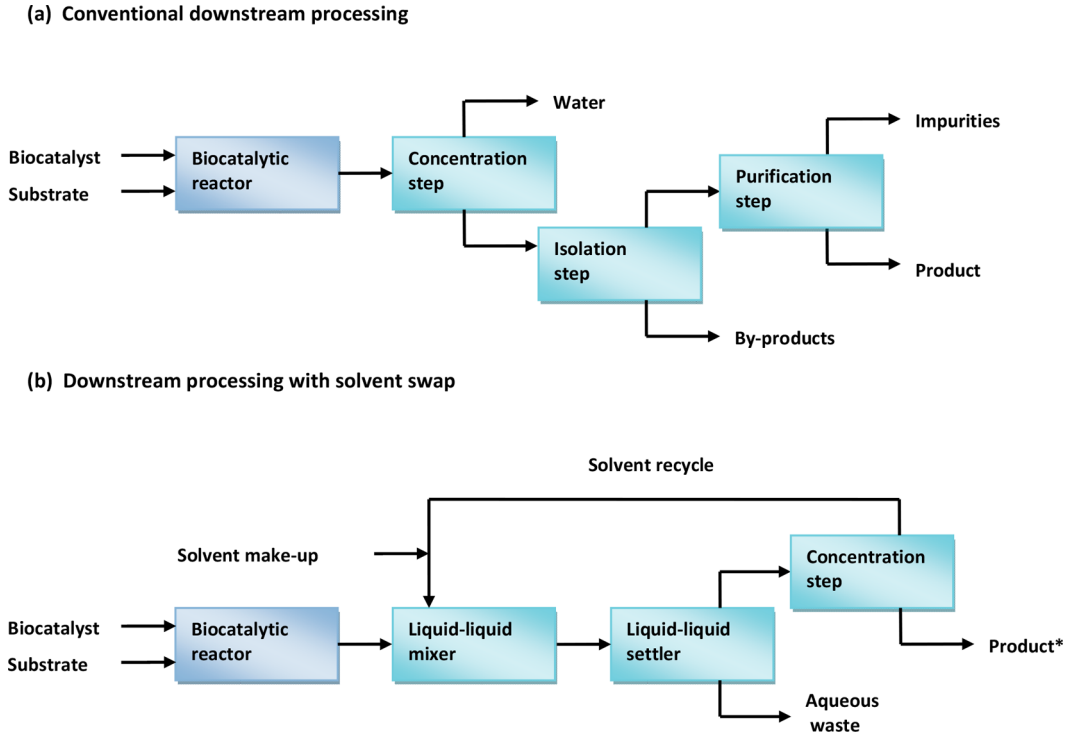

Figure 2. (a) Conventional downstream process. (b) Downstream process with solvent swap.

comparison was undertaken. Whereas the more mature chemical process was found to have a higher yield, it had a significantly lower reaction mass efficiency and half the mass productivity of the enzymatic process. Likewise, the chemical process used more solvent, required about 25% more process energy, and had a greater impact on greenhouse gas (GHG) emissions than the enzymatic route. On the other hand, the enzymatic process used significantly more water, leading to a higher *E* factor if water is included. The chemical route was mature, and further improvement of the biocatalytic route would no doubt reduce the *E* factor, but this also shows the difficulties of comparing two processes at different stages of development.

Integration of mass-based green metrics with LCA affords an extremely useful tool for evaluating the environmental impacts of processes for the manufacture of bulk and fine chemicals and APIs, and the methodology continues to be further refined.[77,78] However, simply integrating mass-based green metrics with LCA environmental indicators is not sufficient for assessing sustainability. For this purpose, an assessment of economic viability is an essential part of the equation. The integration of environmental and economic metrics for assessing fine-chemical processes underlies the above-mentioned BASF eco-efficiency analysis and, more recently, the eight criteria for good chemical manufacturing processes defined by Roschangar and co-workers at Boehringer Ingelheim.[79] For biocatalytic processes, simple process metrics [g of product/(g of substrate), g of product/(g of biocatalyst), g of product/(L h), g of product/(L of reactor)] reflecting the key economic drivers—raw material efficiency, biocatalyst cost, reactor size, and downstream costs—can serve as a guide to biocatalyst development and process improvement.[80]

3. REACTION MEDIA

As with all production processes, solvent usage is an important consideration in biocatalytic transformations. It is generally perceived as an advantage that enzymes function optimally in water, but this can be a serious shortcoming if the organic substrate is only sparingly soluble in water. Moreover, some reactions such as (trans)esterifications and amidations cannot be conducted in water owing to equilibrium limitations and/or product hydrolysis. Hence, there is a long-standing interest in nonaqueous biocatalysis.[81] However, the use of volatile organic solvents in biocatalytic processes is subject to the same caveats as organic syntheses in general. Moreover, highly polar substrates, such as carbohydrates, nucleosides, and peptides, are sparingly soluble in common organic solvents. In addition, enzymes generally denature in polar aprotic solvents such as dimethylformamide (DMF). There is a growing interest, therefore, in the use of avant garde solvents such as ionic liquids (ILs) and deep eutectic solvents (DESs) for conducting enzymatic transformations (see sections 3.4 and 3.5).

3.1. Use of Water as the Solvent for Biocatalysis

Water is the predominant solvent for biologically mediated reactions. This implies that the product needs to be separated from the water following the reaction. However, removing water (the most plentiful component) is expensive on account of its high boiling point. Indeed, a simple economic analysis of many biocatalytic processes reveals the importance of achieving sufficient product concentration leaving the reactor, to reduce the burden of water removal downstream. It should also be mentioned that, if water is used as the solvent, it still has to be treated to remove traces of organics before it can be discharged (and the complete *E* factor includes water).[82] Hence, high substrate concentrations (preferably 10 wt % or more), or even suspensions of substrates, should be used to limit the amount of water. Despite these challenges to the conventional wisdom of replacing some of the water with organic solvent, it remains the case that the majority of biocatalytic processes are carried out in water.

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Chem. Rev. XXXX, XXX, XXX−XXX

Unlike many conventional chemical processes, where the purity of the product leaving the reactor reflects the downstream processing cost, in the case of aqueous-based systems, it is the product concentration that dominates, because the costs of removing water are so significant. General guidelines reflecting the value of the product have been reported between 50 g/L for high-priced products to 300 g/L for low-priced products.[83] For product streams up to 300 g/L, it is always necessary to remove water prior to feeding the stream to product isolation and recovery. This is required to keep the size of the separation steps manageable. The method of Hermann and Patel[84] for estimating the effects of concentration on the recovery costs, discussed by Grotkjaer,[85] provides a good illustration regarding such issues for low-value products. From the perspective of sustainability, a higher product concentration leaving the reactor is also reflected in a smaller amount of water used in the process, namely, in the water intensity (WI) and the PMI and the *E* factor (when water is included). Recently, Ni and co-workers reported an inverse correlation between the *E* factor and substrate concentration.[86] This indicates the great importance of designing processes that achieve adequate product concentration, not only from an economic perspective, but also to ensure that environmental metrics are satisfied. This also provides an important target for protein engineers to improve the tolerance of enzymes against inhibitory and toxic products (which might otherwise limit concentrations).[34]

The use of aqueous solutions for reactions appears highly attractive at first glance, but in reality, it hides the downstream processing problem. The aqueous material containing the product at any concentration less than 300 g/L will need water removal (e.g., by evaporation) ahead of product isolation and purification. Given the high boiling point of water, this makes it expensive and potentially more attractive to extract the product into a lower-boiling solvent (also at a higher concentration), which can then be cheaply evaporated—so-called "solvent swap" (see Figure 2).[87−89] Alternatively, water replacement can be back-integrated into the reactor itself by employing aqueous/organic biphasic reaction media. This has proven attractive in several biocatalytic processes. Methods to improve product concentration more effectively and enhance downstream processing are also a high priority for future research.

3.2. Organic Solvents

The 1984 article of Zaks and Klibanov,[25] showing that enzymes are active in organic solvents, heralded the advent of nonaqueous enzymology.[90] Biocatalysis in organic media has several benefits. Most organic substrates are sparingly soluble in water, and some reactions, such as (trans)esterifications and amidations, cannot be conducted in water owing to competing product hydrolysis. In addition, product recovery is easier from volatile organic solvents, and microbial contamination is eliminated. The reaction medium can also influence enzyme specificities, in particular, it can enhance or even invert the enantioselectivity (Table 2).[91] In short, medium engineering can be used to optimize the synthetic utility of enzymatic transformations.[92] On the other hand, environmental issues associated with many volatile organic solvents and polar aprotic solvents represent a serious drawback of biocatalysis in organic media.

Another drawback is that rates are generally much lower than in water. Enzymes are able to function as suspensions in organic solvents, but as one would a priori expect, the catalytic efficiency is orders of magnitude lower than that of the same enzyme dissolved in water.[93] Hence, if competing hydrolysis is not an issue, it probably makes more sense to conduct the reaction in an aqueous/organic biphasic system.

Table 2. Effect of Solvent on the Enantioselectivity of a Lipase-Catalyzed Kinetic Resolution[91]

solvent	*E*
t-amyl alcohol	518
3-pentanone	212
dioxane	178
acetone	142
vinyl acetate	89
tetrahydrofuran	69

3.3. Supercritical CO_2

A potentially attractive alternative to volatile organic solvents is supercritical carbon dioxide, $scCO_2$. It is nontoxic, noninflammable, readily available, and inexpensive. Moreover, it is supercritical under conditions (31 °C and 7.4 MPa) that are conducive to biocatalysis[94−97] and provides the possibility of using a renewable catalyst in a natural solvent. Furthermore, it combines the solubilizing capacity of a liquid with the low viscosity of a gas, affording high rates of mass transfer and facile product recovery by simple decompression. Enzymes generally exhibit good activity and stability in $scCO_2$, but there are two potentially detrimental properties: (i) Reaction of free amino groups in lysine residues with CO_2 produces carbamates, and (ii) reaction with water affords carbonic acid, resulting in a lowering of pH.

Immobilization of the enzyme by covalent attachment to free amino groups on the enzyme surface can suppress inactivation caused by their reaction with CO_2. For example, in the kinetic resolution of 1-phenylethanol by *Candida antarctica* lipase B-(CaLB-)-cross-linked-enzyme-aggregate- (CLEA-) catalyzed transesterification, reaction rates were higher in $scCO_2$ than in *n*-hexane.[98] Interestingly, the same reaction, catalyzed by Novozym-435 in a packed-bed reactor, was also enhanced in liquid CO_2 compared with organic solvents.[99] A benefit of using liquid rather than supercritical CO_2 is that it requires a lower pressure (4.5 MPa at 10 °C). Interestingly, the enantioselectivity of such kinetic resolutions can be influenced by both the pressure and temperature used.[100] Another recent development involves the transesterification of triglycerides in $scCO_2$ catalyzed by lipases immobilized in monoliths in packed-bed reactors for the continuous production of biodiesel.[101]

3.4. Ionic Liquids (ILs)

The activity of enzymes in organic solvents can be increased by lyophilization in the presence of relatively large amounts of salts,[102] such as potassium chloride. Hence, one might expect that significant rate enhancements, compared to those observed in organic solvents, could be obtained with a suspension of an enzyme in a room-temperature ionic liquid (IL), based on its salt- and water-like character. ILs are liquid at or close to ambient temperature and are composed entirely of ions. Based on their nonvolatility, they have been widely proposed as potentially attractive alternatives to volatile organic solvents, and much attention has been devoted to their use as reaction media for catalytic processes.[103−106]

In the first example of biocatalysis in water-free ILs, suspensions of *Candida antarctica* lipase B (CaLB) in anhydrous 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF_6]) and 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]) were shown[107] to catalyze transesterifications

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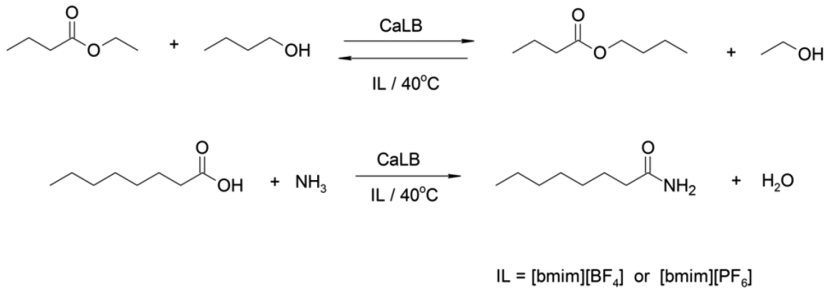

Figure 3. CaLB-catalyzed transesterification and amidation in anhydrous ILs.

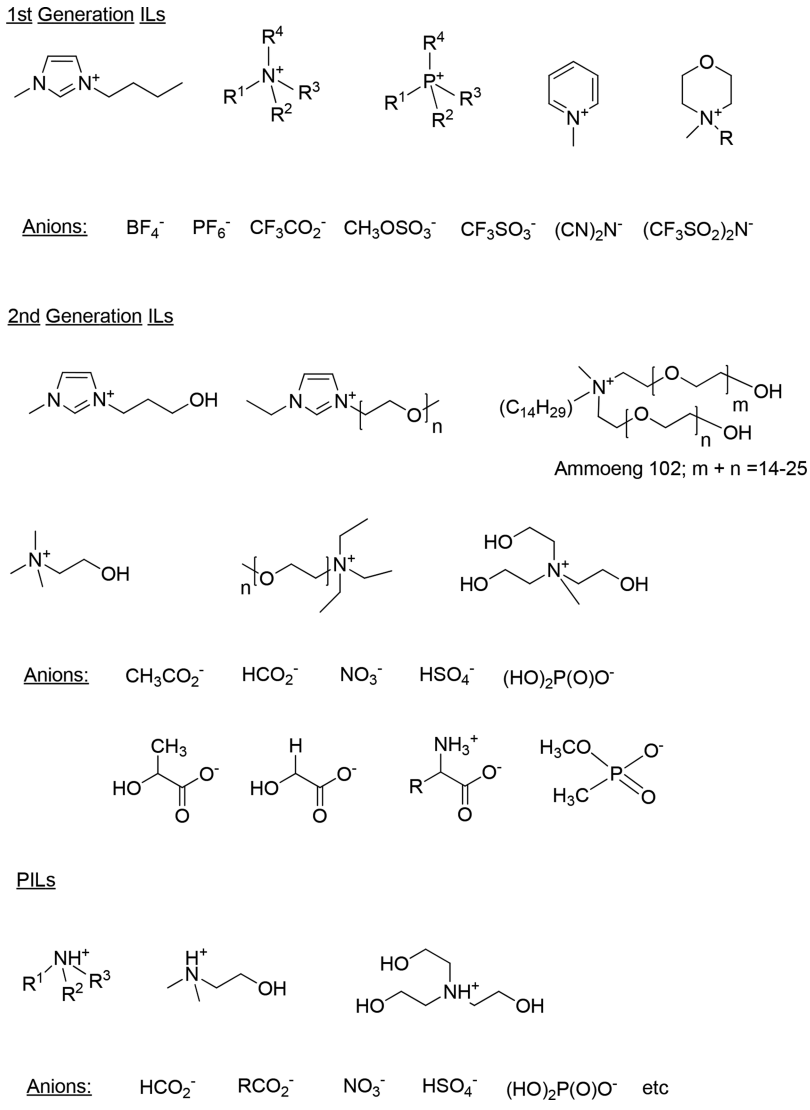

Figure 4. Structures of first- and second-generation ILs and PILs.

and amidations (Figure 3). Both the IL and the enzyme were dried over phosphorus pentoxide beforehand to ensure that they were anhydrous. Although the results were very exciting, the rates were only slightly higher than those observed in the best organic solvents (e.g., *tert*-butanol and toluene). Subsequently, extensive studies over the past two decades have shown that ILs are biocompatible with many enzymes and are eminently suitable as alternative reaction media for biotransformations. Numerous reviews have been published on the subject.[108−114]

The expectation that higher activities would be observed, compared to those in organic solvents, was not realized. The rates were the same as or slightly higher than those observed in the best organic solvents. There have been many reports of remarkable increases in enzyme stabilities in ILs containing noncoordinating anions,[115] and enhanced (enantio)selectivities have been observed in some cases.[116] These probably result from conformational changes of enzymes in IL media. Because ILs are able to dissolve large amounts of highly polar substrates, they are also interesting media for biotransformations of carbohydrates[117]

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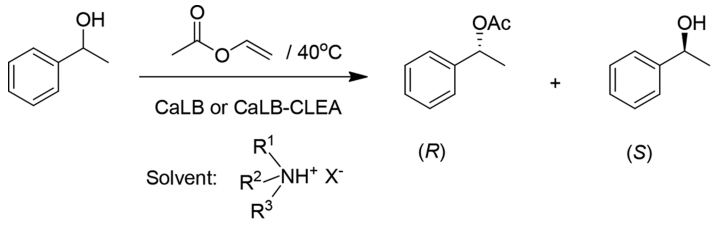

R^1	R^2	R^3	X^-	50% conv. (days)	$ee_{product}$ (%)
t-BuOH				11	>99
CH_3	CH_3	n-C_4H_9	propionate	14	95
CH_3	CH_3	n-C_4H_9	3-hexyldecanoate	2	99
CH_3	CH_3	n-$C_{12}H_{25}$	octanoate	1	99
i-C_4H_9	i-C_4H_9	i-C_4H_9	hexanoate	2	93
n-C_8H_{17}	n-C_8H_{17}	n-C_8H_{17}	acetate	4	>99

Figure 5. CaLB-catalyzed transesterification in PILs.

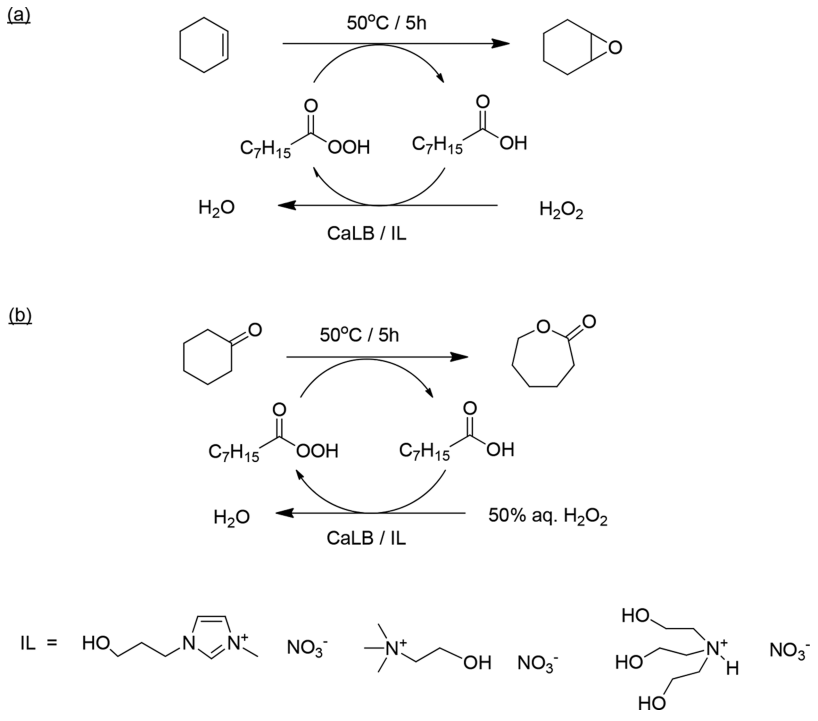

Figure 6. Chemoenzymatic epoxidation and Baeyer−Villiger oxidations in ILs.

and nucleosides,[118] which have low solubilities in most organic solvents.

An important motivation for conducting biocatalysis in ILs was the possibility of replacing environmentally undesirable volatile organic solvents with nonvolatile ILs, thereby reducing the risk of air pollution. However, most tetraalkylammonium and dialkylimidazolium ILs have significant solubilities in water, are poorly biodegradable,[119] and exhibit aquatic ecotoxicity,[120−122] and the anions BF_4 and PF_6 are susceptible to hydrolysis, forming HF. Furthermore, the synthesis of quaternary ILs involves circuitous, High-*E* factor processes, resulting in relatively high costs.[123]

Because of the problems associated with these first-generation ILs, more biocompatible second-generation ILs, derived from relatively inexpensive, ecofriendly natural products,[124] such as carbohydrates[125] and amino acids,[126] were developed (Figure 4). For example, cholinium carboxylates, derived from inexpensive choline hydroxide and a range of carboxylic acids[127−129] or amino acids,[130] exhibit low toxicity and good biodegradability.

Currently, there is a distinct trend toward the rational design of task-specific ILs, with small environmental footprints, that can be

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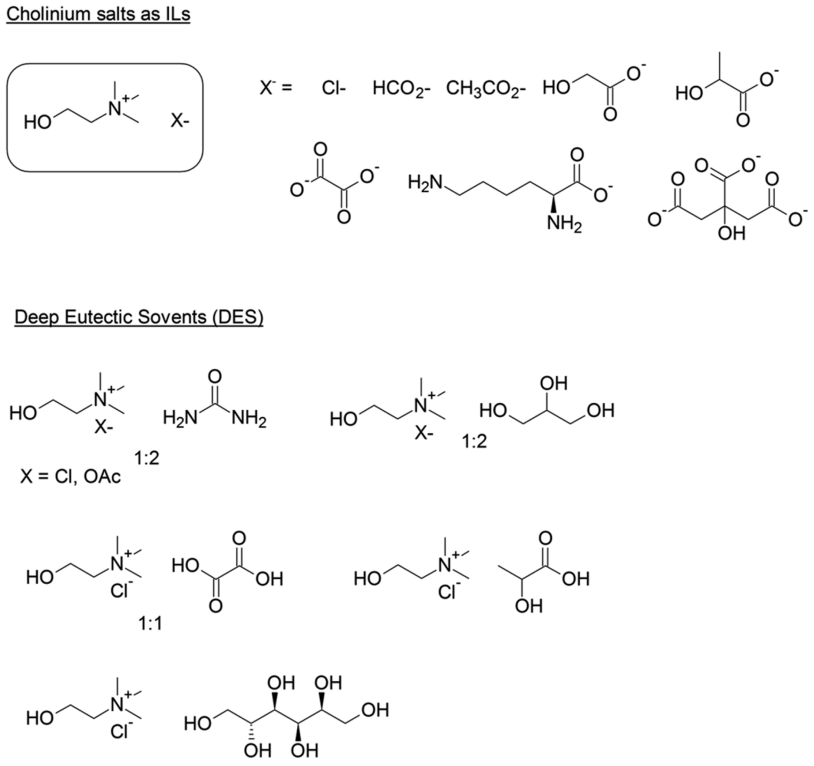

Figure 7. Structures of deep eutectic solvents (DESs).

used for biocatalytic reactions. For example, Zhao et al.[131] designed ether-functionalized ILs by building oligoethylene (or oligopropylene) glycol side chains into the cation (see Figure 4 for structures). The length and steric bulk of the glycol side chains could be designed to dissolve substantial amounts of carbohydrates (including polysaccharides), triglycerides, and amino acids and, at the same time, stabilize CaLB immobilized as Novozym 435. Soluble enzymes, such as nonimmobilized CaLB, dissolve in such designer ILs with retention of activity, thus providing the possibility of conducting homogeneous enzymatic reactions such as the acylation of glucose, sucrose, and cellulose.[132] Similarly, Ammoeng 110, a commercially available quaternary ammonium IL containing oligopropylene glycol side chains, dissolves enzymes with high retention of activity.[121] Such designer ILs, that are able to dissolve substantial amounts of polysaccharides, including lignocellulose, have recently attracted much attention as possible solvents for the pretreatment and further enzymatic hydrolysis (saccharification) of renewable biomass for second-generation fuels and chemicals (see section 7.3).[133−135]

Protic ionic liquids (PILs) are a subclass of ILs with particularly attractive properties. They are exquisitely simple and inexpensive to produce, by merely mixing an amine with an acid, and exhibit reduced toxicity and better biodegradability compared to quaternary ILs.[136] Moreover, their H-bond-donating properties enable interaction with and stabilization of enzymes, and if they contain carboxylate anions, they are self-buffering. 2-Hydroxyethylammonium lactates are examples of nontoxic, readily biodegradable PILs with cations closely resembling choline. They are easily prepared from commodity mono-, di-, and triethanolamines and renewable lactic acid.[137] PILs derived from tertiary amines and carboxylic acids were found to be suitable solvents for the resolution of 1-phenyl-ethanol by CaLB-catalyzed transesterification (Figure 5).[138] The fact that very high enantioselectivities [>99% enantiomeric excess (ee) at 50% conversion] were observed strongly suggests that there is essentially no background nonenzymatic reaction taking place.

The anion of the IL also has a significant influence on the properties of enzymes in these media. Enzymes dissolve well in ILs consisting of coordinating anions but are generally insoluble in ILs consisting of noncoordinating anions such as the originally used BF_4 and PF_6 anions. In those cases where the enzyme dissolves, this often leads to their denaturation. However, when the IL contains a hydrogen-bond-donating (HBD) cation, such as the 1-(3-hydroxypropyl)-3-methylimidazolium cation, the enzyme dissolves while still retaining its activity.[139] In the original study[107] of CaLB catalysis in [bmim][BF_4] and [bmim][PF_6], a suspension of CaLB was shown to catalyze the reaction of hydrogen peroxide with carboxylic acids (perhydrolysis) to give the corresponding percarboxylic acid. In the presence of an olefin substrate, this forms the basis for a chemoenzymatic epoxidation (Figure 6a). More recently, it was shown[140] that better results could be obtained using CaLB dissolved in ILs consisting of HBD cations in combination with nitrate as the coordinating anion. Nitrate is an attractive anion: It is stable under oxidative conditions, nontoxic, biocompatible, and inexpensive. Thus, CaLB was shown to catalyze chemoenzymatic epoxidation (Figure 6a) and Baeyer−Villiger oxidation of ketones (Figure 6b) in a selection of ILs and PILs containing HBD cations and nitrate anions.

An important issue, particularly from a sustainability viewpoint, regarding reactions in ILs is recovery of the product from the IL. It could be extracted with an organic solvent, but that is what one is trying to avoid in the first place. However, replacing an environmentally undesirable reaction medium by an IL and extraction with an environmentally more attractive solvent could result in an improvement overall. Alternatively, $scCO_2$ can be

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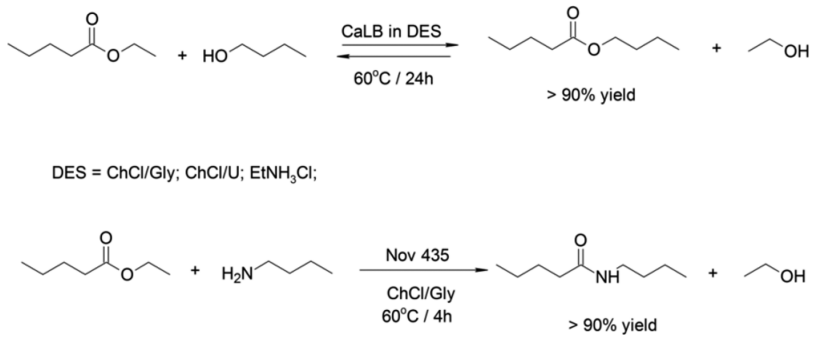

Figure 8. CaLB-catalyzed transesterification and amidation in DESs.

used to extract the product. ILs are generally insoluble in $scCO_2$. In contrast, $scCO_2$ is highly soluble in the IL phase and can, therefore, be used to extract hydrophobic molecules. This provides the possibility for conducting biphasic biocatalysis in which $scCO_2$ is used to continuously extract the products from the IL phase.[141,142] The product is recovered by decompression of the $scCO_2$, and the latter is recycled. For example, this concept was used in the CaLB-catalyzed kinetic resolution of 1-phenylethanol, affording high enantioselectivities (ee > 99.9%) coupled with good operational stability.

Actually, IL/$scCO_2$ mixtures can be mono- or biphasic depending on the pressure. Hence, a miscibility switch can be designed, whereby the reaction is conducted in a single homogeneous phase and subsequent reduction of the pressure affords two phases.[143] The product is separated in the $scCO_2$ phase, and the IL phase is recycled to the reactor. In a further variation on this theme, the free enzyme is absorbed in a supported IL phase (SILP), prepared by covalent attachment of an IL to a polymeric support, such as cross-linked polystyrene, and used in $scCO_2$ as the mobile phase.[144]

Finally, we note that ILs are like any other (organic) solvents. They can be very cheap, and they can be very expensive. In both cases, the price is dependent on the price of the raw materials and the cost of converting them. ILs can be produced from very cheap amines as a source of cations and very cheap anions. Standard ILs have the disadvantage that reaction of the amine with an alkylating agent is needed. In contrast, protic ILs (PILs) are formed by simply mixing an amine with an acid. For example, mixing triethylamine with sulfuric acid affords a PIL with a price of ca. 1 $/kg or less.[145] Similarly, mixing triethanolamine with acetic acid affords a PIL for not much more than 1 $/kg. These are prices that compare favorably with almost any organic solvent.

3.5. Deep Eutectic Solvents (DESs)

Another class of interesting solvents for performing biotransformations comprises the so-called deep eutectic solvents (DESs).[146−148] These are produced by mixing ammonium or phosphonium salts with a hydrogen-bond donor (HBD), XH, such as an alcohol, polyol, carboxylic acid, or amide, and heating gently (Figure 7). The HBD interacts with the anion of the salt, thereby increasing its effective size and causing a decrease in its melting point. For example, mixing choline chloride (mp 302 °C) with urea (mp 132 °C) in a 1:2 molar ratio affords a DES with a melting point of 12 °C. DESs are, strictly speaking, not ILs because they contain uncharged moieties, but they exhibit similar properties, such as low volatility and high thermal stability. More importantly, they are easy to synthesize by simply mixing the two components. Moreover, they are often made from naturally occurring, biocompatible substrates and, hence, are generally nontoxic and biodegradable.

Choline chloride (ChCl), for example, is a readily available, inexpensive feed additive produced in bulk quantities; urea is a common fertilizer; and glycerol is a byproduct of biodiesel manufacture. Hence, mixtures of ChCl with urea (U)[149] and glycerol (Gly),[150] in molar ratios of 1:2, are readily available, inexpensive, biocompatible, and biodegradable.

DESs have also been prepared by mixing ChCl with carbohydrates.[151] So-called natural deep eutectic solvents (NADESs)[152] derived from metabolites, such as sugars, amino acids, choline, and natural organic acids, can actually function as reaction media for the in vivo synthesis of sparingly water-soluble compounds such as flavonoids and steroids in living cells. Indeed, NADESs have been referred to as "solvents for the 21st century".[153]

The first example of biocatalysis in DESs was reported by Kazlauskas and co-workers.[154] Immobilized CaLB (Novozym 435) was shown to catalyze the transesterification and amidation of ethyl valerate with 1-butanol and 1-butylamine, respectively (Figure 8) in ChCl/U (1:2) and ChCl/Gly (1:2), exhibiting conversions comparable to those observed in toluene.

The explanation for this surprising result (urea is a known potent protein denaturant) is that, in the absence of ChCl, the urea molecules diffuse readily into the protein core and denature the enzyme by disrupting intramolecular hydrogen bonds. In contrast, in ChCl/U, the formation of hydrogen bonds between urea and choline and chloride ions prevents its diffusion into the protein core.[155] Moreover, the formation of hydrogen bonds between urea, choline and chloride ions, and surface amino acid residues actually has a stabilizing effect on the enzyme. Interestingly, transesterifications in ChCl/Gly (8 M glycerol) gave <0.5% glyceryl ester formation at more than 90% conversion of the alcohol substrate. This can similarly be explained by assuming that complexation of the glycerol with ChCl prevents it from entering the active site of the enzyme.

Choline-based DESs are excellent reaction media, alone or acting as cosolvents with water, for biocatalytic reactions catalyzed by a variety of enzymes,[156] including lipases,[157−164] proteases,[165,166] epoxide hydrolases,[167] glycosidases,[168] peroxidases,[169,170] and benzaldehyde lyase.[171] For example, ChCl-based DESs containing 10% water proved to be excellent solvents for chymotrypsin-catalyzed peptide synthesis at high substrate loadings.[166] Productivities up to 20 $g \cdot L^{-1} \cdot h^{-1}$ were obtained with reduced competing hydrolysis, and the enzyme was recycled several times. The authors further noted that recycling could probably be significantly improved by immobilizing the enzyme.

A variety of DESs were recently shown to be attractive solvents for the CaLB-catalyzed chemoenzymatic epoxidation of olefins,

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Chem. Rev. XXXX, XXX, XXX−XXX

with the best results being obtained in ChCl mixtures with polyols, in particular, ChCl/sorbitol (1:1).[172] The *E* factor of the epoxidation of styrene in ChCl/sorbitol was calculated to be 7.7 compared with 16.7 for the same olefin in [bmim][BF_4] (as discussed in the preceding section).[140] We note, however, that comparison with one of the nitrate ILs would probably have been more meaningful.

4. BIOCATALYSIS ENGINEERING

In the preceding section, we discussed what can be achieved in biocatalytic reactions by engineering the reaction medium. In this section, we discuss what can be achieved by engineering the other variables in biocatalysis: the substrate, the protein, the biocatalyst once formed, and the reactor configuration and downstream processing. The whole is referred to as biocatalysis engineering.[173] Although, historically, this was the order in which these fields were developed, meaning, for example, that many examples of substrate engineering were at the time restricted by a lack of established methods for protein engineering, in recent years, many of the examples have used engineered proteins. For this reason, we have placed developments in context by starting the discussion about protein engineering, followed by substrate engineering.

4.1. Protein Engineering: Evolution in the Fast Lane

4.1.1. Historical Development. Wild-type enzymes evolved over millions of years to be active and specific with their natural substrates, under ambient conditions in water. In contrast, industrial processes, such as the synthesis of pharmaceutical intermediates, generally involve transformations of non-natural substrates under harsh conditions, namely, elevated temperatures and high substrate concentrations in the presence of organic solvents. Wild-type enzymes are often not effective under such conditions, which results in low selectivities, low activities, and low space-time yields. To exhibit industrially viable space-time yields and high (enantio)selectivities at practical substrate concentrations and low enzyme loadings, the enzymes need to be re-evolved. Moreover, this evolution process should take weeks rather than millions of years. This can be achieved using so-called directed (in vitro) evolution to generate libraries of mutant enzymes that are subsequently screened for improved properties.[174]

Smith and co-workers[175] introduced the genetic engineering tool known as rational design by *site-directed mutagenesis* (SDM) in the late 1970s. With SDM, so-called point mutations are created in a particular protein by replacing an amino acid at a predetermined site in the protein by one of the other 19 canonical amino acids. However, to perform SDM, detailed information regarding the three-dimensional structure and mechanism of the enzyme must be available, which is not always the case. *Random mutagenesis*, in contrast, does not require any structural information. In the early 1990s, random mutagenesis through the error-prone polymerase chain reaction, epPCR, was used to generate libraries of mutant enzymes. A seminal article, published by Chen and Arnold[176] in 1993, reported the use of sequential cycles of epPCR and screening of mutant libraries to evolve the protease, subtilisin E, to be hundreds of times more active than wild-type subtilisin in 60% aqueous dimethylformamide (DMF). Subtilisin E was an industrially important enzyme used, inter alia, in laundry detergents. Similarly, the same group[177] used sequential random mutagenesis and screening to improve the activity of an esterase in the hydrolysis of a *p*-nitrobenzyl ester of a cephalosporin antibiotic intermediate in 30% aqueous DMF.

Although epPCR is an effective means for introducing beneficial mutations into a protein, in natural, Darwinian evolution, such changes are amplified by an additional mechanism: recombination. Hence, the next landmark in the development of directed evolution by random mutagenesis was the invention of DNA shuffling by Stemmer in 1994.[178] DNA shuffling is a method for in vitro homologous recombination of pools of selected mutant genes by random fragmentation and PCR reassembly, which was used to enhance the activity of a β-lactamase. This sequence of random mutagenesis, recombination, and screening can be repeated until mutants with the targeted characteritics are generated in a Darwinian-type evolution in vitro. These powerful methodologies have allowed researchers to alter protein properties such that enzymes can operate effectively (with high activity and stability) with non-natural substrates under harsh reaction conditions.

An important challenge was to develop advanced mutagenesis methodologies that enable the generation of small, high-quality mutant libraries that can be rapidly assessed using automated gas chromatography (GC) or high-performance liquid chromatography (HPLC). For example, semirational approaches to directed evolution involve *site-saturation mutagenesis* (SSM), in which all natural amino acids are tested at residues in or near the active site.[179] This "targeted randomization" can be simultaneously combined with random mutagenesis. Thus, Reetz and co-workers[180] developed a novel strategy in which relatively small focused libraries of mutants are produced by randomization at several sets of two spatially close amino acid positions around the active site. Complete randomization is then performed at each pair in a process termed combinatorial active-site saturation test (CAST), which constitutes a practically useful compromise between conventional site-saturation mutagenesis and simultaneous random mutagenesis at multiple sites. If the degree of improvement is insufficient, further rounds of iterative saturation mutagenesis (ISM) can be used. The CAST methodology has proven highly successful, especially with respect to improvement of asymmetric catalysis by enzymes.[181]

4.1.2. Protein Engineering and Organic Synthesis. The attention of synthetic organic chemists was attracted by the appearance of articles describing the use of directed evolution for the improvement of another important property of enzymes, namely, stereoselectivity and, in particular, enantioselectivity. One of the most important properties of enzymes from a synthetic perspective is their ability to catalyze highly stereospecific reactions, although, in many cases, this applies only to their natural substrates, which clearly limits their scope in organic synthesis. In 1996, Matcham and Bowen[182] reported the use of sequential random mutagenesis and activity screening of a library of 10000 mutants to improve the enantioselectivity of an (*S*)-selective transaminase, in the conversion of a β-tetralone to the corresponding amine, from 65% ee to 94% ee, while maintaining the activity of the wild-type enzyme. The best results were obtained with mutants containing double synergistic substitutions. Subsequently, Reetz and co-workers[183] reported the optimization of a lipase-catalyzed enantioselective hydrolysis of a chiral ester in 1997. This work is of particular importance because it struck a chord with synthetic organic chemists. Using sequential epPCR and rapid colorimetric screening of an initial library of 1000 mutants, followed by further mutagenesis of the most enantioselective clone, they were able to increase the enantioselectivity from 2% to 81% in four generations.

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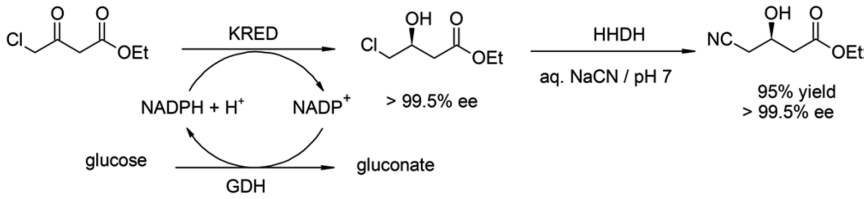

Figure 9. Three-enzyme process for the atorvastatin intermediate.

In the past two decades, DNA shuffling and other directed evolution strategies, often in combination, have been widely applied to improve existing activities[184] and evolve new activities of enzymes.[185] Additionally many other properties such as stability under non-natural conditions can also be improved. This has made it possible to predefine the minimum parameters necessary for a process to be commercially viable and then use directed evolution to modify the biocatalyst to achieve these parameters.[186] This is in complete contrast to the traditional approach of modifying a process to accommodate a commercially available catalyst. It has no real equivalent in chemocatalysis.

An excellent review by Hauer and co-workers[187] outlines the many new possibilities in organic synthesis that have resulted from developments in protein engineering, including selective hydrogenation of C═C bonds, preparation of chiral amines and amino acids, and use of enzymes in alternative syntheses of enantiopure chiral epoxides and the corresponding ring-opened products.

4.1.3. Directed Evolution and the Synthesis of APIs. Combining advances in DNA sequencing and gene synthesis with directed evolution techniques has enabled the widespread industrial application of biocatalysis, particularly in the synthesis of active pharmaceutical ingredients (APIs). The vast majority of processes for the manufacture of enantiomerically pure APIs involve chiral alcohols or chiral amines as key intermediates. Considerable attention has been devoted, therefore, to developing efficient methodologies for their production. The asymmetric reduction of ketones to chiral alcohols using engineered enzymes has been particularly successful in this respect.[188,189] Indeed, the catalytic efficiencies, volumetric productivities, and environmental benefits of these biocatalytic routes to enantiomerically pure chiral alcohols are such that they appear to have totally eclipsed more traditional technologies such as stoichiometric reduction with chiral boranes and noble metal catalyzed asymmetric hydrogenation.[190−192] This success story has been enabled by the development of effective cofactor regeneration systems combined with the use of directed evolution to improve their catalytic efficiency and, hence, commercial viability and sustainability.

The flagship example is definitely the Codexis process for the synthesis of a chiral intermediate for atorvastatin, the active ingredient of Lipitor. As shown in Figure 9, it is a two-step, three-enzyme process involving a ketoreductase- (KRED-) catalyzed asymmetric reduction of an α-chloroketone to the corresponding chlorohydrin accompanied by cofactor regeneration with glucose catalyzed by glucose dehydrogenase (GDH) and followed by a halohydrin dehalogenase- (HHDH-) catalyzed conversion to a cyanohydrin (Figure 9). Proof-of-concept was obtained on a laboratory scale using wild-type KRED, GDH, and HHDH. The chlorohydrin was obtained in the first step in 85% yield and >99.5% ee and was subsequently converted to the cyanohydrin in an overall yield of 95% and an ee of >99.5%.

However, the activities of all three wild-type enzymes were too low for a commercially viable process, and the use of high concentrations of the enzymes led to the formation of emulsions, resulting in problematic product recovery. Consequently, DNA shuffling was used[193] to improve the activity and stability of the KRED/GDH combination while maintaining the near-perfect enantioselectivity observed with the wild-type KRED. Table 3 compares the parameters of the wild-type KRED/GDH combination with those of the best variants, obtained following several rounds of DNA shuffling. No emulsion problems were encountered, because of the much lower enzyme concentrations, and phase separation was achieved in less than 1 min.

Table 3. Evolution of a KRED/GDH Biocatalyst[a]

parameter[b]	wild-type	best variant
TTN catalyst	3000	>100000
TTN NADP	4000	>20000
STY ($g \cdot L^{-1} \cdot h^{-1}$)	3.3	25
yield (%)	80	>95
ee (%)	99.8	>99.9
[enzyme] ($g \cdot L^{-1}$)	100	<1
[substrate] ($g \cdot L^{-1}$)	80	200
reaction time (h)	24	10
phase separation	>1 h	ca. 1 min
workup	complex	very simple

[a]Adapted from Table 1 of ref 173 with permission from the Royal Society of Chemistry. [b]TTN, total turnover number; STY, space-time yield.

Similarly, the activity of the wild-type HHDH in the non-natural hydrocyanation step was extremely low, and the enzyme showed strong product inhibition and poor stability in the presence of both substrate and product. Multiple rounds of DNA shuffling and screening, in the presence of increasing concentrations of product, resulted in virtually complete suppression of product inhibition and afforded a mutant HHDH with a >2500-fold increase in activity. Table 4 compares the characteristics of the wild-type HHDH with those of the best variant. The substrate loading was increased from 20 to 140 $g \cdot L^{-1}$, the enzyme loading was reduced from 30 to 1.2 $g \cdot L^{-1}$, and the reaction time for completion was reduced from 72 to 5 h, giving a space-time yield of 28 $g \cdot L^{-1} \cdot h^{-1}$.

Assessment of this process according to the metrics and 12 principles of green chemistry clearly showed that it is substantially greener than previous processes.[194] The *E* factor (kg of waste per kg of product) for the overall process is 5.8 kg/kg if water is excluded and 18 kg/kg if it is included. The major contributors to the *E* factor are losses of solvent (ethyl and butyl acetates, 51%) and formation of sodium gluconate (25%) and salts (NaCl and Na_2SO_4, combined 22%). The three enzymes and the NADP cofactor accounted for <1% of the waste. The dramatic improvement in the key properties of the three enzymes constitutes a perfect example of the power of directed evolution

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Chem. Rev. XXXX, XXX, XXX−XXX

Table 4. Evolution of an HHDH Biocatalyst by DNA Shuffling[a]

parameter	process design	wild-type	best variant
[substrate] ($g \cdot L^{-1}$)	120	20	140
[enzyme] ($g \cdot L^{-1}$	1.5	30	1.2
catalyst productivity (g/g)	80	0.7	117
STY ($g \cdot L^{-1} \cdot h^{-1}$)	>15	0.3	28
isolated yield (%)	>90	67	92
chemical purity (%)	>98	>98	>98
ee (%)	>99.5	>99.5	>99.5
reaction time (h)	8	72	5
phase separation (min)	<10	>60	<1

[a]Adapted from Table 2 of ref 173 with permission from the Royal Society of Chemistry.

in enabling green-by-design, economically viable biocatalytic processes that would otherwise not have progressed beyond the laboratory stage.

The Codexis process was commercialized in about 2006, and the status of this "Third Wave of Biocatalysis" was reviewed in 2012.[195] In the past 5 years, developments in enzyme engineering have been accelerated through the further integration of directed evolution with newly developed technologies for rational design. The creation of new enzymes with novel catalytic functions has become the norm rather than the exception. Because of the dramatic improvements in stereoselectivity, catalytic efficiency, and stability that can be achieved using protein engineering, the application of wild-type enzymes in processes for API synthesis is more the exception than the rule.[190] The exponential growth of available genomic information provides starting points for enzyme engineering and functional diversity from closely related homologues for recombination. This is supplemented by advanced in silico techniques that further enable the design of libraries of variants.

Another striking example of the use of an evolved KRED is the synthesis of the key chiral alcohol intermediate in the manufacture of montelukast, the active ingredient of the antiasthmatic drug Singulair (Figure 15a).[196] A complicating practical factor was the very low solubility, even in water/organic solvent mixtures, of the ketone substrate. Nonetheless, an initial screening, using isopropanol for cofactor regeneration, afforded a few KREDs exhibiting >99.9% (*S*)-selectivity. However, their activities were 3 orders of magnitude too low for a commercially viable process. Moreover, they were unstable in the presence of the high levels of organic solvents required and were inhibited by the acetone coproduct. DNA shuffling was used to improve both the activity and stability under the reaction conditions. After three rounds of evolution, a 400-fold improvement in product/enzyme mass ratio, from 1:50 to 8:1, was observed. Optimization of the reaction medium to a toluene/water mixture and an increase of the reaction temperature to 40 °C afforded further improvements. The final variant exhibited a 3000-fold improvement under these conditions. The process is performed in a slurry-to-slurry mode at 45 °C using 100 $g \cdot L^{-1}$ substrate and 3 $g \cdot L^{-1}$ enzyme concentrations and a reaction time of 24 h. The (*S*)-hydroxy ester is obtained in >95% yield and >99.9% ee. Another illustrative example is the development of a highly evolved KRED for the challenging asymmetric reduction (Figure 10b)[197] of a near-symmetrical ketone to give an intermediate for the antibiotic Sulopenem.

Yet another example of the power of directed evolution is provided by the biocatalytic process for the manufacture of the key intermediate for sitagliptin, the active ingredient of the antidiabetic agent Januvia. The target molecule was a chiral amine, and Codexis and Merck scientists used a combination of protein engineering techniques to develop a transaminase for the conversion of the ketone precursor, prositagliptin (Figure 11).[198] Their starting point was to identify an (*R*)-selective transaminase (TA) that contained the necessary machinery to catalyze the desired reaction but completely lacked activity toward prositagliptin. They subsequently used a combination of computer-aided catalyst design of the active site and site-saturation mutagenesis to generate a variant that gave 0.7% conversion in 24 h using an enzyme loading of 10 $g \cdot L^{-1}$ and a substrate loading of 2 $g \cdot L^{-1}$. As with the above-discussed example of the montelukast intermediate, prositagliptin is sparingly soluble (<1 $g \cdot L^{-1}$) in water, thus requiring the addition of large amounts of dimethyl sulfoxide (DMSO) as a cosolvent.

To be commercially viable, the enzyme had to be evolved to withstand the harsh reaction conditions of 100 $g \cdot L^{-1}$ substrate, 1 M isopropylamine, >25% DMSO, and a temperature of >40 °C for 24 h and give a product with >99.9% ee. Multiple rounds of directed evolution using, among other protein engineering techniques, DNA shuffling afforded a variant containing 27 mutations. Of the 17 noncatalytically essential amino acids predicted to interact with the substrate, 10 were mutated. Using a 6 $g \cdot L^{-1}$ concentration of this variant in 50% DMSO at 45 °C, 100 $g \cdot L^{-1}$ substrate was converted to sitagliptin at >99.95% ee in 92% yield. Compared with the rhodium-catalyzed asymmetric hydrogenation of an enamine that it replaced, the biocatalytic process provided a 13% increase in overall yield, a 53% increase

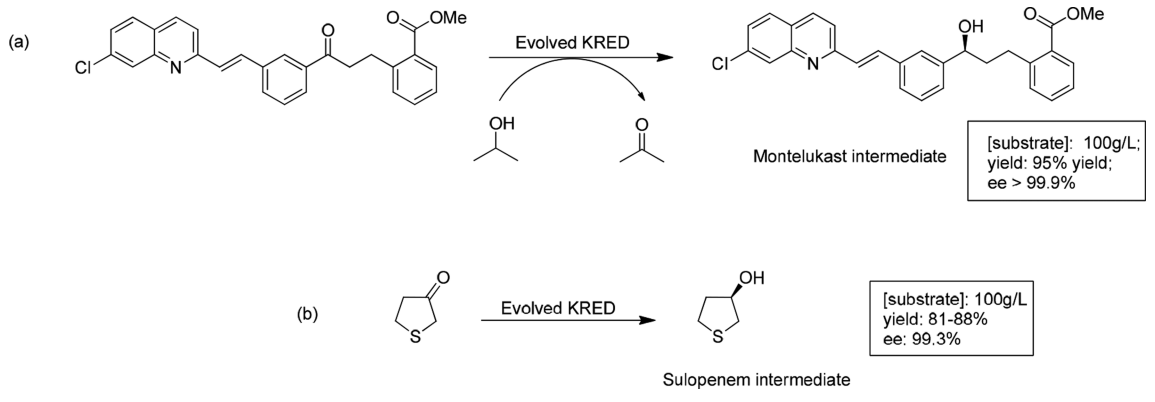

Figure 10. Asymmetric ketone reductions with KREDs for API syntheses.

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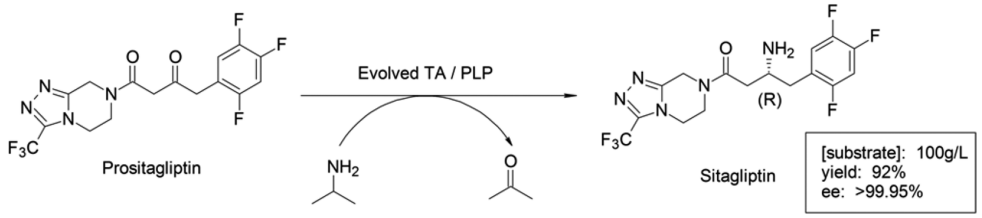

Figure 11. Synthesis of sitagliptin by biocatalytic transamination.

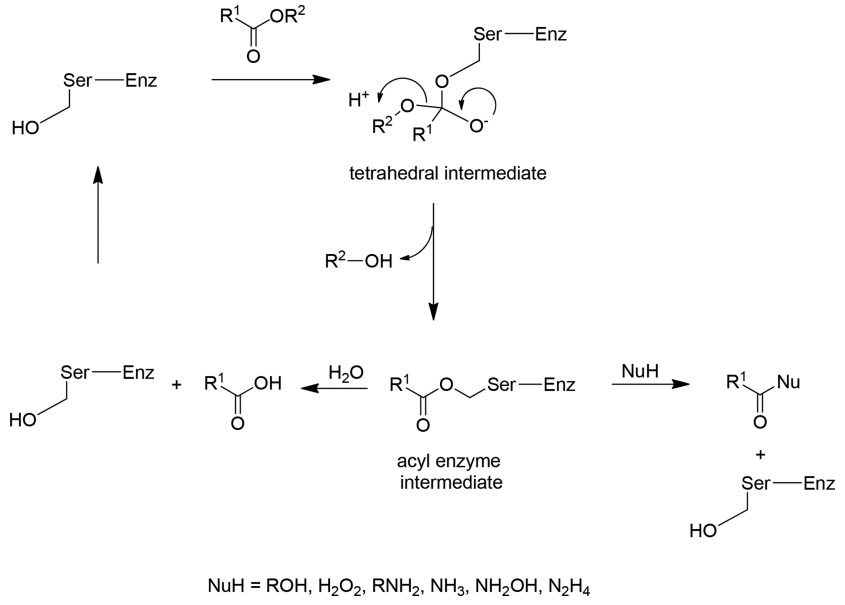

Figure 12. Serine protease mechanism.

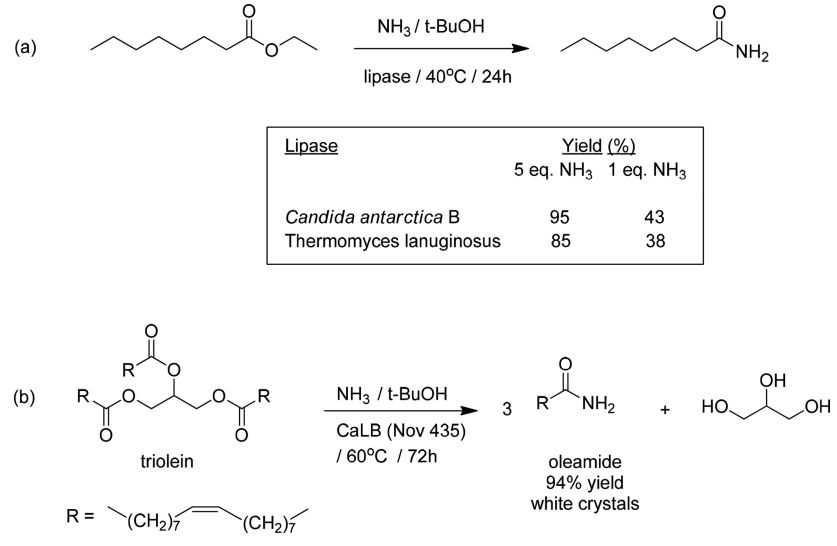

Figure 13. Enzymatic ester ammoniolysis.

in space-time yield ($g \cdot L^{-1} \cdot h^{-1}$), and a 19% reduction in *E* factor. The biocatalytic process is also more cost-effective and eliminated the use of noble metals. Moreover, the process is conducted in standard multipurpose reactors, thus avoiding the need for high-pressure hydrogenation equipment. In addition, the transaminases developed for the sitagliptin synthesis were found to have broad scope in the synthesis of chiral amines, which are of interest as chiral intermediates in the pharmaceutical industry. Similarly, Bornscheuer and co-workers[199] developed a set of highly active (*S*)-selective TAs using extensive protein engineering followed by optimization of the identified motif. The resulting enzymes had an 8900-fold improvement in activity over the starting scaffold and were highly enantioselective (up to >99.9% ee) in the asymmetric synthesis of a set of bulky chiral amines.

4.2. Substrate Engineering and Enzyme Promiscuity

4.2.1. Substrate Engineering. Substrate engineering can lead to both the optimization of existing biocatalytic reactions

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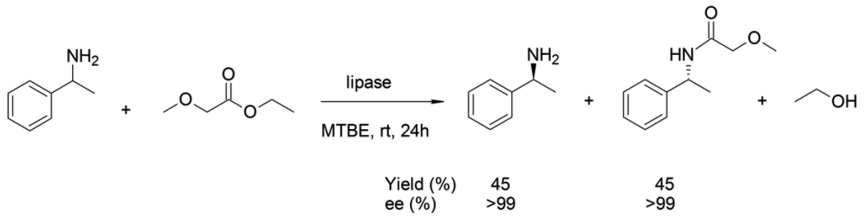

Figure 14. Lipase-catalyzed resolution of chiral amines.

and the invention of completely new transformations, which gave rise to the term *enzyme promiscuity*.[200−203] The simplest examples of substrate engineering are well-known and involve the use of an enzyme in its natural reaction but with a non-natural substrate. For example, lipases catalyze the hydrolysis of triglycerides in vivo, but they can also catalyze the hydrolysis of a wide variety of esters (and the reverse reaction). These constitute examples of substrate engineering. However, in this part of the review, we are mainly concerned with the invention of new reactions through substrate engineering. To design a substrate engineering strategy, it is virtually essential to have knowledge of the reaction mechanism. For example, in vivo lipases catalyze the hydrolysis of triglycerides to a mixture of glycerol and fatty acids by the serine protease mechanism. This mechanism involves the formation of an acyl−enzyme intermediate by reaction of the ester substrate with the OH group of a serine residue in the active site. Subsequent reaction with water leads to the formation of the free fatty acid and regenerates the serine residue in the active site (Figure 12). Based on this mechanism, it is not unreasonable to assume that the water could be replaced by "non-natural nucleophiles", leading to the formation of alternative products.

In the early 1990s, it was known that amines could function as the nucleophile to afford the corresponding amides from esters.[204] However, the use of the simplest nitrogen nucleophile, ammonia, was not known. Hence, when it was shown[205] that lipases catalyze the reaction of ethyl octanoate with a saturated solution of ammonia in *tert*-butanol to give the corresponding amide (Figure 13a), this signaled the invention of a new enzymatic reaction: ester ammoniolysis. *Candida antarctica* lipase B (CaLB) and *Thermomyces lanuginosus* lipase (lipolase) were found to be the most active, but most lipases showed some activity, depending on the ammonia concentration.

The method was used,[206] for example, to convert oleic acid to the corresponding amide, in 94% yield, by the reaction of olive oil (triolein) with ammonia in *tert*-butanol at 60 °C (Figure 13b). Amides were also prepared from the corresponding acids in a cascade of two reactions catalyzed by CaLB: esterification followed by ammoniolysis of the resulting ester.

Enzymatic ammoniolysis can also be used for the kinetic resolution of chiral carboxylic acid esters and amino acid esters through amide formation catalyzed by both lipases and serine proteases.[207,208]

Similarly, enzymatic reactions of esters with amines can also be used to resolve chiral amines. Although the reactions are generally too slow for industrial viability, BASF workers successfully developed, by substrate engineering of the acyl donor, a much faster reaction. Thus, in the BASF Chipros process (Figure 14), an ester of 2-methoxyacetic acid is used as an acyl donor, and the presence of the oxygen atom in the chain results in a dramatic rate increase, affording a highly effective resolution process that is performed at a multithousand-ton-per-year scale.[209,210] The reaction can be conducted solvent-free over a lipase-containing column. Subsequent hydrolysis of the amide was carried out with 50% aqueous sodium hydroxide at elevated temperatures. Such harsh conditions are not compatible with sensitive functional groups and give rise to undesirable salt waste streams. Hence, a fully enzymatic resolution of chiral amines was designed,[211] whereby CaLB was used in the acylation step and, after separation of the unreacted amine enantiomer, in the hydrolysis of the amide. The amidase activity of the lipase, albeit rather low, also constitutes a good example of enzyme promiscuity. Alternatively, when penicillin amidase was used to catalyze the amide hydrolysis step, a much faster reaction was observed.[212]

More recently, the generality of the lipase-catalyzed aminolysis of carboxylic acid esters was further established with the demonstration that *Pseudomonas stutzeri* lipase catalyzes ester aminolysis with a broad range of esters and amines, including bulky esters and secondary amines.[213]

Another pertinent example of an enzymatic reaction involving non-natural nucleophiles is the ring opening of epoxides catalyzed by halohydrin dehalogenases (HHDHs). In vivo, these enzymes catalyze the ring closure of chlorohydrins to epoxides and the ring opening of epoxides with halide ion (Figure 15). Because the mechanism involves a halide-ion

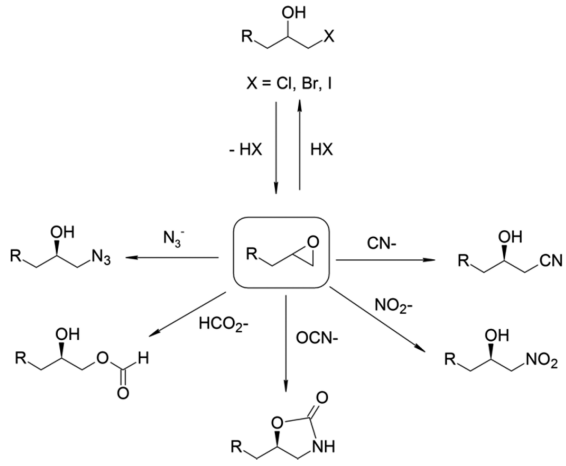

Figure 15. HHDH-catalyzed ring opening of epoxides.

binding site, one might predict that the halide ion could be exchanged with other nucleophiles. Indeed, this is the case. HHDHs are highly promiscuous enzymes that can utilize at least nine different anions as nucleophiles in the enantioselective ring opening of epoxides.[214] This promiscuity provides a method for the synthesis of a broad range of enantiopure β-substituted alcohols. The use of cyanide ion as the nucleophile is particularly interesting because it results in the generation of a new C−C bond. In fact, this reaction forms the basis for the Codexis process for the synthesis of the key atorvastatin intermediate that was discussed in section 4.1.3.

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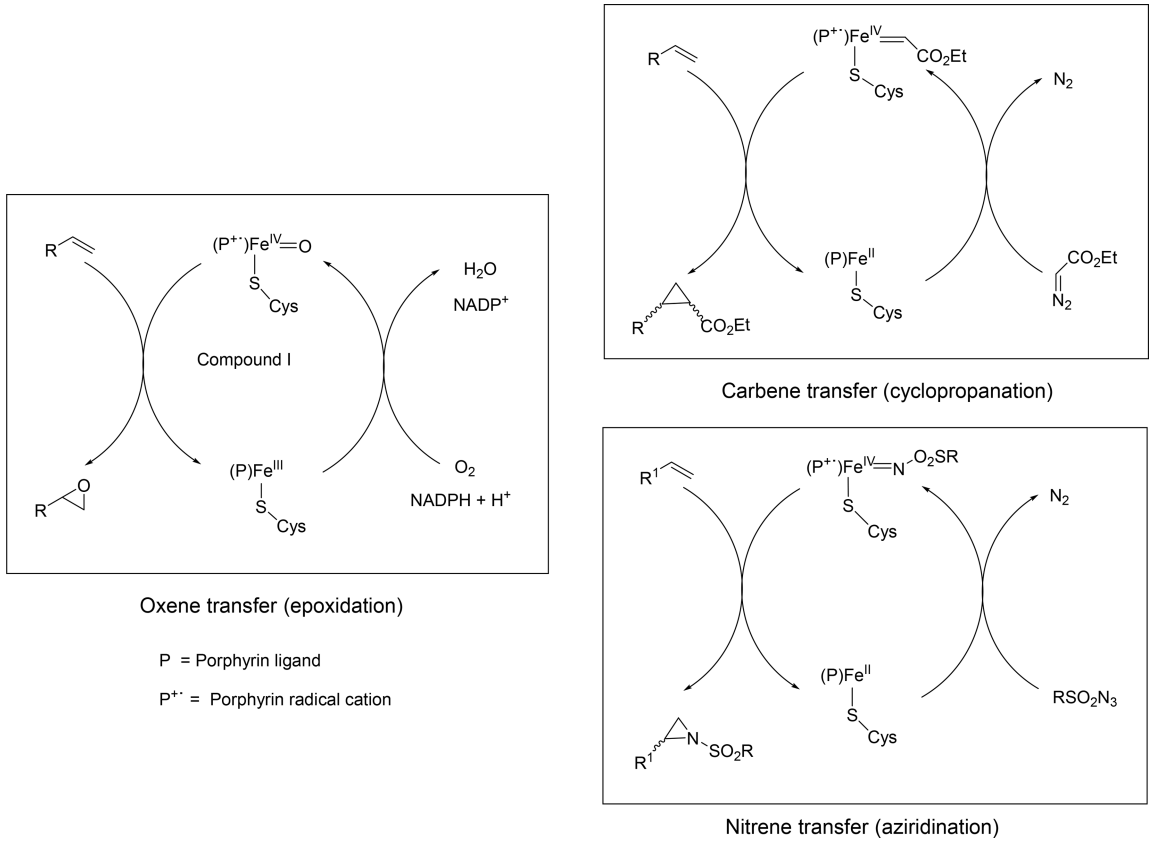

Figure 16. Enzymatic cyclopropanation and aziridination.

4.2.2. Enzyme Promiscuity and Chemomimetic Biocatalysis. Another manifestation of enzyme promiscuity is what was dubbed the chemomimetic biocatalysis approach.[215] Why should Nature have a monopoly on good catalysts? Many of Nature's oxidation catalysts are metalloenzymes based on the abundant elements iron and copper. However, in vitro, we are not limited to this narrow choice, and much effort has been devoted to designing relatively simple metal complexes that are able to emulate the activities of enzymes. Nevertheless, this biomimetic chemocatalysis approach has been singularly unsuccessful in developing industrially viable catalysts from simple metal complexes. Although biomimetic catalysts can be designed, they do not exhibit sufficient specific activity and stability under process conditions for industrially viable use. The three-dimensional structures of proteins are the results of millions of years of evolution and are difficult to emulate with simple metal complexes, which led to the conclusion that a different strategy was needed. Various transition metals are known to catalyze a broad range of oxidations using oxygen or hydrogen peroxide as the primary oxidant. Nesting such a metal in the active site of a readily available hydrolase (analogous to situating a redox element in the framework of a molecular sieve) could afford a semisynthetic metalloenzyme capable of mediating (enantioselective) oxidations.

This chemomimetic biocatalysis strategy, in which a protein scaffold forms the starting point for modification, was successfully used to develop a semisynthetic peroxidase[216] by incorporating vanadate into the active site of the acid phosphatase phytase, an inexpensive, readily available enzyme used in animal feed. In vivo, phytase accommodates a phosphate ester, phytate, in its active site. Because vanadium(V) exhibits coordination geometries similar to those of phosphorus(V), it was reasoned that the addition of vanadate to phytase should afford a robust and inexpensive vanadium peroxidase. This proved to be the case: Vanadate-substituted phytase catalyzed enantioselective sulfoxidations with hydrogen peroxide. Similarly, substitution of the zinc(II) ion in carbonic anhydrase with manganese(II) afforded a semisynthetic enzyme that, in the presence of bicarbonate, acted as a peroxidase.[217] It was further noted[215] that the "combination of the concept of enzyme promiscuity with the technique of directed evolution could provide a powerful tool for the design and optimization of novel reactions with enzymes".

More recently, Arnold and co-workers used heme-dependent enzymes, notably cytochrome P450s, a remarkable class of iron porphyrin-containing enzymes that play key roles in xenobiotic catabolism and natural product biosynthesis, as starting points for chemomimetic biocatalysis.[218−222] In all natural P450s, the iron porphyrin in the active site is ligated by an axial cysteine thiolate residue. The active oxidant in oxidations catalyzed by P450s is an iron(IV) oxo porphyrin radical cation (compound **I**; see Figure 16) formed by the reaction of dioxygen and two hydrogen equivalents from the NAD(P)H cofactor with the iron(II) state of the enzyme. In epoxidations catalyzed by these enzymes, the high-valent iron oxo intermediate transfers an oxygen to the olefinic double bond (oxene transfer). By analogy with metalloporphyrins, which catalyze reactions unknown in vivo such as carbene and nitrene transfer, the authors envisaged that P450s would also catalyze these reactions. This proved to be the case. One of the most widely used P450s, the $P450_{BM3}$ from *Bacillus megaterium*, was shown to catalyze the cyclopropanation and aziridination of olefins by reaction of the iron(II) form with ethyl diazoacetate[219] and tosyl azide,[220] respectively (Figure 16).

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Chem. Rev. XXXX, XXX, XXX−XXX

However, the wild-type $P450_{BM3}$ catalyzed the cyclopropanation of styrene but with very low efficiency (five turnovers) and low levels of diastereo- and enantioselectivity. Protein engineering was used to produce, by mutation of threonine 268 to alanine, a variant with a 60-fold improvement in activity and excellent selectivity to the *trans*-cyclopropane. Other variants were identified that gave the *cis*-cyclopropane in high diastereo- and enantioselectivity, and mutation of the axial cysteine ligand to a serine afforded a variant capable of more than 67000 turnovers that could also be used as a whole-cell biocatalyst. The cyclopropanation methodology has been used in highly diastereo- and enantioselective syntheses of cyclopropane intermediates for levomilnacipran[221] and ticagreclor.[222]

Most recently, the Arnold group showed that an engineered cytochrome *c* from *Rhodothermus marinus* catalyzes carbon−silicon bond formation through carbene insertion into silicon—hydrogen bonds, affording an efficient method for generating optically active organosilicon compounds.[223]

4.3. Biocatalyst Engineering: Enzyme Immobilization

Once an enzyme for the targeted transformation has been identified and subsequently optimized using protein engineering techniques, recombinant DNA techniques are used to express the enzyme in a microbial production host with a GRAS (generally regarded as safe)[224] status. This enables its industrial production at relatively low cost. The following step involves its effective formulation. Enzymes are soluble in water and, hence, difficult and costly to recover from aqueous effluents. Consequently, many enzymes are employed on a single-use, throw-away basis, which is not cost-effective and not in line with current thinking on the circular economy. The enzyme costs per kilogram of product can, therefore, be substantially reduced by immobilization of the enzyme to form a free-flowing powder. Converting a homogeneous catalyst into a heterogeneous catalyst enables catalyst recovery and reuse, resulting in simplification of the process, improvement of the product quality, and reduction of the environmental footprint.

A further benefit of immobilization is generally enhanced stability, toward denaturation by heat or organic solvents or by autolysis, as a result of the decrease in flexibility, which suppresses the propensity of enzymes toward unfolding and the accompanying loss of the tertiary structure necessary for activity. It also allows for a much broader choice of solvents. An illustrative example of the remarkable gains to be obtained by immobilization is provided by the sitagliptin process using the (*R*)-selective TA discussed in section 4.1.3. The optimized process was conducted in aqueous DMSO. However, with the goal of broadening the choice of organic solvents, immobilization of the TA on a variety of polymer-based resins was subsequently investigated,[225] and the results compared with those obtained using the lyophilized free TA. The best results were obtained by adsorbing the TA, at 4% loading, on a highly hydrophobic octadecyl-functionalized polymethacrylate resin, which afforded an activity recovery of 45%. The immobilized TA was active in a variety of organic solvents, with the best results being obtained in the environmentally friendly isopropyl acetate. The immobilized TA was remarkably robust in dry isopropyl acetate at 50 °C, exhibiting a deactivation rate of 0.5%/h over 6 days. Indeed, no deactivation was observed at all when water-saturated isopropyl acetate was used and the immobilized TA was recycled 10 times, with no detectable loss of activity over 200 h. In contrast, the soluble TA was completely denatured with total loss of activity in the organic solvent.

Improved enzyme performance and repeated reuse are reflected in higher biocatalyst productivities [kg of product/(kg of enzyme)], which, in turn, determine the enzyme costs per kilogram of product. In short, enzyme immobilization can enable applications of enzymes that would not have been economically viable using the soluble enzyme. Some loss of activity is usually observed, but the increase in stability coupled with reusability, affording dramatic cost reductions compared with those of the soluble enzyme, more than compensates for such losses.[226] Two important landmarks in industrial biocatalysis were the immobilizations of glucose isomerase and penicillin G amidase. Glucose isomerase is one of the most important industrial enzymes in use today and was first developed in the 1970s.[227] This robust and stable enzyme has even been applied in aqueous ethanol solutions of up to 90%.[228] The stability of the enzyme lends itself to immobilization, which, in turn, facilitates continuous use. Today, the entire process, including plant and factory use (with 10−20 reactors in parallel) has been optimized.[229] The immobilization of glucose isomerase dates back more than 50 years, and all current commercial processes involve the use of an immobilized form. More than 500 tons are used annually to produce ca. 10^6 tons of high-fructose corn syrup, corresponding to productivities of more than 10000 kg/kg. Similarly, the use of immobilized penicillin G amidase in the enzymatic hydrolysis of penicillin G to 6-aminopenicillanic acid (6-APA) was a landmark in both the industrial application of immobilized enzymes and the industrial synthesis of semi-synthetic penicillin antibiotics.[230] Penicillin G amidase (PGA) is used in the manufacture of more than 10000 tons of penicillin and cephalosporin antibiotics annually.[231] Here again, the use of the enzyme in an immobilized form is essential for commercial viability, and productivities of 600 kg/kg or more are the norm.

4.3.1. Methods for Immobilization. Immobilization typically involves the binding of an enzyme to a prefabricated carrier (support), such as an organic resin or silica, or entrapment in a polymeric inorganic or organic matrix that is formed in the presence of the enzyme. Binding to a prefabricated carrier can involve simple adsorption, for example, through hydrophobic or ionic interactions or the formation of covalent bonds.[232] However, physical binding is often too weak to keep the enzyme bound to the carrier in the presence of water and under the rigorous industrial conditions of high substrate and product concentrations and high ionic strength (but, for an exception, see the immobilized TA discussed in the preceding section). In contrast, covalent binding essentially prevents leaching of the enzyme from the surface, but if the enzyme is irreversibly deactivated both the enzyme and the (often-expensive) support are rendered useless. Typical supports comprise synthetic polymers,[233] biopolymers such as polysaccharides, and inorganic solids such as (mesoporous) silicas.[234−236]

Entrapment involves inclusion of an enzyme in an organic or inorganic polymer matrix, such as silica sol−gel or a membrane device.[237] It generally requires the synthesis of the polymeric matrix in the presence of the enzyme. However, the physical constraints are generally too weak to prevent enzyme leakage entirely, and additional covalent attachment is often required.

Immobilization on or in a carrier inevitably leads to a dilution of activity, owing to the introduction of a large portion of noncatalytic ballast, ranging from 90% to >99%, which results in lower space-time yields and catalyst productivities.[238] Increasing the enzyme loading leads to a loss of activity as a result of the inaccessibility of some of the enzyme molecules when they are contained in multiple layers on the carrier surface or are situated

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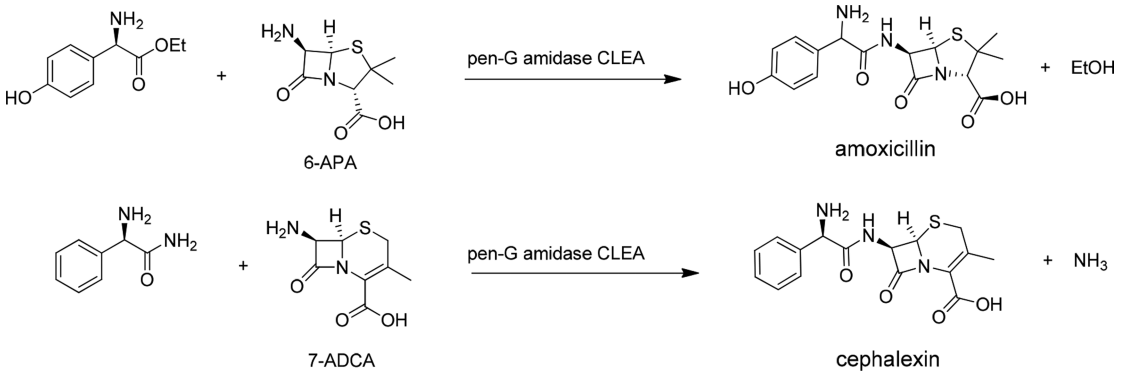

Figure 17. Enzymatic synthesis of penicillin and cephalosporin antibiotics.

deeply within the carrier pores. In contrast, immobilization by cross-linking of enzyme molecules affords carrier-free immobilized enzymes with high productivities and avoids the extra costs of a carrier.

Cross-linked enzymes were already known in the 1960s. They were produced by mixing a solution of the enzyme in aqueous buffer with an aqueous solution of glutaraldehyde. However, they were generally difficult-to-handle, gelatinous materials and exhibited low activity coupled with poor reproducibility and low operational and storage stability. Consequently, carrier-bound enzymes became the norm for the next three decades. In the early 1990s, Altus Biologics introduced the use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts.[239] The methodology had broad scope, and CLECs exhibited excellent operational stability and controllable particle size coupled with high productivity and facile recovery and reuse, making them ideally suited for applications as industrial biocatalysts. However, they had one inherent limitation: the need to crystallize the enzyme, a laborious and costly procedure requiring enzyme of high purity.

In contrast, precipitation of the enzyme from aqueous buffer is a simpler and less expensive method that does not require highly pure enzymes. It is well-known that the addition of salts, or water-miscible organic solvents or nonionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates that are held together by noncovalent bonding without perturbation of their tertiary structure. Addition of water to this precipitate results in redissolution of the enzyme. In contrast, cross-linking of these physical aggregates renders them permanently insoluble while maintaining their preorganized superstructure and, hence, their catalytic activity. The result was a new class of immobilized enzymes: cross-linked enzyme aggregates (CLEAs).[240] Because selective precipitation with ammonium sulfate is commonly used to purify enzymes, the CLEA methodology essentially combines two processes, purification and immobilization, into a single unit operation. The CLEA technology has subsequently been applied to the immobilization of a broad spectrum of enzymes and is the subject of several reviews.[241−246]

4.3.2. Cross-Linked Enzyme Aggregates (CLEAs). Glutaraldehyde is generally the cross-linker of choice, as it is inexpensive and readily available and cross-linking of proteins with glutaraldehyde has GRAS status for use in, inter alia, food and beverage processing.[247,248] Glutaraldehyde has been used for decades to cross-link proteins, but the chemistry is complex and not fully understood.[249] Cross-linking involves the reaction of free amino groups, usually of lysine residues, on the surface of neighboring enzyme molecules with oligomers or polymers of glutaraldehyde.

Immobilization of enzymes as CLEAs can lead to dramatic increases in storage and operational stability, particularly with multimeric enzymes that are unstable outside the cell owing to facile multimer dissociation. Nitrile hydratases (NHases), for example, constitute a class of industrially important enzymes[250] that are multimers and are notoriously unstable outside the cell. Consequently, NHase-mediated industrial biotransformations, such as the manufacturing of acrylamide and nicotinamide from the corresponding nitriles, are conducted as whole-cell processes. In contrast, immobilization of a NHase as a CLEA led to a dramatic increase in storage and operational stability compared to that of the free enzyme.[251] Because the enzyme molecules are bound together by covalent bonds, there is no leaching of enzyme observed with CLEAs in aqueous media, even under drastic conditions such as in the presence of surfactants.

Likewise, important advantages from a cost-effectiveness viewpoint are that CLEAs can be prepared, with high activity recoveries, directly from crude cell lysate obtained from fermentation broth and are easy to recover and reuse by filtration or centrifugation. Furthermore, because they consist mainly of active enzyme, they exhibit high biocatalyst productivities (kg of product/kg of enzyme) compared to those of carrier-bound enzymes, and the costs of the carrier ballast are avoided. A limitation of the technique is that, because every enzyme is a different molecule, the protocol has to be optimized for every enzyme, although, in practice, the protocol can be readily automated. Another limitation is the relatively small particle size, generally 5−50 μm, which can be an issue for processes conducted in fixed-bed reactors, for example, resulting in a high pressure drop. The problem can be alleviated by mixing the CLEA with an inert, less compressible solid, such as controlled microporous glass[252] or perlite.

4.3.3. "Smart" Ferromagnetic Immobilized Enzymes: The New Frontier. A disadvantage of many immobilized enzymes is their relatively small particle size. On the other hand, large particles can cause mass-transfer limitations, and inevitably, a compromise has to be reached between the ease of processing of large particles and the high activities of small particles. Ideally, one would like to have the high activity of small particles while maintaining the ease of processing of large particles. This can be achieved with enzyme−ferromagnetic particle hybrids that can be easily separated magnetically on an industrial scale using standard commercial equipment, enabling a novel combination of biocatalysis and downstream processing. Indeed, the past five years have seen a flourishing of activity in the design of magnetically recoverable catalysts, based on ferromagnetic

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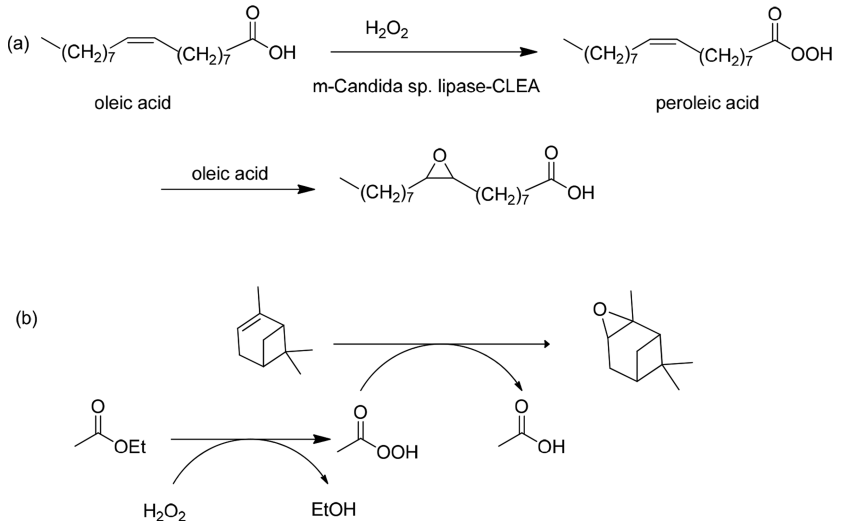

Figure 18. *m*-LipaseCLEA-catalyzed epoxidations.

magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) (nano)particles, both in chemocatalytic[253−256] and biocatalytic[257−261] processes. Such magnetically recoverable catalysts are particularly useful for applications in which an immobilized enzyme has to be separated from other suspended solids.

Recently, magnetic CLEAs (mCLEAs) from a variety of enzymes have been described.[262] For example, an mCLEA of penicillin G amidase was used to catalyze the hydrolysis of penicillin G to 6-APA.[263] In principle, this approach could be easily extended to the synthesis of semisynthetic penicillins and cephalosporins by coupling of the side chain to the 6-APA or 7-aminodesacetoxycephalosporanic acid (7-ADCA) nucleus, as shown in Figure 17. The coupling is conducted in water, and the product crystallizes out of the reaction mixture. Crystallization of the product is essential as, otherwise, the enzyme would catalyze the subsequent hydrolysis of the product, which is the thermodynamically favored reaction. For commercial viability, the enzyme needs to be immobilized to enable its reuse, but then it has to be separated from the solid product, which presents quite a challenge. One possibility, which is practiced on an industrial scale, involves the use of a sieve-bottom reactor whereby the product can diffuse through the sieve bottom whereas the larger catalyst particles are retained in the reactor and can be reused with the following batch.[264] Another possibility is to use an mCLEA of penicillin G amidase and separate it magnetically.

mCLEAs of the lipases CaLB[265] and surfactant-activated *Thermomyces lanuginosus* (TLL)[266] were used in the production of biodiesel from nonedible vegetable oils and waste cooking oils by transesterification with *n*-propanol and methanol, respectively. The mCLEAs could be readily recovered and recycled for at least 10 cycles. An mCLEA of a *Candida sp.* lipase catalyzed the epoxidation of oleic acid with hydrogen peroxide, through the lipase-catalyzed formation of peroxyoleic acid (Figure 18a).[267] The mCLEA exhibited higher thermal and storage stability and better tolerance to hydrogen peroxide than the soluble enzyme and the standard CLEA. Similarly, an mCLEA of an *Aspergillus niger* lipase was used[268] to catalyze the epoxidation of α-pinene with hydrogen peroxide using ethyl acetate as both the solvent and the acetate source (Figure 18b).

mCLEAs of laccase have been used for the decolorization of recalcitrant dyes[269] and the elimination of pharmaceuticals[270,271] in industrial and municipal wastewaters. Residues of pharmaceuticals in hospital and municipal wastewater constitute a pervasive environmental problem.[272] Magnetic CLEAs of other enzymes have been described, such as phenylalanine ammonia lyase (PAL; EC 4.3.1.24),[273] but recent interest in the industrial application of magnetically recoverable immobilized enzymes is primarily in carbohydrate conversions, particularly in the conversion of polysaccharides such as starch and lignocellulose in relation to first- and second-generation biofuels, respectively, and food and beverage processing (see section 7).

4.4. Reactor Engineering

Chemical reactions used for commercial production need to be operated in an efficient manner with respect to starting material [reaction yield, g of product/(g of substrate)], catalyst [catalyst productivity, g of product/(g of catalyst)], and equipment [volumetric productivity, g of product/(L·h)] and in a reactor that suits the characteristics of the reaction. For many high-priced products, such as pharmaceuticals, the reactions will be carried out on a relatively small scale (up to 1000 tonnes per year) and, therefore, in multipurpose reactors, usually stirred tanks equipped with a propeller or turbine stirrer, although there is a marked trend toward the use of flow reactors.[274,275] Stirred-tank reactors (STRs) have several beneficial characteristics such as the ability to mix well, meaning that multiphase reactions and reactions where additions are required (for pH control or substrate feeding) can be carried out effectively. As the production scale increases (more than 10000 tonnes per year), achieving sufficient volumetric productivity becomes critical so as to minimize the size of individual reactors. At a larger scale, mixing becomes expensive and needs to be limited to 0.5−2 W/L, implying that some regions of the tank will be poorly mixed, leading to "hot spots" of reagent concentration and attendant enzyme deactivation. Additionally, the kinetics might also be affected, depending on the rate law for the enzyme. Multiphasic mixing can ensure a high interfacial area between the phases (by dispersing gas as bubbles and immiscible liquids as droplets). Nevertheless, low solubility in the aqueous phase will result in low mass-transfer rates.[276] In general, the flexibility of the stirred tank is a huge advantage in biocatalysis, because similar equipment can be used for a wide range of reaction types. An alternative reactor technology is to use a packed bed of immobilized enzyme in plug-flow mode, which can be beneficial

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Chem. Rev. XXXX, XXX, XXX−XXX

especially for reactions that exhibit poor kinetics at low substrate concentrations (as would otherwise be the case in a well-mixed reactor). Plug-flow mode refers to the conversion of substrate to product from the entry to the exit of the reactor, without mixing with any material preceding or following. It is therefore carried out in tube or packed-bed reactors. Nevertheless, for multiphasic reactions or those that require substrate feeding or pH control, the stirred tank is likely a better choice. Selection of the right reactor is a critical part of biocatalysis engineering because it ensures the best possible use of the reagents, biocatalyst, and equipment, a prerequisite for sustainable processes.

When immobilized enzymes are used in STRs, their recovery and reuse generally involves filtration or centrifugation. However, mechanical attrition, as a result of shear forces caused by the stirrer, can lead to the pulverization of the immobilized enzyme particles, rendering them difficult to separate and reuse. Various reactor engineering solutions to this problem have been reported. For example, a membrane slurry reactor (MSR) retains the immobilized enzyme particles in the reactor by virtue of the fact that they are too large to pass through the pores of a membrane. The membrane consists of a patch in the wall of an existing STR and is relatively easy to implement. The substrate and product, on the other hand, can be pumped in and out of the reactor through the membrane. MSRs can be used in continuous mode with a wide range of catalyst particle sizes. The MSR has many benefits: high catalyst loadings, longer lifetimes owing to less mechanical stress, and high volumetric and catalyst productivities. The biocatalytic step and the separation step are combined into a single operation, and the MSR is suitable for use with the relatively small particles of some CLEAs. Its practical utility was demonstrated in the industrially pertinent hydrolysis of penicillin G to 6-APA catalyzed by a penicillin G amidase CLEA.[277]

Basket reactors (BRs) are a refinement of the "tea-bag" concept.[278] The immobilized biocatalyst is retained in a "basket" that is suspended in the reactor. A BR was used, for example, in the laccase-CLEA-catalyzed degradation of endocrine-disrupting chemicals in aqueous waste streams.[279] The spinning basket reactor is a variation of the BR used in the enzymatic degumming of rice bran oil catalyzed by an immobilized Lecitase.[280] The company SpinChem (www.spinchem.com) commercialized yet another variant: a rotating flow cell reactor, in which the catalyst-containing compartment is attached to a propeller stirrer. Bornscheuer and co-workers used such a reactor, which combines the advantages of a stirred tank with those of a packed bed, in a transamination catalyzed by an immobilized TA.[281] Scaleup beyond, say, 100 L could be an issue, but on the other hand, an important benefit of this novel concept is that, in addition to circumventing mechanical attrition of the biocatalyst, mass transfer is greatly accelerated. This leads to substantially higher reaction rates and creates the possibility of using much smaller reactors. Another new reactor type is the agitated stirred cell reactor,[282] which operates in plug flow and links a series of stirred cells. Again, this would appear to be excellent for smaller scales (otherwise limited by oxygen transfer), screening biocatalysts, and conditions, but scaleup beyond 100 L would likewise appear to be limited.

Alternatively, catalyst attrition can be avoided by using a bubble column reactor (BCR). For example, a bubble column reactor was used to perform the Novozym 435-catalyzed, solvent-free esterification of polyols.[283,284] The products, so-called emollient esters, are highly viscous materials that are used as cosmetic ingredients. Their production is not commercially viable in conventional STRs or fixed-bed reactors (FBRs). The water formed in the reaction is removed by bubbling air under pressure through the column, which also serves to mix the reactants without causing any significant attrition of the catalyst.

In the industrial conversion of oils and fats, continuous processing with immobilized enzymes in fixed-bed reactors (FBRs) is the norm. To circumvent large pressure drops over the column, relatively large particles are used. However, this can lead to mass-transfer limitations, and hence, a compromise generally has to be found. Alternatively, in a fluidized bed, the reactants are introduced at the bottom of the column and flow upward, which dictates that the particles should be of sufficient density to prevent them from being blown out of the column. Alternatively, relatively small magnetic particles, for example, magnetic CLEAs, could be held in place in a magnetically stabilized fluidized bed.

A particularly challenging process is one in which an immobilized enzyme needs to be separated from other suspended solids. This is generally the case in the enzymatic hydrolysis of polysaccharides, such as starch and lignocellulose. In the production of bioethanol from starch, for example, the hydrolysis and subsequent fermentation step can be integrated in a so-called simultaneous saccharification and fermentation (SSF) process. Smart magnetic CLEAs of the hydrolytic enzymes can be used to enable their facile recovery and reuse, with potentially significant reductions in the enzyme cost contribution (see section 7).

Much attention has been focused in recent years on process intensification through the use of microchannel reactors (microfluidic devices). This approach potentially has many advantages compared with traditional batch process technologies: rapid mass and heat transfer and large surface-area-to-volume ratios. Littlechild and co-workers,[252] for example, prepared CLEAs from a thermophilic L-aminoacylase from *Thermococcus litorali* that had been overexpressed in *E. coli* and subsequently mixed them with controlled-pore glass before packing them in a capillary reactor fitted with a silica frit to contain them in the reactor.

4.5. Integrated Product Removal

One of the major limitations for the implementation of many biocatalytic processes is the low concentration of product leaving the reactor. As discussed previously, this leads to large and costly downstream equipment as well as inefficient processes. Despite attempts to improve the tolerance of enzymes to product through protein engineering, this remains difficult, and in many cases, therefore, removal of the product directly from the reactor can prove essential. Numerous documented reports attest to the value of in situ product removal (ISPR), which can afford order-of-magnitude improvements in the process.[285−287] This approach is analogous to that taken in conventional chemical engineering with process intensification technologies, such as reactive distillation.[288,289] The implementation of such schemes is essential to achieving sustainable biocatalytic processes. Some of the requirements of implementation are listed in Table 5 for ISPR (with external recycle, the usual mode of operation in industry).

Some excellent examples of the implementation of ISPR into processes can be found in the literature concerning the enzymatic synthesis of sugars such as L-erythrulose,[290] the whole-cell synthesis of fluorocatechol,[291] and the enzymatic synthesis of cephalexin by complexation[292] and cefaclor,[293] leading to productivity increases. One of the most cited industrial examples concerns the whole-cell reduction of 3,4-methylene dioxyphenol

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Chem. Rev. XXXX, XXX, XXX−XXX

Table 5. Requirements for the Implementation of ISPR[a]

process step	requirement
biocatalyst filtration	ability to remove the biocatalyst, ahead of the ISPR operation
product removal	sufficient selectivity of removal system between products (and other species, in particular, substrate)
	sufficient capacity of removal system to recover product, without the need for multiple elution steps
product elution	sufficient up-concentration during product elution that a high concentration reaches the downstream process

[a]With external recycle.

acetone to its corresponding alcohol for use as an API. The process uses the hydrophobic resin Amberlite XAD both to supply ketone and to recover alcohol in a recycle loop.[294]

4.6. Flow Technology

The application of continuous-flow techniques can favorably influence the sustainability of manufacturing processes. Performing reactions in continuous-flow mode can increase yields, optimize resource utilization, minimize waste generation, and improve process safety.[295−302] This has not gone unnoticed by the pharmaceutical industry in its drive to implement greener, more sustainable processes. Hence, the application of continuous-flow techniques in the industrial synthesis of pharmaceuticals is currently the focus of growing attention.[303−311] For example, the application of continuous-flow chemistry techniques to the synthesis of diazepam and atropine led to substantial reductions in the waste generated, as reflected in much lower *E* factors.[312] Not surprisingly, this interest in continuous-flow operation also applies to the implementation of bioconversion processes. Although several large-scale industrial biocatalytic processes are operated in continuous mode (in a series of stirred tanks or in packed beds) for good economic reasons, the vast majority are still operated in fed-batch mode, mainly because of the necessity of using multipurpose plants. Nonetheless, recent developments in continuous-flow chemistry are stimulating applications in biocatalytic processes.[274,313−319] For example, two research groups have used an immobilized transaminase successfully in the continuous-flow synthesis of a series of amines, integrated with an in-line purification step for recovery of the pure amines.[275,320] A further interesting example addresses the issue of substrate feeding for transketolase-catalyzed carbon−carbon bond formation, to overcome inhibitory effects on the kinetics by using multiple feed points.[321] A particular challenge is how to run multiphasic reactions in flow mode (especially with gases). This is an active area of research in all areas of synthesis.[322] Biocatalytic examples have focused mainly on oxidations, which can be carried out in tube-in-tube reactors,[323] where the outer tube can supply oxygen (under pressure) to biocatalyst in the inner tube.[324] Further applications of continuous-flow biocatalysis are expected to be forthcoming, as this mode of operation results in smaller production units, significant productivity increases (eliminating downtime), and reduced inventories, affording more sustainable processes.

5. CASCADE REACTIONS: CELL-FREE SYNTHETIC BIOLOGY

In traditional organic syntheses, a simple step-by-step approach is used to convert a starting material A into product D, in which intermediates B and C are isolated and purified before going on to the next step (Figure 19). This generally results in low space-time yields, laborious recycle loops, and copious amounts of waste. In contrast, catalytic processes are generally shorter than their classical counterparts. A reduction in the number of steps, what Wender termed step economy,[325] will, in most cases, lead to a reduction in the amounts of reagents and solvents used and, hence, in the amounts of waste generated. Indeed, the ultimate in green catalytic syntheses is to integrate several catalytic steps into step-economical, one-pot procedures without the need for isolation of intermediates,[326] which truly emulates the elegant orchestration of enzymatic steps in metabolic pathways in the living cell.[327] Such "telescoping" of multistep syntheses has several advantages: fewer unit operations, less solvent, smaller reactor volume, shorter cycle times, higher volumetric and space-time yields, and less waste (lower *E* factors). Furthermore, coupling of reactions can be used to drive equilibria toward product, thus avoiding the need for excess reagents. The bottom line is a more sustainable technology with substantial economic and environmental benefits.

A seminal work on biocatalytic cascade processes was the report of Scott and co-workers[328] on the in vitro synthesis of an advanced corrinoid precursor of vitamin B_{12} using a mixture of all of the enzymes and cofactors and cosubstrates involved in the biosynthetic pathway in vivo. Twelve different enzymes were involved in the creation of nine stereocenters. The authors noted that "the practical realization of this goal gives us confidence that the development of genetically engineered syntheses of equally challenging natural product structures will also be practical."[328] The alternative to this in vitro approach of "replacing the cell wall with a glass wall" is to use metabolic engineering to rewire cellular

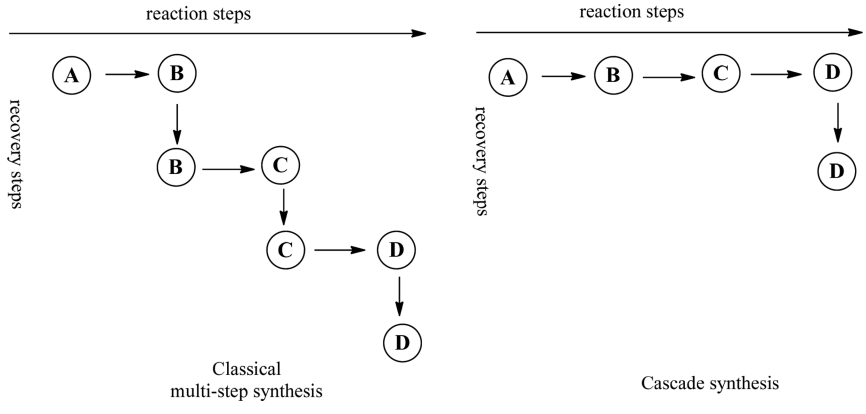

Figure 19. Classical multistep synthesis vs a synthetic cascade process.

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metabolism to enhance the production of native metabolites or to endow cells with the ability to produce new products through existing metabolic pathways.[329] Indeed, metabolic engineering and the underpinning concepts of synthetic biology[330] and designer cells[331] have revolutionized the microbial production of chemical and pharmaceutical products. However, a detailed discussion of such whole-cell approaches falls outside the scope of this review.

The coupling of chemocatalytic steps into cascade processes is fraught with problems: Catalysts are often incompatible with each other, rates and optimum conditions can be very different, and catalyst recovery and recycle can be complicated. In contrast, biocatalytic processes generally proceed under roughly the same conditions—in aqueous media at about ambient temperature and atmospheric pressure—which facilitates their integration in cascade processes.[51] Nature solves problems of compatibility by compartmentalization of the enzymes in different parts of the cell. Hence, compartmentalization through immobilization could be a solution to compatibility problems in biocatalytic cascade processes performed in vitro.

Biocatalytic cascade processes have become a particular focus of attention in recent years,[332−336] motivated largely by potential environmental and economic benefits. Indeed, multienzyme biocatalysis has come to represent the most effective use of biocatalysis. It has also been referred to as systems biocatalysis or cell-free artificial metabolism.[337] That it provides a basis for shifting thermodynamically unfavorable equilibria[338] and cost-effective cofactor recycling is well-established. Many of the reported examples involve the combination of a redox process with a cofactor regeneration step and/or the combination of two or more redox processes to afford an overall redox neutral process,[339,340] for example, for the enantioselective conversion of secondary alcohols to the corresponding chiral amines, involving an overall replacement of an OH by an NH_2 functionality.[341] Examples of such cascade processes involving cofactor regeneration, for example, in conjunction with ketoreductases, were discussed in section 4.1.3. The combination of imine reductase with amine oxidase for deracemization of nitogen hetercycles is another example that illustrates well the economic advantages of combining biocatalytic steps.[342]

Another interesting example is the conversion of D-methionine to L-methionine in a four-enzyme cascade involving a D-amino acid oxidase, an L-amino acid dehydrogenase, formate dehydrogenase, and catalase (Figure 20),[343] which, overall, constitutes the deracemization of DL-methionine to L-methionine.

Combining biocatalytic steps in this way can provide the possibility for rapid proliferation of molecular complexity, including the introduction of multiple asymmetric centers, starting from very simple, inexpensive raw materials. A striking example of this is the four-step, four-enzyme cascade synthesis of non-natural carbohydrates starting from inexpensive and readily available glycerol and an aldehyde (Figure 21).[344] Interestingly, pH adjustment was used to "switch off" the activities of the various enzymes during the process and to switch them on again when needed.

The rates of biocatalytic cascades can be substantially increased by simulating the close proximity of the enzymes present in microbial cells.[345] This can be achieved, for example, through the coimmobilization of two or more enzymes in a so-called combi-CLEA by coprecipitating the enzymes from aqueous buffer and cross-linking the aggregates. Combi-CLEAs of glucose oxidase or galactose oxidase with catalase, for example, were recycled without significant loss of activity.[346] Moreover,

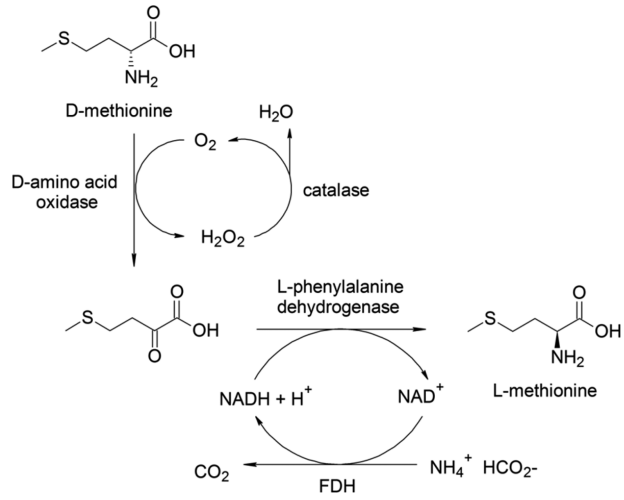

Figure 20. Biocatalytic cascade for the deracemization of methionine.

significantly better activities and stabilities were observed with the combi-CLEA than with an equivalent mixture of the two separate CLEAs. Oxidases catalyze the aerobic oxidations of various substrates, generally with concomitant production of an equivalent of hydrogen peroxide The latter can cause oxidative degradation of the enzyme, and in vivo, oxidases generally occur together with catalase, which catalyzes the spontaneous decomposition of hydrogen peroxide to oxygen and water.

Similarly, a combi-CLEA of a ketoreductase (KRED) and glucose dehydrogenase (GDH) was shown to be a robust system for the regeneration of dihydropyridine nucleotide cofactors. In addition to increased thermal and pH stability, it exhibited high substrate tolerance and long-term operational stability.[347] It was used to catalyze the enantioselective reduction of the prochiral keto ester used in the synthesis of a key atorvastatin intermediate (see section 4.1.3) and was recycled repeatedly with high activity and selectivity retention. The researchers predicted that the system would be widely applied in the manufacture of chiral alcohols. Similarly, a KRED/GDH combi-CLEA displayed superior activity and stability, compared with a mixture of the corresponding free enzymes, in the enantioselective synthesis of a chiral alcohol intermediate for the anticancer drug Crizotinib.[348]

A trienzyme combi-CLEA consisting of an (*S*)-hydroxynitrile lyase, a nonselective nitrilase, and an amidase was shown to catalyze the conversion of benzaldehyde to (*S*)-mandelic acid, in 90% yield and >99% ee at complete benzaldehyde conversion (Figure 22a).[349] The system was originally developed[350] as a hydroxynitrile lyase/nitrilase bienzyme combi-CLEA, but because large amounts of the corresponding amide were formed as a byproduct of the nitrilase-catalyzed hydrolysis, an amidase was added to hydrolyze the amide side product. The rates were higher than with mixtures of the respective CLEAs. Similarly, a combi-CLEA of the (*S*)-hydroxynitrile lyase with an alkaliphilic nitrile hydratase (EC 4.2.1.84) catalyzed the one-pot conversion of aldehydes to (*S*)-α-hydroxycarboxylic acid amides (Figure 22b).[351,352]

Combi-CLEAs have also been widely used in carbohydrate chemistry. For example, Park and co-workers[353] described the production of trehalose from sucrose in a one-pot process (Figure 23) using a trienzyme combi-CLEA of amylosucrase (EC 2.4.1.4), maltooligosyl trehalose synthase (EC 5.4.99.15), and maltooligosyl trehalose hydrolase (EC 3.2.1.141). The CLEA

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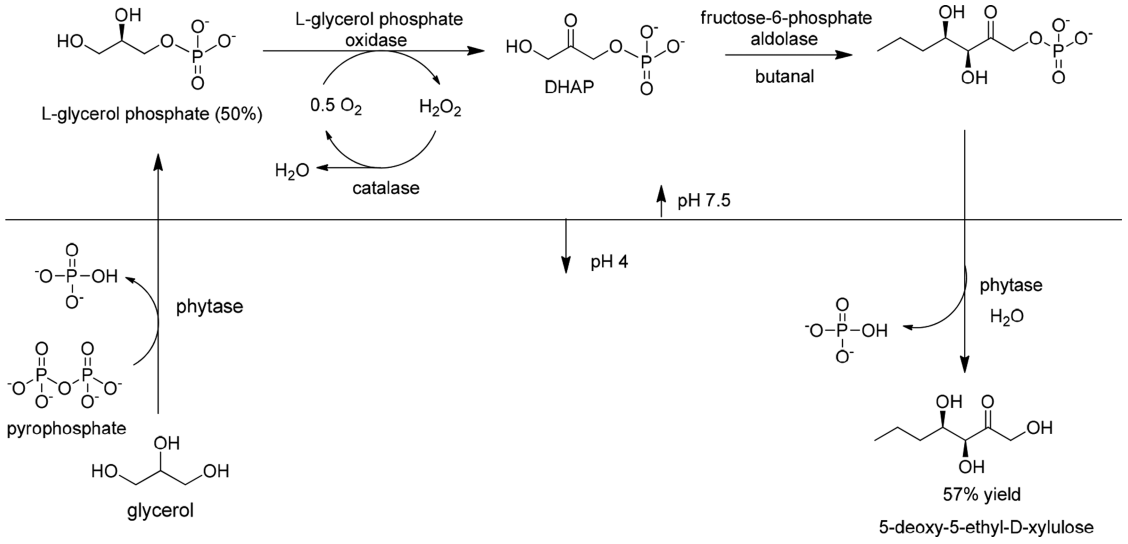

Figure 21. Four-enzyme cascade for the synthesis of non-natural carbohydrates.

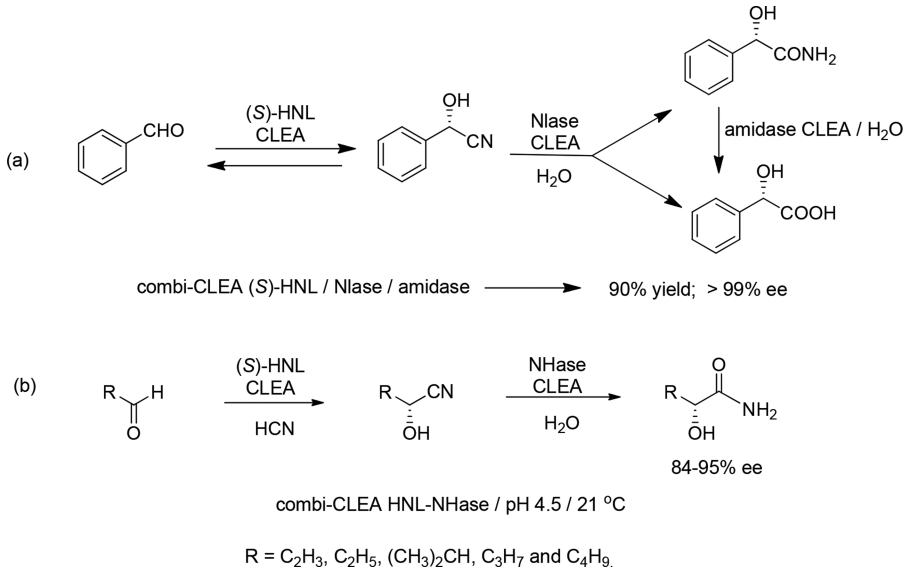

Figure 22. (*S*)-Mandelic acid and amide synthesis using trienzyme combi-CLEAs.

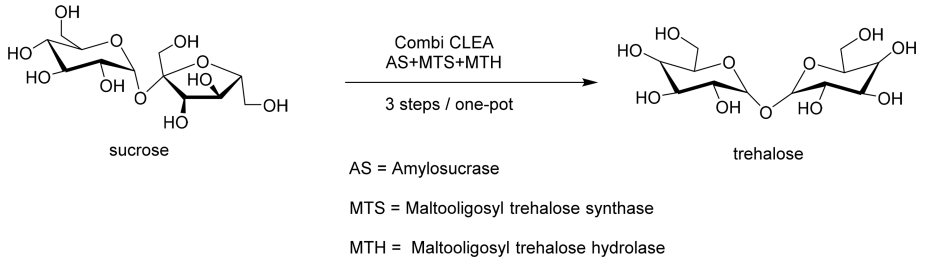

Figure 23. One-pot synthesis of trehalose using a combi-CLEA.

was recycled five times. Trehalose is a starch-derived disaccharide with many applications in the food, pharmaceutical, and cosmetic industries. The conversion of polysaccharides in biocatalytic cascade processes is discussed further in section 7.

Scism and Bachmann[354] reported, already in 2010, a remarkable five-enzyme combi-CLEA that could be prepared from crude cell lysates and was used successfully in the cascade synthesis of nucleotide analogues. Without extensive optimization, the combi-CLEA was shown to be robust and reusable and demonstrated greatly improved stability compared to the free enzyme cascade. The authors noted that further investigations of such "pathway CLEAs" would reveal the potential and limitations of this methodology compared with whole-cell-based synthetic biology approaches.

Finally, in the context of enzymatic cascade syntheses, it is worth mentioning the concept of *reverse biosynthesis*,[355] whereby high-molecular-weight natural products are, with the aid of enzymes, disassembled into a pool of lower-molecular-weight

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Chem. Rev. XXXX, XXX, XXX−XXX

compounds with diverse structures for combinatorial screening as drugs or drug leads.

6. INTEGRATING BIOCATALYSIS INTO SYNTHESIS AND MANUFACTURING

An essential step in enabling the further implementation of biocatalysis into common industrial practice is its integration into the broader synthesis and manufacturing context. Biocatalytic processes frequently operate under mild conditions, as described earlier, which supports the concept of green chemistry for an individual reaction step. However, it is essential that the broadest possible view be taken. In many processes, the enzymatic step represents just one step among many synthetic steps in a complete synthetic scheme. To assess the overall sustainability of a process, the entire synthetic scheme needs to be considered. Changing reaction conditions (e.g., pH or temperature), or changing reaction media, in the middle of a synthetic route (even to enable a more benign individual step) can result in a suboptimal solution overall. One method for overcoming this difficulty is to ensure that the biocatalytic reaction is either the first or last step in a synthesis, but such a choice is rarely available and does not always make sense. A second strategy is to try to bring the conditions closer together, and invariably, this means operating biocatalytic reactions in media other than water. The huge developments in this field (as outlined in section 3) attest to what can be achieved in this respect. Moving from water as the medium of first choice will also have significant benefits for product concentration and, therefore, beneficial effects on the *E* factor (as outlined in section 3.1). Although engineering enzymes to provide the ability to operate at different temperatures and pH values is also possible, the engineered enzymes rarely reach the values characteristic for many catalytic processes. Hence, compromise conditions are difficult to find. Nevertheless, there is a growing area of research focused on the interface between homogeneous/heterogeneous catalysis and biocatalysis.[356,357] A particular challenge is integrating single biocatalytic steps with neighboring chemocatalytic ones, where the conditions might not match and cross-reactivity can be a problem, in addition to poisoning of catalysts or biocatalysts. In recent years, a new focus has begun to address this challenge by considering various options for integration with[358,359] and without[360] compartmentalization. Real success here will also hinge on achieving sufficient stability for the enzyme systems under these unusual and sometimes harsh conditions.[361]

The final strategy is to make the entire synthesis biocatalytic. The developments toward cascade reactions are therefore part of a larger step that will need to be taken to convert the major part of synthetic routes to enzyme-based steps. Such schemes will need to compete with the metabolic engineering approach (which is frequently fraught with complexity in an attempt to control regulation and enable adequate productivity). The ability to develop processes independent of the regulatory constraints in the cell, with suitable loadings of enzyme and matched kinetics (as well as the ability to feed or remove intermediates where necessary), is a formidable task. Nevertheless, achieving such a goal by "in vitro metabolic engineering" (or sometimes in combination with in vivo metabolic engineering and synthetic biology)[362] will lead to many opportunities for new products, as well as new sustainable routes to existing products.

7. BIOCATALYSIS AND THE BIOBASED ECONOMY

It is widely accepted that the use of nonrenewable fossil resources—oil, coal, and natural gas—as raw materials for the manufacture of biofuels, bulk chemicals, and materials is not sustainable in the long term.[363] Hence, there is a growing emphasis on the transition to a carbon-neutral, biobased economy[364−367] that is largely driven by the pressing need for climate-change mitigation and the conservation of natural carbon resources. A switch to the use of renewable biomass in integrated biorefineries[368] will afford environmentally beneficial reductions in the carbon footprints of chemicals, liquid fuels, and materials. A further benefit of the biobased economy could be the substitution of existing products by inherently safer, environmentally friendlier alternatives, such as biocompatible and biodegradable plastics.[369] However, the use of first-generation (1G) biomass feedstocks, such as corn and edible oil seeds, is not perceived to be a sustainable option in the longer term because of direct or indirect competition with food production. An alternative, second-generation (2G) scenario, more in line with the concept of a circular economy, involves the valorization of waste biomass,[370] such as waste lignocellulose derived from agricultural and forestry residues, triglycerides from waste cooking oil, and even food-supply-chain waste.[371] This will require the development of efficient chemo- and biocatalytic methodologies.

A direct result of the impending transition to renewable biomass is that the traditional hydrocarbon feedstocks of the petrochemical industry will be replaced, at least partially, by carbohydrates as basic chemicals in the biobased economy. White biotechnology, with its underpinning tools of enzymes and microorganisms, is a key technological driver of this growing biobased economy.[372] Biocatalysis will be a key enabling technology, both in the production of monosaccharides from polysaccharide feedstocks and in their further conversion to commodity chemicals.[373]

7.1. Starch Hydrolysis

Although 2G lignocellulosic biofuels are seen as the long-term option, in the short term, biofuels will consist primarily of corn- and sugar-based bioethanol. The United States is the largest producer of biofuels, with a production of 14.7 billion gallons in 2015,[374] and uses cornstarch as the feedstock. Starch hydrolysis consists of two steps. The first step, liquefaction, is conducted at 90 °C and pH 7 and is catalyzed by α-amylase (EC 3.2.1.1), which hydrolyzes α-(1−4) glycosidic bonds. This is followed by saccharification to the fermentable sugar glucose, which involves hydrolysis of both α-(1−4) and α-(1−6) glycosidic bonds catalyzed by a second enzyme, glucoamylase (EC 3.2.1.3), at 60 °C and pH 5. Talekar and co-workers[375] prepared mCLEAs of α-amylase with an activity recovery of 100% and used them in starch hydrolysis. Thermal and storage stability was improved compared to that of the free enzyme, and the mCLEA retained 100% of its activity after six recycles. An mCLEA of α-amylase was also prepared using pectin dialdehyde as the cross-linker with 95% activity recovery, compared to 85% using glutaraldehyde as the cross-linker.[376] The higher activity recovery was attributed to better mass transfer of macromolecular substrates in the more open porous structure.

Similarly, glucoamylase from *Aspergillus niger* was immobilized on magnetic polystyrene particles and used successfully in a magnetically stabilized fluidized-bed reactor (MSFBR).[377] It was necessary to use low flow rates in the column to avoid diffusion and channeling problems arising from particle agglomeration.

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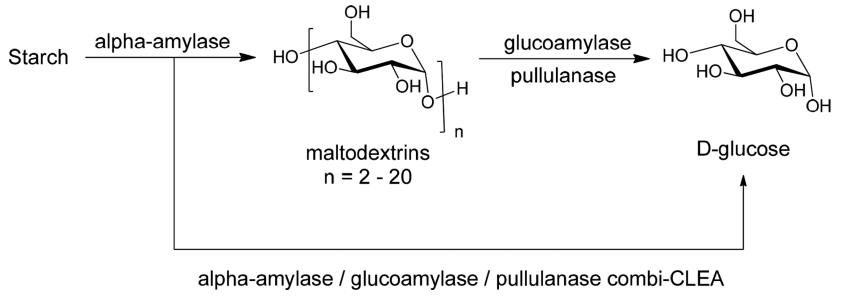

Figure 24. Hydrolysis of starch to glucose.

Glucoamylase was also immobilized, by ionic interaction, on Fe_3O_4/chitosan microspheres formed by coating the chitosan on monodisperse superparamagnetic Fe_3O_4 nanoparticles. The resulting immobilized glucoamylase showed excellent storage and pH stability as well as reusability. Gupta and co-workers[378] also prepared magnetic CLEAs of glucoamylase with 93% activity recovery that showed enhanced thermal and storage stability and reusability.

Because the glucoamylase-catalyzed hydrolysis of the α-(1,6) branches is relatively slow, a second enzyme, pullulanase (EC 3.2.1.41), is sometimes added to facilitate the hydrolysis. Coimmobilization of the two enzymes in a combi-CLEA resulted in a shift in optimum pH (from 5 to 7) and temperature (from 60 to 70 °C).[379] In a batch-mode hydrolysis of starch, the combi-CLEA gave 100% conversion after 3 h compared with 30% for the soluble enzyme. A mixture of the two separate CLEAs gave 80% conversion. The combi-CLEA had good stability, retaining 90% and 85% of the glucoamylase and pullulanase activities, respectively, after eight recycles.

More recently, the same group prepared a trienzyme combi-CLEA containing α-amylase, glucoamylase, and pullulanase from commercially available enzyme preparations.[380] In a one-pot starch hydrolysis (Figure 24) in batch mode, 100%, 60%, and 40% conversions were observed with the combi-CLEA, a mixture of the separate CLEAs, and a mixture of the soluble enzymes, respectively, and the catalytic performance was maintained for up to five cycles. Coimmobilization increased the thermal stability of all three enzymes.

In both 1G and 2G bioethanol production, to improve the cost-effectiveness, the enzyme-catalyzed hydrolysis of the feedstock—starch or lignocellulose—is often combined with the subsequent fermentation step in so-called simultaneous saccharification and fermentation (SSF) processes.[381] The enzyme costs per kilogram of product are crucial for the economic viability of these processes, particularly in the case of lignocellulosic feedstocks. Enzyme manufacturers have achieved remarkable results in reducing enzyme costs by optimizing the production of the enzymes involved, but there is still room for reducing the enzyme cost contribution to total process costs by improving the enzyme usage. The enzyme used in the hydrolysis step is dissolved in the aqueous reaction mixture and, consequently, is discarded with the wash water. Multiple recycling of the enzyme(s) represents a clear opportunity to reduce the enzyme costs and drive competitiveness and sustainability. This can be achieved by using an immobilized solid enzyme to facilitate its recovery and reuse. However, the need to separate the solid immobilized enzyme from substantial amounts of other solids, such as fibers and/or yeasts (in SSF processes), suspended in the reaction mixture prohibits the use of standard immobilized enzymes.

A key enabling technology to solve the problem of separating the solid immobilized enzymes from the liquid phase and other solid particles is to use, for example, ferromagnetic CLEAs (mCLEAs) that can be separated using an external magnetic field in readily available equipment that is already widely used at the industrial scale.

7.2. Lignocellulose Hydrolysis

Probably the most exciting and challenging development is the application of magnetically separable immobilized enzymes to the complex chemistry of lignocellulose conversion, in connection with the production of 2G biofuels and commodity chemicals from waste biomass.[382] The complex, recalcitrant structure of lignocellulose, consisting of lignin (15−25%), cellulose (30−45%), and hemicellulose (25−40%), requires the use of a complex cocktail of cellulolytic and hemicellulolytic enzymes, referred to collectively as cellulase, to catalyze hydrolysis to its constituent sugars.[383] The hydrolysis of cellulose involves catalysis by at least five enzymes, including exo- and endoglucanases and β-glucosidase. Hemicellulose has a more complicated structure than cellulose and requires a diverse suite of enzymes, including xylanase (EC 3.2.1.8) and mannanase (EC 3.2.1.78), to effect its hydrolysis to its constituent sugars, mainly xylose and mannose.

Immobilization of the cellulase enzyme cocktail on magnetic particles has been extensively studied, either on prefabricated magnetic (nano)carriers[384−391] or as magnetic CLEAs,[392−394] but activities were generally measured only in the hydrolysis of the water-soluble carboxymethyl cellulose as a model for the complex mixture derived from lignocellulose. Immobilization of β-glucosidase, one of the enzymes contained in the cellulase cocktail, on magnetic silica-based particles has also been described.[395] It is also worth noting that it might not be essential to immobilize all of the enzymes in the cellulase cocktail. Immobilization and recycling of a selection of the enzymes present could lead to substantial cost reductions.

Bhattacharya and Pletschke[396] prepared mCLEAs of a bacterial xylanase and observed that the incorporation of Ca^{2+} ions in the CLEA led to increased thermal stability. The Ca-mCLEA exhibited 35% more activity than the free enzyme and a 9-fold higher sugar release from ammonia-pretreated sugar cane bagasse. Similarly, Illias and co-workers[397] prepared mCLEAs of a recombinant xylanase from *Trichoderma reesei* using maghemite (γ-Fe_2O_3) rather than the more typical magnetite (Fe_3O_4) nanoparticles.

7.3. Lignocellulose Pretreatment

In addition to enzyme costs, another important cost item that needs to be minimized to achieve economically viable production of 2G biofuels is the pretreatment of the lignocellulose. Although lignocellulose is available in very large quantities, it is much more

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Chem. Rev. XXXX, XXX, XXX−XXX

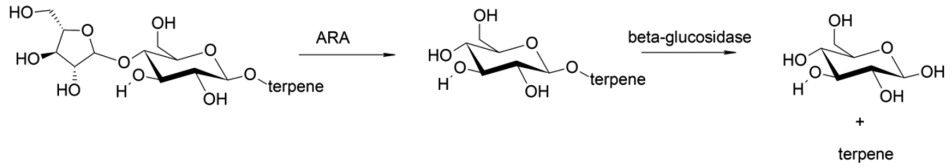

Figure 25. Reactions involved in aroma enhancement in wine.

difficult to process than first-generation feedstocks such as sucrose and starch. Some form of pretreatment, such as a steam explosion, ammonia fiber expansion (AFEX), or treatment with lime, is needed to open up the recalcitrant lignocellulose structure and render it accessible to the enzyme cocktail.[398] Pretreatment generally accounts for a large fraction of the total energy consumption of lignocellulose conversion. The reaction medium is, generally speaking, water, but alternatives have been considered. The organosolv process, for example, involves reaction at elevated temperatures (185−210 °C) in water/organic solvent (e.g., ethanol) mixtures.[399] Alternatively, ILs and DESs are being considered as reaction media for the deconstruction of lignocellulosic biomass.[400]

The ultimate goal is to develop an integrated process for the pretreatment of the lignocellulose and enzymatic hydrolysis of the polysaccharides, coupled with efficient recycling of both the IL or DES and the (immobilized) enzyme.[401] The cellulase enzyme cocktail is known to be active and stable in designer ILs and DESs.[133] For example, cellulase was engineered to improve its stability in mixtures of ChCl/Gly and concentrated seawater as a low-cost solvent for the enzymatic hydrolysis of cellulose.[402]

7.4. Hydrolysis of Polysaccharides in Food and Beverage Processing

Although important applications of combi- and mCLEAs in 1G and 2G biofuels are foreseen, there are also opportunities in food and beverage processing. For example, the turbidity of fruit juices is caused by insoluble polysaccharides such as pectin and (ligno)cellulose, and a pectinase/cellulase combi-CLEA was successfully used in the clarification of grape juice.[403] A trienzyme magnetic combi-CLEA consisting of an α-amylase, pectinase, and cellulase was similarly used to clarify fruit juices.[404]

Wilson and co-workers[405,406] prepared a combi-CLEA from Rapidase AR2000, a commercial enzyme preparation, containing two glycosidases: α-L-arabinofuranosidase (ARA) and β-glucosidase (βG). These enzymes are responsible for the hydrolysis of monoterpene glycoconjugates present during winemaking to afford volatile terpenoids, which provide the aromatic aroma of wines. All grape varieties comprise such precursors, but Muscat grapes, in particular, have the highest concentrations. The enzymatic hydrolysis is a sequential two-step process. In the first step, formation of the monoterpenyl-β-glucoside is catalyzed by ARA and in the second step monoterpenes are liberated by βG-catalyzed hydrolysis (Figure 25).

8. CHALLENGES FOR THE FUTURE

The development and implementation of biocatalytic processes, whether for single synthetic steps or across larger parts of the value chain, requires significant efforts. As with all technologies, a primary challenge is to modify laboratory-scale chemistry to make it suitable for industrial implementation.[44,407] In this review, we have termed such optimization "biocatalysis engineering", which covers protein engineering, biocatalyst engineering (through immobilization), and reactor engineering.[36,173,408] It includes ensuring that the enzymes have sufficient activity and stability under the required conditions and that these conditions match the commercial necessities of sufficient productivity. It is therefore necessary to have guidelines and supporting tools to help determine the extent of improvement required and how to go about achieving it. Alongside this, a major obstacle becomes the necessity for multidisciplinary teams, which need to overcome considerable inertia due to different methods and languages. Setting common goals and targets is what is needed here. Likewise, from an educational perspective, the further development of chemistry, biotechnology, and process engineering curricula to incorporate biocatalysis is also a major target.

A second major challenge is that not enough information is collected on the sustainability of biocatalytic processes. A major driving force for the implementation of biocatalysis should be the improved sustainability of processes, but without the measurement and quantification of waste and resultant calculation of sustainability metrics, it is hard to measure the extent of improvement in a given case. Benchmarking biocatalytic processes with the aim of understanding when such processes are sustainable is a major need. Very few studies of side-by-side life-cycle analyses of a chemical route and its biocatalytic counterpart have been undertaken. The synthesis of 7-amino-cephalosporic acid (7-ACA) by the conventional chemical route and a two-enzyme route, discussed in section 2.3, forms an interesting exception.[76]

Another challenge to be overcome is the development process itself. It is clear that the development of synthetic strategies needs to achieve a certain speed (especially in the pharmaceutical sector),[409] and it is equally clear that, although the development of new biocatalytic routes has been hugely successful in several well-publicized cases, there are also many examples where the speed of development, or the resources required, could not be justified. Faster and more efficient methods of process design and development will require the use of computational tools and knowledge about the enzyme[410] to be altered, just as much as the process requirements.

In the future, guidelines for implementing sustainable syntheses should be developed and should, of course, emphasize the importance of biocatalysis. Sustainable implementation will often favor biocatalysis (renewable catalyst, used under mild conditions) but should also emphasize the need for several other features that capitalize on its benefits.

(1) It is necessary to operate with a sufficient product concentration, to obtain a realistic *E* factor. This is required for both economic and environmental reasons. In this way, research should use the extensive power of protein engineering, not only to create new catalysts (with a wide substrate reservoir), but also to ensure that they operate effectively in terms of economic and environmental metrics. The developments in protein engineering have been spectacular, but it is necessary for renewed attention to be paid to the translation from laboratory catalyst to industrial catalyst.

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Chem. Rev. XXXX, XXX, XXX−XXX

(2) It is necessary to operate the biocatalytic reaction in the right medium, taking into account the solubilities of the compounds involved and the requirements of downstream processing. More research is required here to understand when to use slurry-to-slurry reactors and when to use biphasic media, as well as how to choose solvents and ensure adequate stability of the enzymes in such media.

(3) Particularly careful consideration should be given to cofactor recycle. For instance, it is clear that the use of NADH oxidases (NOXs)[411] is an excellent way to regenerate NAD^+ from an environmental perspective because they use molecular oxygen as a cosubstrate and yield water (or hydrogen peroxide, which can be converted to water by catalase) as a coproduct. However, such systems need significant protein engineering to improve activity and stability, so they can be made economical. For example, when coupled with dehydrogenases for the oxidation of alcohols (rather than the usual reduction of ketones), relatively poor process performance was observed, which was attributed, in part, to NOX inactivation.[411]

(4) Care should also be taken with equilibrium-controlled conversions, where excess cosubstrates will lead to high *E* factors and ISPR will lead to further downstream complications. Use of alternative substrates should be a better strategy to improve the reaction ΔG value.[412]

These are important research targets for the future to ensure that the sustainable benefits of biocatalysis are fully exploited. This will enable still further the implementation of biocatalytic syntheses across all industries.

9. CONCLUDING REMARKS

Industrial biocatalysis has come a long way in the past two decades. Thanks to the sequencing of large numbers of microbial genomes, and the availability of this information in the public domain, coupled with advances in gene synthesis, it is now possible to readily access a broad range of wild-type enzymes. The properties of potentially interesting enzymes can then, with the aid of directed evolution tools, be fine-tuned to fit seamlessly into a predefined process. Thanks to recombinant DNA technology, the promising candidates can then be produced, cost-effectively, on an industrial scale. This has provided the foundations for the widespread application of biocatalysis in sustainable chemicals manufacture. In the United States, the prestigious Presidential Green Chemistry Challenge Awards, instituted by the Environmental Protection Agency (EPA) in 1996 and cosponsored by the American Chemical Society Green Chemistry Institute, recognize chemical technologies that incorporate the principles of green chemistry into chemical design, manufacture, and use. Categories include greener synthetic pathways and greener reaction conditions. Over the years, more than 20 of these awards have involved biocatalytic or biotechnology-based methods of synthesis, including fermentation. For example, the Codexis process for a key intermediate for atorvastatin and the Merck−Codexis process for sitagliptin won in 2006 and 2010, respectively (https://www.epa.gov/greenchemistry/presidential-green-chemistry-challenge-winners). There can hardly be a better measure of the important impact that biocatalysis has on sustainability. Still further penetration of biocatalysis into the chemical and allied industries and into more traditional areas such as food and beverage processing is being further stimulated by the transition from an unsustainable economy based on fossil resources to a more sustainable biobased economy. In short, biocatalysis is playing a key role in sustainable chemistry. In the words of the eminent Russian chemist and Nobel laureate, N. N. Semenov,[413] who was writing about the chemistry of the future four decades ago: "By applying the ideas of biochemistry, chemical science may solve the energy crisis, make industrial production infinitely more efficient, and provide mankind with wings."

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