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Recent preparative applications of redox enzymes

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Redox enzymes offer many powerful transformations for the efficient industrial-scale synthesis of diverse chemicals desired by society. Here we survey recent preparative applications of redox enzymes, highlighting both mature enzyme platforms and promising technologies for future applications. While in some cases commercial enzymes can be employed directly on industrial scales, in other cases protein engineering is necessary to evolve an enzyme fit for non-biological substrates and conditions. Both approaches require the input of process engineering to properly balance the needs of the enzymatic chemistry with the requirements for an industrial process. A convergence of advances in enzyme discovery, protein engineering, and process engineering is expected to fuel a more rapid development of enzymatic synthetic processes and a wider adoption of biocatalysis on industrial scales.

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Introduction

Biocatalysis has become a state of the art technology for synthesis in the pharmaceutical, fine chemical, agriculture, food, and polymer industries. The excellent catalytic efficiencies and selectivities of enzymes offer direct and cost-effective routes to many valuable chemical products, and the ability to ferment enzymes from simple renewable feedstocks provides straightforward and scalable access to biocatalysts. New transformations and novel applications of enzymes are being reported in ever-increasing numbers, filling the chemist's toolbox with a multitude of options for employing biocatalysis [1]. These advances in enzyme discovery are complemented by recent progress in protein engineering, enabling rapid optimization of enzyme properties to fit the desired reaction and process parameters, and critically allowing the implementation of enzymatic processes with the speed required in industry (Figure 1) [2•]. As a technology that shortens synthetic sequences, lowers resource demands, and employs non-toxic catalysts, biocatalysis also meets industrial and societal demands for increasingly green and sustainable chemical manufacturing [3].

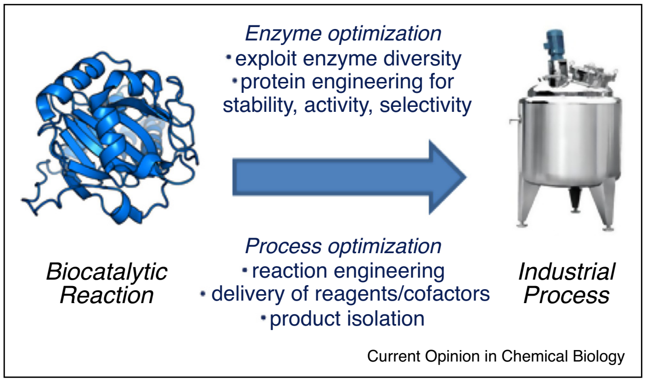
Redox enzymes, those that catalyze a reduction or oxidation of their substrate, can offer resource-efficient solutions to long-standing chemical problems. This class of enzymes includes some of the most widely employed industrial biocatalysts, as well as a range of less frequently employed—but potentially valuable—technologies. To be useful on scale, an enzymatic process must meet certain requirements: the transformation should proceed at high substrate concentrations (in general, at least 50 g/L), cofactors and other reagents must be economically supplied or regenerated, the reaction must be highly regioselective, chemoselective, and/or stereoselective, and the process must allow for straightforward and clean product isolation [4,5]. The enzyme must possess sufficient activity, stability, and kinetics under the required reaction conditions and be readily overexpressed in a common host organism. The protein is used without purification, as this would incur unacceptable costs.

Here we evaluate progress in the use of redox enzymes on preparative scale over the last two years. We focus on reports describing the preparation of at least one gram of product, and in particular on those that describe a complete process, from reaction execution to work up and product isolation. We focus on the peer-reviewed literature, as patents on biocatalysis in manufacturing have been recently reviewed [6]. We aim to highlight the broad utility of redox enzymes for industrial applications, as well as to suggest future opportunities for industrial biocatalysis.

Ketoreductases and transaminases

Ketoreductases (KREDs), which catalyze the transfer of a hydride from NAD(P)H to ketones and aldehydes, have historically been some of the most frequently employed biocatalysts on industrial scale. Their use has continued in recent years, with several reports on the preparation of chiral benzylic alcohols [7–9]. As one example, Han *et al.* employed a KRED to reduce the cyclic ketone **1** to the *trans*-configured product en route to the kinase inhibitor ipatasertib (Scheme 1a) [10]. The use of ketoreductases to prepare (*S*)-3-hydroxypiperidine, an intermediate in

Figure 1



Development of a potentially useful biocatalytic reaction into an efficient industrial process requires a combination of enzyme optimization (identification of the best existing enzyme or engineering of a new one) and process engineering (development of a robust protocol for transforming starting materials into isolated products).

the synthesis of the anticancer drug ibrutinib, has also been the subject of recent reports [11–13]. Other notable applications include the regioselective and diastereoselective reduction of a steroid diketone in the synthesis of dehydroepiandrosterone [14] and the KRED-catalyzed desymmetrization of *meso*-cyclohexanones to produce chiral 4-aminocyclohexanols [15]. In many of these examples, ketoreductases identified from commercial enzyme panels were directly translated to large-scale chemical production, without any further protein engineering required.

A particularly useful class of biocatalytic reductions occur as dynamic kinetic resolutions (DKRs), allowing two stereocenters to be set in a single reaction. Hyde *et al.* reported a ketoreductase-catalyzed dynamic kinetic resolution of γ-ketoester **2** (Scheme 1b) [16]. Starting with a KRED that was only modestly selective for producing the desired *trans*-diastereomer, directed evolution delivered the more selective enzyme KRED-264. Under conditions employing a nitrogen sweep to remove acetone and thereby limit the reverse reaction, KRED-264 generated *trans*-alcohol **3** with excellent stereoselectivity. This γ-hydroxyester was then transformed to a cyclopropane present in a GPR40 partial agonist. Another KRED-promoted DKR was demonstrated in the synthesis of vibregon (Scheme 1c) [17••]. The racemization of the desired substrate **4** required high pH and elevated temperature, under which conditions the instability of the cofactor NADPH was identified as a limiting factor. Evolution of a KRED to improve cofactor binding, and thereby protect NADPH from degradation, was successful at delivering an enzyme that is highly active and selective under the required process conditions.

Amine transaminases (ATAs), enzymes for the conversion of ketones and aldehydes to amines, are also well-established for use on preparative scale. Productive reaction conditions are widely recognized, and enzyme evolution over the past decade has significantly improved the substrate scope as well as the enzymes' tolerance to organic co-solvents and elevated temperatures. Multiple equilibrium displacement techniques have been developed to overcome unfavorable thermodynamic equilibria as well as substrate or product inhibition [18,19••].

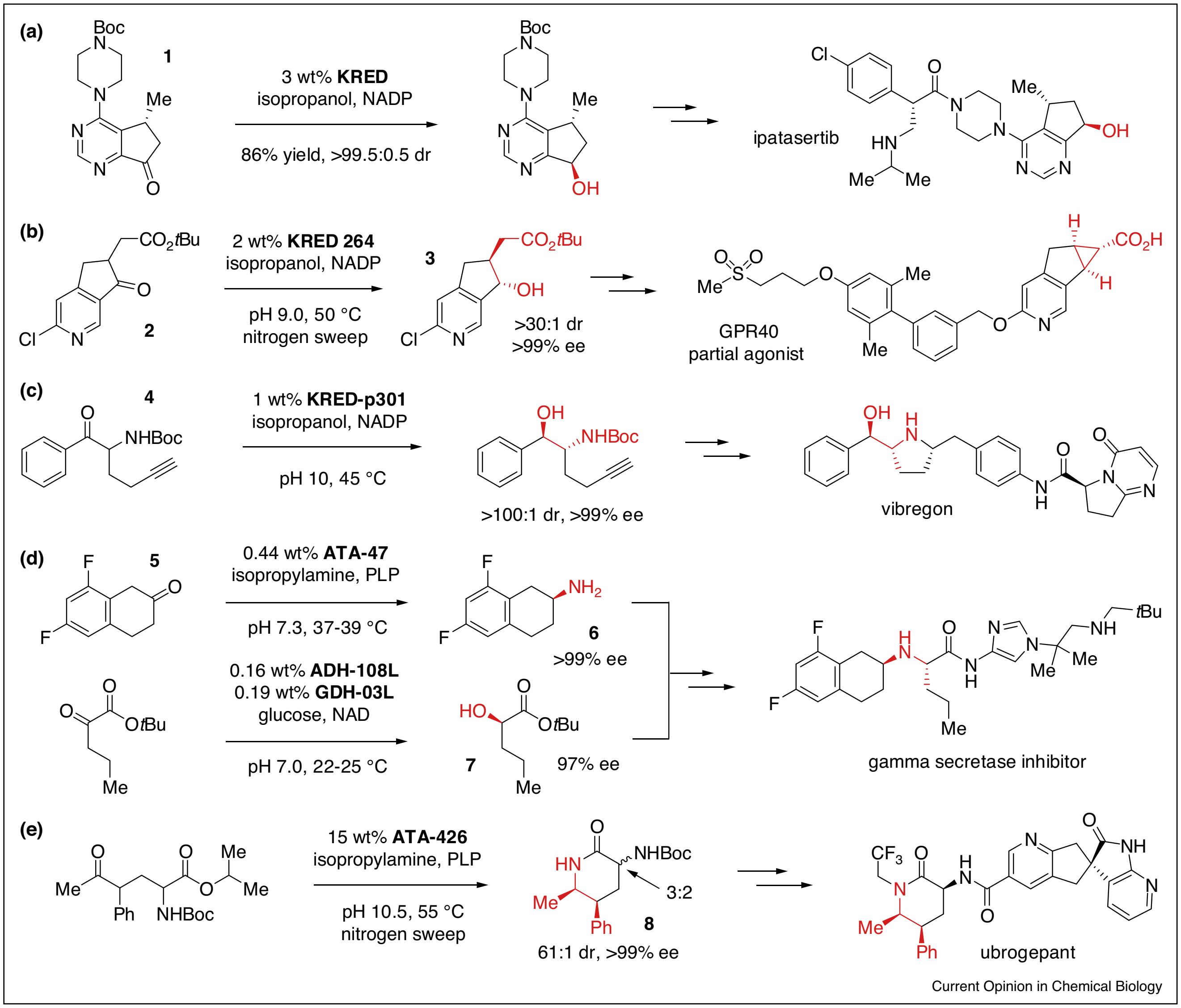
Cyclic ketones are particularly well-accepted transaminase substrates; Feng *et al.* recently developed an efficient synthesis of the building block 3-aminoazepane using a transaminase from a commercial kit without the need for enzyme evolution [20]. Similarly, another enzyme from a commercial screening panel was used to transaminate difluorinated tetralone **5** with excellent enantioselectivity and yield [21•]. The aminotetralin **6** precipitates as the phosphate salt from the reaction mixture, driving the equilibrium and enabling product isolation via simple filtration. The amine **6** was subsequently coupled with hydroxyester **7** derived from an enzymatic ketone reduction to form the key precursor to a gamma secretase inhibitor (Scheme 1d). Yasuda *et al.* have recently presented a dynamic kinetic transamination enabling the preparative synthesis of a lactam with multiple stereocenters (**8**, Scheme 1e) [22]. A single round of directed evolution improved the moderate *syn*-selectivity of the initial transaminase 9-fold. Additional protein engineering in parallel with process development enabled a practical synthesis of lactam **8** employing high DMSO levels and elevated temperature. Another class of enzymes capable of amination are the amino acid dehydrogenases (AADHs); these have been used less often on scale, but were recently employed for the synthesis of a trifluorinated phenylglycine [23].

All of the ketoreductase and transaminase chemistry described here was performed on at least multi-gram scale, and in many cases on hundreds of grams scale. In some cases the enzymes were evolved specifically for activity and selectivity on the required substrate or for tolerance of the required conditions, and in other cases pre-existing commercial enzymes were sufficient for large-scale use. From our perspective, both enzyme classes represent mature technologies that can be readily applied on manufacturing scale for the production of diverse chiral alcohols and amines, and their derivatives.

Flavin-dependent enzymes

Flavin-dependent and nicotinamide-dependent ene reductases (EREDs) selectively reduce activated C=C

Scheme 1

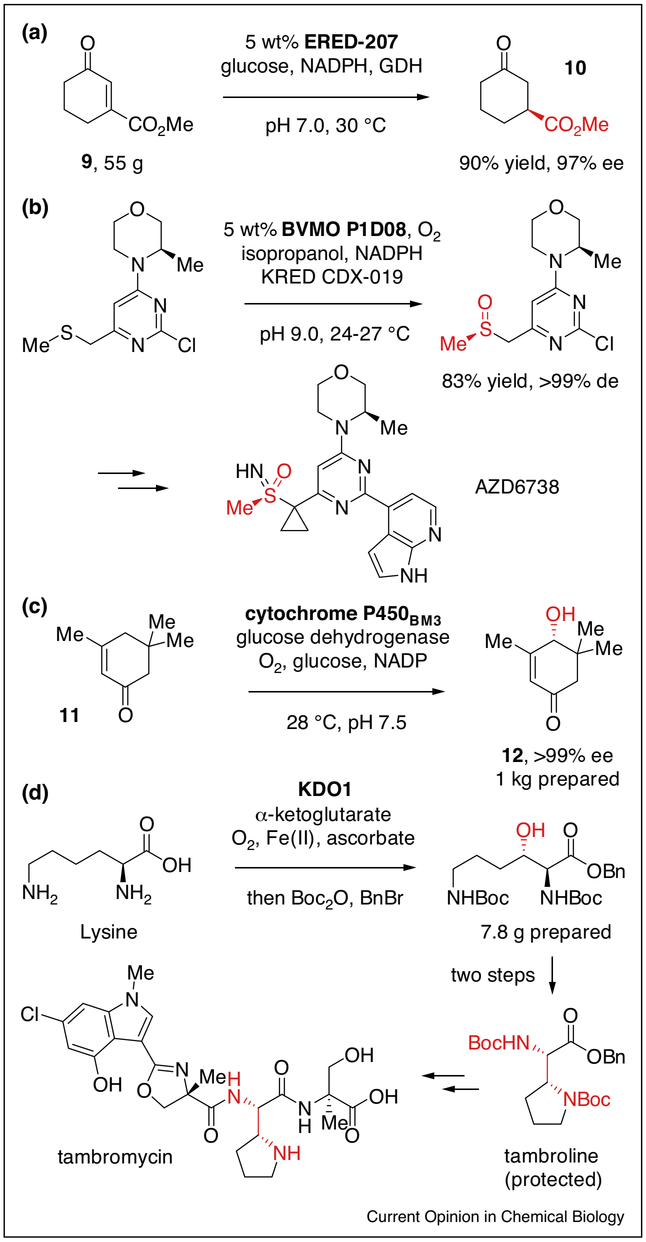


Ketoreductases and transaminases recently employed on industrial scale. **(a)** A KRED was used to install the *trans*-alcohol moiety in ipatasertib, overriding the preference of the substrate for reduction from the opposite face. **(b)** A dynamic kinetic resolution using an evolved KRED was employed in the synthetic route to a GPR40 partial agonist. **(c)** Evolution of a KRED for tighter NADPH binding enabled a DKR reaction for the preparation of vibregon. **(d)** A route for the multikilogram-scale preparation of a gamma secretase inhibitor employs both a transaminase and ketoreductase to install the chiral centers in two key intermediates. **(e)** A transaminase was evolved for the dynamic kinetic amination of an α-aryl ketone including spontaneous lactamization of the product under high pH conditions.

bonds, providing access to molecules with up to two new stereocenters [24–26]. Industrial applications, however, are rare. Gröger and Iding employed an ERED to synthesize cyclohexyl methyl ketone in quantitative yield on multi-gram scale, notably at a high substrate loading (100 g/L) in a bi-phasic system [27•]. More recently, Hadi *et al.* demonstrated a robust process for the reduction of enone **9** on >50-gram scale to give chiral β-substituted cyclohexanone **10** (Scheme 2a) [28]. The wider adoption of ene reductases in industry will be aided by the ongoing discovery of novel EREDs with improved stability and scope as well as directed evolution for the acceptance of larger and more complex substrates [26].

In addition to the above *reductive* processes, efficient *oxidative* biocatalytic transformations are highly sought-after as alternatives to existing chemical methods. The flavin-dependent Baeyer-Villiger monooxygenases (BVMOs) are a promising class of enzymes for this

Scheme 2



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Flavin-dependent and iron-dependent enzymes employed on preparative scale. **(a)** Chiral cyclohexanone building blocks were produced on multi-gram scales using ene reductases. **(b)** A Baeyer-Villiger monooxygenase was used in the synthesis of a chiral sulfoxide intermediate for the oncology candidate AZD6738. **(c)** Hydroxylation of α-isophorone was performed on large scale using cytochrome $P450_{BM3}$. **(d)** The α-ketoglutarate-dependent enzyme KDO1 was employed to access multi-gram quantities of 3-hydroxylysine. Subsequent protection and cyclization provided the amino acid tambroline, which was then converted to the natural product tambromycin.

purpose, catalyzing a number of oxidative transformations with molecular oxygen including Baeyer-Villiger oxidation, epoxidation, and sulfoxidation. In contrast to KREDs and transaminases, however, biocatalysis with BVMOs is underdeveloped on industrial scale, in part as a result of challenges presented by oxygen delivery and the formation of reactive oxidizing species.

Li *et al.* have used directed evolution approaches to adjust or invert the stereoselectivity of thermostable BVMOs, increasing their utility for asymmetric oxidations [29]. Goundry *et al.* recently showcased reaction engineering as the key to achieving a BVMO-catalyzed synthesis of a chiral sulfoxide en route to the drug candidate AZD6738 [30••] (Scheme 2b). Efficient oxygen delivery via air sparging and optimized agitation proved more important in reducing enzyme loading than any changes to co-solvent, pH, or substrate concentration, enabling enzymatic sulfoxidation on multi-kilogram scale. A BVMO-catalyzed sulfoxidation for the production of esomeprazole has been reported by Bong *et al.*; in this case, extensive protein evolution was critical to achieving high activity and stereoselectivity of the enzyme, limiting the formation of a sulfone by-product, and reducing the required loading of NADP [31••]. Process development was also required to manage the low aqueous solubilities of both substrate and product, and ensure product stability. BVMOs have also been employed on multi-gram scales in the synthesis of lactone building blocks for biodegradable polymers; slow feeding of the substrate and/or removal of the product were beneficial in some cases [32–34]. We envision that greater biochemical characterization of BVMOs, together with research in reaction engineering for oxygen mass transfer [35], will lead to a rise in practical applications of enzymatic oxidation.

Iron-dependent hydroxylases

Cytochrome P450s, while capable of a wide breadth of oxidative chemistries using molecular oxygen, have generally been very challenging to use on large scale, typically suffering from low total turnover numbers and poor stability [36]. In a remarkable recent report from Kaluzna *et al.*, the widely studied enzyme cytochrome $P450_{BM3}$ was employed to hydroxylate α-isophorone (**11**) to give 4-hydroxy-α-isophorone (**12**) on a multi-hundred gram scale (Scheme 2c) [37••]. The wild-type $P450_{BM3}$ was co-expressed with glucose dehydrogenase for turnover of the cofactor NADPH, and permeabilized whole cells were used for the biotransformation. Optimization of process conditions, in particular the mode of oxygen delivery, resulted in a process that delivered one kilogram of product from two reaction batches. Although the substrate concentration employed was relatively low (10 g/L of **11**), this work indicates that under properly designed process conditions, cytochrome P450s may be viable catalysts for large-scale chemical production.

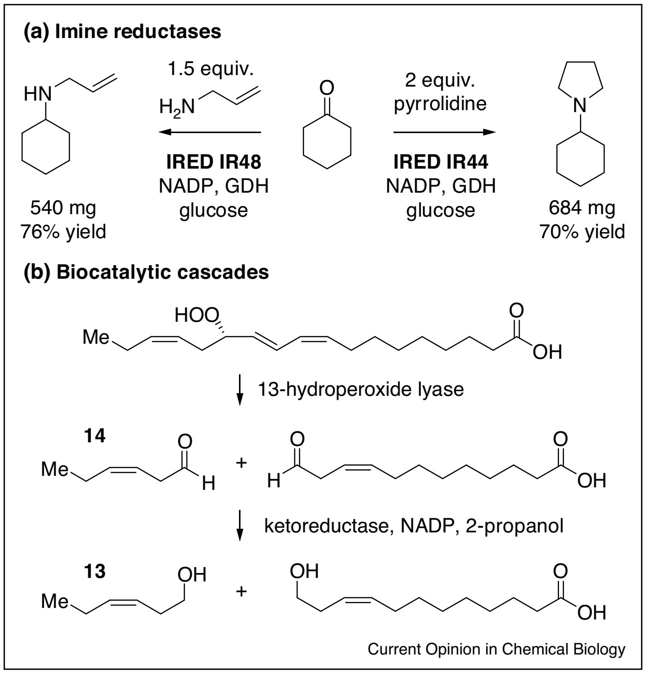
Non-heme iron-dependent hydroxylases have also been the focus of increasing research. In particular, α-ketoglutarate-dependent enzymes offer a variety of oxidative transformations requiring only oxygen and α-ketoglutarate, without the need for the reductase partners and cofactors required by P450s [38]. Renata *et al.* recently employed one such enzyme on preparative scale in the total synthesis of tambromycin [39•]. The hydroxylase KDO1 performs regioselective and stereoselective β-hydroxylation of lysine; using a clarified cell lysate preparation of the enzyme, the reaction could be readily performed on multi-gram scale (Scheme 2d). Zwick and Renata have also reported gram-scale hydroxylation of leucine by the Fe(II)/α-ketoglutarate-dependent enzyme GriE [40]. Previous studies by industrial groups on proline hydroxylases, also members of this enzyme class, have demonstrated approaches for using α-ketoglutarate-dependent enzymes on larger scales [41,42]. Further investigation will be necessary to determine if more non-heme iron oxygenases are practical for preparative use.

Future opportunities

Several additional classes of redox enzymes also have the potential for application on industrial scale. Peroxygenases, which require only peroxide as the terminal oxidant, represent a promising class of heme-dependent oxygenases that may have advantages over cytochrome P450s [43]. Given the abundance of amines in the pharmaceutical industry, enzymes for the synthesis of secondary and tertiary amines—which cannot be directly prepared using transaminases—will be especially valuable. Monoamine oxidases, while not the subject of any recent large scale reports, have been previously used in industry [44,45]. A class of enzymes with great potential are the imine reductases (IREDs), and their sub-family the reductive aminases (RedAms), which perform the coupling of carbonyls with amines using NADPH (Scheme 3a) [46,47]. Although preparative reactions have been reported only on roughly one-gram scale [48–52], their synthetic utility and relatively straightforward reaction conditions suggest they are ripe for larger-scale application.

Biocatalytic cascades, in which multiple enzymes act in the same reaction vessel to perform multiple bond formations, also represent a major opportunity for biocatalysis. Cascades have the potential to reduce the number of steps and product isolations in synthetic sequences, improving the efficiency and lowering the cost of biocatalytic manufacturing, and may even enable new chemistry by allowing reaction sequences that proceed through unstable intermediates or unfavorable equilibria [53]. To date there are few examples of applying biocatalytic cascades on scale. In one recent report, Brühlmann and Bosijokovic described the production of (*Z*)-3-hexenol (**13**) via a cascade employing an engineered hydroperoxide lyase and a ketoreductase; performing the reaction as a cascade prevents isomerization of the intermediate (*Z*)-3-hexenal (**14**) to the undesired *E*-alkene (Scheme 3b) [54••]. Additionally, Li *et al.* have constructed multi-enzyme cascades for the synthesis of α-hydroxy acids, 1,2-amino alcohols, and α-amino acids from styrenes [55•]. The cascades involve multiple redox enzymes, all starting with a styrene monooxygenase, and were demonstrated *in vivo* to synthesize up to one gram of product.

Scheme 3



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Future opportunities for industrial biocatalysis include the use of recently discovered enzyme classes and the construction of biocatalytic cascades. **(a)** Imine reductases perform the coupling of carbonyls with amines to give secondary or tertiary amines, and have been demonstrated at gram scale. **(b)** An enzymatic cascade was implemented for the synthesis of (*Z*)-3-hexenol to avoid isolation and isomerization of an aldehyde intermediate.

Conclusion

Ketoreductases and transaminases are established technologies; in both cases, the available enzymes are robust, highly active, have broad substrate scope, generally act with high selectivity, and work well with established cofactor regeneration systems, allowing for a rapid transition from a screening hit to large scale. Other classes of redox enzymes can be considered technologies under development—the diversity of known enzymes may be lacking, their suitability for preparative use may be highly substrate-specific, or best practices for performing the reactions on scale have not yet been elucidated. Certain enzyme classes (such as α-ketoglutarate-dependent hydroxylases) will benefit tremendously from the discovery of new enzymes, which is proceeding rapidly. While most applications of preparative biocatalysis to date have been in the pharmaceutical industry, recent applications in other sectors [54••] indicate that the scope of biocatalysis has great potential to grow. The increasing speed of protein engineering has also made it possible to evolve enzymes in the timeframes necessary for commercial applications, making it possible to select enzymes with poor activity or selectivity as starting points for developing new processes. Process engineering is a critical component of transitioning biocatalysis to industrial scales, as demonstrated by the optimization of oxygen delivery required to achieve efficient reactions with BVMOs and cytochrome P450s. Implementing new biocatalytic redox technologies will thus require continued close collaboration between fundamental biochemistry, protein engineering, and process engineering.

Conflict of interest statement

Nothing declared.

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