



# Practical chiral alcohol manufacture using ketoreductases

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Over the past two years the application of ketoreductases in the commercial synthesis of chiral alcohols has undergone a revolution. Biocatalysts are now often the preferred catalyst for the synthesis of chiral alcohols via ketone reduction and are displacing reagents and chemocatalysts that only recently were considered break-through process solutions themselves. Tailor-made enzymes can now be generated from advanced, non-natural variants using HTP screening and modern molecular biology techniques. At the same time, global economic and environmental pressures direct industrial process development toward versatile platforms that can be applied to the different stages of product development. We will discuss the technologies that have emerged over the past years that have guided biocatalysis from the bottom of the toolbox, to the power tool of choice.

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## Current Opinion in Chemical Biology 2010, 14:122–129

This review comes from a themed issue on  
Biocatalysis and Biotransformation  
Edited by Roger Sheldon and Jean-Louis Reymond

Available online 12th January 2010

1367-5931/\$ – see front matter

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DOI 10.1016/j.cbpa.2009.12.003

Ketoreductases (KREDs) or carbonyl reductases are ubiquitous in nature and new members of this family are identified in the growing number of genome sequences that are becoming available. At the same time, advanced enzyme engineering technologies have provided many KREDs with improved and expanded performance characteristics. Matsuda *et al.* recently published an exhaustive review with 180 references on the use of KREDs, covering mechanism, substrate range and much more [1<sup>\*</sup>]. Scientists from the fine chemical [2] and the pharmaceutical industry [3] have provided review articles exulting the virtues of biocatalysis in general and KREDs in particular [4,5] as tools for synthesizing compounds of pharmaceutical interest. KREDs ('Group 4'/carbon-based catalysts) can now be considered fully complementary for large-scale industrial pharmaceutical manufacturing, in terms of both cost and operation, to the traditional 'Group 3' (e.g. boron-

based reagents/catalysts) and 'Group 5' (phosphine ligands) options in the chemists' toolbox.

For practical applications biocatalysts need to be as cost-effective, robust, safe, and environmentally friendly as traditional methods. Pollard and Woodley [6] provided an excellent review outlining the need for readily available biocatalyst early in the development of a pharmaceutical candidate and high-performance biocatalyst at the late stages of development where cost is an important factor. Access to readily available catalysts in early development was often accomplished via the generation of kits of microbes [7], followed by using the most active hit from such screens for manufacturing initial quantities of compound. These biocatalysts were often of insufficient quality for large-scale manufacture and this led to a common belief that biocatalysts were inferior to other synthetic options despite early success. However, recent developments in the biocatalysis field have transitioned trial-and-error approaches into approaches based on result-oriented, engineering principles [8<sup>\*\*</sup>]. As the pharmaceutical industry is adapting to increased globalization and commoditization, new business models are emerging and with them the offering of new process technologies such as HTP experimentation for selection of (bio)catalysts [9<sup>\*</sup>]. Modern biocatalysis fits very well in the new business models for chemical process development.

## Enzymes out of their natural environment

Enzymes in nature have evolved over millions of years to optimally support *the fitness of the host*. To optimally support *the processes they catalyze*, industrial enzymes need to be tolerant to substrate and product concentrations that are often more than 100-fold higher than within the natural host [10]. Commercial chemical processes are characterized by their high volumetric productivity. Product concentrations of at least 10% (w/v), corresponding to concentrations in excess of 500 mM, are the norm. Because pharmaceutical products tend to be hydrophobic in nature, the solubility of the substrate is often low, adding challenges to the enzyme to cope with: tolerance to solvents that are needed to increase substrate solubility, tolerance to increased temperatures needed to increase substrate solubility, a high substrate affinity, and combinations thereof. Besides being tolerant to the reaction conditions, it is required that the enzyme is tolerant to the conditions of enzyme manufacture, formulation, transportation, and storage as well. To adapt enzymes to this diverse set of non-natural conditions, advanced enzyme engineering technologies have been applied.

Table 1

Considerations for cofactor recycling					
Enzyme (technology)	Reductant	Product	Advantages	Disadvantages	Scalability
Whole cells	Glucose (typically)	Gluconic acid	Irreversible reaction. One catalyst system. Applicable for both NADH-dependent and NADPH-dependent enzymes.	Potential for by-products from endogenous enzymes. Whole cell catalyst often sensitive to reaction conditions.	Proven.
Glucose dehydrogenase	Glucose	Gluconic acid	Irreversible reaction. Highly active. Applicable for both NADH-dependent and NADPH-dependent enzymes.	Causes pH of reaction to drop, requiring pH control system. Needs significant aqueous phase. Water-soluble substrate and product may impact solubility of desired substrate. Makes process a two-enzyme process.	Proven.
Formate dehydrogenase	Sodium formate	CO <sub>2</sub>	Irreversible reaction. Volatile product simplifies work-up.	Low activity and stability. Works best for NAD-dependent KREDs although mutants known that work with NADP. Makes process a two-enzyme process.	Proven.
KRED	Isopropanol	Acetone	Same enzyme can be used for desired conversion and cofactor regeneration. Often a good substrate for KREDs. Can be used for both NADH-dependent and NADPH-dependent enzymes. Robust enzymes available.	May require acetone removal to shift equilibrium toward desired product.	Proven
Phosphite dehydrogenase	Sodium phosphite	Sodium phosphate	Irreversible reaction. Mutant works with either NAD <sup>+</sup> and NADP <sup>+</sup> . Decent activity.	High ionic strength reaction medium. High phosphate waste stream. Phosphate destabilizes cofactor at low pH. Makes process a two-enzyme process.	Not demonstrated
Hydrogenase	H <sub>2</sub>		Irreversible reaction. Simple reagents.	Hazardous. Makes process a two-enzyme process.	Not demonstrated
Electrochemical	Rhodium complex		Clean, sustainable	Low productivity. Insufficient stability.	Not demonstrated
Photochemical	Photosynthetic microorganism		Sustainable	Low productivity.	Not demonstrated

Practical KRED processes require efficient cofactor regeneration systems to recycle this expensive cosubstrate [1<sup>•</sup>]. Reductions that run at 1 M substrate concentrations produce (or consume) similar concentrations of recycling substrate/product and can cause a significant change in the ionic strength of the reaction medium. Such changes in ionic strength can have a potentially destructive effect on the activity of the reductases enzyme as well as the cofactor regenerating enzyme and may also impact substrate solubility. The various options for cofactor recycling need to be considered in the context of the

desired process (Table 1). The intricacies of developing a scaleable KRED process are exemplified for the preparative synthesis of a chiral intermediate for a chemokine receptor inhibitor [12<sup>••</sup>]. After screening a panel of (semi)-purified enzymes [11] a specific recombinant KRED was identified for further process development. Fine tuning the process to minimize cost, by identifying process conditions (pH, temperature, nature of the base for pH control, and mixing speed) resulted in a high yielding process that was used to manufacture 80 kg of the chiral intermediate in >99% e.e. [12<sup>••</sup>] (Table 2, entry A).

Table 2

## KRED reactions described in the recent literature

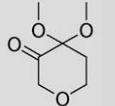
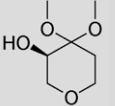
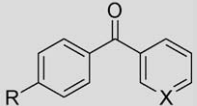
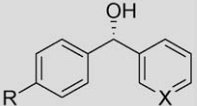
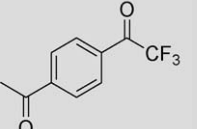
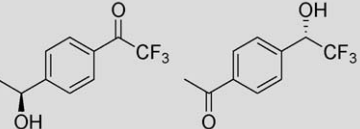
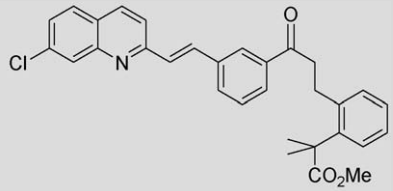
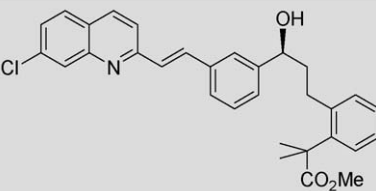
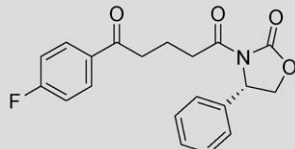
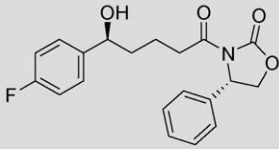
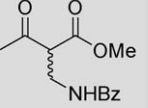
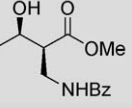



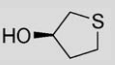
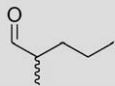
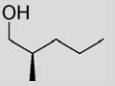
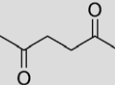
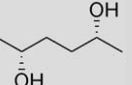
Entry	Substrate	Product	Comments
A			Description of process development from catalyst identification to scale-up.
B			Both enantiomers produced in high e.e. with a variety of KREDs.
C			Regiospecific KREDs produce a variety of enantiomers in high e.e.
D			A chiral montelukast intermediate was produced using an engineered KRED giving the product in >99% e.e. at a substrate loading of 100 g/L.
E			A chiral precursor to ezetimibe was produced in >99% e.e. using an engineered KRED.
F			A highly enantioselective and enantiospecific KRED was developed given the desired diastereomer in >99% d.e.
G			An enantioselective KRED was identified to manufacture this small chiral molecule in high e.e.
H			A KRED was engineered to convert this almost symmetrical substrate to product of >99% enantiopurity.
I			An efficient resolution of 2-methylpentanal was accomplished using an engineered KRED.
J			The desired diastereomer was produced in a continuous process.

Table 3

## The guiding principles to optimize enzymes for large-scale application

Guiding principle	Components
Fitness function	<p>The enzyme optimization objective is expressed via definition of the desired process, for example &gt;95% conversion of substrate to product having &gt;99.5% e.e. in a 24-hour reaction with [Substrate] &gt;100 g/L, a substrate-to-enzyme ratio of &gt;50, and an [NADP] &lt;0.5 g/L.</p> <p>The fitness function of individual genes is evaluated via HTP screening techniques where the enzyme variants expressed from the individual genes are tested under conditions that represent the desired process as closely as possible. Analytical methods used for such evaluations range from colorimetric assays to more involving methods such as HPLC, GC, MS, and CE.</p> <p>It may be that either substrate or product is unstable under typical reaction conditions in which case the optimization objective may have additional requirements for a specific pH and <i>T</i> range, or the inclusion of organic solvents.</p>
Diversity generation	<p>A pool of possible amino acid changes that may lead to improved function of the enzyme under study is required. This pool is obtained by applying methods to mutate a gene encoding the starting enzyme thereby generating variants that may be improved for the desired function or may contain beneficial mutations for the desired process. Methods include random mutagenesis, site-directed mutagenesis, and site saturation mutagenesis.</p>
Search algorithm	<p>The methods used to efficiently sift through the available amino acid diversity to identifying variants with improved function:</p> <p>Iterative EP-PCR, DNA shuffling via single gene, family, semisynthetic and synthetic approaches [10], amplified by bioinformatics tools such as ProSAR [38].</p>

### Engineering enzymes toward function in chemical processes

Advanced enzyme engineering technologies are used to generate enzyme variants that are optimized to the reaction conditions of a chemical manufacturing process. As discussed above, the reaction conditions in a chemical process are not constant over time and engineering of enzymes for large-scale application is a complex problem. Directed evolution encompasses modern protein engineering technologies that rely on high-throughput screening methodology as a means to direct the experiment to provide the best enzymes for the desired conditions from a pool of variants [8<sup>•</sup>,10]. The efficiency and outcome of a directed evolution project are determined by the quality of the three guiding principles, the fitness function, diversity generation, and the search algorithm (Table 3). Fundamental to these guiding principles are chemical process development, molecular biology, and HTP screening and the overarching need for scientists in the respective disciplines to work closely together and to speak a common language.

### Recent developments in KRED engineering

Ketone reduction processes can be engineered via a variety of means, including changing the nature of the catalyst, improving the catalyst, and changing the process itself, by either reaction engineering or overall process engineering. In this section we will describe the identification of new KREDs, improvement of known KREDs, identification of KRED/process combinations (e.g. solvents), as well as developments with continuous KRED processes.

Extremophilic microbes have been considered as useful sources for industrial enzymes. Indeed, the exploration of thermophilic KREDs has demonstrated that these

enzymes are highly tolerant to temperatures as high as 70°C and this property may relate to improved tolerance to solvents as well. Enzymes were identified and isolated from organism from *Picrophilus torridus* [13], *Thermus thermophilus* [14], *Pyrococcus furiosus* [15,16]. From these studies it appears that thermostability and solvent tolerance correlate and these enzymes may provide benefits for the processes that run under very demanding conditions. A solvent tolerant KRED was identified in a microbe isolated from a chemically polluted site and is quite tolerant to 50% DMSO [17]. Unfortunately, the substrate loading used in these studies is generally low and it is difficult to assess how well these desired properties translate to real life process in chemical manufacturing. ADH from *Rhodococcus ruber* on the other hand, functions well in micro-aqueous organic systems with up to 99% organic solvents and up to 2 M substrate suggesting that it may be useful at larger scale [18].

A number of recent reports have described the optimization of several KRED characteristics via directed evolution, such as thermostability, enantioselectivity, and cofactor specificity [19,20<sup>•</sup>,21]. Techniques used included rational mutagenesis based on substrate docking studies, as well as random mutagenesis and combining mutations using traditional molecular biology tools. Although the reported findings underscore the ability to improve KRED characteristics in general, they do not necessarily provide practical advances in engineering KREDs to enable commercially relevant processes in terms of volumetric productivity.

Enzymatic manufacture of a large number of chiral alcohols has been described in the recent literature and these synthons are now available as building blocks for drug discovery efforts (Table 2). Truppo *et al.* demonstrated

the synthesis of a range of diarylmethanols (Table 2B) via the reduction of substituted benzophenone and benzoylpyridine derivatives with a range of commercially available KREDs. The chiral alcohols were obtained with high yield (>90%) and enantiomeric excess (up to >99%) in reactions with 25 g/L ketone substrate. Various substituents at the *ortho*, *meta*, and *para* substitutions were accepted and the selectivity was not dependent on substitution at the *ortho* position, and/or highly electronically asymmetric molecules as is the case with conventional chemical catalyst reductions [22]. In studies using the KRED from *Sporobolomyces salmonicolor* the range of products was further expanded and their chiral purity studied by using site-directed mutants of the natural enzymes [23]. The reactions performed in these studies were run at a relatively low substrate loading of ~2 g/L and substrate-to-catalyst ratios varying from 1 to 10, but the work demonstrates that these chiral molecules are now readily available for medicinal chemistry studies and early clinical trials, while their manufacture can be further optimized using enzyme engineering technology for late stage trials and commercial manufacture. Grau *et al.* have shown that KREDs can reduce *p*-trifluoroacetyl-acetophenones and *m*-trifluoroacetyl-acetophenones regioselectively and stereoselectively providing either chiral methyl hydroxyketones, chiral trifluoromethyl hydroxyketones, or the chiral diols (Table 2C) [24].

Montelukast and ezetimibe are two large volume pharmaceutical products that involve a chiral alcohol intermediate. Scientists at Merck recognized that a bioreduction of a ketone could provide an important chiral intermediate for montelukast, an anti-asthmatic compound marketed as Singulair® (Table 2D). After screening 80 randomly chosen strains *Microbacterium campuquemaensis* and *Mucor hiemalis* cultures showed activity for the conversion of interest and provided the desired (*S*)-hydroxy ester at a titer of 500 mg/L in >95% e.e. after a 280-hour reaction [25]. The poor solubility of the ketone substrate in water was regarded as a limiting factor for an enzymatic process and with a volumetric productivity of <2 mg/L.hr this process was not commercially viable. The currently employed process uses a superstoichiometric amount (1.8 equiv.) of hazardous (-)-DIP-Cl in THF at -25°C.

Despite this unfavorable precedent, a biocatalytic process was recently enabled by KRED engineering [26,27]. A non-natural KRED was identified and optimized to manufacture the (*S*)-hydroxy ester with >99.9% e.e. at a volumetric productivity of >4 g/L.hr, thereby circumventing the use of toxic reagents and hazardous conditions. This >1000-fold improvement is not only because of the improved catalytic proficiency of the enzyme, but also a result of robustness to high organics content (40% isopropylalcohol, 10% toluene), thermal robustness, reactivity at low dissolved substrate concen-

tration and relief of competitive inhibition by acetone. Under these reaction conditions, the ketone substrate is still poorly soluble (<10 mg/L), but given that conversion of 100 g/L is obtained in a 24-hour reaction, the solubility is not the limiting factor for an enzymatic process, but the rate of solubilization is. While substrate solubilization is a physical phenomenon that cannot be improved by enzyme engineering, the enzyme was optimized for improved function (fitness) under conditions where solubilization was sufficient to provide the desired rate of reaction.

In search of an efficient process for manufacturing ezetimibe, an active ingredient in Zetia®, biotransformation was explored as a route to introduce the chiral alcohol moiety [28] (Table 2E). The economics of these processes were unfavorable however in comparison with the chemocatalytic reduction of the oxazolidinone ketone that uses stoichiometric BH<sub>3</sub>-THF with the chiral catalyst (*R*)-MeCBS. Applying directed evolution technologies a KRED for the large-scale reduction of the oxazolidinone ketone was recently described (J Grate, Presentation at Green Chemistry and Engineering Conference, Washington, DC June 29, 2007). Glucose is used to provide the reducing equivalents in this process that also uses a previously evolved glucose dehydrogenase. In contrast to the chemocatalytic process, the chemical and chiral purity of the product is excellent and the need for hazardous and toxic MeCBS/BH<sub>3</sub> circumvented.

*AOSA for penem antibiotics.* Takasago's dynamic kinetic resolution with asymmetric ketone reduction for the diastereomeric alcohol for carbapenem antibiotics uses a Ru-BINAP catalyst under 100 atm H<sub>2</sub> (Table 2F). Unfortunately, the use of methylene chloride as solvent is required to achieve even imperfect 96:4 syn:anti stereospecificity. KRED activity for this ketone reduction was recently identified by screening a set of non-natural KREDs [29]. While the best enzyme for the desired diastereomer gave perfect stereoselectivity for the 3*R*-alcohol, it needed ~1000-fold activity improvement as well as improved stereospecificity for the 2*S*-enantiomer of the racemizing substrate to enable the process performance targets. Using the sequence-activity and sequence-stereospecificity data, a small semisynthetic library recombining the beneficial mutations was generated. It provided variants with perfected stereospecificity, diastereoselectivity, and activity exceeding the original design target.

Small chiral molecules are often more difficult to manufacture than larger ones where the carbonyl substituents are significantly different in size and electronics. 2-Butanol and 3-hydroxytetrahydrothiophene exemplify products where the enantioselectivity of the catalysts is really challenged. US7371903 describes the use of a KRED from *C. parapsilosis* to produce (*S*)-2-butanol

(Table 2G) at 10 kg scale in 98.4% e.e. [30]. WO2009029554 describes enzymes generated from a *Lactobacillus kefir* KRED to provide (*R*)-3-hydroxythiolane (Table 2H) in high stereopurity. The wild-type enzyme provided the desired enantiomer in 65% e.e., but after iterative rounds of optimization, variants with improved enantioselectivity were obtained that provided (*R*)-3-hydroxythiolane in >99 e.e. [31,32<sup>••</sup>]. Similarly, resolution of chiral primary aldehydes is difficult. Several biocatalytic routes have been pursued for the manufacture of (*R*)-2-methylpentanol (Table 2I) an intermediate for pharmaceutical products as well as liquid crystals. A highly enantiospecific KRED was engineered from a natural enzyme that gave the (*R*)-enantiomer in 85% e.e. ( $E \sim 20$ ) [33<sup>••</sup>]. After three rounds of enzyme optimization a KRED variant was identified that provide (*R*)-2-methylpentanol in >98% e.e. ( $E > 200$ ) at a substrate loading of 220 g/L.

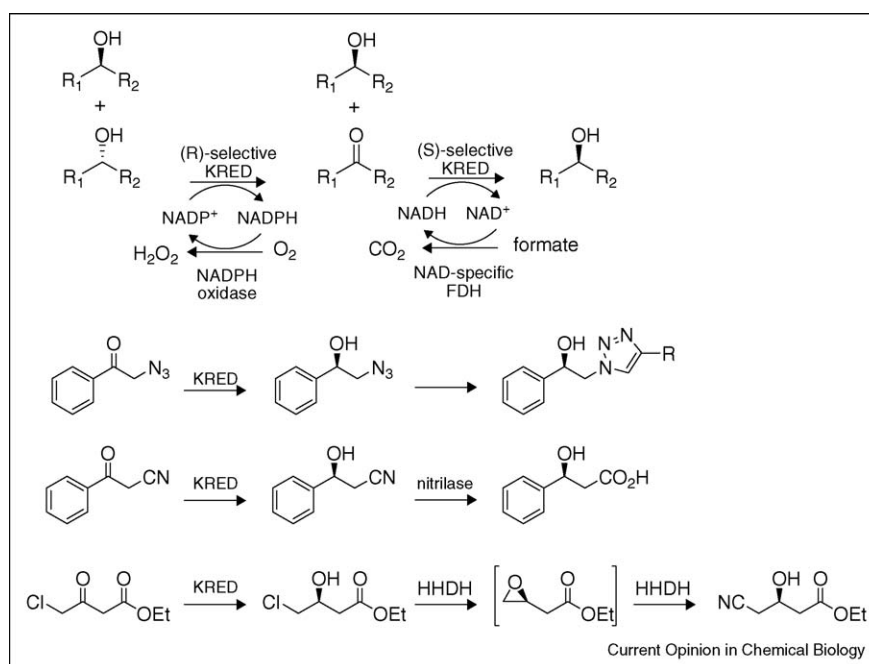
### Recent developments in KRED process engineering

Enzymatic ketone reduction processes can be run batchwise or in continuous processes. In a 10 ml packed bed reactor, 1 g of dry *E. coli* cells containing a recombinantly expressed ADH from *Leifsonia* produced 250 g of 99% e.e. (*R*)-1,3-butanediol over 500 hours using 2-propanol for cofactor regeneration [34]. Further improvements in the process have been made by changing the *E. coli* host and it is to be expected that space-time yields of this processes will exceed the 3.6 g/L.day in the near future.

Reduction of methyl acetoacetate (MAA) to methyl (*R*)-3-hydroxybutyrate (MRHB) using 2-propanol for cofactor recycling was achieved in a cell retention system with a maximum space-time yield of about 700 g/L day [35]. This one-enzyme-two-substrate process outperformed other catalytic configurations where the cofactor was recycled using GDH-based or FDH-based systems. The recombinant *E. coli* cells harboring a KRED from *Lactobacillus brevis* were retained in the reactor by employing an ultrafiltration membrane. Building on this concept, Schroer and Luetz developed a process for the reduction of 2,5-hexanedione to (*R,R*)-2,5-hexanediol (Table 2J), a precursor for certain chemocatalysts, that utilized both an ultrafiltration membrane for cell retention and a pervaporation membrane for the removal of acetone thereby moving the thermodynamic equilibrium toward the product [36]. In contrast to the MRHB process, the equilibrium constant for this process is  $\sim 0.1$  requiring product removal to attain relevant conversions.

KREDs can easily be combined with other enzymes for the synthesis of interesting compounds (Figure 1). Two KREDs can be used for the deracemization of racemic alcohols [37<sup>••</sup>]. KRED-catalyzed reduction of 2-azide-1-arylethanones provides chirally pure intermediates that can be further converted to triazole-containing  $\beta$ -blocker antagonists [38]. Such intermediates can also be generated by KRED-catalyzed reduction of chloroketones followed by regioselective, halohydrin dehalogenase-catalyzed synthesis of cyanohydrins or azidohydrins. Such a

Figure 1



Recent examples of concerted chemoenzymatic processes evolving KREDs.

process has been enabled for the manufacture of the key chiral precursor to atorvastatin [39] which was enabled by an optimized search algorithm process, ProSAR [40]. Cyanohydrins formed by KRED-catalyzed reduction of cyanoketones or via HHDH-catalyzed chemistry are also substrates for nitrile converting enzymes such as nitrilases and nitrile hydratases [41].

## Conclusion

Given the complexity of pharmaceuticals, KRED technology combined with state-of-the-art enzyme engineering provides tremendous opportunity for developing efficient, scalable routes for drug manufacturing. The combination of such enzyme engineering technology with reactor engineering is expected to provide dramatic opportunity for the enablement of highly efficient, green manufacturing processes. Indeed, all previous notions about the limitations of enzymes for large-scale use have now been disposed off as a result of new enzyme engineering technologies. HC Brown, EJ Corey, and R Noyori were awarded Nobel prizes for developing a broad range of technologies that have found application in the manufacture of some of the pharmaceutical compounds described here; the momentum that Pim Stemmer's seminal paper [42] on molecular breeding provided has now resulted in new methodologies for chemical manufacture that are not only efficient and robust, they also provide safety advantages for plant operators and environmental benefits for society.

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Papers of particular interest, published within the period of review, have been highlighted as:

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  - of outstanding interest
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