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Highly engineered biocatalysts for efficient small molecule pharmaceutical synthesis

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Technologies for the engineering of biocatalysts for efficient synthesis of pharmaceutical targets have advanced dramatically over the last few years. Integration of computational methods for structural modeling, combined with high through put methods for expression and screening of biocatalysts and algorithms for mining experimental data, have allowed the creation of highly engineered biocatalysts for the efficient synthesis of pharmaceuticals. Methods for the synthesis of chiral alcohols and amines have been particularly successful, along with the creation of non-natural activities for such desirable reactions as cyclopropanation and esterification.

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In 2012, 'The Third Wave of Biocatalysis' [1] reviewed progress in engineering enzymes for the efficient synthesis of pharmaceuticals. Since that time, enzyme engineering has been accelerated by the integration of newly developed technologies for rational design and directed evolution. Creation of new enzymes with novel catalytic function, rare in the recent past, is now the norm. It is uncommon for wild-type enzymes to be used in the synthesis of a pharmaceutical given the dramatic enhancements in catalytic efficiency, stereoselectivity and stability that can be had through protein engineering.

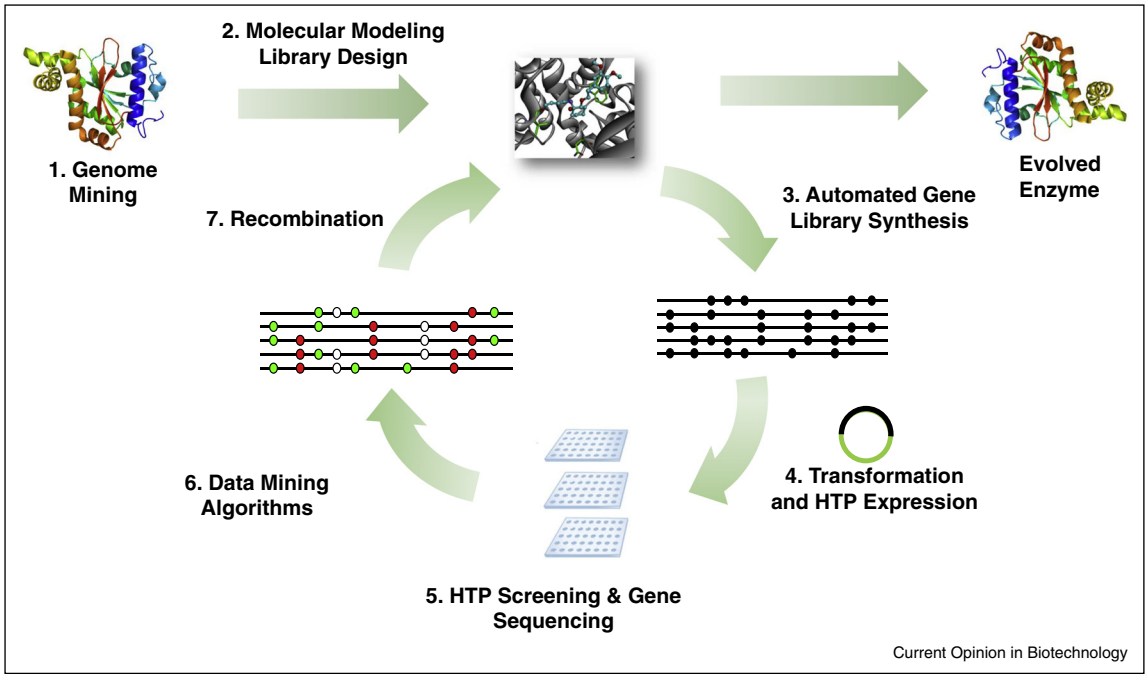
Advances in enzyme engineering

Advanced enzyme engineering technologies make possible the development of enzymes that are highly active on non-natural targets at high concentrations, in the presence of organic solvents, and even enzymes for chemical transformations not found in nature. These technologies help narrow the search of the immensity of protein sequence-activity space (estimated at $\sim 10^{11}$ for a 300 amino acid enzyme) [2] and focus on regions more likely to give improvements in enzyme activity and stability, to do so more rapidly and with much more information per experiment. The high levels of mutation needed to convert wildtype enzymes to suitable manufacturing catalysts means that simple random mutation cannot be used. Advanced engineering technologies, rather than being competitive or exclusive, can be powerfully combined (Scheme 1) to give enzymes with large changes in functional properties. Firstly, the exponential growth of genomic information provides starting points for enzyme engineering along with functional diversity from closely related homologs for recombination [3]. While we are far from the *de novo* design of enzymes [4,5], protein structural modeling, computational enzyme design [6,7] *in silico* substrate docking methods, and molecular dynamic simulation methods [8] serve to focus the design of libraries of variants. High through-put automation of enzyme test methods has been developed for activity, expression [9] protein analytics [10], and ultra high through-put selection methods [11•]. Advances in DNA technologies for low cost synthesis, PCR based library construction and microfluidics chip-based DNA sequencing methods [12], computational data analytics including machine learning algorithms [13•,14,15] substantially increase the information content/experiment. Application of these advanced engineering technologies to the creation of biocatalysts for pharmaceutical manufacturing typically starts with an integrated approach with establishment of initial catalytic activity for the reaction of interest through screening or design, followed by iterative cycles of mutation and selection to create highly stable and active enzymes with novel function.

Asymmetric ketone reduction (KREDs)

Syntheses of pharmaceutical intermediates and active pharmaceutical ingredients (APIs) have incorporated highly engineered enzymatic steps most commonly for the installation of chirality and the elaboration of scaffolds. The asymmetric reduction of ketones to chiral alcohols using engineered enzymes has been particularly successful. The efficiency and volumetric productivity of these biocatalytic routes to chiral alcohols is such that it appears that this technology has eclipsed all chemocatalytic methods for asymmetric reduction of ketones. The scope of the well-established enzyme platforms of ketone reductases has been expanded by numerous groups [16,17] and there has been broad adoption of these biocatalysts in synthetic routes to APIs. Development

Scheme 1



Integration of advanced technologies for enzyme engineering.

of a highly evolved KRED for the asymmetric reduction of a particularly challenging substrate; a near symmetrical ketothiolane [18•] for the antibiotic sulopenem is illustrative. The backbone of the KRED derived from a *Lactobacillus kefir* KRED and the closely related *L. brevis*, has since proven to be highly malleable with engineered variants providing efficient routes to several pharmaceutical targets such as S-licarbazepine [19], and montelukast [20]. Recent kinetic characterization and molecular dynamic studies on this family of enzymes provides some insight into the source of this malleability [21••]; a flexible loop encompasses one side of active site and its flexibility can be controlled through mutations in and near this loop. Similarly, Reetz has developed KREDs for the enantiospecific reduction of a near symmetrical ketooxolane by engineering the active site of the enzyme from *Thermoethanolicus brockii* [22].

Transaminases (TAs)

There has been a tremendous amount of activity in the development of enzymes for chiral amine synthesis in the last three years. While a large proportion of pharmaceutical actives contain chiral amines, most of these were synthesized using wasteful kinetic resolutions until very recently [23]. Novel biocatalytic approaches for the efficient asymmetric synthesis of chiral amines are outlined in Scheme 2. The most established of these is the use of engineered ω-transaminases [24], which catalyze the conversion of ketones to chiral amines using a donor such as isopropylamine and pyridoxidal phosphate as a cofactor as exemplified by the large scale manufacture of sitagliptin [25]. Such evolution programs can provide tens of thousands of variants as progeny for subsequent development. A highly selective transaminase for production of rivastigmine [26], for example, was derived by evolution of progeny from sitagliptin evolution.

Truppo *et al.* [27,28] have reported an improved asymmetric synthesis of vernakalant, an investigational antiarrhythmic agent, using an evolved transaminase dynamic kinetic resolution (DKR) process. Under alkaline conditions, the α-ether chiral center is rapidly equilibrating between the two enantiomers. The enzyme was evolved for selectivity for both the desired configuration at the α-ether center and the newly formed C–N bond to set both chiral centers in a single step. A DKR approach was also used by Wong *et al.* at Pfizer [29] using an evolved transaminase to enable a concise five step synthesis of an investigational leukemia treatment **1** in which the two chiral centers are set in the transamination step with >10/1 diastereoselectivity and >99% e.e.

Asymmetric reductive amination

Transaminases, while broadly used, suffer from the need to shift the relatively flat equilibrium. The position of equilibrium is such that a high degree of conversion is only possible if the equilibrium is shifted, for example, by removal of product. Reductive amination in contrast, in