

Enzyme engineering for biocatalysis

Paul, Caroline; Hanefeld, Ulf; Hollmann, Frank; Qu, Ge; Yuan, Bo; Sun, Zhoutong

DOI

[10.1016/j.mcat.2024.113874](https://doi.org/10.1016/j.mcat.2024.113874)

Publication date

2024

Document Version

Final published version

Published in

Molecular Catalysis

Citation (APA)

Paul, C., Hanefeld, U., Hollmann, F., Qu, G., Yuan, B., & Sun, Z. (2024). Enzyme engineering for biocatalysis. *Molecular Catalysis*, 555, Article 113874. <https://doi.org/10.1016/j.mcat.2024.113874>

Important note

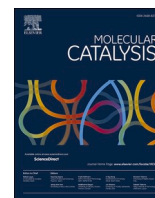
To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.



Enzyme engineering for biocatalysis

Caroline Paul^a, Ulf Hanefeld^a, Frank Hollmann^{a,*}, Ge Qu^{b,c}, Bo Yuan^{b,c}, Zhoutong Sun^{b,c}

^a Department of Biotechnology, Delft University of Technology, the Netherlands

^b Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

^c Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

ARTICLE INFO

Keywords:

Biocatalysis
Directed evolution
Semi-rational design
Iterative saturation mutagenesis

ABSTRACT

Contemporary Biocatalysis heavily relies on enzyme engineering as natural enzymes frequently lack the requisite attributes for effective organic synthesis. The inherent limitations in stability, catalytic activity, and selectivity of wild-type enzymes often hinder their suitability for chemical synthesis.

Over the past 25 years, there has been an unprecedented advancement in protein engineering tools, empowering enzymologists to customise enzymes to precisely meet the demands of organic synthesis.

In this discussion, we delineate some of the most crucial techniques in enzyme engineering and their significance in facilitating chemical synthesis.

1. From random mutagenesis to (semi)rational design - a short historical outline

Darwinian Evolution, the concept of biological evolution through variation, selection and inheritance has had a profound and lasting impact on modern biology. Also modern biotechnology is founded on *Darwinism*.

The first *in vitro* evolution experiment was performed by Mills et al. [1], evolving a dwarf RNA genome of 218 bases from originally 4500 bases (Spiegelmann's monster). The term *Directed Evolution* was used for the first time by Francis and Hansche while adjusting the pH optimum of a *Saccharomyces* phosphatase [2]. Later Eigen and Gardiner first formulated the concept of non-natural *Darwinian Evolution* by gene mutagenesis, amplification and selection [3]. Liao et al. were the first to reported two recurring mutagenesis/screening cycles (to increase the thermal stability of kanamycin nucleotidyltransferase) [4]. The dawn of Directed Evolution was enabled by the development of molecular tools such as *Oligonucleotide Directed Site-Directed Mutagenesis* [5] and the *Polymerase Chain Reaction* (PCR) [6]. The low-fidelity, error-prone variant of the PCR (epPCR) [7] has for a long time been the preferred method for diversity generation [8,9]. epPCR mimics prokaryotic evolution based on cell duplication. Stemmer introduced the eukaryotic (sexual replication) variant in form of *Gene Shuffling* [10]. Also known as molecular breeding or DNA recombination, gene shuffling is a powerful technique used in molecular biology and biotechnology to create genetic

diversity through the recombination of existing, homologous DNA sequences. Hence, it recombines 'already proven' sequences and produces structurally more relevant diversity [11]. There are mainly three gene shuffling types: (1) shuffling of a single gene; (2) shuffling of a group of mutants that are rendered from a single gene, and (3) shuffling of two or more homologous sequences from the same protein family [12]. Especially family shuffling can dramatically increase the sequence diversity of the variants. In addition to DNA shuffling, other recombination techniques, including the staggered extension process (StEP) [13] and random chimeragenesis on transient templates (RACHITT) [14] as well as iterative truncation for the creation of hybrid enzymes (ITCHY) [15] deserve to be mentioned as they are used very often in the realm of protein engineering.

Another milestone was the development of *Site Saturation Mutagenesis* (SSM) enabling the randomisation of selected sites within a protein [16–18]. The *Megaprimer method* [19] enabled the development of the so-called QuikChange™ protocol [20], which today is a standard technique in enzyme engineering.

In 1997 Reetz et al. reported the first example of directed evolution to modulate the enantioselectivity of an enzyme (lipase) [9]. By repeating the evolutionary cycle of diversity creation (epPCR) and selection (based on photometric activity testing of each mutant on both *p*-nitrophenol ester enantiomers) for four times, the power of *Directed Evolution* was impressively demonstrated. A major disadvantage of random methods for diversity generation is the sheer number of possible

* Corresponding author.

E-mail address: f.hollmann@tudelft.nl (F. Hollmann).

<https://doi.org/10.1016/j.mcat.2024.113874>

Received 28 November 2023; Received in revised form 21 January 2024; Accepted 22 January 2024

Available online 31 January 2024

2468-8231/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

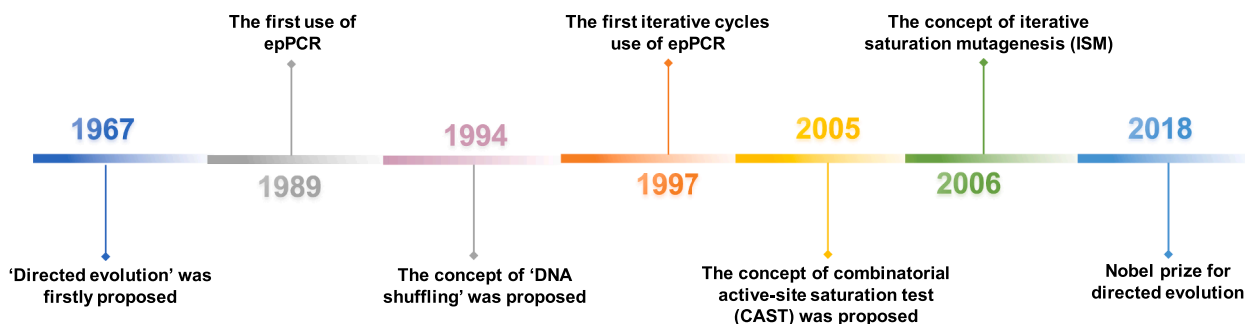


Fig. 1. Some key milestones for the development of enzyme engineering.

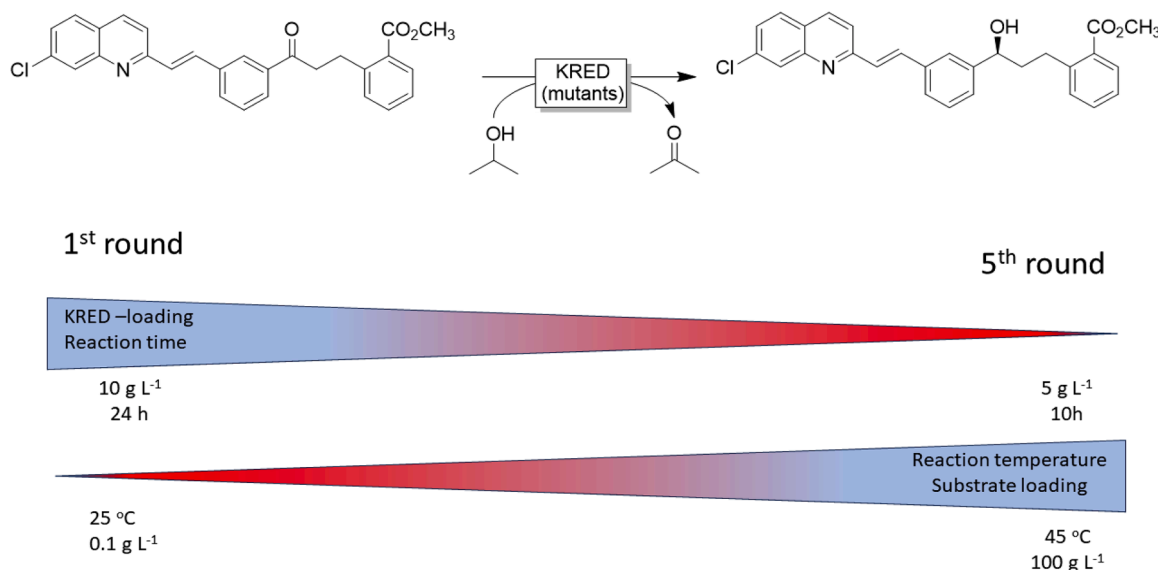


Fig. 2. Co-evolution of various desirable enzyme properties by gradual increasing the selective pressure.

mutations. To find the needle in the haystack, massive screening efforts are inevitable, which make random *Directed Evolution* experiments based on random mutagenesis expensive and cumbersome. A breakthrough occurred in 2005 when the concept of the *Combinatorial Active-Site Saturation Test* (CAST) [21] and *Iterative Saturation Mutagenesis* (ISM) [22] were developed. CAST/ISM strategies can be used in combination to improve the stereoselectivity, activity and robustness. Fig. 1 shows several key milestones for the development of enzyme engineering.

The importance of biocatalysis for a more sustainable chemical industry is unquestioned and has been reviewed in some seminal review articles [23–26]. In this contribution, we highlight the power of modern enzyme engineering to tailor natural enzymes for the needs of organic chemists. As this contribution is part of an article collection honouring Prof. Manfred T. Reetz, a particular focus has been put on his contributions to the field.

2. Enzyme engineering for stability

Enzyme stability is a crucial factor in any application, as the effectiveness of enzymes depends on their ability to maintain their structure and activity over time. Various factors are known to negatively impact enzyme stability such as thermal inactivation, inactivation by non-aqueous cosolvents or inactivation by chemical modification. Apparently, making enzymes more robust in hostile reaction conditions has been a preferred target for Enzyme Engineering from the very beginning [8,27].

2.1. Enzyme engineering for improved resistance against organic solvents

Organic cosolvents are commonly used in biocatalysis to increase the solubility of hydrophobic reactants in aqueous reaction media. Water-soluble solvents can disrupt the native structure of enzymes by altering the hydrogen bonding and hydrophobic interactions that maintain their tertiary and quaternary structures. This disruption can lead to protein denaturation, causing a loss of enzymatic activity. Next to enzyme immobilisation (vide infra) and solvent engineering (e.g. by addition of stabilising additives) enzyme engineering has emerged as tool to improve enzyme robustness against organic solvents [28].

Chen and Arnold were amongst the first ones to use enzyme engineering to improve enzyme activity and stability in organic solvents [8, 27]. After several rounds of directed evolution using epPCR to randomise subtilisin E, the authors achieved an approx. 250-fold increased activity of the enzyme in the presence of 60% (v/v) of dimethyl formamide (DMF). This strategy has also been applied successfully to other enzyme classes [28] such as laccases, [29] proteases [30], esterases [31], or P450 monooxygenases [32].

The random nature of this engineering approach, however, also necessitates screening of large libraries to cover the vast sequence space. Hence, high throughput screening assays are inevitable. As HTP experimentation can be very cumbersome and time- and resource-intensive, the interest is shifting towards more rational approaches allowing for smaller libraries. Today, our fundamental understanding of the interactions between enzyme and solvent molecules, renders rational design of more solvent-stable enzymes difficult. Martinez et al.

Table 1
Selection of available online tools for the prediction of thermal stability.

Software	Input	Availability	Refs.
I-Mutant	Sequence or structure	https://folding.biofold.org/i-mutant/i-mutant2.0.html	[46]
MAESTRO	Structure	https://pbwww.services.came.sbg.ac.at/?page_id=416	[47]
FoldX	Structure	http://foldx.embl.de/	[36]
HotSpotWizard	Sequence and structure	http://loschmidt.chemi.muni.cz/hotspotwizard	[48]
FastML	Multiple sequence alignment phylogenetic tree	http://fastml.tau.ac.il/	[49]
Ancestors	Multiple sequence alignment and phylogenetic tree	http://ancestors.bioinfo.uqam.ca/ancestorWeb/	[50]
FireProt	Sequence and structure	http://loschmidt.chemi.muni.cz/fireprot	[51, 52]
PROSS	Structure	http://pross.weizmann.ac.il	[53]
FRESCO	Structure	Stand alone	[54]

recommended reducing the surface charge in e.g. subtilisin E to increase robustness against polar organic solvents [27]. Similarly, exchanging charged surface lysines by more hydrophobic alanines, penG acylase could be stabilised against DMF [33]. Surface amino acids exhibiting hydrogen bonds with solvent molecules are also attractive targets for substitution [34]. More recently, also disulfide bridges are in focus for enzyme stabilisation. *In silico* design tools such as Modelling of Disulfide Bonds in Proteins (MODIP) [35], Modeling of Disulfide Bonds in Proteins (DbD2) and FoldX [36] can be used to design residue pairs to form disulfide bonds. Using these tools, double mutants of lipase from the thermophilic bacterium *Geobacillus stearothermophilus* T6 (LipT6) were constructed to form disulfide bonds. As a result, their stability in 70 % methanol was improved 5.5-fold [37]. Despite these success examples, rational engineering approaches for increasing solvent stability are scarce.

Semi-rational approaches are more common. A number of studies have suggested that thermal stability and stability towards organic solvents go hand in hand [38–42]. Therefore, it is not astonishing that the B-FIT method, originally developed by Reetz et al. to identify enzyme regions to target thermal stability (vide infra) [43], has also been successfully applied to engineer more solvent resistant enzyme variants [38].

When engineering an enzyme for increased stability often a trade-off with activity is observed. Partially, this can be assigned to the generally

reduced flexibility of an engineered enzyme. Therefore, it is advisable to screen libraries aiming at more stable enzyme mutants not only for stability but also for activity. This strategy has been applied frequently by the company CODEXIS to engineer more solvent stable enzymes [44]. For example, engineering an ADH for the reduction of (*E*)-methyl 2-(3-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-oxopropyl)benzoate consisted of several rounds of evolution with increasing selection pressure (by increasing the solvent concentration as well as the reaction temperature in each consecutive screening step) and selecting for most active mutants (Fig. 2) [44]. A similar approach of sequential increase of the selection pressure was also applied for the evolution of a transaminase [45].

2.2. Enzyme engineering for improved thermostability

Thermal stability represents another enzyme property frequently addressed in enzyme engineering studies. Elevated temperatures are desirable e.g. in the case of poorly water soluble reactants. Also increased self-lifetimes and /or stability under process conditions motivate for engineering more thermostable enzymes.

2.2.1. Rational design of thermostability

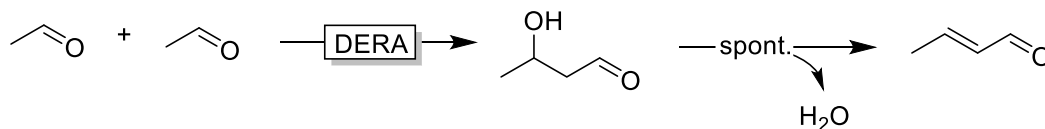
Various *in silico* methods to predict thermostability are currently available (Table 1). Each method has been demonstrated to be beneficial in at least one literature example, however a generally applicable tool remains to be developed. According to Reetz et al. [12], a practitioner should focus on those methods that have already been successfully applied to the superfamily of the enzyme in question.

There are reasons to assume that early life may have been developing under higher average temperatures than modern life faces. For example, indications exist that life developed at oceanic hot vents [55] and the assumption that Precambrian oceans were considerably hotter than today [56]. Hence, ancestral proteins may be assumed to have been intrinsically more thermostable than modern ones. Therefore, the so-called *Ancestral Sequence Reconstruction* (ASR) method has been receiving considerable attention to design thermoresistant enzymes [57, 58]. In fact, this approach has been successfully demonstrated on various enzyme classes such as alcohol dehydrogenases [59], β -lactamases [60], haloalkane dehalogenases [61], manganese peroxidases [62], photodecarboxylases [63], ene reductases [64] and many others [57].

2.2.2. Improving thermal stability using the B-FIT method

One plausible model for thermal unfolding and denaturation (their loss of their quaternary and tertiary structure) of proteins is to assume a

Crotonaldehyde formation



Combined Schiff base formation and Michael addition

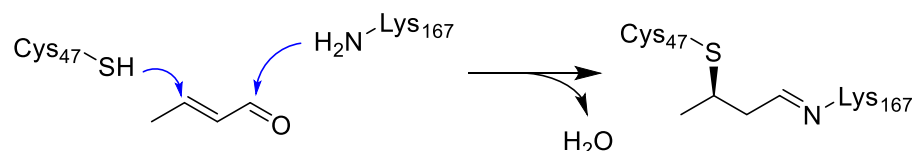


Fig. 3. Molecular basis for the acetaldehyde-related inactivation of DERA.

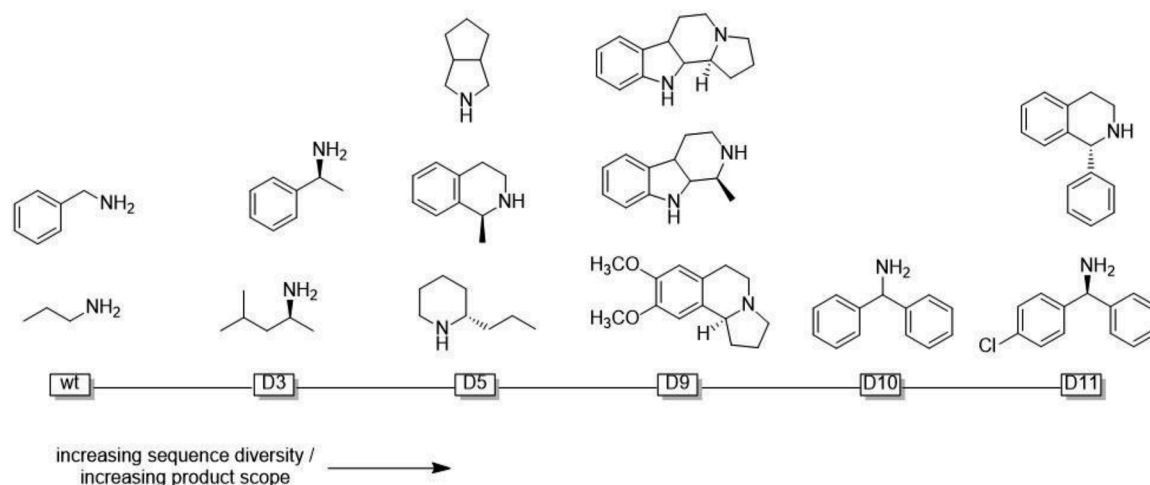


Fig. 4. The evolutionary history of monoamine oxidase (MAO). Starting from a wild-type MAO with a very narrow substrate scope, Turner et al. have evolved over the years a portfolio of synthetically highly relevant mutants.

dynamic equilibrium of fully folded and partially folded enzymes. The latter can further unfold irreversibly to the catalytically inactive, denatured enzyme. Hence, rigidifying the enzyme especially by reducing the movement of the most flexible units within an enzyme represents a promising strategy to increase thermal stability. To identify such flexible regions, Reetz et al. proposed using the so-called B-factors of enzyme X-ray data [43]. B-factors indicate the dispersion of atomic electron densities from their stable positions due to thermal motion. Hence amino acids and polypeptide regions with high B-factors are likely to be the most flexible regions within a given enzyme and therefore attractive targets for enzyme engineering. Within lipase A from *Bacillus subtilis* (Lip A) eight regions with high flexibility were identified (based on the B-factors) and subjected to iterative saturation mutagenesis (ISM, vide infra). While individual hits from the first libraries exhibited rather meagre improvements by a few degrees, their successive combination eventually resulted in mutants with exceptionally high stabilities even around boiling temperature. The B-FIT approach (B-factor iterative test) has ever since been applied to a broad range of enzymes (with B-factors obtained both, from X-ray structures and homology models) [12]. In a recent study the effectiveness of B-FIT was compared with computational methods [65] showing that B-FIT gave better results. This underlines the (still) superiority of semi-rational approaches in enzyme engineering over *in silico* methods. The future will tell if machine learning algorithms [66] will become an effective tool to engineer thermal stability.

2.2.3. Engineering enzyme stability towards reagents

Next to hostile reaction conditions also the reagents themselves can represent an issue for enzyme stability. Class I aldolases such as the synthetically very relevant deoxyribose-5-phosphate aldolase (DERA) for example utilise reactive aldehydes as starting materials. Aldehydes, however, are also highly reactive molecules, which may e.g. form imines with lysine residues and thereby modify the polarity and charge distribution of the enzyme. Provided, the modified amino acid residue plays a role in the catalytic mechanism, complete inactivation of the enzyme is not rare [67].

Dick et al. for example analysed the acetaldehyde-dependent inactivation of DERA from *Corynebacterium bovis* [68]. The authors demonstrated that crotonaldehyde (obtained from the DERA-catalysed dimerisation of acetaldehyde) cross-linked Lys167 and Cys47 accounting for the inactivation of the enzyme (Fig. 3). Mutation of the latter to a methionine residue (Cys47Met) resulted in a highly acetaldehyde-resistant DERA mutant.

3. Enzyme engineering for selectivity

Selectivity is one of the prime properties of enzymes valuable for organic synthesis. Envisioning efficient synthesis of highly added-value fine- and commodity chemicals as well as active pharmaceutical intermediates (APIs) especially stereoselectivity is desired. Naturally occurring enzymes do not necessarily exhibit sufficient (*enantio*)selectivity towards a given starting material of interest, which is why the advent of enzyme engineering undoubtedly marked a landmark in biocatalysis [24]. It was the group of Reetz pioneering enzyme engineering for selectivity [69]. In his ground breaking contribution the enantioselectivity of *Pseudomonas fluorescens* lipase A towards α -methyl carboxylic acid esters was increased from $E = 1$ (i.e. non-enantioselective) to $E = 11.3$ in four rounds of random mutagenesis (via epPCR) and selection (via UV/Vis determination of the mutants' activity on the single substrate enantiomers) [9]. In total 7600 mutant enzymes were tested in this study marking the overall bottleneck of the directed evolution approach. Therefore, work in the following years focused on the development of fast, reliable and (wherever possible) quantitative high throughput screening (HTS) assays [70]. A broad variety of analytical techniques have been evaluated for their suitability for HTS of mutant libraries comprising UV/Vis spectroscopy [71,72], capillary array electrophoresis [73], circular dichroism [74], IR spectroscopy, NMR spectroscopy [75], mass spectrometry [76–78], FACS [79] and many others [70,80].

The concept of Darwinian Evolution in the test tube to control the enantioselectivity was soon taken up by leading research groups in the field of biocatalysis. For example, Alexeeva et al. evolved monoamine oxidase from a practically useless wildtype enzyme into an industrially relevant biocatalyst (Fig. 4) [81,82].

Fong et al. successfully applied directed evolution on aldolases [83], the Bornscheuer group on hydrolases [84,85] and Peters et al. on P450 monooxygenases [86,87]. Despite these successes of directed evolution, its inherent numbers problem (random mutagenesis in a vast sequence space) cannot be satisfactorily solved. The desired mutant remained to be the proverbial needle in the haystack.

With the advent of better bioinformatic tools, advanced DNA technology, crystal structures and homology models, semi-rational approaches kick-started their success [68]. Especially structural information about amino acids residing in the enzyme active site is invaluable if targeting catalyst properties such as substrate scope, activity and selectivity. As today's computational methods are yet not reliable enough for *in silico* prediction of successful mutants, a semi rational approach focusing on the randomisation of relevant amino acid

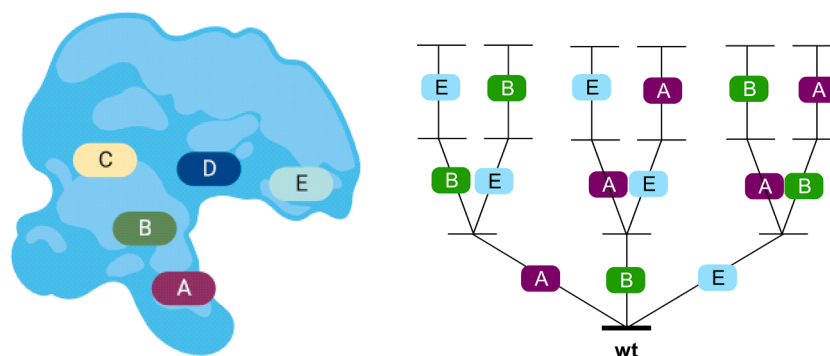


Fig. 5. General representation of the CAST/ISM strategy. As an example, amongst the relevant regions (A–E) A, B and E were selected for ISM.

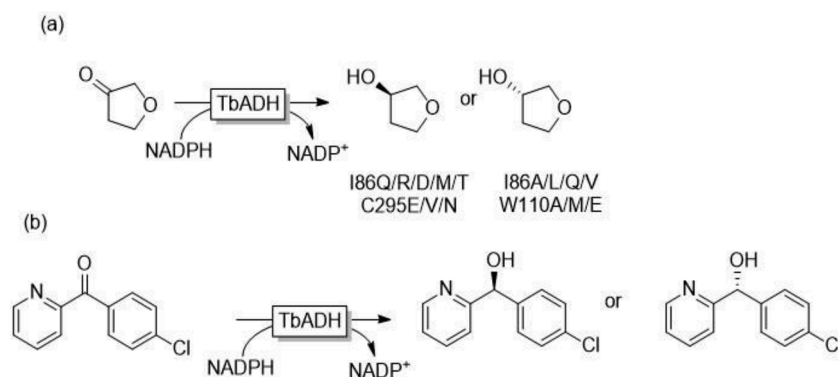


Fig. 6. Examples of 'difficult' stereoselective reductions accomplished with engineered TbADH.

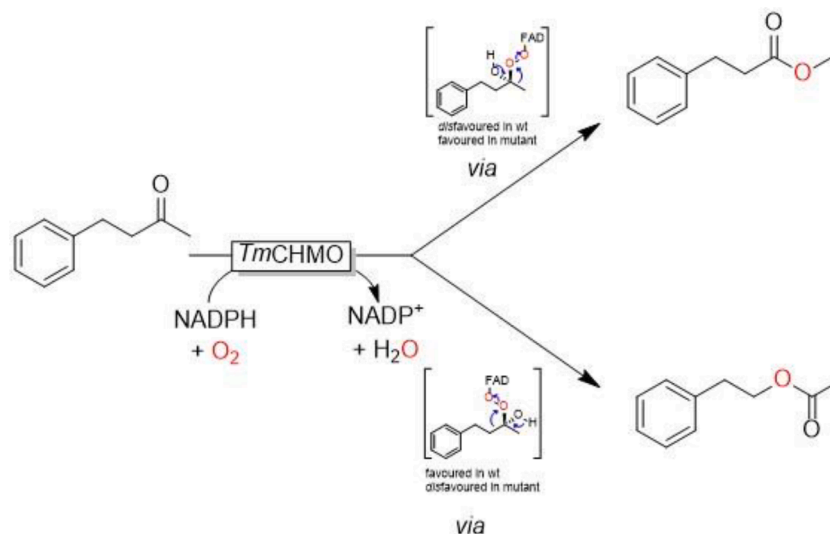


Fig. 7. Controlling the migrational tendency of carbonyl substituents in the enzymatic BV oxidation.

residues is most promising. Again, the Reetz group took a leading role here by developing the *Combinatorial Active-site Saturation Test* (CAST) (Fig. 5) [21]. In contrast to traditional saturation mutagenesis, various residues in selected regions are randomised simultaneously thereby giving access to synergistic mutations. This can be performed in various regions and the best mutants identified from each region can be combined (Fig. 5).

Iterative saturation mutagenesis methods have been successfully applied to engineer the enantioselectivity of many enzymes, and a few examples will be discussed in the following section.

Heterocyclic ketones such as dihydrofuran-3-one are notoriously difficult targets for stereoselective reduction using chemical reducing agents since the pro-*R* and pro-*S* sides are sterically and electronically very similar. Sun et al. chose the ADH from *Thermoethanolicus Brockii* (*TbADH*) as a starting point for the evolution of stereoselective enzymes for the reduction of dihydrofuran-3-one [88]. The wild-type *TbADH* exhibited modest enantioselectivity towards this substrate yielding the (*R*)-product at 23 % ee. Screening only 576 transformants, highly enantioselective mutants exhibiting *R*- and *S*-selectivity (ee > 99 % in both cases) were identified (Fig. 6a). Notably, Codexis evolved the ADH

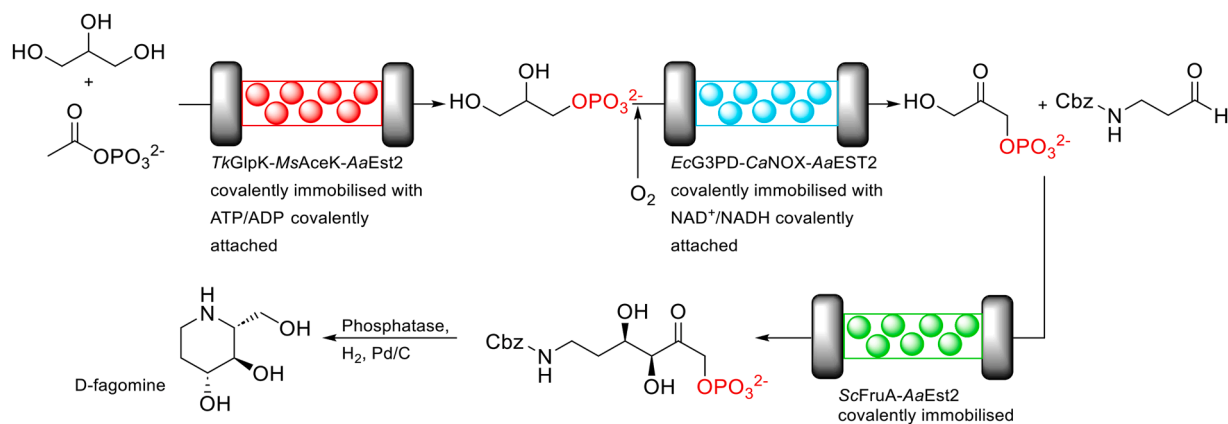


Fig. 8. Three-step continuous flow of the immobilised enzyme assemblies for the conversion of glycerol to D-fagomine.

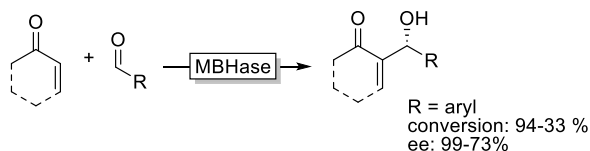


Fig. 9. Newly designed enzyme to catalyse Morita-Baylis-Hillman reactions.

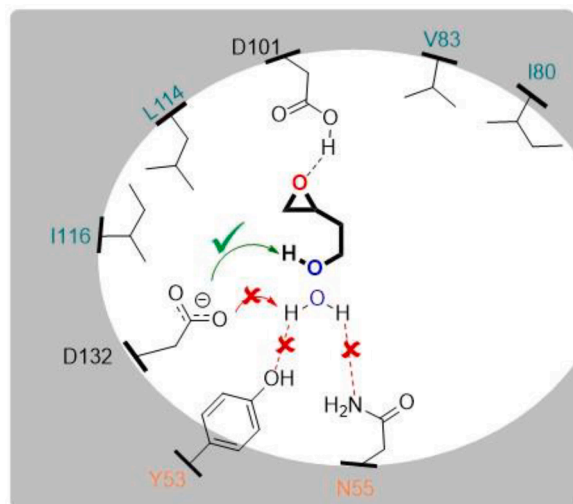


Fig. 10. Epoxide hydrolase engineered to catalyse a Baldwin-type cyclisation.

from *LactoBacillus brevis* for the stereoselective reduction of the tetrahydrothiophen-3-one homologue [89].

Often, active site amino acid residues are chosen for ISM because of their direct contact with the starting material. Modifying amino acid residues, however, also bears the risk of impairing the catalytic mechanism. An alternative approach is to target flexible loop regions near to the active site. Qu et al. therefore developed the proline-induced loop engineering test (PiLoT) to target particularly loop regions [90]. They demonstrated the potential of this strategy at the example of *TbADH*-catalysed reduction of yet another ‘difficult’ substrate (Fig. 6b) identifying P84S and Δ P84 as highly enantioselective mutants.

Another difficult reaction to control the selectivity is the Baeyer-Villiger oxidation. The regioselectivity of the O-insertion is chemically defined by the migrational tendency of the carbonyl substituents. As a rule of thumb, substituents stabilising carbocations migrate more readily and thereby control the regioselectivity of the O-insertion. The Reetz group engineered the Baeyer-Villiger monooxygenase (BVMO)

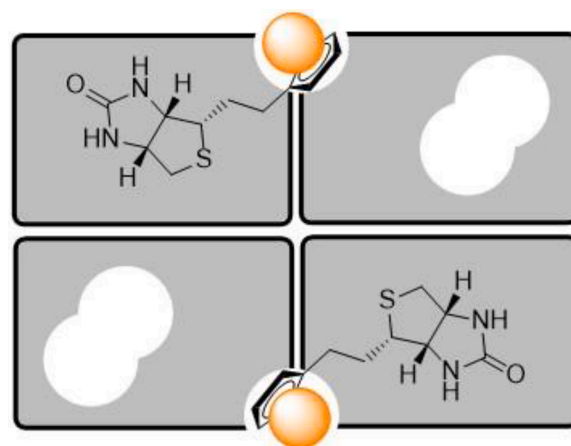


Fig. 11. Schematic representation of a metal-loaded biotin binding to streptavidin and thereby placing it in a chiral environment.

from *Thermococcus municipale* DSM 44,069 (*TmCHMO*) to override the substrate-dictated selectivity by destabilising the Criegee intermediate leading to the ‘normal’ ester (Fig. 7) [91].

4. Enzyme engineering for immobilisation

Enzymes are the work horses in industrial biotechnology, and in order to achieve efficient reusability, stability and activity, immobilisation techniques are commonly applied in the industrial processes. This has led to huge successes. Two are in particular worth mentioning, the immobilisation and recycling of PenG for hundreds of times for penicillin production [111] and the long term application of glucose isomerase in the high fructose corn syrup production, the largest enzyme catalysed process [112]. However, for immobilisations based on adsorptions or encapsulations, enzyme loss caused by leaching often raises concerns. Covalent bonding between the surface groups of the enzyme and the carriers has been a focus to improve the immobilised activity of the enzymes, but lower activities were often observed after immobilisation. This has been addressed in different manners and one of the first was to remove the lysine residues on the enzyme surface by genetic modification [113]. For two lipases it was shown that selective removal could orient the immobilised lipase such, that the active site was directed towards the reaction mixture and away from the carrier [114]. Following this line of orienting the enzyme by single attachment, tags were introduced to the enzyme. They followed the His-tag model, popular for enzyme purification. Therefore, a few engineering techniques for the enzymes to adopt better immobilisation efficiency were developed, such

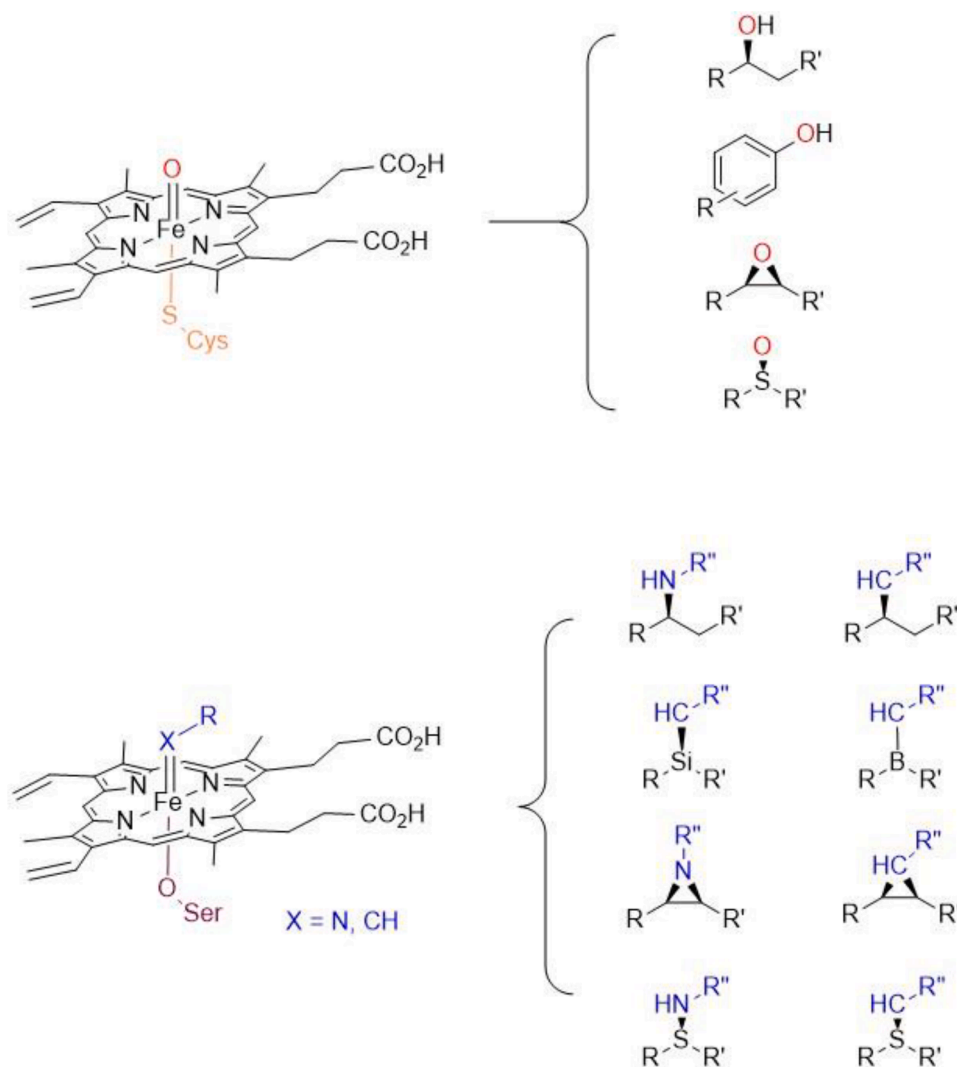


Fig. 12. Comparison of the native reactivity of P450 monooxygenases with the engineered P411 enzymes.

as the utilisation of Spy or Halo-based tags to create fused proteins, or linkers between the enzyme and the carriers were investigated.

Peschke et al. genetically fused three enzymes using high-affinity tags: the alcohol dehydrogenase *Lb*ADH, the methylglyoxal reductase Gre2p and the NADP(H) regeneration enzyme glucose dehydrogenase GDH. A 39 amino acid streptavidin-binding peptide (SBP) tag that binds with high affinity to the protein streptavidin (STV) was employed, and the other systems such as SpyTag/SpyCatcher and Halo-tag were also used. Comparative studies demonstrated that the three systems can be used for immobilisation in a flow reactor [115].

In addition to affinity tags, linkers such as peptides [116,117] or polymers [118] have been used to create hybrid catalyst with enzymes and other catalysts. An additional approach for directed immobilisation is to use a functional group in the active site of the enzyme in combination with a suicide inhibitor attached to the carrier. In this way for instance the serine in a serine hydrolase can be utilised to attach this enzyme on a carrier decorated with an irreversible serine hydrolase inhibitor. Hartley et al. reported the use of engineered enzymes with this attachment methodology and at the same time they elegantly enable the recycling of cofactors without loss of activity [119]. Three modules of three biocatalyst have been assembled with flexible linkers in between to allow movement. The approach is thus a mixture of genetic engineering and chemical modification. First the enzymes are linked by genetic modification, mimicking the polyketide synthases type 1 [120].

The modules were conversion of glycerol to DHAP via phosphorylation and oxidation steps using a *Thermococcus kodakarensis* glycerol kinase (GlpK_{Tk}) and a *Mycobacterium smegmatis* acetate kinase (AceK_{Ms}); *E. coli* glycerol-3-phosphate dehydrogenase (G3PD_{Ec}) and NADH oxidase from *Clostridium aminovalericum* (NOX_{Ca}) were utilised for the production of DHAP from glycerol -3-phosphate (G3P). Finally a cofactor-independent fructose aldolase (FruA) from *Staphylococcus carnosus* was utilised to produce the *N*-Cbz-(3*S*,4*R*)-amino-3,4-dihydroxy-2-oxyhexyl phosphate (*N*-Cbz-3*S*,4*R*-ADHOP). To these assemblies of enzymes the cofactor was chemically attached. These assemblies of enzymes were immobilised via suicide inhibition. A three-step continuous flow reactor system was designed using the single-molecule multi-enzymatic reactions and superior space-time yields and TTNs to the synthesis of *D*-fagomine were achieved compared to the chemical synthesis (Fig. 8).

5. Enzyme engineering for non-natural reactions

The repertoire of enzyme catalysis covers a broad range of synthetically useful transformations. Nevertheless, several transformations within chemical space remain out of scope for naturally occurring enzymes. However, the line between naturally occurring and new to nature is not always straightforward to draw.

The Morita-Baylis-Hillman reaction is such an example. Reetz described that proteins such as serum albumin from diverse sources can

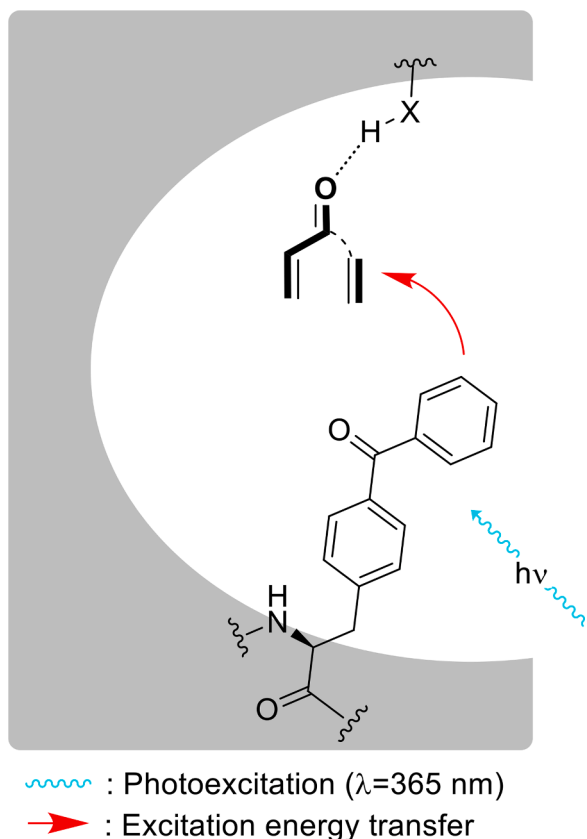


Fig. 13. Design of an artificial Diels Alderase by genetically incorporating a photosensitizer.

catalyse this reaction, while enzymes display even lower activity [121]. More recently this catalytic activity was engineered successfully into a protein and a Morita-Baylis-Hillmanases (MBHase) are today a fact (Fig. 9) [122,123].

Epoxide hydrolases catalyse the selective hydrolysis of epoxides by simultaneous activation of the oxirane moiety and the water nucleophile. Recently Sun et al. repurposed the epoxide hydrolase from *Rhodococcus erythropolis* to catalyse Baldwin-type cyclisation reactions (Fig. 10) [124]. The engineering strategy based on the dissolution of the water activation network within the active site (Y53 and N55) as well as re-positioning the catalytic base D132 to facilitate the deprotonation of the alcohol moiety. The e. r. increased from 50:50 to 90:10 for the model substrate using the mutant SZ612. The highest stereoselectivity and conversions for the N- and O-heterocycles were up to 99:1 and 99 %, respectively.

Despite the enormous successes of enzyme engineering in expanding the chemical space of biocatalysis there are still some chemical

transformations remaining out of scope for enzymes composed of the canonical amino acids and the known cofactors. Transition metal-catalysed cross-coupling reactions for example can (so far) only be performed using non-biogenic metals such as Pt or Pd. To bridge this ‘reactivity gap’, the so-called hybrid catalyst concept has been developed initially by Wilson and Whitesides [125].

The Whitesides approach consisted of chemically modifying biotin and introducing this modified chemical catalyst into biotin-binding streptavidin and thereby into the chiral environment of a protein. Later, Ward successfully developed this concept further by establishing efficient expression systems for streptavidin and engineering it (Fig. 12).

This approach proved to be very successful for a broad range of metals and the engineering of their activity and selectivity in non-natural reactions such as nitrene insertions (Ir) [126] addition to $C\equiv C$ -triple bonds [127], metathesis reactions (Ru) [128,129], $C=C$ -double bond isomerisation [130], Suzuki couplings (Pd) [131], cyclopropanations (Rh) [132] and allylic alkylation [133]. Further reactions such as carbonyl reduction (Mn) [134], imine reductions, (Ir) [135] hydroxylation reactions (Fe) [136], *cis*-dihydroxylations (Os) [137] or sulfoxidations (V) [138] have been reported as well.

Naturally, P450 monooxygenases catalyse the reductive activation of molecular oxygen to form a reactive oxyferryl species (compound I, Cpd I). The latter is responsible for the well-known oxyfunctionalisation reactions mediated by P450 monooxygenases (Fig. 11) [139–141].

Substituting the Fe central ion by other transition metals gives access to non-natural transformations using the protein scaffold as chiral ligand. Artificial Ir porphyrin-containing myoglobins are capable of carbene-type insertion reactions [142,143].

However, substituting the conserved, Fe-coordinating cysteine by a serine not only brings about a change of the spectral properties of the resulting variants (explaining their name as P411 enzymes) but, more importantly, to a dramatic change in reactivity. P411-enzymes are capable of forming Fe^{II} -centred carbenes and nitrenes catalysing C- or N-insertion (Fig. 11) [144–147]. Undoubtedly, these works have revolutionised biocatalysis opening up new, previously not known to nature transformations.

Recently, the expansion of the natural amino acid alphabet by non-canonical amino acids has opened up new avenues for enzyme engineering and biocatalysis [148]. One particularly interesting approach was reported by Trimble et al. [149]. Installation of a genetically encoded photosensitizer into the scaffold of *Methanococcus jannaschii* tyrosyl-tRNA synthetase resulted in an artificial Diels Alderase capable of [2 + 2] cycloaddition reactions (Fig. 13). Structure-guided saturation mutagenesis further improved the activity and stereoselectivity of the artificial enzyme in some intramolecular [2 + 2] cycloaddition reactions.

Expanding the reaction scope of enzymes by photoexcitation is a current research trend worth mentioning [150,151]. This approach was pioneered by Hyster who demonstrated that photoactivated NADH in alcohol dehydrogenases is capable of enantioselective, reductive dehalogenation reactions (Fig. 14) [152,153]. Later on, this approach was

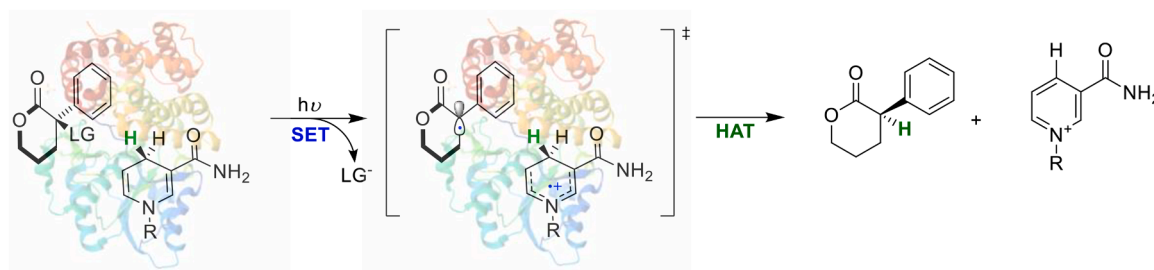


Fig. 14. The charge-transfer complex of enzyme-bound NAD(P)H and substrate can be photoexcited resulting in a single electron transfer (SET) and mesolytic cleavage of the leaving group (LG). The resonance stabilised substrate radical then selectively receives a hydrogen atom transfer (HAT) from the NAD(P)H radical cation, delivering the chiral product.

Table 2
Further studies for the applications and developments of ISM.

Entry	Enzyme class	Type of selectivity	Method applied	Comments	Refs.
1	Lipase	diastereo- & enantioselectivity	FRISM	stereodivergent study using FRISM, 4 stereoisomers were obtained with 95–99 % selectivity	[92]
2	Uridine diphosphate (UDP)-glycosyltransferases	regioselectivity	FRISM	rational design of the building blocks with <100 variants screened	[93]
3	P450BM3	catalytic mechanism	FRISM	P450 monooxygenase was converted to peroxidase	[94]
4	P450 from <i>Deinococcus apachensis</i>	Enantioselectivity	ISM	enantiodivergent control	[95]
5	Photodecarboxylase (<i>Chlorella variabilis</i>)	substrate scope, activity, stereoselectivity	FRISM	kinetic resolution	[96, 97]
6	Monoamine oxidase (<i>Aspergillus niger</i>)	substrate scope, enantioselectivity	ISM	synthesis of (R)-mexiletine	[98]
7	Lipase A (<i>Bacillus subtilis</i>)	enantioselectivity	ISM	stereodivergent synthesis	[99]
8	Lipase (CALB)	enantioselectivity	ISM	kinetic resolution of profen-esters	[100]
9	Limonene epoxide hydrolase	enantioselectivity	ISM	very small amino acid alphabets	[101]
10	Limonene epoxide hydrolase	enantioselectivity	ISM	reduced amino acid alphabet	[102]
11	Laccase (<i>Pycnoporus cinnabarinus</i>)	activity	ISM	oxidation of ligninolytic sinapic acid	[103]
12	Imine reductase	activity, thermostability	CAST, SM	3 CAST cycles	[104]
13	Halogenase Wel05	regio- & diastereoselectivity	CAST/ISM	Non-heme iron dependent halogenase; site-directed SM at 9 residues; additional SKA and VNT codons at two sites; late installation of chlorines to indole alkaloids	[105]
14	Halohydrin dehalogenase (<i>Arthrobacterium radiobacter hhec</i>)	enantioselectivity, activity	ISM	DC-analyser software tool used	[106]
15	Galactase oxidase (<i>Fusarium</i> sp.)	substrate scope	CAST/ISM	glycoprotein labelling	[107]
16	Carboxylic acid reductase (<i>Segniliparus rugosus</i>)	activity	CAST	NDT, VMA, ATG, TGG codons	[108, 109]
17	Ene reductase	Enantioselectivity	ISM	Unnatural photoenzymatic reactions	[110]

extended to flavin-dependent enzymes [154]. The radical character of this reaction can also be applied to catalyse *intra*-[155] and *intermolecular* [156] C—C-bond forming reactions.

The recent developments in enabling unnatural reactions in biocatalysis are exciting and impressive. Although it is relevant to highlight that the current state of the art of these approaches resembles more organocatalytic reaction conditions with catalyst loadings in the mol% range and several hours of reaction time, than native enzymatic reactions. Such reactions certainly pose a challenge of economic relevance and should be addressed further by enzyme engineering.

6. Future aspects of enzyme engineering

Enzyme engineering has been the primary tool to make biocatalysts suitable for the synthesis of industrially relevant products. Shifting from trial and error to rational design, we observe a trend to more mechanism- and structure-guided design. Computational tools are constantly improving: the Rosetta algorithms [157,158] and the Damborsky metrics [52,159] can be applied in this endeavour. Meanwhile, the general trend for artificial intelligence (AI) assisted protein engineering [159, 160], as exemplified by recent breakthroughs such as AlphaFold2 [161], will continue to spark the biocatalysis community. Nevertheless, accurate predictions of sequence-function relationships remain challenging. Suitable AI-based algorithms that are specifically designed for protein engineering in improving the stereoselectivity, activity and stability are not yet fully established.

In the past decades biocatalysis has been firmly established as a tool for the synthesis of chiral, high value-added products [162]. Yet, biocatalytic syntheses of bulk chemicals such as acrylamide remain scarce. Here, however, biocatalysis could really unfold its potential for greener, less energy- and resource-consuming syntheses. An increased focus of the biocatalysis community on such transformations is urgently needed. This also implies intensified interdisciplinary exchange with chemists and chemical engineers, both from industry and academia (Table 2).

CRedit authorship contribution statement

Caroline Paul: Writing – review & editing, Writing – original draft.
Ulf Hanefeld: Writing – review & editing, Writing – original draft.

Frank Hollmann: Writing – review & editing, Writing – original draft.
Ge Qu: Writing – review & editing, Writing – original draft.
Bo Yuan: Writing – review & editing, Writing – original draft.
Zhoutong Sun: Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

- [1] D.R. Mills, R.L. Peterson, S. Spiegelman, An Extracellular Darwinian Experiment with a Self-Duplicating Nucleic Acid Molecule, *Proc. Natl. Acad. Sci. U. S. A.* 58 (1967) 217–224, <https://doi.org/10.1073/pnas.58.1.217>.
- [2] J.C. Francis, P.E. Hansche, Directed Evolution of Metabolic Pathways in Microbial Populations. Modification of Acid-Phosphatase pH Optimum in *S. cerevisia*, *Genetics* 70 (1972) 59–73, <https://doi.org/10.1093/genetics/70.1.59>.
- [3] M. Eigen, W. Gardiner, Evolutionary Molecular Engineering Based on Rna Replication, *Pure Appl. Chem.* 56 (1984) 967–978, <https://doi.org/10.1351/pac198456080967>.
- [4] H. Liao, T. McKenzie, R. Hageman, Isolation of a thermostable enzyme variant by cloning and selection in a thermophile, *Proc. Natl. Acad. Sci. U. S. A.* 83 (1986) 576–580, <https://doi.org/10.1073/pnas.83.3.576>.
- [5] M. Smith, Synthetic DNA and Biology (Nobel Lecture), *Angew. Chem. Int. Ed.* 33 (1994) 1214–1221, <https://doi.org/10.1002/anie.199412141>.
- [6] K.B. Mullis, The Polymerase Chain Reaction (Nobel Lecture), *Angew. Chem. Int. Ed.* 33 (1994) 1209–1213, <https://doi.org/10.1002/anie.199412091>.
- [7] R.C. Cadwell, G.F. Joyce, Randomization of genes by PCR mutagenesis, *Genome Res.* 2 (1992) 28–33, <https://doi.org/10.1101/gr.2.1.28>.
- [8] K. Chen, F.H. Arnold, Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 5618–5622, <https://doi.org/10.1073/pnas.90.12.5618>.
- [9] M.T. Reetz, A. Zonta, K. Schimossek, K.E. Jaeger, K. Liebeton, Creation of Enantioselective Biocatalysts for Organic Chemistry by *In Vitro* Evolution, *Angew. Chem. Int. Ed.* 36 (1997) 2830–2832, <https://doi.org/10.1002/anie.199728301>.
- [10] W.P.C. Stemmer, DNA shuffling by random fragmentation and reassembly - *in vitro* recombination for molecular evolution, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 10747–10751, <https://doi.org/10.1073/pnas.91.22.10747>.

- [11] R. Petri, C. Schmidt-Dannert, Dealing with complexity: Evolutionary engineering and genome shuffling, *Curr. Opin. Biotechnol.* 15 (2004) 298–304, <https://doi.org/10.1016/j.copbio.2004.05.005>.
- [12] M.T. Reetz, Z. Sun, G. Qu, *Enzyme engineering: selective catalysts for applications in biotechnology. Organic Chemistry, and Life Science*, Wiley-VCH, 2023.
- [13] H. Zhao, L. Giver, Z. Shao, J.A. Affholter, F.H. Arnold, Molecular evolution by staggered extension process (StEP) in vitro recombination, *Nat. Biotechnol.* 16 (1998) 258–261, <https://doi.org/10.1038/nbt0398-258>.
- [14] W.M. Coco, W.E. Levinson, M.J. Crist, H.J. Hektor, A. Darzins, P.T. Pienkos, C. H. Squires, D.J. Monticello, DNA shuffling method for generating highly recombined genes and evolved enzymes, *Nat. Biotechnol.* 19 (2001) 354–359, <https://doi.org/10.1038/10338/70754>.
- [15] M. Ostermeier, J.H. Shim, S.J. Benkovic, A combinatorial approach to hybrid enzymes independent of DNA homology, *Nat. Biotechnol.* 17 (1999) 1205–1209, <https://doi.org/10.1038/10338/70754>.
- [16] R. Bruce Wallace, M. Schold, M.J. Johnson, P. Dembek, K. Itakura, Oligonucleotide directed mutagenesis of the human β -globin gene: a general method for producing specific point mutations in cloned DNA, *Nucleic Acids Res.* 9 (1981) 3647–3656, <https://doi.org/10.1093/nar/9.15.3647>.
- [17] D.A. Estell, T.P. Graycar, J.A. Wells, Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation, *J. Biol. Chem.* 260 (1985) 6518–6521, [https://doi.org/10.1016/S0021-9258\(18\)88811-1](https://doi.org/10.1016/S0021-9258(18)88811-1).
- [18] S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene* 77 (1989) 51–59, [https://doi.org/10.1016/0378-1119\(89\)90358-2](https://doi.org/10.1016/0378-1119(89)90358-2).
- [19] M. Kammann, J. Laufs, J. Schell, B. Gronenbom, Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR), *Nucleic Acids Res.* 17 (1989) 5404, <https://doi.org/10.1093/nar/17.13.5404>.
- [20] H.H. Hogrefe, J. Cline, G.L. Youngblood, R.M. Allen, Creating Randomized Amino Acid Libraries with the QuickChange® Multi Site-Directed Mutagenesis Kit, *BioTech* 33 (2002) 1158–1165, <https://doi.org/10.2144/02335p01>.
- [21] M.T. Reetz, M. Bocola, J.D. Carballeira, D. Zha, A. Vogel, Expanding the Range of Substrate Acceptance of Enzymes: Combinatorial Active-Site Saturation Test, *Angew. Chem. Int. Ed.* 44 (2005) 4192–4196, <https://doi.org/10.1002/ange.200500767>.
- [22] M.T. Reetz, J.D. Carballeira, Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes, *Nat. Protoc.* 2 (2007) 891–903, <https://doi.org/10.1038/nprot.2007.72>.
- [23] D. Yi, T. Bayer, C.P.S. Badenhorst, S. Wu, M. Doerr, M. Höhne, U.T. Bornscheuer, Recent trends in biocatalysis, *Chem. Soc. Rev.* 50 (2021) 8003–8049, <https://doi.org/10.1039/D0CS01575J>.
- [24] S. Wu, R. Snajdrova, J.C. Moore, K. Baldenius, U.T. Bornscheuer, Biocatalysis: Enzymatic Synthesis for Industrial Applications, *Angew. Chem. Int. Ed.* 60 (2021) 88–119, <https://doi.org/10.1002/anie.202006648>.
- [25] B. Hauer, Embracing Nature's Catalysts: A Viewpoint on the Future of Biocatalysis, *ACS Catal.* 10 (2020) 8418–8427, <https://doi.org/10.1021/acscatal.0c01708>.
- [26] B.M. Nestl, S.C. Hammer, B.A. Nebel, B. Hauer, New Generation of Biocatalysts for Organic Synthesis, *Angew. Chem. Int. Ed.* 53 (2014) 3070–3095, <https://doi.org/10.1002/anie.201302195>.
- [27] P. Martinez, M.E. Van Dam, A.C. Robinson, K. Chen, F.H. Arnold, Stabilization of subtilisin E in organic solvents by site-directed mutagenesis, *Biotechnol. Bioeng.* 39 (1992) 141–147, <https://doi.org/10.1002/bit.260390204>.
- [28] V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova, J. Damborsky, Strategies for Stabilization of Enzymes in Organic Solvents, *ACS Catal.* 3 (2013) 2823–2836, <https://doi.org/10.1021/cs400684x>.
- [29] M. Alcalde, T. Bulter, M. Zumáruga, H. García-Arellano, M. Mencía, F.J. Plou, A. Ballesteros, Screening Mutant Libraries of Fungal Laccases in the Presence of Organic Solvents, *SLAS Discov.* 10 (2005) 624–631, <https://doi.org/10.1177/1087057105277058>.
- [30] T. Takahashi, K.K.S. Ng, H. Oyama, K. Oda, Molecular Cloning of the Gene Encoding *Vibrio* Metalloproteinase Vimelysin and Isolation of a Mutant with High Stability in Organic Solvents, *J. Biochem.* 138 (2005) 701–710, <https://doi.org/10.1093/jb/mvi173>.
- [31] J.C. Moore, F.H. Arnold, Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents, *Nat. Biotechnol.* 14 (1996) 458–467, <https://doi.org/10.1038/nbt0496-458>.
- [32] T. Seng Wong, F.H. Arnold, U. Schwaneberg, Laboratory evolution of cytochrome P450 BM-3 monooxygenase for organic cosolvents, *Biotechnol. Bioeng.* 85 (2004) 351–358, <https://doi.org/10.1002/bit.10896>.
- [33] S. Yang, L. Zhou, H. Tang, J. Pan, X. Wu, H. Huang, Z. Yuan, Rational design of a more stable penicillin G acylase against organic cosolvent, *J. Mol. Catal. B Enzym.* 18 (2002) 285–290, [https://doi.org/10.1016/S1381-1177\(02\)00108-X](https://doi.org/10.1016/S1381-1177(02)00108-X).
- [34] H.J. Park, J.C. Joo, K. Park, Y.H. Kim, Y.J. Yoo, Prediction of the solvent affecting site and the computational design of stable *Candida antarctica* lipase B in a hydrophilic organic solvent, *J. Biotechnol.* 163 (2013) 346–352, <https://doi.org/10.1016/j.jbiotec.2012.11.006>.
- [35] V.S. Dani, C. Ramakrishnan, R. Varadarajan, MODIP revisited: re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins, *Prot. Eng. Des. Select.* 16 (2003) 187–193, <https://doi.org/10.1093/proeng/gzg024>.
- [36] J. Schymkowitz, J. Borg, F. Stricher, R. Nys, F. Rousseau, L. Serrano, The FoldX Web server: an online force field, *Nucl. Acids Res.* 33 (2005) W382–W388.
- [37] A. Dror, E. Shemesh, N. Dayan, A. Fishman, Protein Engineering by Random Mutagenesis and Structure-Guided Consensus of Lipase T6 for Enhanced Stability in Methanol, *Appl. Environ. Microbiol.* 80 (2014) 1515–1527, <https://doi.org/10.1128/Aem.03371-13>.
- [38] M.T. Reetz, P. Soni, L. Fernández, Y. Gumulya, J.D. Carballeira, Increasing the stability of an enzyme toward hostile organic solvents by directed evolution based on iterative saturation mutagenesis using the B-FIT method, *Chem. Commun.* 46 (2010) 8657–8658, <https://doi.org/10.1039/C0CC02657C>.
- [39] E. Vazquez-Figueroa, V. Yeh, J.M. Broering, J.F. Chaparro-Riggers, A. S. Bommarius, Thermostable variants constructed via the structure-guided consensus method also show increased stability in salts solutions and homogeneous aqueous-organic media, *Protein Eng. Des. Sel.* 21 (2008) 673–680, <https://doi.org/10.1093/protein/gzn048>.
- [40] E. Vazquez-Figueroa, J. Chaparro-Riggers, A.S. Bommarius, Development of a thermostable glucose dehydrogenase by a structure-guided consensus concept, *ChemBioChem* 8 (2007) 2295–2301, <https://doi.org/10.1002/cbic.200700500>.
- [41] M. Wehrmann, J. Klebensberger, Engineering thermal stability and solvent tolerance of the soluble quinoprotein PdeE from *Pseudomonas putida* KT2440 with a heterologous whole-cell screening approach, *Microb. Biotechnol.* 11 (2018) 399–408, <https://doi.org/10.1111/1751-7915.13036>.
- [42] A. Cerdbobbel, K. De Winter, D. Aerts, R. Kuipers, H.J. Joosten, W. Soetaert, T. Desmet, Increasing the thermostability of sucrose phosphorylase by a combination of sequence- and structure-based mutagenesis, *Prot. Eng. Des. Select.* 24 (2011) 829–834, <https://doi.org/10.1093/protein/gzr042>.
- [43] M.T. Reetz, J.D. Carballeira, A. Vogel, Iterative Saturation Mutagenesis on the Basis of B Factors as a Strategy for Increasing Protein Thermostability, *Angew. Chem. Int. Ed.* 45 (2006) 7745–7751, <https://doi.org/10.1002/anie.200602795>.
- [44] J. Liang, J. Lalonde, B. Borup, V. Mitchell, E. Mundorff, N. Trinh, D.A. Kochrekar, R.N. Cherat, G.G. Pai, Development of a Biocatalytic Process as an Alternative to the (-)-DIP-Cl-Mediated Asymmetric Reduction of a Key Intermediate of Montelukast, *Org. Proc. Res. Dev.* 14 (2010) 193–198, <https://doi.org/10.1021/op900272d>.
- [45] C.K. Saville, J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J. C. Colbeck, A. Kriebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, G. J. Hughes, Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture, *Science* 329 (2010) 305–309, <https://doi.org/10.1126/science.1188934>.
- [46] E. Capriotti, P. Fariselli, R. Casadio, I-Mutant 2.0: predicting stability changes upon mutation from the protein sequence or structure, *Nucl. Acids Res.* 33 (2005) W306–W310, <https://doi.org/10.1093/nar/gki375>.
- [47] J. Laimer, H. Hofer, M. Fritz, S. Wegenkittl, P. Lackner, MAESTRO - multi agent stability prediction upon point mutations, *BMC Bioinform.* 16 (2015) 116, <https://doi.org/10.1186/s12859-015-0548-6>.
- [48] J. Bendl, J. Stourac, E. Sebestova, O. Vavra, M. Musil, J. Brezovsky, J. Damborsky, HotSpot Wizard 2.0: automated design of site-specific mutations and smart libraries in protein engineering, *Nucl. Acids Res.* 44 (2016) W479–W487, <https://doi.org/10.1093/nar/gkx416>.
- [49] H. Ashkenazy, O. Penn, A. Doron-Faigenboim, O. Cohen, G. Cannarozzi, O. Zomer, T. Pupko, FastML: a web server for probabilistic reconstruction of ancestral sequences, *Nucl. Acids Res.* 40 (2012) W580–W584, <https://doi.org/10.1093/nar/gks498>.
- [50] A.B. Diallo, V. Makarenkov, M. Blanchette, Ancestors 1.0: a web server for ancestral sequence reconstruction, *Bioinform.* 26 (2009) 130–131, <https://doi.org/10.1093/bioinformatics/btp600>.
- [51] M. Musil, J. Stourac, J. Bendl, J. Brezovsky, Z. Prokop, J. Zendlulka, T. Martinek, D. Bednar, J. Damborsky, FireProt: web server for automated design of thermostable proteins, *Nucl. Acids Res.* 45 (2017) W393–W399, <https://doi.org/10.1093/nar/gkx285>.
- [52] M. Musil, A. Jezik, J. Horackova, S. Borko, P. Kabourek, J. Damborsky, D. Bednar, FireProt 2.0: web-based platform for the fully automated design of thermostable proteins, *Brief. Bioinform.* 25 (2023), <https://doi.org/10.1093/bib/bbad425>.
- [53] A. Goldenzweig, M. Goldsmith, Shannon E. Hill, O. Gertman, P. Laurino, Y. Ashani, O. Dym, T. Unger, S. Albeck, J. Prilusky, Raquel L. Lieberman, A. Aharoni, I. Silman, Joel L. Sussman, Dan S. Tawfik, Sarel J. Fleishman, Automated Structure- and Sequence-Based Design of Proteins for High Bacterial Expression and Stability, *Mol. Cell* 63 (2016) 337–346, <https://doi.org/10.1016/j.molcel.2016.06.012>.
- [54] H.J. Wijma, R.J. Floor, P.A. Jekel, D. Baker, S.J. Marrink, D.B. Janssen, Computationally designed libraries for rapid enzyme stabilization, *Prot. Eng. Des. Select.* 27 (2014) 49–58, <https://doi.org/10.1093/protein/gzt061>.
- [55] W. Martin, J. Baross, D. Kelley, M.J. Russell, Hydrothermal vents and the origin of life, *Nature Rev. Microbiol.* 6 (2008) 805–814, <https://doi.org/10.1038/nrmicro1991>.
- [56] F. Robert, M. Chausson, A palaeotemperature curve for the Precambrian oceans based on silicon isotopes in cherts, *Nature* 443 (2006) 969–972, <https://doi.org/10.1038/nature05239>.
- [57] V.A. Rizzo, J.M. Sanchez-Ruiz, S.B. Ozkan, Biotechnological and protein-engineering implications of ancestral protein resurrection, *Curr. Opin. Struct. Biol.* 51 (2018) 106–115, <https://doi.org/10.1016/j.sbi.2018.02.007>.
- [58] M.A. Spence, J.A. Kaczmarek, J.W. Saunders, C.J. Jackson, Ancestral sequence reconstruction for protein engineers, *Curr. Opin. Struct. Biol.* 69 (2021) 131–141, <https://doi.org/10.1016/j.sbi.2021.04.001>.
- [59] X.Y. Chen, Z. Dou, T.W. Luo, Z.W. Sun, H.M. Ma, G.C. Xu, Y. Ni, Directed reconstruction of a novel ancestral alcohol dehydrogenase featuring shifted pH-profile, enhanced thermostability and expanded substrate spectrum, *Biores. Technol.* 363 (2022), <https://doi.org/10.1016/j.biortech.2022.127886>, 127886.
- [60] V.A. Rizzo, J.A. Gavira, D.F. Mejia-Carmona, E.A. Gaucher, J.M. Sanchez-Ruiz, Hyperstability and Substrate Promiscuity in Laboratory Resurrections of

- Precambrian β -Lactamases, *J. Am. Chem. Soc.* 135 (2013), <https://doi.org/10.1021/ja404387m>, 10580–10580.
- [61] P. Babkova, E. Sebestova, J. Brezovsky, R. Chaloupkova, J. Damborsky, Ancestral Haloalkane Dehalogenases Show Robustness and Unique Substrate Specificity, *ChemBioChem* 18 (2017) 1448–1456, <https://doi.org/10.1002/cbic.201700197>.
- [62] I. Ayuso-Fernández, A.T. Martínez, F.J. Ruiz-Dueñas, Experimental recreation of the evolution of lignin-degrading enzymes from the Jurassic to date, *Biotechnol. Biofuels* 10 (2017) 67, <https://doi.org/10.1186/s13068-017-0744-x>.
- [63] Y. Sun, E. Calderini, R. Kourist, A Reconstructed Common Ancestor of the Fatty Acid Photo-decarboxylase Clade Shows Photo-decarboxylation Activity and Increased Thermostability, *ChemBioChem* 22 (2021) 1833–1840, <https://doi.org/10.1002/cbic.202000851>.
- [64] J. Livada, A.M. Vargas, C.A. Martínez, R.D. Lewis, Ancestral Sequence Reconstruction Enhances Gene Mining Efforts for Industrial Ene Reductases by Expanding Enzyme Panels with Thermostable Catalysts, *ACS Catal* (2023) 2576–2585, <https://doi.org/10.1021/acscatal.2c03859>.
- [65] A. Chen, Y.M. Li, J.Q. Nie, B. McNeil, L. Jeffrey, Y.K. Yang, Z.H. Bai, Protein engineering of pullulanase for enhanced thermostability using data driven rational design methods, *Enzyme Microb. Technol.* 78 (2015) 74–83, <https://doi.org/10.1016/j.enzmictec.2015.06.013>.
- [66] A. Kunka, S.M. Marques, M. Havlasek, M. Vasina, N. Velatova, L. Cengelova, D. Kovar, J. Damborsky, M. Marek, D. Bednar, Z. Prokop, Advancing Enzyme's Stability and Catalytic Efficiency through Synergy of Force-Field Calculations, Evolutionary Analysis, and Machine Learning, *ACS Catal* 13 (2023) 12506–12518, <https://doi.org/10.1021/acscatal.3c02575>.
- [67] J. Rouvinen, M. Andberg, J. Pääkkönen, N. Hakulinen, A. Koivula, Current state of and need for enzyme engineering of 2-deoxy-D-ribose 5-phosphate aldolases and its impact, *Appl. Microbiol. Biotechnol.* 105 (2021) 6215–6228, <https://doi.org/10.1007/s00253-021-11462-0>.
- [68] M. Dick, R. Hartmann, O.H. Weiergräber, C. Bisterfeld, T. Classen, M. Schwarten, P. Neudecker, D. Willbold, J. Pietruszka, Mechanism-based inhibition of an aldolase at high concentrations of its natural substrate acetaldehyde: structural insights and protective strategies, *Chem. Sci.* 7 (2016) 4492–4502, <https://doi.org/10.1039/c5sc04574f>.
- [69] C.G. Acevedo-Rocha, F. Hollmann, J. Sanchis, Z. Sun, *ACS Catal* (2020) 15123–15139, <https://doi.org/10.1021/acscatal.0c04108>.
- [70] J.L. Reymond, V.S. Fluxà, N. Maillard, Enzyme assays, *Chem. Comm.* (2009) 34–46.
- [71] M. Baumann, R. Stürmer, U.T. Bornscheuer, A High-Throughput-Screening Method for the Identification of Active and Enantioselective Hydrolases, *Angew. Chem. Int. Ed.* 40 (2001) 4201–4204, [10.1002/1521-3773\(20011119\)40:22<4201::AID-ANIE4201>3.0.CO;2-V](https://doi.org/10.1002/1521-3773(20011119)40:22<4201::AID-ANIE4201>3.0.CO;2-V).
- [72] S. Hecko, A. Schiefer, C.P.S. Badenhorst, M.J. Fink, M.D. Mihovilovic, U. T. Bornscheuer, F. Rudroff, Enlightening the Path to Protein Engineering: Chemoselective Turn-On Probes for High-Throughput Screening of Enzymatic Activity, *Chem. Rev.* 123 (2023) 2832–2901, <https://doi.org/10.1021/acs.chemrev.2c00304>.
- [73] M.T. Reetz, Klaus M. Kühling, A. Deege, H. Hinrichs, D. Belder, Super-High-Throughput Screening of Enantioselective Catalysts by Using Capillary Array Electrophoresis, *Angew. Chem. Int. Ed.* 39 (2000) 3891–3893, [10.1002/1521-3773\(20001103\)39:21<3891::AID-ANIE3891>3.0.CO;2-1](https://doi.org/10.1002/1521-3773(20001103)39:21<3891::AID-ANIE3891>3.0.CO;2-1).
- [74] M.T. Reetz, K.M. Kühling, H. Hinrichs, A. Deege, Circular dichroism as a detection method in the screening of enantioselective catalysts, *Chirality* 12 (2000) 479–482, [10.1002/\(SICI\)1520-636X\(2000\)12:5<479::AID-CHIR32>3.0.CO;2-#](https://doi.org/10.1002/(SICI)1520-636X(2000)12:5<479::AID-CHIR32>3.0.CO;2-#).
- [75] S. Bhattacharya, E.G. Margheritis, K. Takahashi, A. Kulesha, A. D'Souza, I. Kim, J. H. Yoon, J.R.H. Tame, A.N. Volkov, O.V. Makhlynets, I.V. Korodovych, NMR-guided directed evolution, *Nature* 610 (2022) 389–393, [10.1038/s41586-022-05278-9](https://doi.org/10.1038/s41586-022-05278-9).
- [76] R. Mao, D.J. Wackelin, C.S. Jamieson, T. Rogge, S. Gao, A. Das, D.M. Taylor, K. N. Houk, F.H. Arnold, Enantio- and Diastereoenriched Enzymatic Synthesis of 1,2,3-Polysubstituted Cyclopropanes from (Z/E)-Trisubstituted Enol Acetates, *J. Am. Chem. Soc.* 145 (2023) 16176–16185, <https://doi.org/10.1021/jacs.3c04870>.
- [77] A. Knorrscheidt, J. Soler, N. Hünecke, P. Püllmann, M. Garcia-Borràs, M. J. Weissenborn, Simultaneous screening of multiple substrates with an unspecific peroxxygenase enabled modified alkane and alkene oxyfunctionalisations, *Catal. Sci. Technol.* 11 (2021) 6058–6064, <https://doi.org/10.1039/D0CY02457K>.
- [78] X.W. Diefenbach, I. Farasat, E.D. Guetschow, C.J. Welch, R.T. Kennedy, S. Sun, J. C. Moore, Enabling Biocatalysis by High-Throughput Protein Engineering Using Droplet Microfluidics Coupled to Mass Spectrometry, *ACS Omega* 3 (2018) 1498–1508, <https://doi.org/10.1021/acsomega.7b01973>.
- [79] Y. Zhang, X. Zhang, M. Wang, Product-driven high-throughput screening of industrial filamentous actinomyces, *Trends Biotechnol* 41 (2023) 1109–1112, <https://doi.org/10.1016/j.tibtech.2023.02.004>.
- [80] M.T. Reetz, An overview of high-throughput screening systems for enantioselective enzymatic transformations, *Methods Mol. Biol.* 230 (2003) 259–282, [10.1385/1-59259-396-8:259](https://doi.org/10.1385/1-59259-396-8:259).
- [81] M. Alexeeva, A. Enright, M.J. Dawson, M. Mahmoudian, N.J. Turner, Deracemization of alpha-methylbenzylamine using an enzyme obtained by in vitro evolution, *Angew. Chem. Int. Ed.* 41 (2002) 3177–3179, [10.1002/1521-3773\(20020902\)41:17<3177::aid-anie3177>3.0.co;2-p](https://doi.org/10.1002/1521-3773(20020902)41:17<3177::aid-anie3177>3.0.co;2-p).
- [82] D. Ghislieri, N.J. Turner, Biocatalytic Approaches to the Synthesis of Enantiomerically Pure Chiral Amines, *Top. Catal.* 57 (2014) 284–300, <https://doi.org/10.1007/s11244-013-0184-1>.
- [83] S. Fong, T.D. Machajewski, C.C. Mak, C.H. Wong, Directed evolution of D-2-keto-3-deoxy-6-phosphogluconate aldolase to new variants for the efficient synthesis of D- and L-sugars, *Chem. Biol.* 7 (2000) 873–883, [https://doi.org/10.1016/S1074-5521\(00\)00035-1](https://doi.org/10.1016/S1074-5521(00)00035-1).
- [84] M. Schmidt, D. Hasenpusch, M. Kahler, U. Kirchner, K. Wiggenhorn, W. Lange, U. T. Bornscheuer, Directed evolution of an esterase from *Pseudomonas fluorescens* yields a mutant with excellent enantioselectivity and activity for the kinetic resolution of a chiral building block, *ChemBioChem* 7 (2006) 805–809, <https://doi.org/10.1002/cbic.200500546>.
- [85] G.P. Horsman, E.Henke Liu, U.T. Bornscheuer, R.J. Kazlauskas, Mutations in Distant Residues Moderately Increase the Enantioselectivity of *Pseudomonas fluorescens* Esterase towards Methyl 3-Bromo-2-methylpropanoate and Ethyl 3-Phenylbutyrate, *Chem. Eur. J.* 9 (2003) 1933–1939, <https://doi.org/10.1002/chem.200204551>.
- [86] M.W. Peters, P. Meinhold, A. Glieder, F.H. Arnold, Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3, *J. Am. Chem. Soc.* 125 (2003) 13442–13450, <https://doi.org/10.1021/ja0303790>.
- [87] A. Glieder, E.T. Farinas, F.H. Arnold, Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase, *Nature Biotechnol* 20 (2002) 1135–1139, <https://doi.org/10.1038/nbt744>.
- [88] Z.T. Sun, R. Lonsdale, A. Ilie, G.Y. Li, J.H. Zhou, M.T. Reetz, Catalytic Asymmetric Reduction of Difficult-to-Reduce Ketones: Triple-Code Saturation Mutagenesis of an Alcohol Dehydrogenase, *ACS Catal* 6 (2016) 1598–1605, <https://doi.org/10.1021/acscatal.5b02752>.
- [89] J. Liang, E. Mundorff, R. Voladri, S. Jenne, L. Gilson, A. Conway, A. Krebber, J. Wong, G. Huisman, S. Truesdell, J. Lalonde, Highly Enantioselective Reduction of a Small Heterocyclic Ketone: Biocatalytic Reduction of Tetrahydrothiophene-3-one to the Corresponding (R)-Alcohol, *Org. Proc. Res. Dev.* 14 (2010) 188–192, <https://doi.org/10.1021/op9002714>.
- [90] G. Qu, Y. Bi, B. Liu, J. Li, X. Han, W. Liu, Y. Jiang, Z. Qin, Z. Sun, Unlocking the Stereoselectivity and Substrate Acceptance of Enzymes: Proline-Induced Loop Engineering Test, *Angew. Chem. Int. Ed.* 61 (2022) e202110793, [10.1002/anie.202110793](https://doi.org/10.1002/anie.202110793).
- [91] G. Li, M. Garcia-Borràs, M.J.L.J. Fürst, A. Ilie, M.W. Fraaije, K.N. Houk, M. T. Reetz, Overriding Traditional Electronic Effects in Biocatalytic Baeyer–Villiger Reactions by Directed Evolution, *J. Am. Chem. Soc.* 140 (2018) 10464–10472, <https://doi.org/10.1021/jacs.8b04742>.
- [92] J. Xu, Y. Cen, W. Singh, J. Fan, L. Wu, X. Lin, J. Zhou, M. Huang, M.T. Reetz, Q. Wu, Stereodivergent Protein Engineering of a Lipase To Access All Possible Stereoisomers of Chiral Esters with Two Stereocenters, *J. Am. Chem. Soc.* 141 (2019) 7934–7945, <https://doi.org/10.1021/jacs.9b02709>.
- [93] J. Li, G. Qu, N. Shang, P. Chen, Y. Men, W. Liu, Z. Mei, Y. Sun, Z. Sun, Near-perfect control of the regioselective glycosylation enabled by rational design of glycosyltransferases, *Green Synth., Catal* 2 (2021) 45–53, <https://doi.org/10.1016/j.gresc.2021.01.005>.
- [94] N. Ma, W. Fang, C. Liu, X. Qin, X. Wang, L. Jin, B. Wang, Z. Cong, Switching an Artificial P450 Peroxygenase into Peroxidase via Mechanism-Guided Protein Engineering, *ACS Catal* 11 (2021) 8449–8455, <https://doi.org/10.1021/acscatal.1c02698>.
- [95] X.J. Zhou, M.Y. Wang, L. Zhao, Y.Q. He, Z.Q. Wang, J.J. Li, G.Z. Deng, N.W. Wan, Y.Z. Chen, Enantiodivergent Synthesis of Halohydrins by Engineering P450DA Monooxygenases, *ACS Catal* (2023) 15948–15955, <https://doi.org/10.1021/acscatal.3c04742>.
- [96] J. Xu, J. Fan, Y. Lou, W. Xu, Z. Wang, D. Li, H. Zhou, X. Lin, Q. Wu, Light-driven decarboxylative deuteration enabled by a divergently engineered photodecarboxylase, *Nat. Commun.* 12 (2021) 3983, <https://doi.org/10.1038/s41467-021-24259-6>.
- [97] K. Mou, Y. Guo, W. Xu, D. Li, Z. Wang, Q. Wu, Stereodivergent Protein Engineering of Fatty Acid Photodecarboxylase for Light-Driven Kinetic Resolution of Sec-Alcohol Oxalates, *Angew. Chem. Int. Ed.* 63 (2024) e202318374, [10.1002/anie.202318374](https://doi.org/10.1002/anie.202318374).
- [98] Z. Chen, Y. Ma, M. He, H. Ren, S. Zhou, D. Lai, Z. Wang, L. Jiang, Semi-rational Directed Evolution of Monoamine Oxidase for Kinetic Resolution of rac-Mexiletine, *Appl. Biochem. Biotechnol.* 176 (2015) 2267–2278, <https://doi.org/10.1007/s12010-015-1716-x>.
- [99] D. Li, X. Chen, Z. Chen, X. Lin, J. Xu, Q. Wu, Directed evolution of lipase A from *Bacillus subtilis* for the preparation of enantiocomplementary sec-alcohols, *Green Synth., Catal* 2 (2021) 290–294, <https://doi.org/10.1016/j.gresc.2021.07.003>.
- [100] B. Qin, P. Liang, X. Jia, X. Zhang, M. Mu, X.Y. Wang, G.Z. Ma, D.N. Jin, S. You, Directed evolution of *Candida antarctica* lipase B for kinetic resolution of profen esters, *Catal. Commun.* 38 (2013) 1–5, <https://doi.org/10.1016/j.catcom.2013.03.040>.
- [101] Z. Sun, R. Lonsdale, X.D. Kong, J.H. Xu, J. Zhou, M.T. Reetz, Reshaping an Enzyme Binding Pocket for Enhanced and Inverted Stereoselectivity: Use of Smallest Amino Acid Alphabets in Directed Evolution, *Angew. Chem. Int. Ed.* 54 (2015) 12410–12415, <https://doi.org/10.1002/anie.201501809>.
- [102] H. Zheng, M.T. Reetz, Manipulating the Stereoselectivity of Limonene Epoxide Hydrolase by Directed Evolution Based on Iterative Saturation Mutagenesis, *J. Am. Chem. Soc.* 132 (2010) 15744–15751, <https://doi.org/10.1021/ja1067542>.
- [103] I. Pardo, G. Santiago, P. Gentili, F. Lucas, E. Monza, F.J. Medrano, C. Galli, A. T. Martínez, V. Guallar, S. Camarero, Re-designing the substrate binding pocket of laccase for enhanced oxidation of sinapic acid, *Catal. Sci. Technol.* 6 (2016) 3900–3910, <https://doi.org/10.1039/C5CY01725D>.
- [104] J. Zhang, D. Liao, R. Chen, F. Zhu, Y. Ma, L. Gao, G. Qu, C. Cui, Z. Sun, X. Lei, S. S. Gao, Tuning an Imine Reductase for the Asymmetric Synthesis of

- Azacycloalkylamines by Concise Structure-Guided Engineering, *Angew. Chem. Int. Ed.* 61 (2022) e202201908.10.1002/anie.202201908.
- [105] T. Hayashi, M. Ligibel, E. Sager, M. Voss, J. Hunziker, K. Schroer, R. Snajdrova, R. Buller, Evolved Aliphatic Halogenases Enable Regio-complementary C–H Functionalization of a Pharmaceutically Relevant Compound, *Angew. Chem. Int. Ed.* 58 (2019) 18535–18539, <https://doi.org/10.1002/anie.201907245>.
- [106] C. Guo, Y. Chen, Y. Zheng, W. Zhang, Y. Tao, J. Feng, L. Tang, Exploring the Enantioselective Mechanism of Halohydrin Dehalogenase from *Agrobacterium radiobacter* AD1 by Iterative Saturation Mutagenesis, *Appl. Environ. Microbiol.* 81 (2015) 2919–2926, <https://doi.org/10.1128/AEM.04153-14>.
- [107] J.B. Rannes, A. Ioannou, S.C. Willies, G. Grogan, C. Behrens, S.L. Flitsch, N. J. Turner, Glycoprotein Labeling Using Engineered Variants of Galactose Oxidase Obtained by Directed Evolution, *J. Am. Chem. Soc.* 133 (2011) 8436–8439, <https://doi.org/10.1021/ja2018477>.
- [108] G. Qu, B. Liu, K. Zhang, Y. Jiang, J. Guo, R. Wang, Y. Miao, C. Zhai, Z. Sun, Computer-assisted engineering of the catalytic activity of a carboxylic acid reductase, *J. Biotechnol.* 306 (2019) 97–104, <https://doi.org/10.1016/j.jbiotec.2019.09.006>.
- [109] G. Qu, M. Fu, L. Zhao, B. Liu, P. Liu, W. Fan, J.A. Ma, Z. Sun, Computational Insights into the Catalytic Mechanism of Bacterial Carboxylic Acid Reductase, *J. Chem. Inf. Model.* 59 (2019) 832–841, <https://doi.org/10.1021/acs.jcim.8b00763>.
- [110] Y. Ouyang, J. Turek-Herman, T. Qiao, T.K. Hyster, Asymmetric Carbohydroxylation of Alkenes Using Photoenzymatic Catalysis, *J. Am. Chem. Soc.* (2023), <https://doi.org/10.1021/jacs.3c06618>.
- [111] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, *Chem. Soc. Rev.* 42 (2013) 6223–6235, <https://doi.org/10.1039/c3cs60075k>.
- [112] R. DiCosimo, J. McAuliffe, A.J. Poulou, G. Bohlmann, Industrial use of immobilized enzymes, *Chem. Soc. Rev.* 42 (2013) 6437–6474, <https://doi.org/10.1039/c3cs35506c>.
- [113] U. Hanefeld, L. Gardossi, E. Magner, Understanding enzyme immobilisation, *Chem. Soc. Rev.* 38 (2009) 453–468, <https://doi.org/10.1039/b711564b>.
- [114] A. Svendsen, M. Skjot, J. Brask, J. Vind, S.A. Patkar, L.D. Maria, Immobilised Enzymes, WO2007080197A2.
- [115] T. Peschke, M. Skoupi, T. Burgahn, S. Gallus, I. Ahmed, K.S. Rabe, C.M. Niemeyer, Self-immobilizing Fusion Enzymes for Compartmentalized Biocatalysis, *ACS Catal.* 7 (2017) 7866–7872, <https://doi.org/10.1021/acscatal.7b02230>.
- [116] J. Liang, F. Mazur, C. Tang, X. Ning, R. Chandrawati, K. Liang, Peptide-induced super-assembly of biocatalytic metal–organic frameworks for programmed enzyme cascades, *Chem. Sci.* 10 (2019) 7852–7858, <https://doi.org/10.1039/C9SC02021G>.
- [117] A. Ledesma-Fernandez, S. Velasco-Lozano, J. Santiago-Arcos, F. López-Gallego, A. L. Cortajarena, Engineered repeat proteins as scaffolds to assemble multi-enzyme systems for efficient cell-free biosynthesis, *Nat. Commun.* 14 (2023) 2587, <https://doi.org/10.1038/s41467-023-38304-z>.
- [118] X. Li, Y. Cao, K. Luo, Y. Sun, J. Xiong, L. Wang, Z. Liu, J. Li, J. Ma, J. Ge, H. Xiao, R.N. Zare, Highly active enzyme–metal nanohybrids synthesized in protein–polymer conjugates, *Nat. Catal.* 2 (2019) 718–725, <https://doi.org/10.1038/s41929-019-0305-8>.
- [119] C.J. Hartley, C.C. Williams, J.A. Scoble, Q.I. Churches, A. North, N.G. French, T. Nebl, G. Coia, A.C. Warden, G. Simpson, A.R. Frazer, C.N. Jensen, N.J. Turner, C. Scott, Engineered enzymes that retain and regenerate their cofactors enable continuous-flow biocatalysis, *Nat. Catal.* 2 (2019) 1006–1015, <https://doi.org/10.1038/s41929-019-0353-0>.
- [120] J. Staunton, K.J. Weissman, Polyketide biosynthesis: a millennium review, *Nat. Prod. Rep.* 18 (2001) 380–416, <https://doi.org/10.1039/A909079G>.
- [121] M.T. Reetz, R. Mondière, J.D. Carballeira, Enzyme promiscuity: first protein-catalyzed Morita–Baylis–Hillman reaction, *Tetrahedron Lett.* 48 (2007) 1679–1681, <https://doi.org/10.1016/j.tetlet.2007.01.063>.
- [122] T. Ütner, N. Çelebi-Ölçüm, Morita–Baylis–Hillman reaction: how do optimal enzyme active sites compare with organocatalysts, *Catal. Sci. Technol.* 13 (2023) 329–341, <https://doi.org/10.1039/D2CY00929C>.
- [123] R. Crawshaw, A.E. Crossley, L. Johannissen, A.J. Burke, S. Hay, C. Levy, D. Baker, S.L. Lovelock, A.P. Green, Engineering an efficient and enantioselective enzyme for the Morita–Baylis–Hillman reaction, *Nat. Chem.* 14 (2022) 313–320, <https://doi.org/10.1038/s41557-021-00833-9>.
- [124] J.K. Li, G. Qu, X. Li, Y. Tian, C. Cui, F.G. Zhang, W. Zhang, J.A. Ma, M.T. Reetz, Z. Sun, Rational enzyme design for enabling biocatalytic Baldwin cyclization and asymmetric synthesis of chiral heterocycles, *Nat. Commun.* 13 (2022) 7813, <https://doi.org/10.1038/s41467-022-35468-y>.
- [125] M.E. Wilson, G.M. Whitesides, Conversion of a protein to a homogeneous asymmetric hydrogenation catalyst by site-specific modification with a diphosphinerhodium(I) moiety, *J. Am. Chem. Soc.* 100 (1978) 306–307, <https://doi.org/10.1021/ja00469a064>.
- [126] K. Yu, Z. Zou, N.V. Igareta, R. Tachibana, J. Bechter, V. Köhler, D. Chen, T. R. Ward, Artificial Metalloenzyme-Catalyzed Enantioselective Amidation via Nitrene Insertion in Unactivated C(sp³)–H Bonds, *J. Am. Chem. Soc.* 145 (2023) 16621–16629, <https://doi.org/10.1021/jacs.3c03969>.
- [127] F. Christoffel, N.V. Igareta, M.M. Pellizzoni, L. Tiessler-Sala, B. Lozhkin, D. C. Spiess, A. Lledós, J.D. Maréchal, R.L. Peterson, T.R. Ward, Design and evolution of chimeric streptavidin for protein-enabled dual gold catalysis, *Nat. Catal.* 4 (2021) 643–653, <https://doi.org/10.1038/s41929-021-00651-9>.
- [128] M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke, T.R. Ward, Directed evolution of artificial metalloenzymes for *in vivo* metathesis, *Nature* 537 (2016) 661–665, <https://doi.org/10.1038/nature19114>.
- [129] S. Wu, Y. Zhou, D. Gerngross, M. Jeschek, T.R. Ward, Chemo-enzymatic cascades to produce cycloalkenes from bio-based resources, *Nat. Commun.* 10 (2019) 5060, <https://doi.org/10.1038/s41467-019-13071-y>.
- [130] T. Heinisch, F. Schwizer, B. Garabedian, E. Csibra, M. Jeschek, J. Vallapurackal, V.B. Pinheiro, P. Marliere, S. Panke, T.R. Ward, E. coli surface display of streptavidin for directed evolution of an allylic deallylase, *Chem. Sci.* 9 (2018) 5383–5388, <https://doi.org/10.1039/c8sc00484f>.
- [131] A. Chatterjee, H. Mallin, J. Klehr, J. Vallapurackal, A.D. Finke, L. Vera, M. Marsh, T.R. Ward, An enantioselective artificial Suzukiase based on the biotin–streptavidin technology, *Chem. Sci.* 7 (2016) 673–677, <https://doi.org/10.1039/C5SC03116H>.
- [132] J.M. Zhao, D.G. Bachmann, M. Lenz, D.G. Gillingham, T.R. Ward, An artificial metalloenzyme for carbene transfer based on a biotinylated dirhodium anchored within streptavidin, *Catal. Sci. Technol.* 8 (2018) 2294–2298, <https://doi.org/10.1039/c8cy00646f>.
- [133] J. Pierron, C. Malan, M. Creus, J. Gradinaru, I. Hafner, A. Ivanova, A. Sardo, T. R. Ward, Artificial Metalloenzymes for Asymmetric Allylic Alkylation on the Basis of the Biotin–Avidin Technology, *Angew. Chem. Int. Ed.* 47 (2008) 701–705, <https://doi.org/10.1002/anie.200703159>.
- [134] W. Wang, R. Tachibana, Z. Zou, D. Chen, X. Zhang, K. Lau, F. Pojer, T.R. Ward, X. Hu, Manganese Transfer Hydrogenases Based on the Biotin–Streptavidin Technology, *Angew. Chem. Int. Ed.* 62 (2023) e202311896.10.1002/anie.202311896.
- [135] M. Hestericová, T. Heinisch, L. Alonso-Cotchico, J.D. Maréchal, P. Vidossich, T. R. Ward, Directed Evolution of an Artificial Imine Reductase, *Angew. Chem. Int. Ed.* 57 (2018) 1863–1868.
- [136] J. Serrano-Plana, C. Rumo, J.G. Rebelein, R.L. Peterson, M. Barnett, T.R. Ward, Enantioselective Hydroxylation of Benzylic C(sp³)–H Bonds by an Artificial Iron Hydroxylase Based on the Biotin–Streptavidin Technology, *J. Am. Chem. Soc.* 142 (2020) 10617–10623, <https://doi.org/10.1021/jacs.0c02788>.
- [137] V. Köhler, J.C. Mao, T. Heinisch, A. Pordea, A. Sardo, Y.M. Wilson, L. Knörr, M. Creus, J.C. Prost, T. Schirmer, T.R. Ward, OsO₄•Streptavidin: A Tunable Hybrid Catalyst for the Enantioselective Dihydroxylation of Olefins, *Angew. Chem. Int. Ed.* 50 (2011) 10863–10866, <https://doi.org/10.1002/anie.201103632>.
- [138] A. Pordea, M. Creus, J. Panek, C. Duboc, D. Mathis, M. Novic, T.R. Ward, Artificial metalloenzyme for enantioselective sulfoxidation based on vanadyl-loaded streptavidin, *J. Am. Chem. Soc.* 130 (2008) 8085–8088, <https://doi.org/10.1021/ja8017219>.
- [139] V.B. Urlacher, M. Girhard, Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology, *Trends Biotechnol.* 37 (2019) 882–897, <https://doi.org/10.1016/j.tibtech.2019.01.001>.
- [140] G.C. Schröder, M.S. Smit, D.J. Opperman, Harnessing heme chemistry: Recent advances in the biocatalytic applications of cytochrome P450 monooxygenases, *Curr. Opin. Green Sust. Chem.* 39 (2023) 100734, <https://doi.org/10.1016/j.cogsc.2022.100734>.
- [141] R. Fasan, Tuning P450 Enzymes as Oxidation Catalysts, *ACS Catal.* 2 (2012) 647–666, <https://doi.org/10.1021/cs300001x>.
- [142] H.M. Key, P. Dydio, D.S. Clark, J.F. Hartwig, Abiological catalysis by artificial haem proteins containing noble metals in place of iron, *Nature* 534 (2016) 534–537, <https://doi.org/10.1038/nature17968>.
- [143] P. Dydio, H.M. Key, A. Nazarenko, J.Y.E. Rha, V. Seyedkazemi, D.S. Clark, J. F. Hartwig, An artificial metalloenzyme with the kinetics of native enzymes, *Science* 354 (2016) 102–106, <https://doi.org/10.1126/science.aah4427>.
- [144] K. Chen, F.H. Arnold, Engineering new catalytic activities in enzymes, *Nat. Catal.* 3 (2020) 203–213, <https://doi.org/10.1038/s41929-019-0385-5>.
- [145] P.S. Coelho, Z.J. Wang, M.E. Ener, S.A. Baril, A. Kannan, F.H. Arnold, E. M. Brustad, A serine-substituted P450 catalyzes highly efficient carbene transfer to olefins *in vivo*, *Nat. Chem. Biol.* 9 (2013) 485–487, <https://doi.org/10.1038/nchembio.1278>.
- [146] C.C. Farwell, J.A. McIntosh, T.K. Hyster, Z.J. Wang, F.H. Arnold, Enantioselective Imitation of Sulfides via Enzyme-Catalyzed Intermolecular Nitrogen-Atom Transfer, *J. Am. Chem. Soc.* 136 (2014) 8766–8771, <https://doi.org/10.1021/ja503593n>.
- [147] S.B.J. Kan, X.Y. Huang, Y. Gumulya, K. Chen, F.H. Arnold, Genetically programmed chiral organoborane synthesis, *Nature* 552 (2017) 132–138, <https://doi.org/10.1038/nature24996>.
- [148] J. Zhao, A.J. Burke, A.P. Green, Enzymes with noncanonical amino acids, *Curr. Opin. Chem. Biol.* 55 (2020) 136–144, <https://doi.org/10.1016/j.cbpa.2020.01.006>.
- [149] J.S. Trimble, R. Crawshaw, F.J. Hardy, C.W. Levy, M.J.B. Brown, D.E. Fuerst, D. J. Heyes, R. Obexer, A.P. Green, A designed photoenzyme for enantioselective [2 + 2] cycloadditions, *Nature* 611 (2022) 709–714, <https://doi.org/10.1038/s41586-022-05335-3>.
- [150] M.A. Emmanuel, S.G. Bender, C. Bilodeau, J.M. Carceller, J.S. DeHovitz, H. Fu, Y. Liu, B.T. Nicholls, Y. Ouyang, C.G. Page, T. Qiao, F.C. Raps, D.R. Sorigué, S. Z. Sun, J. Turek-Herman, Y. Ye, A. Rivas-Souchet, J. Cao, T.K. Hyster, Photobiocatalytic Strategies for Organic Synthesis, *Chem. Rev.* (2023), <https://doi.org/10.1021/acs.chemrev.2c00767>.
- [151] V. Alphan, W.J.H. van Berkel, V. Jurkaš, S. Kara, R. Kourist, M. Kroutil, F. Mascia, M.M. Nowaczyk, C.E. Paul, S. Schmidt, J. Spasic, P. Tamagnini, C.K. Winkler, Exciting Enzymes: Current State and Future Perspective of Photobiocatalysis, *ChemPhotoChem* 7 (2023) e202200325.10.1002/cptc.202200325.

- [152] M.A. Emmanuel, N.R. Greenberg, D.G. Oblinsky, T.K. Hyster, Accessing non-natural reactivity by irradiating nicotinamide-dependent enzymes with light, *Nature* 540 (2016) 414–417, <https://doi.org/10.1038/nature20569>.
- [153] L. Schmermund, V. Jurkaš, F.F. Özgen, G.D. Barone, H.C. Büchschütz, C. K. Winkler, S. Schmidt, R. Kourist, W. Kroutil, Photo-Biocatalysis: Biotransformations in the Presence of Light, *ACS Catal* (2019) 4115–4144, <https://doi.org/10.1021/acscatal.9b00656>.
- [154] B.A. Sandoval, A.J. Meichan, T.K. Hyster, Enantioselective Hydrogen Atom Transfer: Discovery of Catalytic Promiscuity in Flavin-Dependent ‘Ene’-Reductases, *J. Am. Chem. Soc.* 139 (2017) 11313–11316, <https://doi.org/10.1021/jacs.7b05468>.
- [155] K.F. Biegasiewicz, S.J. Cooper, X. Gao, D.G. Oblinsky, J.H. Kim, S.E. Garfinkle, L. A. Joyce, B.A. Sandoval, G.D. Scholes, T.K. Hyster, Photoexcitation of flavoenzymes enables a stereoselective radical cyclization, *Science* 364 (2019) 1166–1169, <https://doi.org/10.1126/science.aaw1143>.
- [156] H. Fu, H. Lam, M.A. Emmanuel, J.H. Kim, B.A. Sandoval, T.K. Hyster, Ground-State Electron Transfer as an Initiation Mechanism for Biocatalytic C–C Bond Forming Reactions, *J. Am. Chem. Soc.* (2021), <https://doi.org/10.1021/jacs.1c04334>.
- [157] F. Richter, A. Leaver-Fay, S.D. Khare, S. Bjelic, D. Baker, De Novo Enzyme Design Using Rosetta, *PLoS One* 6 (2011), <https://doi.org/10.1371/journal.pone.0019230> e19230.
- [158] S.L. Lovelock, R. Crawshaw, S. Basler, C. Levy, D. Baker, D. Hilvert, A.P. Green, The road to fully programmable protein catalysis, *Nature* 606 (2022) 49–58, <https://doi.org/10.1038/s41586-022-04456-z>.
- [159] S. Mazurenko, Z. Prokop, J. Damborsky, Machine Learning in Enzyme Engineering, *ACS Catal* 10 (2020) 1210–1223, <https://doi.org/10.1021/acscatal.9b04321>.
- [160] T. Yu, A.G. Boob, M.J. Volk, X. Liu, H. Cui, H. Zhao, Machine learning-enabled retrobiosynthesis of molecules, *Nat. Catal.* 6 (2023) 137–151, <https://doi.org/10.1038/s41929-022-00909-w>.
- [161] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Židek, A. Potapenko, A. Bridgland, C. Meyer, S. A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Highly accurate protein structure prediction with AlphaFold, *Nature* 596 (2021) 583–589, <https://doi.org/10.1038/s41586-021-03819-2>.
- [162] U. Hanefeld, F. Hollmann, C.E. Paul, Biocatalysis making waves in organic chemistry, *Chem. Soc. Rev.* 51 (2022) 594–627, <https://doi.org/10.1039/D1CS00100K>.