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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>

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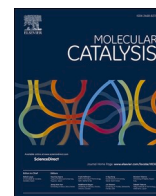
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Enzyme engineering for biocatalysis

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

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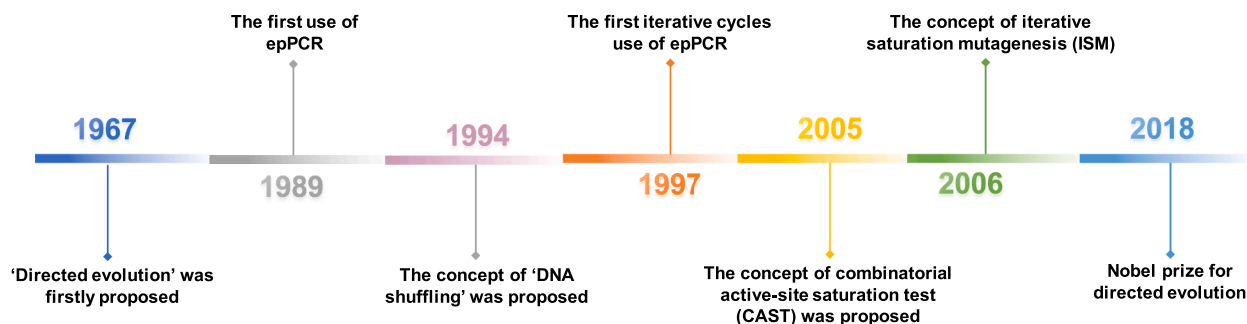


Fig. 1. Some key milestones for the development of enzyme engineering.

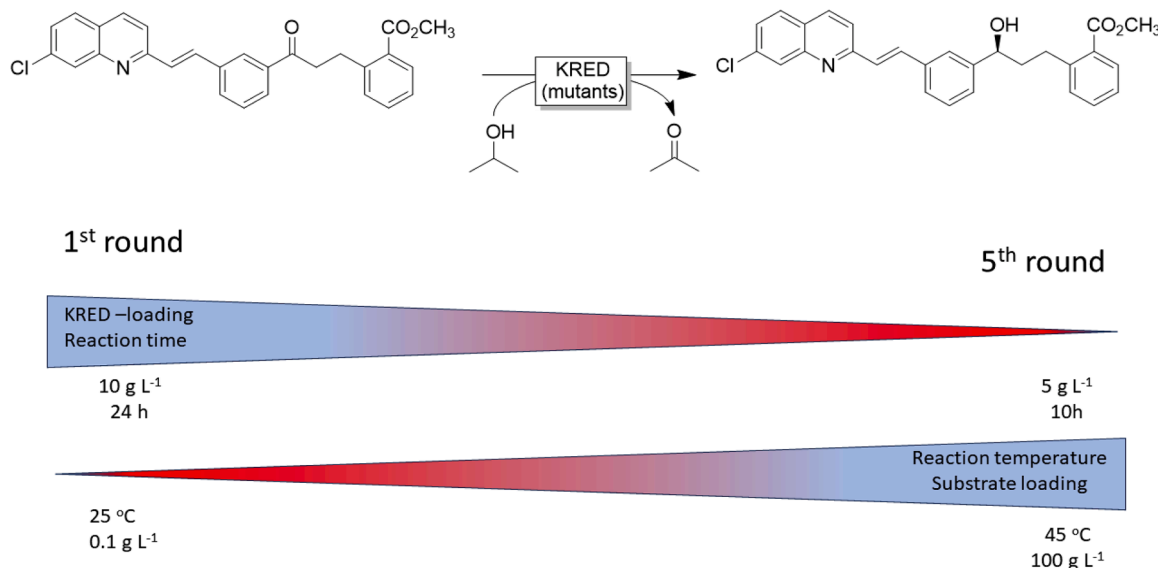


Fig. 2. Co-evolution of various desirable enzyme properties by gradual increasing the selective pressure.

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.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

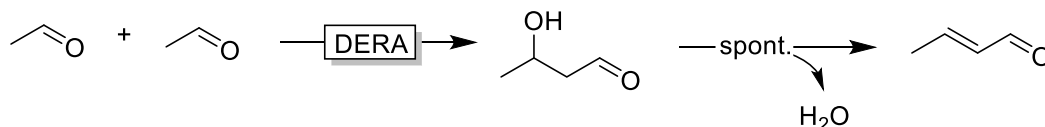
Various *in silico* methods to predict thermostability are currently available (Table 1). Each method has been demonstrated to be beneficial in at least one literature example, however a generally applicable tool remains to be developed. According to Reetz et al. [12], a practitioner should focus on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

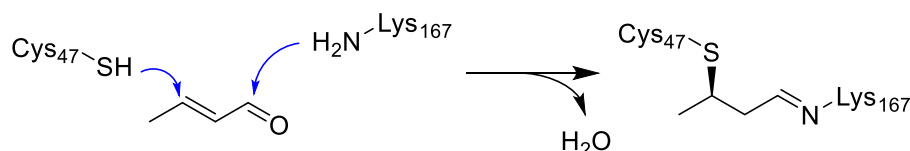


Fig. 3. Molecular basis for the acetaldehyde-related inactivation of DERA.

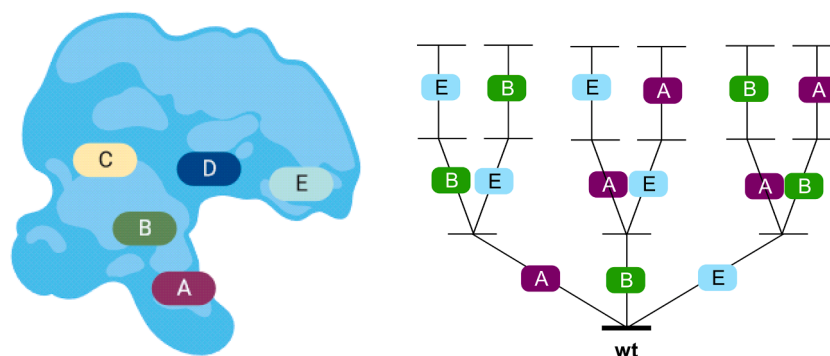


Fig. 5. General representation of the CAST/ISM strategy. As an example, amongst the relevant regions (A–E) A, B and E were selected for ISM.

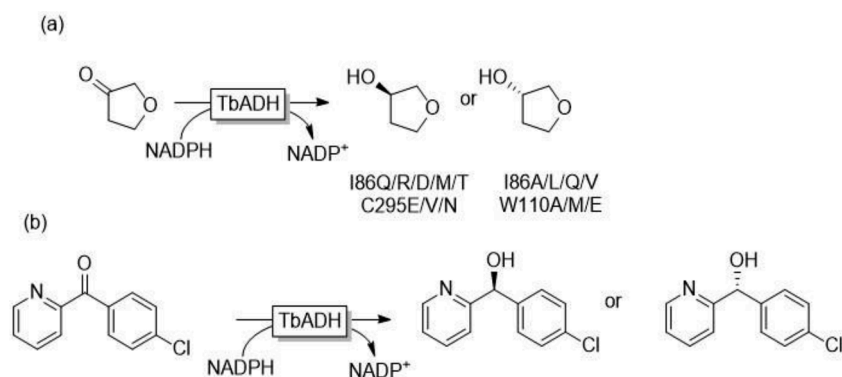


Fig. 6. Examples of 'difficult' stereoselective reductions accomplished with engineered TbADH.

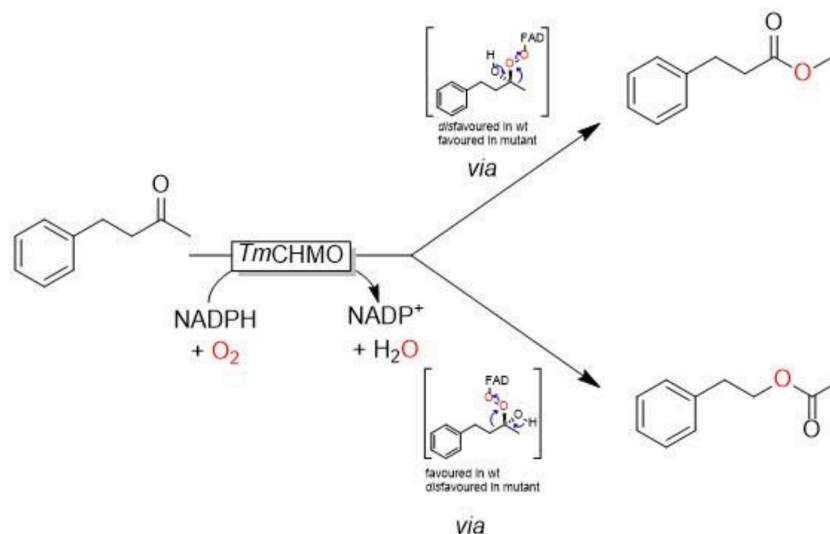


Fig. 7. Controlling the migrational tendency of carbonyl substituents in the enzymatic BV oxidation.

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

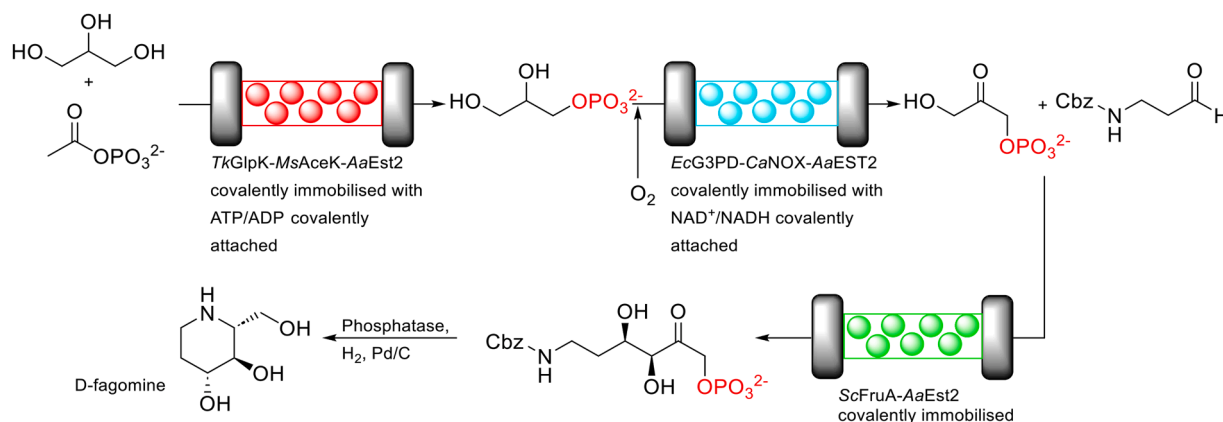


Fig. 8. Three-step continuous flow of the immobilised enzyme assemblies for the conversion of glycerol to D-fagomine.

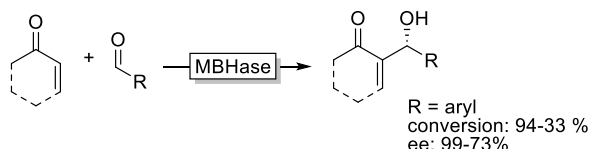


Fig. 9. Newly designed enzyme to catalyze Morita-Baylis-Hillman reactions.

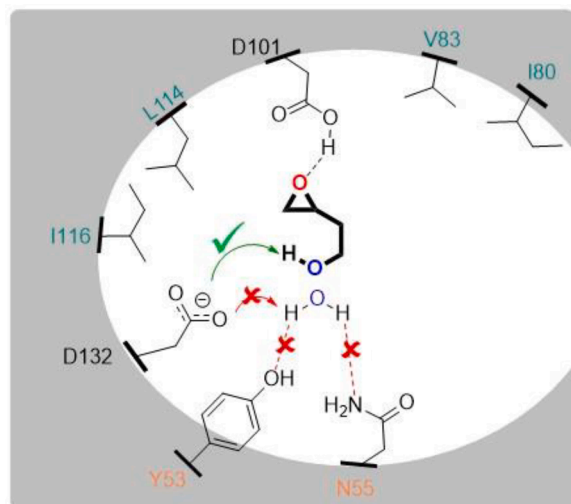


Fig. 10. Epoxide hydrolase engineered to catalyze a Baldwin-type cyclisation.

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 ΔP84 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)

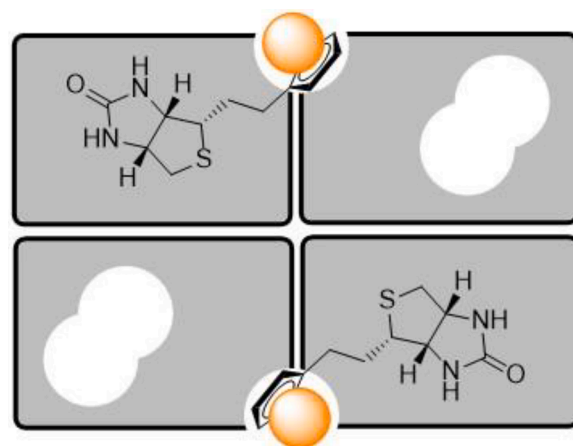


Fig. 11. Schematic representation of a metal-loaded biotin binding to streptavidin and thereby placing it in a chiral environment.

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

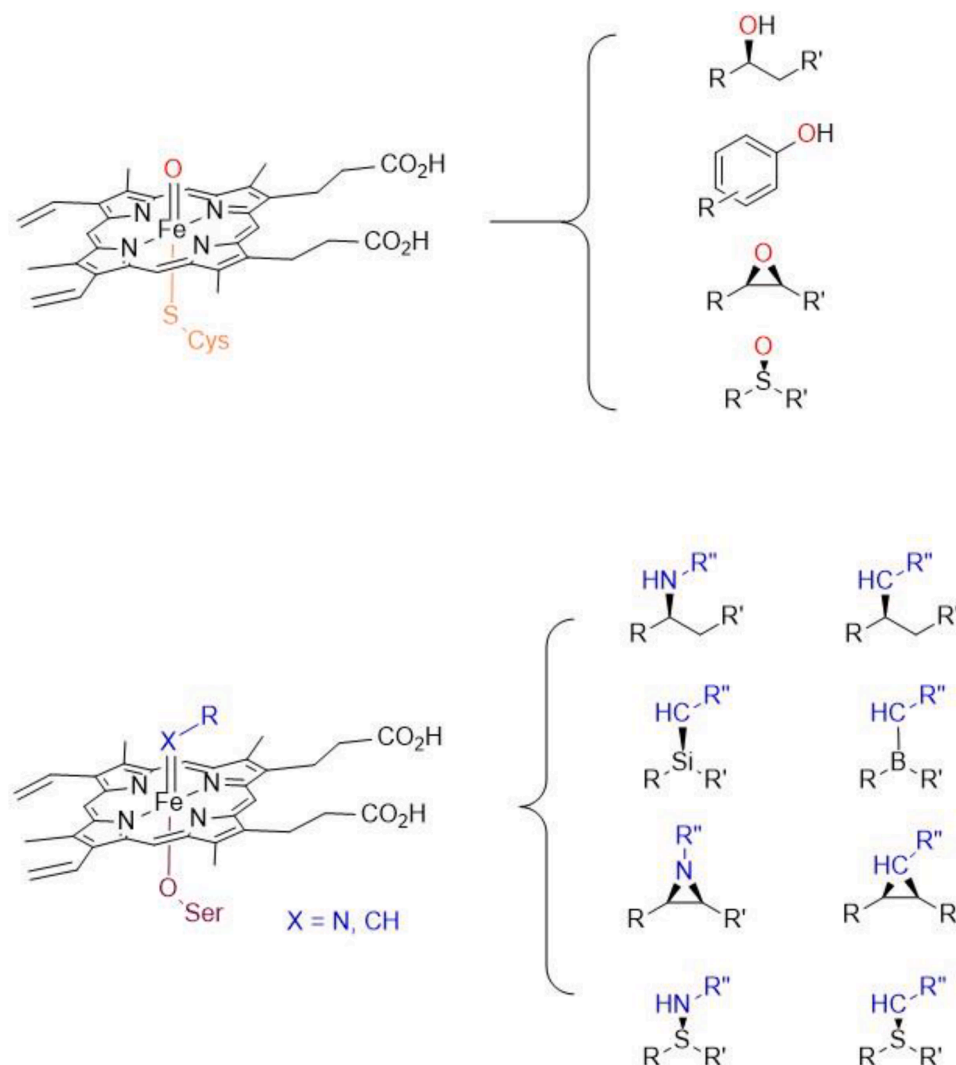


Fig. 12. Comparison of the native reactivity of P450 monooxygenases with the engineered P411 enzymes.

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

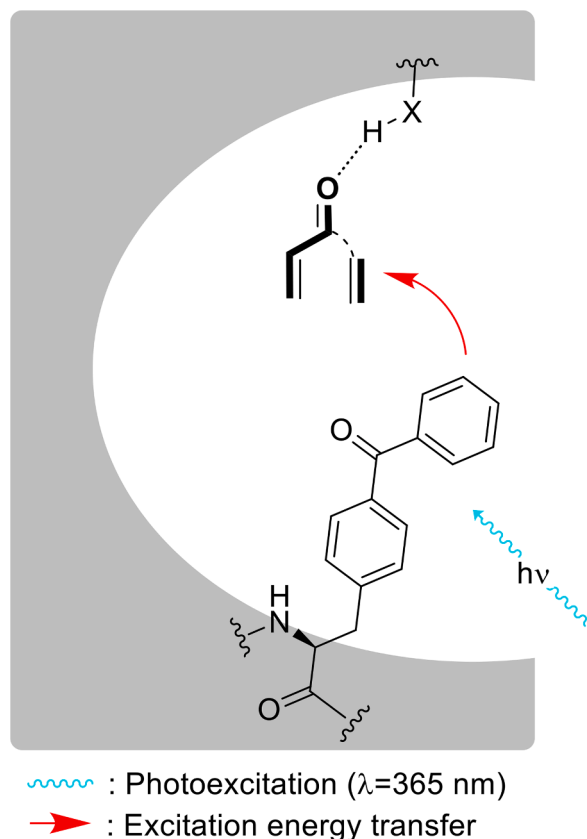


Fig. 13. Design of an artificial Diels Alderase by genetically incorporating a photosensitizer.

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 reaction 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

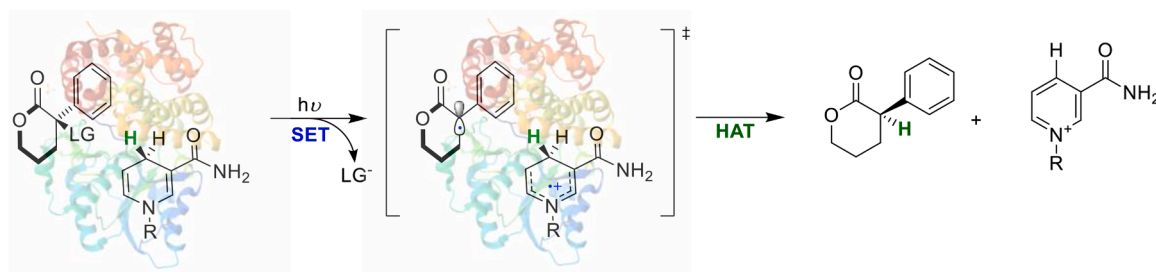


Fig. 14. The charge-transfer complex of enzyme-bound NAD(P)H and substrate can be photoexcited resulting in a single electron transfer (SET) and mesolytic cleavage of the leaving group (LG). The resonance stabilised substrate radical then selectively receives a hydrogen atom transfer (HAT) from the NAD(P)H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

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