ENGINEERING BACTERIAL NITROREDUCTASES FOR ANTICANCER GENE THERAPY AND TARGETED CELL ABLATION

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Key Words: nitroreductase, prodrug, directed evolution, gene therapy, cell ablation

Bacterial nitroreductases are members of a diverse family of oxidoreductase enzymes that can catalyse the bioreductive activation of nitroaromatic compounds, including anti-cancer and antibiotic prodrugs. Nitroreductases have diverse applications in medicine and research, including anti-cancer gene therapy and targeted ablation of nitroreductase-expressing cells in transgenic zebrafish to model degenerative diseases. Research in these fields to date has focused almost exclusively on the canonical nitroreductase NfsB from *Escherichia coli* (NfsB_Ec), which is a relatively inefficient choice for most applications. By exploring alternative nitroreductase candidates from a variety of bacterial species, in concert with enzyme engineering to fine-tune specific activities, we have generated improved prodrug-activating enzymes. The nitroreductase NfsB from *Vibrio vulnificus* (NfsB_Vv) has been central to our efforts, and following solution of its crystal structure, was selected as a scaffold for directed evolution via site-saturation mutagenesis. By applying library screening strategies that involved rounds of both positive and negative selection, several mutants that displayed improved activity with a promising next-generation cancer prodrug were identified. In parallel work, an engineered NfsB_Vv variant from the same library was found to be substantially improved in activation of the antibiotic prodrug metronidazole, which is widely used for targeted cell ablation in transgenic zebrafish. Current methods of ablation employing NfsB_Ec require high, near lethal concentrations of metronidazole to achieve total ablation of nitroreductase-expressing cells. A transgenic zebrafish line expressing a lead NfsB_Vv variant was generated and we found we could achieve robust ablation of nitroreductase-expressing cells at a 100-fold reduced metronidazole concentration compared to the NfsB_Ec line (0.1 mM challenge for 24 hours vs 10 mM challenge for 48 hours respectively). The identification of these superior nitroreductase variants offers improved tools for researchers aiming to achieve targeted cell ablation in either a cancer therapy or degenerative disease-modelling context.

QM/MM MD STUDIES OF POLYESTER SYNTHESIS/HYDROLYSIS

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Key Words: Biodegradable polyesters, polycaprolactone, enzymatic synthesis, AfEST, lipase, carboxylesterase, CaLB, QM/MM MD sim-ulations..

The world is suffering the consequences of plastic pollution, however modern societies remain heavily reliant on plastics. More sustainable alternatives are actively being sought-after. Enzymatic synthesis can offer a more sustainable route for polyester synthesis. Nevertheless, there are still limitations, such as limited activity and selectivity for some monomers, unfavorable compatibility in chemoenzymatic reactions and low stability under harsh reactions conditions. We have studied the catalytic mechanisms for polycaprolactone hydrolysis/synthesis by the wildtype enzymes Archaeoglobus fulgidus carboxylesterase (AfEST) and Candida antarctica lipase B (CaLB) and respective enzyme variants by performing Quantum Mechanics/Molecular Mechanics Molecular Dynamics simulations [1-3]. Our results give important insights towards the design of new enzyme variants combining good activity with high thermostability.

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DIRECTED EVOLUTION OF THE NON-RIBOSOMAL PEPTIDE SYNTHETASE BpsA TO ENABLE RECOGNITION BY THE HUMAN Sfp-LIKE PPTase

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Key Words: Directed evolution, NRPS, high throughput screening, error-prone PCR

Non-ribosomal peptide synthetases (NRPSs) are large, modular enzymes that have an assembly line architecture and synthesise a diverse range of compounds such as antibiotics, siderophores and immunosuppressants. Within the assembly line, the peptidyl carrier protein (PCP) domain has a crucial role in shuttling substrates between the different catalytic domains. The PCP domain is a small four-helix bundle that requires a phosphopantetheinyl moiety to be attached to a conserved serine on the second alpha helix for functionality. This post-translational modification is catalysed by a family of enzymes called the phosphopantetheinyl transferases (PPTases).

Due to their central role in activating enzymes involved in both primary (e.g., fatty acid synthetases) and secondary metabolism (e.g., NRPSs), PPTases have been identified as a promising antibiotic target in bacterial species such as *Mycobacterium tuberculosis*. We have previously developed a high-throughput enzymatic screen for PPTase inhibitors based on co-incubation of a target PPTase with a blue-pigment synthesising NRPS, BpsA. As part of the development of a complete screening platform, we also wanted to be able to rapidly counter-screen inhibitors for cross-inhibition of the endogenous human PPTase, as this is a potential source of toxicity. We found we were unable to use the native BpsA enzyme for this, as the human PPTase is incapable of recognising the PCP domain of BpsA.

To improve with the ability of BpsA to be activated by the human PPTase, a directed evolution campaign was undertaken. Firstly, error-prone PCR was used to introduce mutations into the PCP domain of BpsA. Approximately 200,000 variants were screened using a high-throughput plate-based assay. Forty ‘hits’ were then characterized in a semi-quantitative liquid assay. Based on the pattern of amino acid substitutions in the most active variants, specific combinations of substitutions were rationally introduced into BpsA. The top variant identified was now capable of being rapidly phosphopantetheinylated by the human PPTase and we have shown this can be used to quickly screen bacterial PPTase inhibitors for cross-reactivity with the human PPTase. This work illustrates the flexible nature of the PCP domain and provides further evidence that only a few point mutations may be sufficient to dramatically change the specificity of PCP domains for different PPTases.

BROADENING SUBSTRATE SPECIFICITY ACROSS SHORT-CHAIN DEHYDROGENASE REDUCTASES (SDRS)

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Short-chain dehydrogenase reductases (SDRs) form a structural motif for enzymes that catalyze a wide variety of reactions[1], even if sequence identity often is low. Substrate promiscuity across SDRs so far has been found in a small number of cases, such as between glucose dehydrogenases and imine reductases.[2] The current work demonstrates both experimental and computational protein engineering towards broadening substrate specificity across SDRs with hitherto distinct substrate profiles.

As an example, we have investigated two classical SDR groups of enzymes: (GDH) glucose 1-dehydrogenase (EC 1.1.1.47), a thermostable protein which catalyzes oxidation of glucose to gluconic acid with NAD(P)H[3] and alcohol dehydrogenases (ADHs), which catalyze the reversible reduction of ketones or aldehydes to alcohols, the former often with exquisite stereoselectivity. In this work, we demonstrate not only broadening of substrate specificity of ADHs but, in addition, the interconversion of GDHs and ADHs.[4]

We have employed site-directed mutagenesis and combinatorial mutagenesis with focused libraries of the active site environment, as well as structure-guided consensus aided by computational modelling to residues affecting the accessibility of the active site.[5] We have tracked thermal and process stability of the generated variants via a novel set of tools, especially Differential Scanning Fluorimetry (DSF) with internal aromatic residue standard and SEC-multi-angle laser scattering (SEC-MALLS) with 4 detectors.

Lastly, the structural basis for substrate specificity of proteins with GDH and ADH functionality as well as GDH vs ADH functionality will be discussed.

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ENZYMATIC BIOTRANSFORMATION OF ADIPIC ACID TO 6-AMINOCAPROIC ACID AND 1,6-HEXAMETHYLENEDIAMINE USING ENGINEERED CARBOXYLIC ACID REDUCTASES AND AMINOTRANSFERASES

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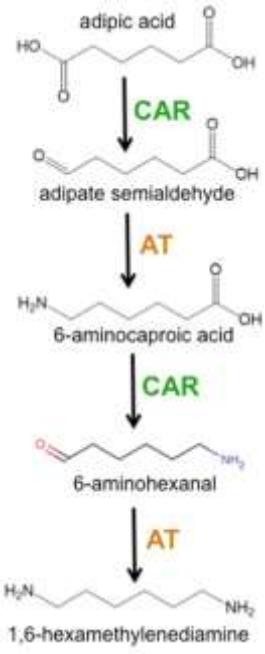
Key Words: adipic acid, hexamethylenediamine, 6- aminocaproic acid, carboxylic acid reductase, aminotransferase.



Biocatalytic reduction of carboxylic acids is gaining importance for the production of polymer precursors and different chemicals. Carboxylic acid reductases (CARs) reduce carboxylic acids to aldehydes using ATP and NADPH as cofactors under mild conditions. Recently, we demonstrated that several bacterial CARs can reduce a broad range of bifunctional carboxylic acids containing amino group or second carboxylic group including adipic acid, which is a precursor for nylon-6-6 (Khusnutdinova et al., 2017). In this project, we demonstrate application of CARs and aminotransferases for further bioconversion of adipic acid to 6-aminocaproic acid and hexamethylenediamine, two other important precursors for nylon synthesis. Based on the crystal structure of the adenylating domain of the CAR enzyme MCH22995 from *Mycobacterium chelonae*, we generated a structural model of the CAR enzyme MAB4714 from *M. abscessus*, which is active toward adipic acid. Aiming at improving MAB4714 activity toward 6-aminocaproic acid, we used structure-based protein engineering and generated 16 MAB4714 mutant proteins. Screening of 16 purified MAB4714 variants against 6-aminocaproic acid,identified one protein, which was 10 times more active than the wild-type protein. We also identified several bacterial aminotransferases producing 6-aminocaproic acid from adipic acid in combination with CARs. Further optimization of reaction conditions and application of cofactor regeneration systems resulted in efficient biotransformation of adipic acid to 6-aminocaproic acid (88% conversion) and further to 1,6-hexamethylenediamine (78% conversion).

Figure 1 – Biotransformation of adipic acid to 6-aminocaproic acid and 1,6-hexamethylenediamine using carboxylic acid reductases (CAR) and aminotransferases (AT).

Khusnutdinova, AN; Flick, R; Popovic, A; Brown, G; Tchigvintsev, A; Nocek, B; Correia, K; Joo, JC; Mahadevan, R; and Yakunin, AF (2017) Exploring bacterial carboxylate reductases for the reduction of bifunctional carboxylic acids. *Biotechnology J*, 12, 1600751.

DESIGN AND EVOLUTION OF ENZYMES WITH NON-CANONICAL CATALYTIC MECHANISMS

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Key Words: enzyme design, non-canonical amino acid, directed evolution, expanded genetic code.

Enzyme design and evolution strategies rely exclusively on Nature's standard amino acid alphabet of twenty canonical residues which contain limited functionality. Here we demonstrate that incorporation of non-canonical amino acids into enzyme active sites provides a fruitful avenue to probe complex biological mechanisms and can lead to the creation of designed enzymes with wholly new catalytic functions. Significantly, optimization of enzyme activity can be achieved using directed evolution workflows adapted to an expanded genetic code. We are optimistic that this integration of enzyme design, genetic code expansion and laboratory evolution can provide a versatile strategy for creating enzymes with catalytic functions not accessible to Nature.

NEW PATHWAYS FOR SUSTAINABLE TERPENE MATERIALS FROM WOOD

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Key Words: Chemo-enzymatic Synthesis, Polymers, Terpenes.

Polymers play an essential role in everyday life as materials in automotive, packaging and electronics to compounds in medicine. Nevertheless, the finite supply of fossil fuels and lacking biodegradability of most synthetic polymers leads to an increased need for the development of more sustainable materials from renewable sources. The use of renewable natural products from forestry, especially hemicellulose and terpenes,1 offers a highly versatile platform for green building blocks. By using Nature's own biofunctionalizations, enzymes can be used as green catalysts for the valorization of abundant terpenes from pine wood extractives. Enzymatic biotransformations enable mild processes for "activating" inert molecular building blocks to afford renewable monomers. By combining in vitro synthetic biology and polymer chemistry, we have generated a new bio based polymer starting from, a naturally abundantterpene found in wood. Specifically, acrylation of the terpene Sobrerol, which can be achieved§ both enzymatically and by using traditional chemistry, constituted a suitable synthon for Atom Transfer Radical Polymerization (ATRP); a common and widely used, controlled polymerization technique. Furthermore, we demonstrate that sobrerol can be generated from other abundant monoterpenes using P450-based biocatalysis.

Thus, it could be shown that polymers with different targeted lengths could be realized in a highly controlled manner. Thermal analysis showed high stability and glass transition temperatures above 120 °C, which is in good agreement with other polymethacrylates. Besides using ATRP, the methacrylated Sobrerol was also successfully polymerized using RAFT (Reversible addition−fragmentation chain-transfer polymerization) as well as conventional free radical polymerizations.

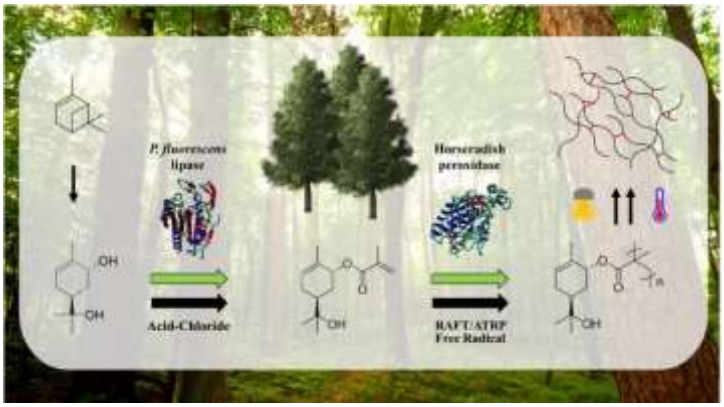

Figure 2 – Chemoenzymatic pathways from (−)α-pinene to PSobMA.

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HIGHLY OXYGEN-STABLE CO_2 REDUCTASE-CATALYZED BIOCONVERSION OF CARBON DIOXIDE INTO FORMATE IN ELECTROCHEMICAL REACTOR

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Electrochemical reduction of carbon dioxide (CO_2) into value-added chemicals have been one of promising issues to utilize greenhouse gas for the storage of hydrogen, bioplastic, fuel cell et al. Among many potential candidates such as formate, alcohols, ethylene, etc., formate was known one of most promising chemicals through the addition of two elections and one proton during electro-catalytic reduction of CO_2.

Here, our group has developed efficient CO_2 reductase-catalyzed conversion of CO_2 into formate in electrochemical reactor. Electro-biocatalytic reduction of CO_2 into formate have three main challenges. Formate dehydrogenase(FDH) have been one of potential CO_2 reductase candidates but the activity of many FDHs for formate oxidation was superior than that of CO_2 reduction. However, recombinant MeFDH1 in our study showed significantly higher preference for CO_2 reduction with 209 (±10.66) s^{-1} of turnover rate than that of formate oxidation with 85.62 (± 5.76) s^{-1}. In addition, the value of kinetic equilibrium constant (K_{eq}), which determines the direction of reaction in reversible catalysis, implied that kinetic preference for CO_2 reduction is 62.3-folds higher than that for formate oxidation. This kinetics analysis allowed MeFDH1 to be referred to as the CO_2 reductase. As another challenge, Mo- or W-containing formate dehydrogenase was known vulnerable against oxygen molecule. As contrasted with the high oxygen-sensitivity of FDHs from other strains, MeFDH1 was not deactivated even under 0.13 mM of dissolved oxygen. At a range of oxygen level (0.1 % to 4 %) in the composition of Carbon Capture and Storage (CCS) gas, MeFDH1 was consequently stable without considerable decreased activity. Lastly, the long-term stability of CO_2 reduction reaction system has been indispensable issue in both biocatalysts and chemical catalysts. Through the immobilization of MeFDH1, the enhanced stability for long-term operation leaded the formate formation over 500 mM with high volumetric productivity for several days.

COBALAMIN DEPENDENT METHYLATION AND DEMETHYLATION BY VERATROL O-DEMETHYLASE

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Key Words: methyl transferases, cobalamin, methylation, demethylation

The formation and breakage of C-O ether bonds are valuable synthetic transformations contributing to the structural diversification of natural products and pharmaceuticals [1-3]. Moreover, O-methylated phenol derivatives are useful building blocks for the manufacture of antioxidants, flavoring agents, fragrances, dyes, agrochemicals and fine chemicals [4,5]. Despite the large variety of chemical reactions for methylation and demethylation, none reaction is reversible and sustainable. They often lack chemo-, regio- and stereoselectivity and rely on harsh reaction conditions [6]. Thus, the development of milder alternatives such as biocatalytic methylation and demethylation reactions is of high interest [7].

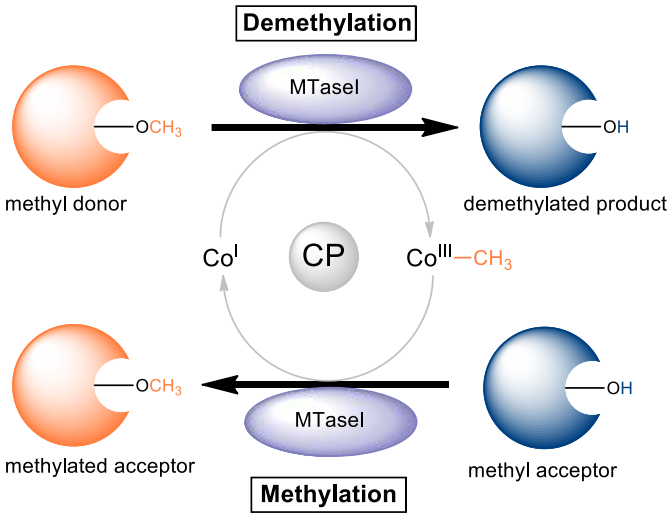

Figure 1: The cobalamin dependent methyltransferase (MTaseI) catalyzes the demethylation of a methyl donor and the methylation of a methyl acceptor alongside with the corrinoid protein (CP) acting as a methyl shuttle.

We present an enzymatic system utilizing cobalamin (vitamin B12) dependent methyl transferases (MTases) derived from the anaerobic bacteria which enable both methylation and demethylation of heteroatoms [8,9]. Naturally, the bacterial system involves four proteins for these reactions whilst our concept requires only two proteins (see Figure 1): the veratrol O-demethylase (MTaseI) from *Acetobacterium dehalogenans* and the corrinoid protein (CP) derived from another origin. The CP incorporates a cobalamin prosthetic group functioning as methyl carrier. Along with the CP the MTaseI catalyzes both, the demethylation of a donor and the methylation of an acceptor substrate in a reversible manner. The activity of the MTaseI relies on the amount of CP and zinc, because it incorporates a zinc binding motif [10]. The approach represents a substrate promiscuous alternative to common chemical and enzymatic methyl transfer methodologies and a valuable extension for the toolbox of available biocatalysts for ether bond formation as well as cleavage.

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LIGHT-DRIVEN KINETIC RESOLUTION OF A-FUNCTIONALIZED ACIDS ENABLED BY ENGINEERED FATTY ACID PHOTODECARBOXYLASE

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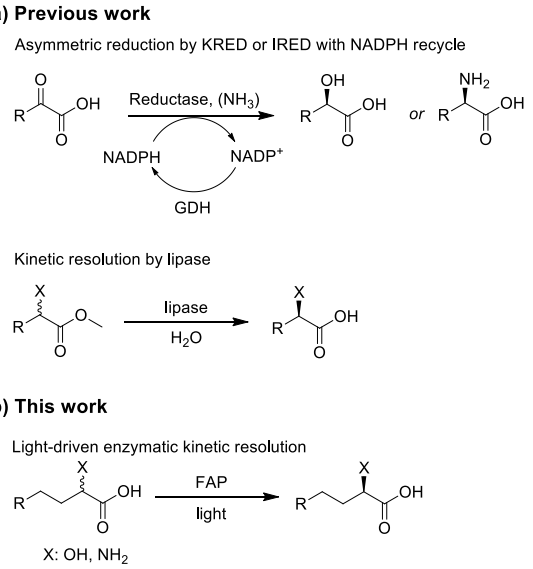
Key Words: photoenzyme; biocatalysis; α-functionalized acids; kinetic resolution; directed evolution

Multifunctional chiral molecules such as unnatural α-amino acids and α-hydroxy acids are valuable precursors to a variety of medicines and natural products.[1] The biocatalysis provides a greener and more sustainable process than transition metal catalysts and complex chiral ligands. For example, keto reductases (KRED) and imine reductases (IRED) have been successfully used to convert α-keto acids into α-hydroxy/amino acids.[2] Another widely used method was kinetic resolution (KR) or dynamic kinetic resolution (DKR) by employing lipases.[3] Herein, we described the variants of fatty acid photodecarboxylase (C*v*FAP), which was used to convert long-chain fatty acids into hydrocarbons,[4] catalyze kinetic resolution of α-amino acids and α-hydroxy acids with high conversion and excellent nonreacted (*R*)-configured substrate stereoselectivity (ee up to 99%). This efficient light-driven process does not require NADPH recycle nor prerequisite preparation of esters in contrast with other biocatalytic methods (Scheme 1). To our delight, although most biocatalysts are hardly to be universal, the best mutant G462Y displayed a satisfactory substrate scope (Figure 1). The structure-guided engineering strategy was introduced by large-size amino acid scanning at hot position to narrow the substrate binding tunnel. We believed that this research conformed to the conference topic of Enzyme promiscuity, evolution and dynamics.



Scheme 1. Enzymatic asymmetric synthesis of α-hydroxy/amino acids

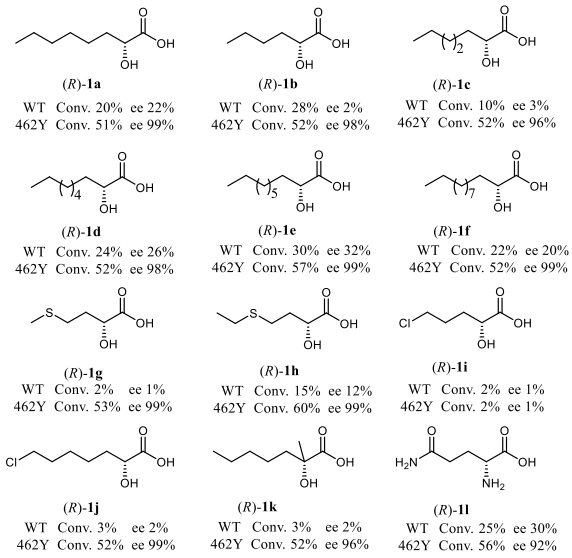


Figure 1. Substrate scope of light-driven kinetic resolution enabled by WT CvFAP and variant G462Y

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HIGH-THROUGHPUT ENZYME DISCOVERY AND ENGINEERING FOR BIOPROCESS OPTIMIZATION

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Key Words: metagenomics, directed evolution, automation

At Zymergen, we have developed a platform to engineer microbes for manufacturing chemicals and novel materials with unprecedented flexibility, efficiency, and reliability. This platform integrates several core technologies including high-throughput laboratory automation, custom software, and machine learning algorithms. We have recently extended this platform to bring enzyme discovery and engineering workflows to bear on both strain improvement projects and development of enzymes for industrial biocatalysis. These projects draw on two new capabilities that leverage our existing platform. First, novel enzyme homologs are identified from our proprietary metagenomics database, then tested for a desired activity using our high throughput platforms for strain build and screening. Second, leading enzyme candidates can be further optimized for a specified process using classical directed evolution methods. Each of these steps can be executed in a host chosen from panel of microbes that are compatible with Zymergen infrastructure; alternatively, our automation workflows can be modified to use a non-standard microbe that is currently being employed at scale. Using case studies, we will describe how we can apply these approaches to engineer enzymes with improved performance for a variety of applications.

ENGINEERING PET-DEGRADING ENZYMES FOR BIORECYCLING AND BIOREMEDIATION

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Key Words: plastic biodegradation, enzymatic recycling

Plastics, due to their inert properties and resistance to biodegradation, have been ravaging ecosystems worldwide and are especially harmful to aquatic wildlife. Plastics in the environment wear and tear into micron sized particles, termed microplastics, which are ingested and/or affect organisms at every level of the food chain. Recently, microplastics have been reported in human feces, and their potential health hazards remain unknown. In 2010, 4.8-12.7 million metric tons entered the oceans due to mismanagement and leakage, with an additional 31 million metric tons when terrestrial and freshwater ecosystems are considered. Both food and water supplies are likely contaminated with microplastics, and we need technologies to decrease formation of microplastics and remove these particulates from the environment. One of the most synthesized plastics is poly(ethylene terephthalate) (PET), an aromatic polyester with extremely low degradation rates. Due to the huge negative environmental impact of PET products, efficient recycling strategies need to be designed to “close the loop”’ to reduce dependence on petroleum feedstocks and decrease economic loss through single-use practices. The recent discovery of a PET-consuming bacteria *Ideonella sakaiensis* and its PET hydrolases has shown potential for enzyme-mediated recycling and bioremediation. Here, we present preliminary characterization of the catalytic rate of the newly discovered PETase and its behaviour over time, with perspective into future engineering potential for the enzyme for use in industrial processes.

GENETIC BIOSENSOR ENABLES IN VIVO GLYCOSYLTRANSFERASE SCREENING

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Key Words: Biosensor, Glycosyltransferase, High-throughput screening, Enzyme discovery

Glycosylation of natural products can alter their solubility and bioavailability, among other properties, which makes glycosyltransferases useful tools for increasing the production and/or generating novel compounds in microbial cell factories. However, the discovery and screening of new enzymes and engineered variants is often a low-throughput endeavor due to the need for over-expression and purification prior to *in vitro* experiments, which do not necessarily represent the *in vivo* activities of the enzyme. Therefore, a genetic biosensor controlling GFP expression was developed based on the flavonoid responsive transcriptional-repressor QdoR and expressed in *E. coli*. Due to the induced fluorescent response upon feeding the flavonoids Quercetin and Kaempferol, but not to their glucosides, the activity of UDP-dependent glycosyltransferases (UGTs) could be screened *in vivo*. Furthermore, a variant of QdoR was generated by directed evolution that showed greater dose-responsiveness and proved to allow greater discrimination of cellular populations and was thus more useful for *in vivo* UGT screening. The designed biosensor-based method will greatly increase the throughput of glycosyltransferase discovery and engineering.

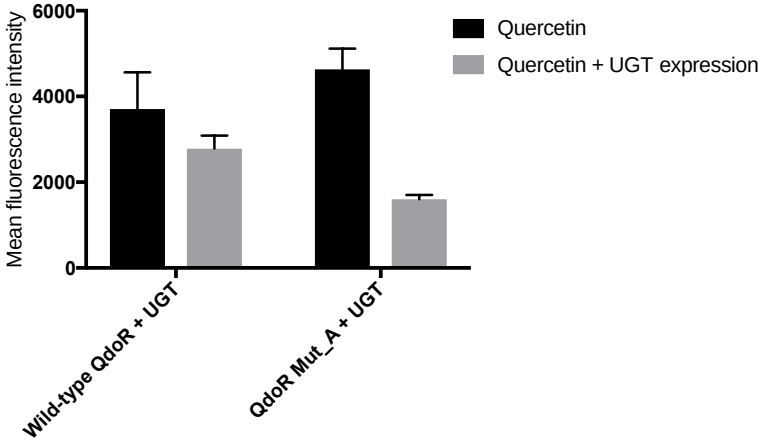

Figure 1 – Expression of flavonoid UGT results in significant decrease of fluorescence due to glycosylation of the flavonoid Quercetin.

COFACTOR SWITCH: DEVELOPMENT OF A NAD$^+$-DEPENDENT CASCADE FOR THE PRODUCTION OF URSODEOXYCHOLIC ACID (UDCA)

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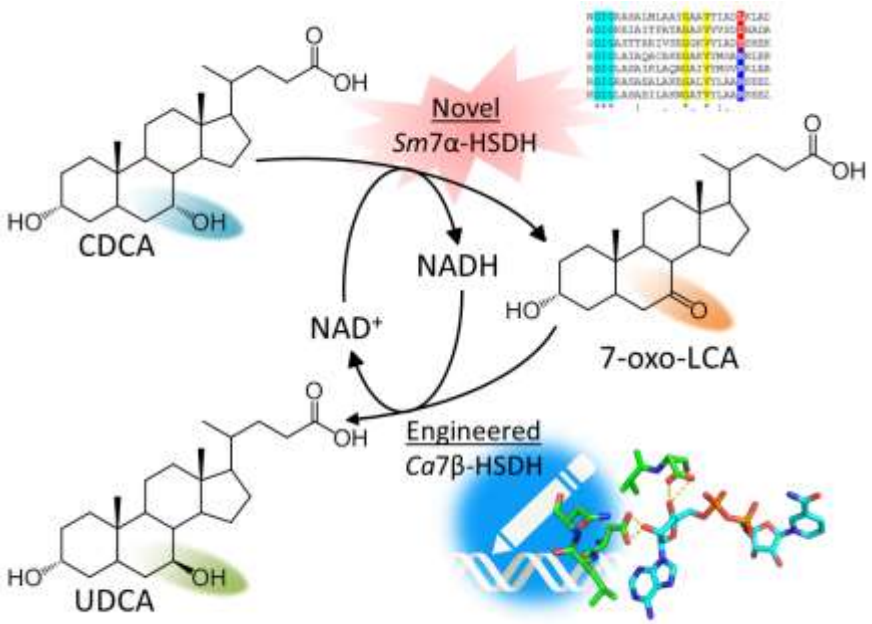
Key Words: Cofactor switching, hydroxysteroid, Ursodeoxycholic acid, redox-neutral, dehydrogenase

The employment of alcohol dehydrogenases in cascade reactions is often limited by the different cofactor specificity of the enzymes involved: the employment of additional cofactor regeneration systems and the excess amount of sacrificial substrates frequently increase the environmental impact and the costs of biocatalytic processes. Additionally, a NADP$^+$-dependent process is generally less desirable, inasmuch this cofactor is more expensive, unstable and less naturally available than NAD$^+$, leading to an increase of the process costs. Nowadays, protein engineering offers the possibility to switch the cofactor dependency of enzymes introducing few targeted mutation[1].

We applied this methodologies for the development of the first fully NAD$^+$ mediated cascade for the production of Ursodeoxycholic acid (UDCA), a widely used pharmaceutical ingredient for the clinical treatment of cholesterol gallstones and liver diseases[2]. The enzymatic epimerization of CDCA into UDCA, can be carried out with two specific hydroxysteroid dehydrogenases (HSDH): the 7α-OH group is firstly oxidized to the ketone by 7α-HSDH and subsequently re-reduced with opposite stereochemistry (7β-OH) by 7β-HSDH. All procedures up to now require a set of a respectively NAD$^+$ and NADP$^+$ dependent enzymes. Nevertheless, gene sequences of NAD$^+$-dependent 7β-HSDH were not reported. In order to obtain a full-NAD$^+$-dependent process, the NADP$^+$ dependent 7β-HSDH from *Clostridium absonum* was engineered. Employing a semi-rational mutagenesis approach, we obtained a variant with shifted cofactor preference. Importantly, this study allows to identify the residues responsible of the cofactor recognition in other 7β-HSDH homologues and, thus, to the identification of the gene coding for a wild-type NADH-dependent homologue from *Lactobacillus spicheri* (*Ls*7β-HSDH). These novel NAD$^+$-dependent 7β-HSDH enzymes in combination with 7α-HSDH from *Stenotrophomonas maltophilia* permitted the redox-neutral biotransformations of CA and CDCA in the presence of catalytic amounts of NAD$^+$, resulting in high yields (>90 %) of UCA and UDCA[3].



Further studies are underway to develop a flow process for this industrially relevant biotransformation.

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CAVERDOCK: SOFTWARE TOOL FOR FAST SCREENING OF UN/BINDING OF LIGANDS IN PROTEIN ENGINEERING

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Key Words: CaverDock; Caver; Tunnels; Docking;

Protein tunnels, channels and gates are important for enzymatic catalysis and also represent attractive targets for rational protein design and drug design [1]. Drug molecules blocking the access of natural substrate or release of products are very efficient modulators of biological activity. Here we demonstrate the application of newly *in-house* developed software tool CaverDock [2,3] for the analysis of the transport of ligands through tunnels in biomolecular targets. Caverdock is a new addition to the Caver Suite [4-6]. We performed virtual screening of large databases of drugs against two pharmacologically relevant targets. We have used FDA-approved drugs for both targets. Oncological drugs (133 molecules), taken from the NIH website, and anti-inflammatory (56 molecules), taken from the Drugbank website, as the libraries of ligands for the two molecular targets: (i) cytochrome P450 17A1 and (ii) leukotriene A4 hydrolase/aminopeptidase. Moreover, we will also show the unbinding of the 2,3-dichloropropan-1-ol product from a buried active site of an haloalkane dehalogenase and its variant. With this study we identified hot-spots that may be used for directed evolution or site-directed mutagenesis to create new variants for faster 2,3-dichloropropan-1-ol release [7]. Finally, we will show the difference on ligand transportation when a protein is in an open and closed conformations [8]. We will show how CaverDock tackles the problem of protein flexibility.

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PRENYLATED FLAVIN-DEPENDENT DECARBOXYLASES: STRUCTURE-GUIDED ENGINEERING AND SYNTHETIC APPLICABILITY

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Key Words: Biocatalysis, enzyme engineering, (de)carboxylation, Prenylated flavin, decarboxylases

The recently discovered prenylated flavin (prFMN) cofactor is utilised by UbiD enzyme family to catalyse non-oxidative decarboxylation of precursors of respiratory quinones. Mechanistic aspect of prFMN-mediated decarboxylation has attracted interests and studies of fungal ferulic acid decarboxylases (FDCs), members of the UbiD enzyme family reveal a rare enzymatic 1,3-dipolar cycloaddition mechanism for the decarboxylation of cinnamic aid.

We have demonstrated the applicability of prFMN-dependent FDCs for both isolated enzyme and whole-cell biocatalysis. Using in-vitro (de)carboxylase activity of prFMN bound-FDCs, we performed a substrate profiling study of four FDCs, providing insights into structural determinants of activity (Figure 1). The FDC-UbiX system enables access to a variety of industrially relevant terminal alkenes from acrylic acid derivatives bearing (hetero)cyclic or olefinic substituents at C3; affording conversions of up to >99 %.

Importantly, the application of the FDC in carboxylation direction has been achieved allowing the functionalisation of unactivated terminal alkenes under mild reaction conditions. By applying structure-guided protein engineering, we have developed FDC variants acting on a wide range of unactivated (hetero)aromatic carboxylic acids, hence expanding the product profile of enzymatic (de)carboxylation. Development of efficient prFMN-dependent enzymes will immensely expand the scope of biocatalytic (de)carboxylation reactions.

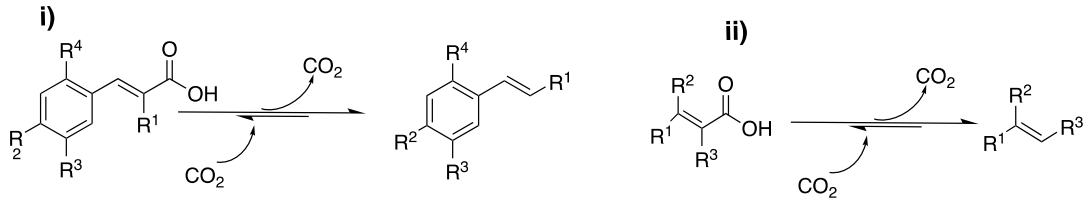

Figure 1. Overview of substrate specificity of prFMN-dependent ferulic acid decarboxylases (FDCs).

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THE ROLE OF CAVITY RESIDUE LEUCINE 95 AND CHANNEL RESIDUES GLUTAMINE 204, ASPARTIC ACID 211, AND PHENYLALANINE 269 ON TOLUENE *O*-XYLENE MONOOXYGENASE ACTIVITY AND REGIOSPECIFICITY

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Key Words: Hydrophobic cavity, Regiospecificity, Saturation mutagenesis, Substrate channel, Toluene *o*-xylene monooxygenase.

The biocatalyst toluene-*o*-xylene monooxygenase (ToMO) of *Pseudomonas* sp. OX1 belongs to a remarkable family of bacterial multicomponent monooxygenases and has been shown to have a great potential for biotechnological and environmental applications. Structural analysis of ToMO hydroxylase revealed the presence of three hydrophobic cavities, a channel, and a pore leading from the protein surface to the active site. A structural study with the related enzyme toluene-4-monooxygenase (T4MO) hydroxylase with its respective regulatory protein confirmed that the channel undergoes extensive structural changes upon binding of the regulatory protein and transiently opens and closes during catalysis (Figure 1). Here, saturation mutagenesis was used to investigate the catalytic roles of alpha-subunit (TouA) second cavity residue L95 and TouA channel residues Q204, D211, and F269. By testing the substrates toluene, phenol, nitrobenzene, and/or naphthalene, these positions were found to influence the catalytic activity of ToMO. Several regiospecific variants were identified from TouA positions Q204, F269, and L95. For example, TouA variant Q204H had the regiospecificity of nitrobenzene changed significantly from 30 to 61 % *p*-nitrophenol. Interestingly, a combination of mutations at Q204H and A106V altered the regiospecificity of nitrobenzene back to 27 % *p*-nitrophenol. TouA variants F269Y, F269P, Q204E, and L95D improved the *meta*-hydroxylating capability of nitrobenzene by producing 87, 85, 82, and 77 % *m*-nitrophenol, respectively. Here, two additional TouA residues, S222 and A106, were also identified that may have important roles in catalysis. Most of the isolated variants from D211 remained active, whereas having a hydrophobic residue at this position appeared to diminish the catalytic activity toward naphthalene. The mutational effects on the ToMO regiospecificity described here suggest that it is possible to further fine tune and engineer the reactivity of multicomponent diiron monooxygenases toward different substrates at positions that are relatively distant from the active site.

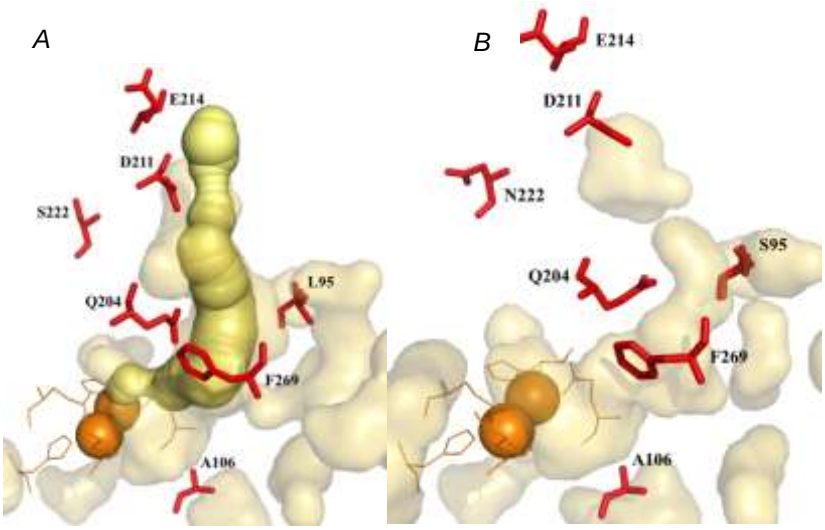

Figure 3– Structural comparison of ToMO hydroxylase (PDB: 1T0Q) (A) and T4MO hydroxylase/T4MO regulatory protein complex (PDB: 3I5J) (B). Residues L95, A106, Q204, D211, E214, S222, and F269 are labeled and represented as sticks. Two iron atoms are shown as spheres. Residues depicted as lines are the iron-binding residues. The solvent accessible tunnel of ToMO, calculated and visualized by the PyMOL Caver 3.0 plugin, is represented as spheres. The surface of the hydrophobic cavities and channel detected by PyMOL are also shown.

Reference

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RATIONAL ENGINEERING OF A HYPERSTABLE GLYCOSYLTRANSFERASE FOR BLUE DENIM DYEING

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Key Words: Rational engineering, Thermostability, Glycosyltransferases

Indigo is one of the most used dyes to produce the textile blue denim worldwide[1]. Its synthesis and the dyeing process require chemical steps that are environmentally damaging, including the use of reducing agents for indigo solubilization. The glycosyltransferase PtUGT1 is able to add a glucose moiety to the reactive indigo precursor indoxyl to form indican, preventing spontaneous oxidation and keeping it soluble[2]. This strategy could be used in a chemoenzymatic approach to replace the use of reducing agents, but in order to do this it's necessary that PtUGT1 resist, and to be active under the harsh conditions used in the industrial process, including high pH and high temperature. We have characterized the activity profile of PtUGT1 at different pH values and temperatures and have determined the enzyme stability by differential scanning fluorometry (DSF) and residual activity. Leveraging the structure information of PtUGT1 obtained by X-ray crystallography (PDB ID: 5nlm)[2], we have rationally designed different mutants to develop a variant adapted to higher temperatures and pH values, including hypothetical residue pair mutants that could lead to the formation of intramolecular disulfide bridges, and mutants that could either improve the hydrophobic packing, lead to formation of polar interactions or improve Pro/Gly ratio, consequently increasing the rigidity/stability of PtUGT1. As a result we have developed several active PtUGT1 variants with up to 15°C increase in their melting temperature (TmB) (Fig. 1), the highest ever reported for an UDP-dependent glycosyltransferase.

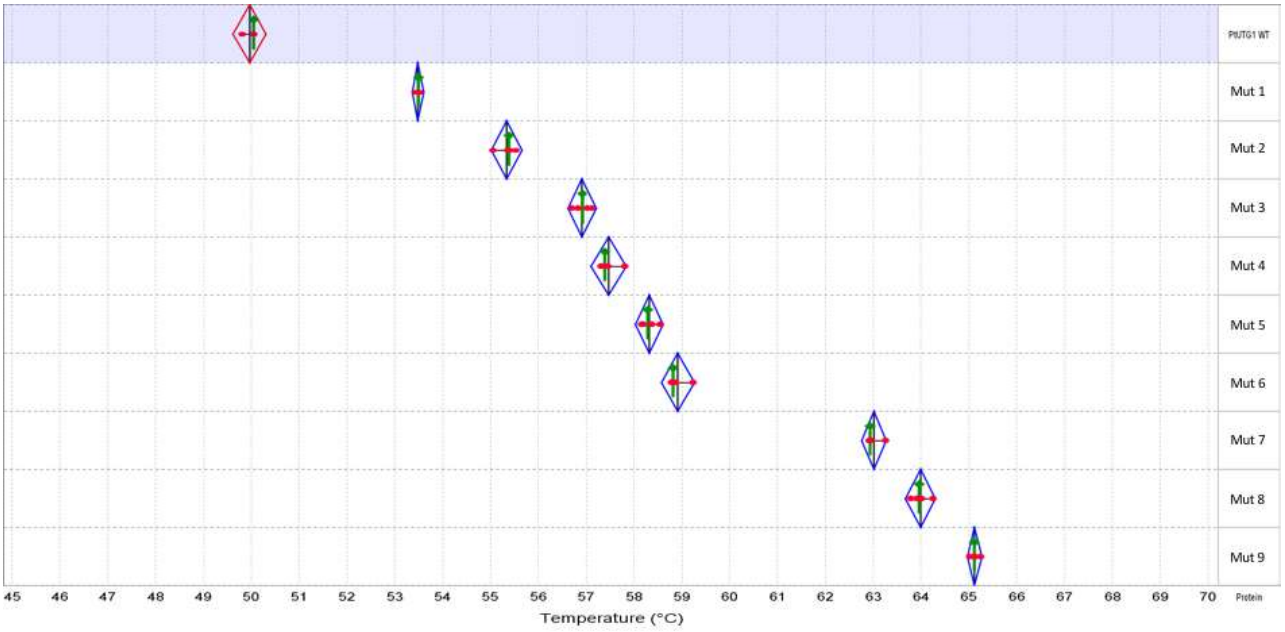

Figure 4 – DSF comparing TmB of PtUGT1 WT and Mutants designed.

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DIRECTED EVOLUTION OF GLYCOSYLTRANSFERASE FOR THE ARTIFICIAL BIOSYNTHESIS OF NATURAL PRODUCT GLYCOSIDES

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Key Words: glycosyltransferase, directed evolution, high-throughput screening, natural products, biosynthesis

Over one fifth natural product drugs (including protein biopharmaceuticals), cosmetics, and nutraceuticals have a diverse set of sugars in their structures. These glycosylations dramatically influence the physicochemical and pharmacological properties of these compounds. Glycosyltransferases (GTs) offer very attractive approaches to the biosynthesis of complex glycosylated natural products. However, the limited number of available GTs, together with their instability and strict substrate specificity, have severely hampered the broad application of these enzymes. In the past few years, we have used directed evolution as a tool to tailor the GTs with desired substrate specificity and higher catalytic activity. Here I will introduce some of our efforts in 1) the semi-rational design of a glucosyltransferase UGT51 from *S. cerevisiae* to repurposing its promiscuous activity towards the biosynthesis of rare ginsenoside Rh2; and 2) the directed evolution of an α1,3-fucosyltransferase using a single-cell ultrahigh-throughput screening method. I will also discuss the development of new tools for the high-throughput screening method for GTs and the mechanistic insight we found during the evolution of these enzymes.

ACCESS TUNNEL ENGINEERING TO OPTIMIZE THE CATALYTIC CYCLE OF CARBOHYDRATE HYDROLASES WITH BURIED ACTIVE SITE

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The active site of many enzymes is buried inside the protein core and is connected with the surrounding solvent by access tunnels. An emerging approach to optimize these enzymes properties is the engineering of structural features governing the exchange of ligands between the active sites and bulk solvent. However, it is still challenging to redesign the access tunnels of enzymes catalyzing biopolymers like carbohydrate hydrolases because of the extremely complicated substrate structure. In this study, structure-guided saturated mutagenesis was performed to reconstruct all three access tunnels of xylanase S7-xyl from *Bacillus halodurans* S7, which results in a mutant 254-RL1 with 3.4-fold increase in specific activity. Structural comparison and kinetic analysis revealed that products egress is the rate-limiting step in the catalytic cycle of S7-xyl. The products release tunnel in S7-xyl was experimentally validated, and not the tunnel radius but the length determining the products release efficiency. Application assessment showed that relieving the inhibition of reducing sugars on mutant 254-RL1 could accelerate the hydrolysis efficiency of cellulase on different pretreated lignocellulose materials, representing a good candidate in enzyme cocktails for lignocellulose biodegradation. In addition, the same strategy was successfully utilized to improve the specific activities of three other xylanases with buried active site, suggesting the general application of tunnel engineering to optimize carbohydrate hydrolases with buried active site.

SUSTAINABLE BIOCATALYTIC SYNTHESIS OF B-HYDROXYL-A-AMINO ACIDS ON AN INDUSTRIAL SCALE

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Key Words: Smart Enzyme Engineering, Directed Evolution, Biocatalysis, Sustainable Chemistry.

The development and commercialization of sustainable enzymatic and microbial catalysis technologies is gaining increasing priority to reduce the environmental impact of chemical and related industries.

Enzymes offer, as common platform, significant opportunities for innovations to enhance production capabilities to meet these new reduced impact demands, whilst retaining product quality and keeping costs down. To enable cost efficient biocatalytic processes, however, high selectivity, high activity, high substrate loadings and tolerance to organic solvents are required. This is in general not sufficiently displayed by natural enzymes, despite providing high selectivity and activity on native substrates under physiological conditions. As a solution, enzyme engineering allows us to optimize any enzyme to a powerful catalyst that overcomes these boundaries and can be conveniently applied under industrial conditions.

As an industrial example, we will discuss the synthesis of β-hydroxyl-α-amino acids, important chiral building blocks in pharmaceutical and fine chemical industry. Current chemical synthesis protocols employ hazardous and environmentally unfriendly methods, thus, a replacement by safe and green biocatalysis is desired. However, wild type aldolases do not meet the anticipated target criteria for selectivity, activity and solvent tolerance under process conditions. Nonetheless, smart bioinformatics guided directed evolution, enabled us to develop an enzyme variant which meets the industrial target for the sustainable (βR)-β-Hydroxy-4-nitro-L-phenylalanine production at multi ton scale, which is a precursor for chloramphenicol. Remarkably, this new synthetic route to chloramphenicol, enabled by our engineered aldolase, is 5 steps shorter than the traditional chemical route, avoiding the use of various hazardous and environmentally unfriendly reactants.

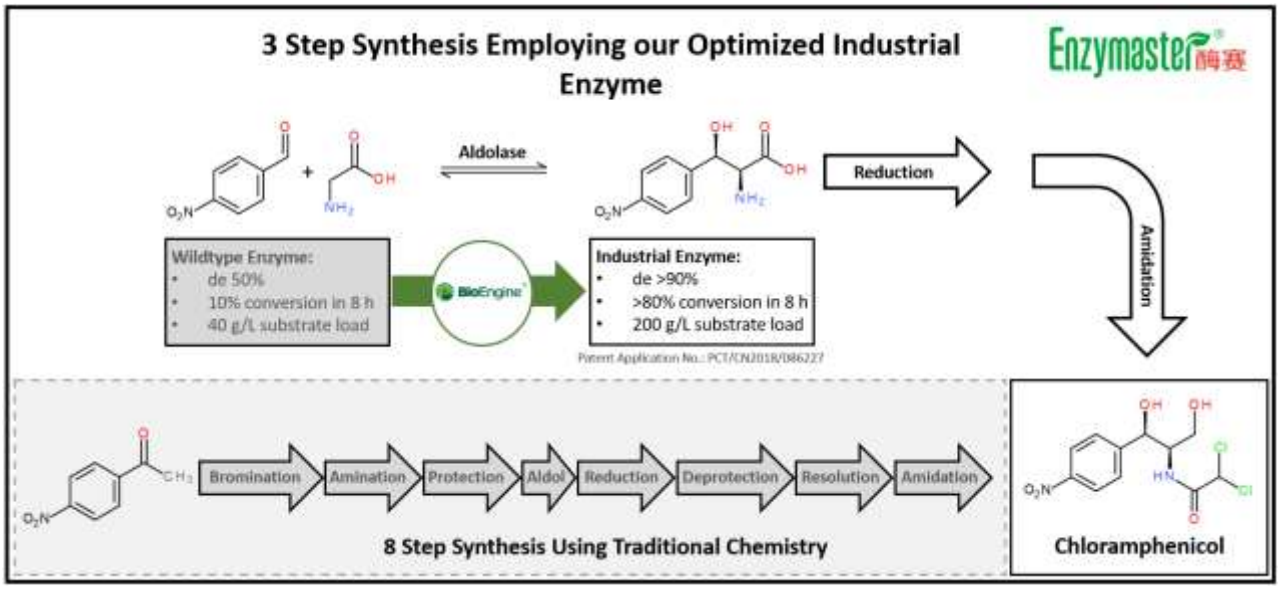

Figure 5 – Comparison of semi-enzymatic and traditional synthesis of Chloramphenicol. Upper case: Enzymatic aldol reaction shortens synthetic route by 5 steps and enzyme engineering allows for strongly improved stereo selectivity, conversion and substrate loading. Lower case (grey box): Inefficient chemical route, suffering from the necessity of activation and protecting/deprotecting chemistries and lack of stereoselectivity.

SMART ENGINEERING OF VARIOUS ENZYMES FOR ASYMMETRIC SYNTHESIS OF CHIRAL MOLECULES ON INDUSTRIAL SCALE

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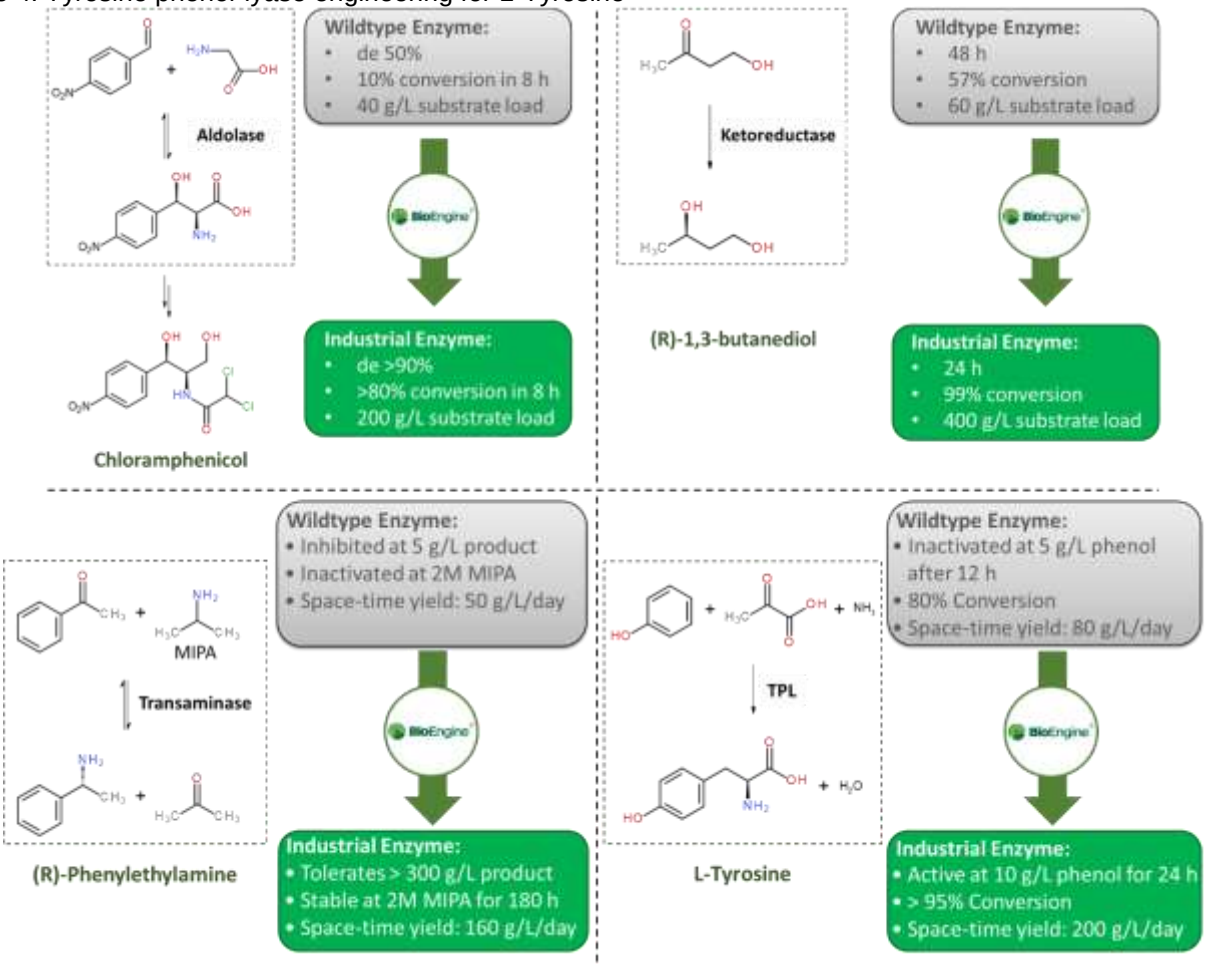
Key Words: Platform Technology, Directed Evolution, Smart Enzyme Engineering, Commercialization

The commercialization of sustainable enzymatic and microbial catalysis technology is gaining increasing priority for the synthesis of chiral compounds from achiral precursors that require high selectivity and high substrate load. Thus, the fast and cost-effective development of novel biocatalytic processes using an integrated platform offers significant opportunities to fine chemical, pharmaceutical, food & feed, material and related industries.

BioEngine® is such an integrated platform that offers the full-spectrum solution from enzyme discovery, enzyme engineering, enzymatic process development all the way to qualified product manufacture. As industrial examples, we will discuss four cases where wild type enzymes that do not meet the desired target criteria for selectivity, activity and solvent tolerance under process conditions, whereas after applying bioinformatics guided directed evolution and *in silico screening*, the evolved variants meet the industrial targets for the sustainable production of the desired molecules at multi ton scale.

Case 1: Aldolase engineering for Chloramphenicol intermediate
Case 2: Ketone reductase engineering for (R)-1,3-Butanediol
Case 3, Transaminase engineering for (R)-Phenylethylamine
Case 4: Tyrosine phenol-lyase engineering for L-Tyrosine



STRUCTURAL SYNTHETIC BIOLOGY STRATEGY FOR THE DESIGN OF A NEW METABOLIC PATHWAY

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Key Words: Rational enzyme design, synthetic biology, metabolic pathway, L-methionine.

To date, notable successes have been made in producing valuable chemicals and fuels from renewable resources by simply modifying and optimizing the metabolic pathways in microorganisms. However, to design a more efficient and desirable pathway with high efficiency from ubiquitously existing multi-branched and multi-level regulated ones, a new approach is needed other than conventional systematic analysis of every bottlenecks embedded in the biosynthetic pathways. Here, we present a strategy combining rational enzyme design and synthetic biology to construct a new metabolic pathway which evades from the highly regulated nature. As a proof-of-concept, we implemented our approach to the design of a new L-methionine biosynthetic pathway. To this end, structure of the MetZ enzyme, which is a key to the construction of new biosynthetic pathway in *Corynebacterium glutamicum*, was modelled, and its substrate specificity was rationally altered toward a substrate required for redirecting the metabolic flux in the pathway. Furthermore, we used mutational approach to relieve feedback inhibition of other enzymes which regulate the metabolic flux in the methionine biosynthetic pathway. As a result, the L-methionine level reached a gram scale in flask culture by recombinant Corynebacterium glutamicum with the methionine biosynthetic pathway. We demonstrate that the “structural synthetic biology” strategy can boost our ability to generate a more efficient metabolic pathway for the production of valuable chemicals.

ENZYME ENGINEERING TOWARDS A FULLY BIOCATALYTIC MANUFACTURING ROUTE FOR MK-8591

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Key Words: HIV; MK-8591; manufacture; enzyme cascade; enzyme engineering.

With 37 million people around the world infected with HIV—and this number rising by almost 2 million a year—the search for newer, better medicines to treat this deadly disease remains an important goal. MK-8591 is a nucleoside reverse transcriptase inhibitor currently undergoing clinical trials for the treatment of HIV.

In order to achieve a world-class manufacturing route to this target that is also green and sustainable, the process chemistry team has turned to biocatalysis. Specifically, the 4'-ethynyl-2'-deoxyribose sugar portion of the molecule is prepared via a highly efficient three-enzyme cascade sequence. Another multi-enzyme reaction system is then used to append the 2-fluoroadenine base, overcoming a challenging glycosylation reaction.

This bold endeavor has posed numerous technical challenges in the optimization of complex multi-enzyme systems, the need for directed evolution of multiple enzymes, the detection and analysis of polar chromophore-free molecules and the isolation of unstable and highly water-soluble intermediates. An overview of the ongoing development and unique challenges—and opportunities—of this unprecedented fully-enzymatic manufacturing route will be presented.

IDENTIFICATION OF THE RESIDUES THAT ARE RESPONSIBLE FOR IMPROVING THE ACTIVITIES OF CYANOBACTERIAL ENZYMES FOR HYDROCARBON BIOSYNTHESIS

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Key Words: Cyanobacteria, Hydrocarbons, Aldehyde deformylating oxygenase, Acyl-ACP reductase, Protein engineering

Cyanobacteria can produce hydrocarbons corresponding to diesel fuels via a reaction catalyzed by two enzymes, acyl-ACP reductase (AAR) and aldehyde deformylating oxygenase (ADO). Because *Escherichia coli* coexpressing these enzymes can produce and secrete hydrocarbons, both AAR and ADO are key enzymes for hydrocarbon biosynthesis. However, the activities of AAR and ADO are low. Therefore, construction of highly active mutants of AAR and ADO is necessary for industrial application of these enzymes for producing hydrocarbons. Our purpose in this study is to identify the residues that are responsible for improving the activities of AAR and ADO. First, we compared the activity of AARs from several cyanobacteria and detected a highly active AAR. Second, we introduced various single amino acid substitutions into a poorly active AAR, to make its sequence close to that of the highly active AAR. When we constructed and analyzed 40 mutants of AAR, we succeeded in identifying the residues that are important for high activity of AAR and those important for high expression level of soluble AAR (*Figure. 1*). Combination of single mutations greatly improved the aldehyde productivity. Similarly, we also identified the residues that are important for high activity of ADO and those important for high expression level of soluble ADO (*Figure. 2*). Mutational analysis of ADO revealed that high productivity of hydrocarbons can be achieved by increasing both the activity and amount of soluble ADO. Our data will be useful for producing higher amount of hydrocarbons using highly active mutants of AAR and ADO created by protein engineering.

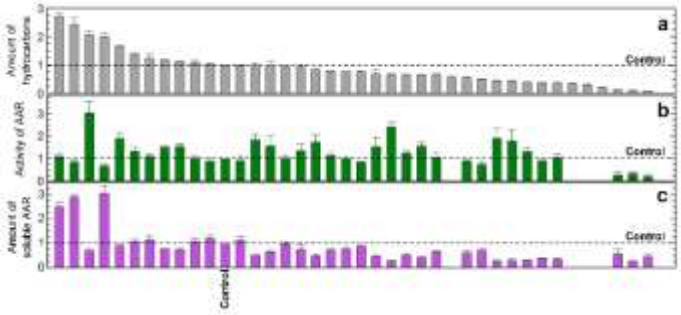

Figure 6. Characteristics of single mutants of AAR. a. Amount of hydrocarbons. b. Activity. c. Amount of soluble AAR.

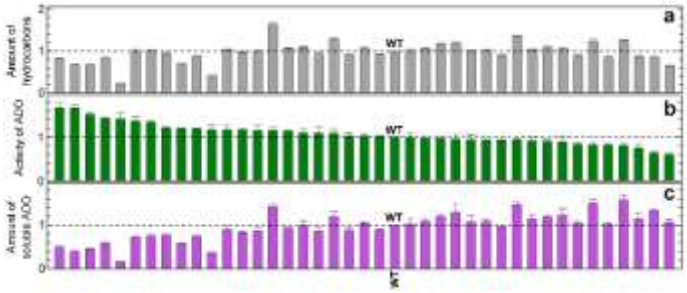

Figure 2. Characteristics of single mutants of ADO. a. Amount of hydrocarbons. b. Activity. c. Amount of soluble ADO.

A COUPLED CHLORINASE-FLUORINASE SYSTEM WITH HIGH EFFICIENCY OF TRANS-HALOGENATION AND A SHARED SUBSTRATE TOLERANCE[1]

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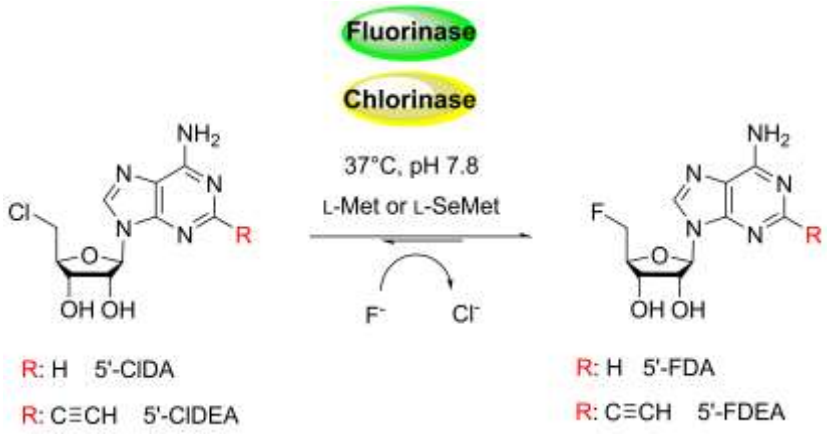
Key Words: Fluorinase, Chlorinase, Trans-halogenation, *S*-adenosyl-L-methionine.

Enzymatic trans-halogenation enables radiolabeling under mild and aqueous conditions, but rapid reactions are desired. We discovered two new *S*-adenosyl-L-methionine (SAM)-dependent chlorinases from soil bacteria and developed a coupled chlorinase-fluorinase system for highly improved trans-halogenation reactions. The chlorinase was for the first time demonstrated to tolerate the modification at the C-2 position of the adenine ring and act cooperatively with the fluorinase to accelerate the trans-halogenation of 5'-chlorodeoxy-2-ethynyladenosine (5'-ClDEA) to 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA). The acetylene group will enable the linkage with an azide tethered peptide via a "click" reaction. The coupled chlorinase-fluorinase system offers the prospect of developing rapid radiolabeling protocols under mild and aqueous conditions.



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A NAKED-EYE DETECTION OF CHOLESTEROL USING ENZYME CASCADE REACTIONS ON CHITOSAN BEADS

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Cholesterol is an important biomarker that is involved in the implication of many diseases including coronary heart disease, hypertension, arteriosclerosis and etc. Faster assessment of the cholesterol level in the body may help the users to monitor their health status and allow faster action for the appropriate treatments. A hydrogel based enzyme cascade system using multiple enzymes was developed to detect the cholesterol levels with a naked-eye. Cholesterol esterase, cholesterol oxidase, and horseradish peroxidase were immobilized onto chitosan beads through a glutaraldehyde cross-linkage. The cholesterol level was monitored through the oxidation of ABTS in the assay system. The optimum temperature of the immobilized chitosan beads was 30ºC and the beads retained its activity up to 9 successive operations. We found that glycerol coated immobilized chitosan beads to be more stable in retaining its structure and activity at room temperature over time. Because enzyme has great specificity against its substrate molecules, we believe that the chitosan beads provide an excellent platform for the enzyme cascade system that may be applied in detecting the small molecule biomarkers.

TOWARDS THE *DE NOVO* DESIGN OF METALLOHYDROLASES

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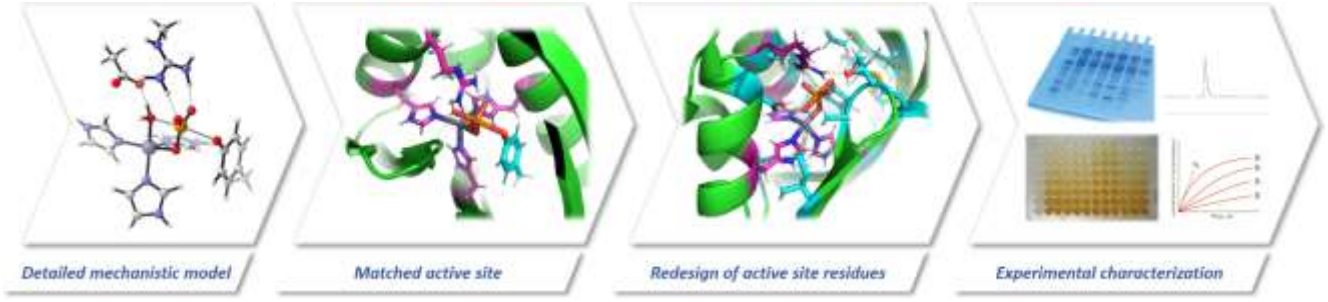
Key Words: Rosetta, metalloprotein, hydrolase

De novo design of proteins has enabled the exploration of vast regions of sequence space previously inaccessible by nature.[1] These proteins are computationally designed based on physical principles of protein structure and folding, and are tailored according to a specific function or property. Great advances have been made in the design of novel highly stable protein folds capable of hosting enzyme active sites.[2] That has in turn allowed us to undertake larger scale efforts of installing various enzymatic activity into these scaffolds. As a proof of principle we have undertaken efforts towards designing hydrolase (esterase and phosphatase) activity into the NTF2[2b] and the TIM-barrel[3] folds through the introduction of metal binding sites.

The computational enzyme design process has been performed at multiple scales, starting with high level quantum chemical computations that were used to obtained a detailed mechanistic model of the reaction in the form of an idealized enzyme active site (theozyme).[4] These insights have then been transferred to the enzyme design process where the algorithms in the Rosetta software have been employed to match and design the corresponding active sites into thousands of *de novo* scaffolds. Combined with smart filtering methods this approach has enabled us to narrow in on the most promising computational designs that were then carried forward to experimental testing. These proteins have been expressed in *E. coli* using the corresponding custom genes, isolated, their biophysical properties spectroscopically determined, and lastly their enzymatic activities measured using appropriate screening assays. The obtained information then feeds back into further rounds of computational design aimed at improving the activity and stability, as well as understanding what mutations are additionally beneficial for the desired activity.

Through the use of computational de novo enzyme design we are aiming to open up new avenues for mechanism-based development of novel enzymatic reactions for which currently no evolutionary trajectories exist.



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THERAPEUTIC PROTEIN EXPRESSION PLATFORM OF MICROBIAL SYSTEM

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Key Words: Pichia pastrois, E. coli, secretion, methanol, scFv, antibody, Therapeutic protein.

A number of expression systems have been developed for the production of pharmaceutical products. *Pichia pastoris* and *Escherichia coli* expression system operate in our lab and express antibody fragment (scFv), cytokine, protein base adjuvant and vaccine and process enzyme. The expression platform are consisted of three part, first is strain generation , the second is fermentation process development in 250 ml fermentor and the last is process scale-up to 5 litter fermentor.

Here are two examples to introduce the platform capability. A recombinant scFv went through *Pichia pastoris* expression system clone generation, include signal peptide tool box, normally yield 2.5 mg/L titer in deep well with promoter AOX1 drive. Through the fermentation process development of induction medium composition and feeding strategy by Eppendorf Dasgip parallel 250 ml mini fermentor. The productivity is increased form deep well scale to 250 ml mini-fermentor scale cane achieved 50 mg/L to ~1000 mg/L with the yield improved in 20-400 folds. Furthermore, the process parameter can be scale-up to 5 L fernentor achieving > 1 g/L.

The *Escherichia coli* expression system is an inclusion body production process. The expression is driven by constitutive promoter. At the batch fermentation process, the optical density only can achieve 5~8 at 600nm with 2.5~3.1 g wet cell weight (WCW) in 200 ml medium. To increase productivity, the two days fed-batch process has tested and can achieve 60~70 OD_{600nm} with 13.6 g WCW in 151 ml final volume. To maintain optical density level and sorter process time are more benefit for future manufacturing production. Thus, increase three folds feeding rate maintain optical density but lower specific productivity (~3 mg/g WCW). Therefore, fine tune feeding rate not only maintain optical density but also got the highest specific productivity (~10 mg/g WCW) in one day 250 ml fermentation process. The fermentation process was scale-up to 5 L fermentor, the optical density is 60~70 at 600nm with 400~450 g WCW in ~4 L final volume and specific productivity is 20 mg/g WCW.

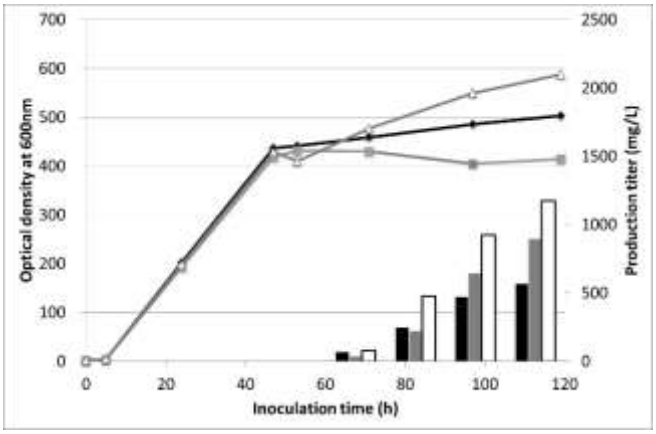

Figure 1. Biomass optical density profile and production titer using different feeding strategy.

White column and triangle, methanol and sorbitol mixture feed by constant feeding rate.
Gray column and squared, methanol and sorbitol i feed separately by using constant feeding rate and DO-stat feeding strategy, respectively;
Black column and diamond, methanol and sorbitol mixture feed by using DO-stat feeding strategy.

FUNCTIONAL ASSESSMENT OF HYDROPHILIC DOMAINS OF LEA PROTEINS FROM DISTANT ORGANISMS

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Key Words: LEA protein, Deinococcus radiodurans, protein chaperone, stress-response

Late embryogenesis abundant (LEA) proteins play a protective role during desiccation and oxidation stresses. LEA3 proteins are a major group characterized by a hydrophilic domain (HD) with a highly conserved repeating 11-amino acid motif. We compared four different HD orthologs from distant organisms: (i) DrHD from the extremophilic bacterium *Deinococcus radiodurans*; (ii) CeHD from the nematode *Caenorhabditis elegans*; (iii) YlHD from the yeast *Yarrowia lipolytica*; and (iv) BnHD from the plant *Brassica napus*. Circular dichroism spectroscopy showed that all four HDs were intrinsically disordered in phosphate buffer and then folded into a-helical structures with the addition of glycerol or trifluoroethanol. Heterologous HD expression conferred enhanced desiccation and oxidation tolerance to Escherichia coli. These four HDs protected the enzymatic activities of lactate dehydrogenase (LDH) by preventing its aggregation under desiccation stress. The HDs also interacted with LDH, which was intensified by the addition of hydrogen peroxide (H_2O_2), suggesting a protective role in a chaperone-like manner. Based on these results, the HDs of LEA3 proteins show promise as protectants for desiccation and oxidation stresses, especially DrHD, which is a potential ideal stress-response element that can be applied in synthetic biology due to its extraordinary protection and stress resistance ability.

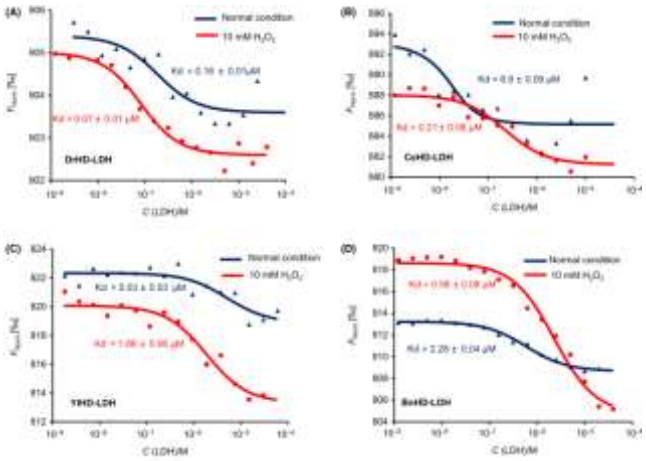

Figure1．Interaction of HDs with LDH.

A protein–protein interaction method, microscale electrophoresis, was employed to characterize the relationship between LDH and DrHD (A), CeHD (B), YlHD (C) and BnHD (D). The Kd value represents the dissociation constant indicating their affinity. The binding curve under normal conditions (phosphate buffer) is shown in dark blue, and the binding curve under 10 mM H_2O_2 treatment is shown in red

IN-SILICO BASED REDESIGN OF CO-DEHYDROGENASE CATALYZING THE OXIDATION OF TOXIC WASTE CO GAS FOR IMPROVED O_2 RESISTANCE AND MEDIATOR AFFINITY

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Carbon monoxide (CO) harmful to most creatures, is largely discharged by industrial processes in steel mill and thermal power plant. Conversion of toxic waste CO gas to safe gas or more valuable chemicals will be a great worth at this point. Interestingly, carbons and high potential electrons from CO-oxidation can be resourced as essential core parts for the chemical products by using CO-dehydrogenase (CODH) and artificial mediator. For industrial application of the enzymatic CO-oxidation, however, key issues remain that most CODHs show oxygen (O_2) sensitivity and low-affinity for artificial mediator. Because steel mill waste gas such as blast furnace gas (BFG) commonly contains a little O_2 and higher affinity is required to achieve higher reaction rate.

In this research, *in-silico* based approach was used to redesign *Carboxydothermus hydrogenoformans* CODH (*Ch*CODH) II, capable of increasing O_2 resistance and affinity to ethyl viologen (EV) mediator. *Ch*CODHs belong to a group of Ni-Fe containing CODH. Among five known *Ch*CODHs (*Ch*CODH I-V), *Ch*CODH II shows the highest activity toward CO but more O_2 sensitive than *Ch*CODH IV. The artificial mediator of EV functions as an electron acceptor for *Ch*CODH II but the affinity of *Ch*CODH II to EV mediator is known poor. As our result, more than 10 folds increase of O_2 resistance was achieved for the redesigned *Ch*CODH II enzyme, which will be definitely a working horse in the conversion of waste CO gas into value-added chemicals.

KINETICS OF GLYCOSYL HYDROLASE FAMILY 2 β-GLUCURONIDASES

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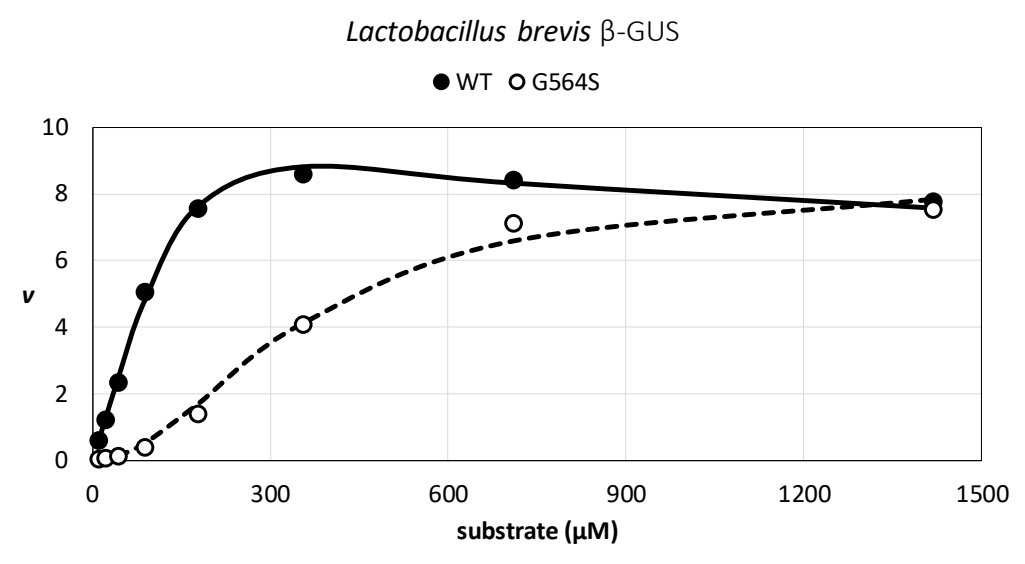
Key Words: Enzymology; Kinetics; Glucuronidase; GH2; Allosteric

Glucuronidases serve important roles in physiology, research, and commercial applications. Bacteria of the microbiome produce β-glucuronidase (β-GUS) to scavenge glucuronic acid from compounds excreted via phase II metabolism. In research, β-GUS is commonly used as a reporter for gene expression. Commercially, β-GUS is used in clinical and forensic drug testing to cleave glucuronic acid from drug molecules making them easier to quantify by LC-MS. Minimally, the enzyme must form a homodimer for activity, and the naturally occurring active form is believed to be a tetramer. Past studies have investigated hydrolysis activity of the enzyme and modeled it as simple Michaelis-Menten kinetics. In our search for new enzymes and enzyme variants, we discovered a point mutation that radically alters the kinetics of the enzyme and points to a strong allosteric interaction between enzyme subunits. We further observe substrate inhibition and consider inhibition by competing substances found in clinical samples. Here we survey several different enzymes and variants across multiple substrates and present models for evaluating enzyme performance.



VARIANTS OF GLYCOSYL HYDROLASE FAMILY 2 β-GLUCURONIDASES

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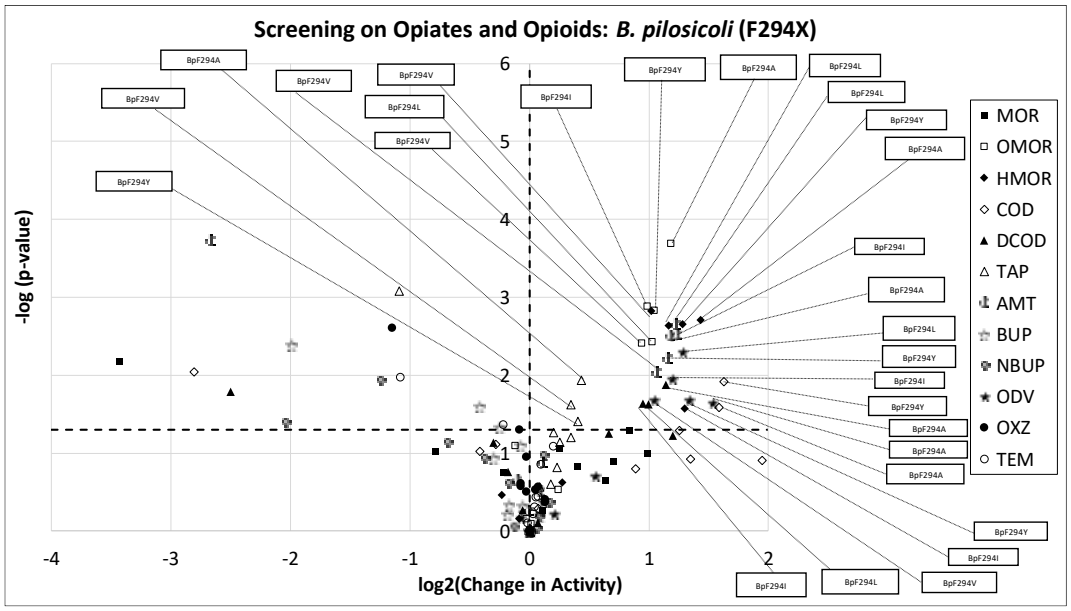
Key Words: Enzymology; Directed Evolution; Glucuronidase; GH2

Mammals remove toxic metabolites and xenobiotics by attaching glucuronic acid to compounds targeted for excretion. The glucuronide increases the solubility of the compound, enabling secretion in urine or bile. Metabolites of opiates and opioids can be quantified from urine by hydrolyzing conjugates with β-glucuronidase (β-GUS) and separating free drug molecules by liquid chromatography and mass spectrometry (LC-MS). Importantly, the hydrolysis step increases both detection and quantitation because free drug molecules ionize better than conjugates in MS (Sitasuwan *et al*., 2019). However, not all glucuronidases have the same activity across a range of potential substrates. Therefore, we generated β-GUS variants with improved activity on a broader range of drug metabolites by using efficient domain swapping and site-saturation mutagenesis techniques.

Sitasuwan P, Melendez C, Marinova M, Spruill M, and LA Lee. (2019) Comparison of purified β-glucuronidases in patient urine samples indicates a lack of correlation between enzyme activity and drugs of abuse metabolite hydrolysis efficiencies leading to potential false negatives. *J. Anal. Toxicol*. 43: 221-227.



THE GROWING NEED TO ASSESS THE KINETIC STABILITY OF ENZYMES

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Key Words: Enzyme stability, NAD(P)H oxidases, Gas-liquid interface, Mixing

Enzyme stability has long been an important topic of great scientific interest[1] as well as of practical importance in the application of enzymes to synthetic problems[2]. Despite great progress in recent years developing protein engineering algorithms for improving stability, this is often assessed solely by measuring increased melting temperature. Nevertheless, industrial reactor conditions can vary greatly and are frequently very different from those found in Nature, meaning that other methods of assessment such as direct kinetic stability measurements are still required. This motivated us to examine the effect of the gas-liquid interface on the kinetic stability of oxidase enzymes, which require molecular oxygen as a second substrate. In order to achieve adequate mass transfer of oxygen from the gas to the liquid phase (where the reaction occurs), bubbled systems are usually employed, with a high gas-liquid interfacial area. Therefore, we explored the kinetic stability of oxidases in an aerated stirred tank. As in previous studies[3], the effect of the interface was found to be inactivating and likewise the damaging effect of oxygen to be more significant than nitrogen. Nevertheless, we have also determined now that the agitation of the solution itself appears to inactivate the enzyme. While the size of the protein is too small for the Kolmogorov scale mixing to affect the enzyme itself[4], it was found that secondary effects of such mixing do have a significant role. This has important implications for the application of enzymes in industrial reactors. In this paper, we present work on kinetic stability measurements of NAD(P)H oxidase (NOX) in an aerated stirred tank using image analysis methods linked with computational tools to clarify the effects of the gas-liquid interface, and thereby differentiate the effects of mixing alone. NOX is an increasingly important enzyme in synthetic applications to allow regeneration of expensive $NAD(P)^+$ cofactors[5], which are necessary for the enzymatic oxidation of alcohols to their corresponding carbonyl compounds using alcohol dehydrogenases. Aside from the specific observations on NOX stability, the results also show the importance of measuring the kinetic stability of enzymes and the impact of enzyme 'lifetime' on reactor design. Furthermore, the wider implications for laboratory testing and process development will be outlined.

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MOLECULAR DYNAMICS PROVIDES INSIGHTS INTO AN ENGINEERED OXIDOREDUCTASE WITH ALTERED COFACTOR SPECIFICITY

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Key Words: cofactor specificity, oxidoreductase, semirational enzyme engineering, molecular dynamics

The aldehyde dehydrogenase from *Thermoplasma acidophilum* is one of the key enzymes in a previously established synthetic cell-free reaction cascade for the production of alcohols. In order alter the cofactor specificity of this enzyme from $NADP^+$ to NAD^+, we applied the CSR-salad tool and investigated further amino acid positions based on its crystal structure. Introduction of five point mutations reduced the K_m for NAD^+ from 18 mM to 0.6 mM and simultaneously increased the activity for D-glyceraldehyde from 0.4 U/mg to 1.5 U/mg. In order to understand the structural basis of the beneficial mutations, we performed molecular dynamics simulations that showed a significant flexibility gain at the cofactor binding site of the final variant. This increased flexibility facilitates a loop movement that largely contributes to the gain in activity and cofactor specificity. We envision a future optimization potential for aldehyde dehydrogenases based on our results.

ENGINEERING SUBSTRATE SPECIFICITY INTO A PROMISCUOUS ANCESTRAL DITERPENE SYNTHASE

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Key Words: Terpene biocatalysis, ancestral sequence reconstruction, enzyme specificity

Terpene synthases are a class of enzymes that catalyse the cyclisation of linear unsaturated hydrocarbons into a plethora of cyclic structures, of which many are industrially relevant as fragrances, flavours or due to their medical properties. Despite the structural and functional diversity of their products, members of this enzyme family show a high degree of structural and functional similarity, which is why the rules defining product and substrate specificity are not fully understood. Recently, we have used ancestral sequence reconstruction to design a hypothetical molecular ancestor of spiroviolene synthase - a diterpene synthase from *Streptomyces violens* that converts the linear C-20 precursor geranylgeranyl pyrophosphate (GGPP) to spiroviolene[1]. This ancestral enzyme shares 77 % sequence identity with the modern wild-type enzyme from *S. violens*. Compared to the modern enzyme, the ancestral enzyme showed increased thermostability and an additional new reactivity with the shorter C-15 substrate farnesyl pyrophosphate (FPP) that the modern enzyme did not display. To the best of our knowledge, this is the first time ancestral sequence reconstruction was used on a diterpene cyclase. Based on the ancestral enzyme, a library of enzyme-variants was designed with the aim to influence the substrate specificity of the promiscuous ancestral enzyme. We identified several variants that showed substantial preference for the native substrate GGPP (modern enzyme-like), which demonstrates that the subtle GGPP-preference of the ancestral enzyme could be “evolved” to the GGPP-specific modern enzyme phenotype. Most interestingly, we were also able to identify a few variants that showed reversed substrate preference for FPP over GGPP and thus demonstrated “evolvability” of the ancestor towards the unpreferred shorter substrate. Taken together these findings suggest that the hypothesis of promiscuous evolvable ancestral enzymes might be appropriate for this member of the diterpene synthase family. Moreover, it opens up the exciting prospect of using ancestral sequence reconstruction as a tool to engineer enzyme specificity – either by introducing new desired functionalities next to an already existing one or by reprogramming existing promiscuity towards a desired substrate specificity.

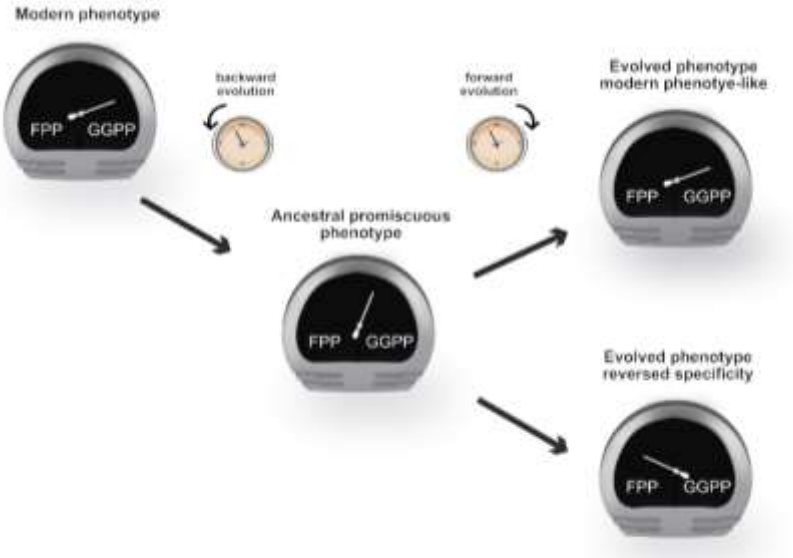

Figure 7 – Concept of redirecting substrate specificity by mimicking the process of evolution

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TREASUREDROP – ENZYME ENGINEERING FOR APPLIED BIOCATALYSIS USING MICROFLUIDICS

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Key Words: enzyme engineering, alcohol dehydrogenase, microfluidics

Enzymes have established as a new class of catalysts in the field of modern synthetic chemistry. Engineering is arguable the most promising approach to generate desired catalytic activities and its success directly correlates with the library size that can be screened. One of the most powerful technologies enabling the quick and cost-effective testing of millions of enzyme variants is the recently introduced microfluidic droplet-based screening. Interestingly, even though numerous publications highlight its potential, an unambiguous evidence of its ability to provide synthetically relevant biocatalysts still needs to be furnished. We present the engineering of an alcohol dehydrogenase for the challenging enantioselective reduction of a prochiral ketone targeting an important key building block for biologically active compounds. The final aim is not only to obtain an improved variant which allows to perform the selected biotransformation efficiently, but also a comparison of varying evolution paths.

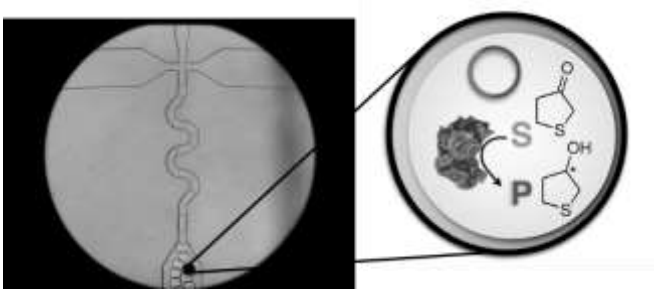

Figure 8 – droplet generation & detected enzymatic reaction in droplets

Directed evolution & semi-rational design of an ADH using ultra high-throughput droplet-based microfluidic screening of single cells.

ACTIVE-SITE STRUCTURE OF D-THREONINE ALDOLASE FROM A GREEN ALGA CHLAMYDOMONAS REINHARDTII

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The unicellular green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) has served as a useful experimental system for many fundamental biological processes. We demonstrated that D-threonine has no inhibitory effect on the cell growth and D-threonine aldolase (DTA) activity exists in *C. reinhardtii*. DTA is a pyridoxal 5'-phophate (PLP) dependent enzyme and catalyzes cleavage of D-form of β-hydroxy amino acids into glycine and corresponding aldehydes. We have reported cloning, purification, characterization, and crystallization of DTA from *C. reinhardtii* (1,2). DTA could catalyze the reverse reaction to produce D-threonine and D-*allo*-threonine from glycine and acetaldehyde. This result shows that the DTA of *C. reinhardtii* has the potential for a useful enzyme catalyzing asymmetric synthesis of various D-form of β-hydroxy amino acids. The structure of the DTA dimer involves non-covalent interactions between both protomers in a head-to-tail arrangement. Each protomer comprises a PLP-binding TIM barrel domain together with a β-strand domain as same as other fold-type-III-PLP-dependent enzymes. In this study, we demonstrated a model structure of enzyme-substrate complex, predicted a catalytic residue and a substrate-binding pocket, and made and characterized mutant enzymes of active-site residues.

The D-threonine-DTA complex structure was built *in silico*, and a putative catalytic residue His216 was proposed. His216 was predicted to be the catalytic base that withdraws the hydrogen of β-hydroxy group of substrates. Demonstration of the model structure also predicted a presumptive pocket in which the side chain of D-*allo*-threonine is located. Gly212 is located at the entrance of the pocket and we predicted that replacement of Gly212 to bulky residues reduces enzymatic activity against D-*allo*-threonine.
Molecular manipulations were performed on the plasmid pCrDTA harboring the native DTA gene using a QuickChange II site-directed mutagenesis kit. The generated plasmids, pCrDTA_H216A and pCrDTA_H212L, were transformed into *E. coli* BL21 (DE3) cells. After cultivation, the recombinant cells were disrupted by ultrasonication and centrifuged. The gene products were purified to electrophoretic homogeneity from the supernatant using ammonium sulfate fractionation and DEAE-Sepharose and Q-stat column chromatographies. Activity of DTA was assayed using alcohol dehydrogenase coupling method. H216A mutant DTA exhibited no activity indicating that His216 residue is the catalytic base in the active site of DTA. Crystallization of substrate-H216A mutant enzyme is in progress. The activity against D-*allo*-threonine of H212L mutant DTA was reduced by approximately 90%. But thermostability of the mutant enzyme also reduced.

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CONSTRUCTION OF THERMOSTABLE ENZYMES

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Key Words:protein engineering, structural analysis, thermophiles, biomass, archaea

The hyperthermophilic archaea were collected from the deep-sea in extreme environment, and the structures and functions of their hyperthermostable enzymes have been characterized. From our studies, the rules for the construction of thermostable enzymes have been discovered. Thermostable enzymes can be used for a number of industrial processes at higher temperature, taking advantage of reduced the risk of microbial contamination and solution viscosity as well as increase in the enzyme reaction rate. Using the information of sequence and structural homology among hyperthermostable enzymes, many types of thermostable enzymes have been constructed with protein engineering method.

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USING *E. COLI* NFSA AS A MODEL TO IMPROVE OUR UNDERSTANDING OF ENZYME ENGINEERING

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Key Words: nitroreductase, directed evolution, epistasis, cell-ablation

There is a substantial gap between the levels of enzyme activity that nature can achieve and those that scientists can evolve in the lab. This suggests that conventional directed evolution techniques involving incremental improvements in enzyme activity may frequently fail to ascend even local fitness maxima. This is most likely due to the difficulty for step-wise evolutionary approaches in effectively retaining mutations that are beneficial in combination with one another, but on an individual basis are neutral or deleterious (i.e., exhibit positive epistasis). We sought to determine whether a superior enzyme identified using a simultaneous mass site directed mutagenesis approach could have been identified using a step-wise approach. We conducted simultaneous mass randomisation of eight key active site residues in *Escherichia coli* NfsA, a nitroreductase enzyme that has diverse applications in biotechnology. Using degenerate codons, we generated a diverse library containing 394 million unique variants. We then applied a powerful positive selection using chloramphenicol which is toxic to *E. coli* but can be detoxified via nitro-reduction. This has enabled us to recover a diverse range of highly active nitroreductase variants. For two of the most active variants, we have created all possible combinations of single mutations. This allowed us to examine whether a step-wise mutagenesis pathway could have also yielded these enzymes. As anticipated, we identified complex epistatic interactions between residues in these enzyme variants. We have also investigated the “black-box” effect of enzyme engineering, examining the consequences that evolving NfsA towards one specialist activity had on the other promiscuous activities of NfsA. Variants generated in this study have also had practical applications, in particular for targeted cell ablation in zebrafish. We have identified NfsA variants that are highly active with nil-bystander prodrugs that can selectively ablate nitroreductase expressing cells without harm to adjacent cells. In ongoing work, our lead variants are being evaluated for their utility in transgenic zebrafish models of degenerative disease.

IN VIVO AND *IN VITRO* FMN PRENYLATION AND (DE)CARBOXYLASE ACTIVATION UNDER AEROBIC CONDITIONS

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Key Words: prenylated FMN, FMN prenyltransferase, UbiX, UbiD, ferulic acid decarboxylase, cofactor regeneration

Prenylated FMN (prFMN) is a newly discovered redox cofactor required for activity of the large family of reversible UbiD (de)carboxylases involved in biotransformation of aromatic, heteroaromatic, and unsaturated aliphatic acids (White et al., 2015). Despite the growing demand for decarboxylases in the pulp/paper industry and in forest biorefineries, the vast majority of UbiD-like decarboxylases remain uncharacterized. Functional characterization of the novel UbiD decarboxylases is hindered by the lack of prFMN generating system. prFMN cofactor is synthesized by the UbiX family of FMN prenyltransferases, which use reduced FMN as substrate under anaerobic conditions and dimethylallyl-monophosphate (DMAP) as the prenyl group donor. Here, we report the *in vivo* and *in vitro* biosynthesis of prFMN and UbiD activation under aerobic conditions. For *in vivo* biosynthesis, we used newly discovered UbiX proteins from *Salmonella typhimurium* and *Klebsiella pneumonia*, which activated ferulic acid UbiD decarboxylase Fdc1 from *Aspergillus niger* under aerobic conditions (0.5-1.5 U/mg). For *in vitro* biosynthesis of prFMN and UbiD activation, we established a one-pot enzyme cascade system that uses prenol, polyphosphate, formate, and riboflavin as starting substrates and (re)generates DMAP, ATP, FMN and NADH. The system contains 6 different enzymes: prenol kinase, polyphosphate kinase, formate dehydrogenase, FMN reductase, riboflavin kinase and FMN prenyltransferase. Under aerobic conditions, this system showed up to 80% conversion of FMN to prFMN and generated active Fdc1 decarboxylase (0.2-1 U/mg). Thus, both systems represent robust approaches for *in vivo* and *in vitro* prFMN biosynthesis and UbiD activation under aerobic conditions. The developed FMN prenylation systems will facilitate the exploration and biochemical characterization of UbiD-like decarboxylases and their applications in biocatalysis.

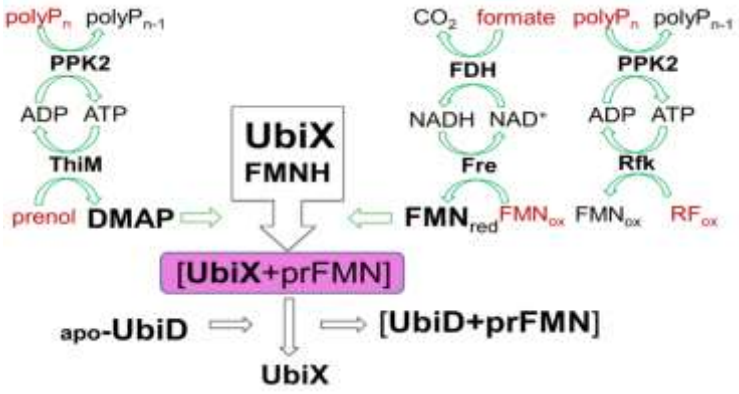

Figure 9 – Schematic diagram showing the in vitro cascade system for prFMN biosynthesis and UbiD decarboxylase. The system includes cyclic and linear (re)generation reactions for ATP, NADH, DMAP and FMN, as well as for enzymatic oxygen removal (FDH).

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PROTEIN DYNAMICS AT SLOW TIMESCALES IN ENGINEERED ß-LACTAMASES DOES NOT LIMIT EVOLVABILITY

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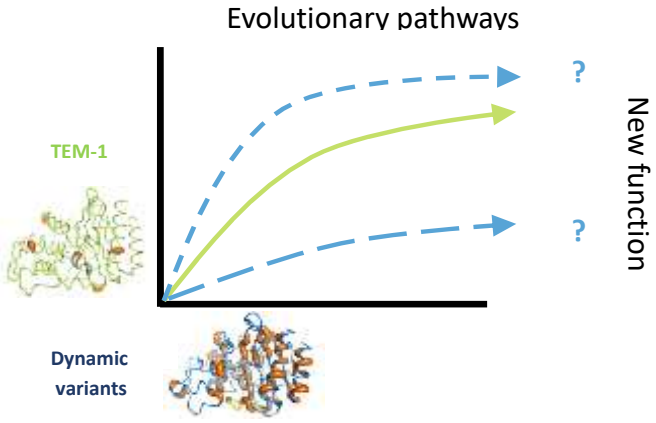
Key Words: Epistasis, protein dynamics, ß-lactamase

Understanding the underlying mechanisms in the evolution of new protein functions is key to better directing enzyme engineering efforts. Intragenic epistasis (the non-additive interaction of mutations affecting function) is a key feature of protein evolution. For example, in TEM-1 ß-lactamase, the mutations E104K and G238S show positive epistasis in that their combination show a greater than expected increase in antibiotic resistance[1]. Here, we aim to understand the impact of protein dynamics at slow timescales on epistasis. Large conformational rearrangements associated with ligand-binding, turnover of substrate or allostery occur at this timescale[2]. The readily evolvable antibiotic-resistant TEM-1 ß-lactamase and two of its variants that are more dynamic at slow timescales serve as models for this study. Our models show similar catalytic activity and substrate recognition, thermal stability, as well as conserved motions in fast (ps-ns) and intermediate (ns-μs) timescales but different motions at slow timescales (μs-ms)[123].

In this study, we use two different approaches to examine the effect of protein dynamics on epistasis. First, we introduced the epistatic mutations E104K and G238S into our dynamic variants. These mutations confer high resistance to the antibiotic cefotaxime in TEM-1, increasing catalytic efficiency ~250-fold. The dynamic variants present similar kinetic values and increase in catalytic efficiency as does TEM-1 when the epistatic mutations are introduced. Molecular dynamic simulations in the presence of cefotaxime support these observations, as the presence of the epistatic mutations correlates with an increase in catalytically-competent conformers. Secondly, we performed directed molecular evolution in TEM-1 and its dynamic variants towards the hydrolysis of the antibiotic cefotaxime. We examined whether mutational pathways accessible to TEM-1 are also available in the context of increased dynamics at the timescale of turnover.
Overall, our work highlights that protein dynamics at slow timescales does not hinder the evolution of new activity in TEM-1 ß-lactamase engineered variants. Furthermore, epistasis can be maintained despite differences in dynamics at slow timescales.



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CONTROLLING THE FATTY ACID HYDROXYLATION REGIOSELECTIVITY OF CYP152A1 (P450BSβ) BY ACTIVE SITE ENGINEERING

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Key Words: Biocatalysis, C-H Activation, Cytochrome P450, Enzyme Engineering, Site-directed Mutagenesis

Regioselective hydroxylation on inactivated C-H bonds is among the dream reactions of organic chemists. Cytochrome P450 enzymes (CYPs) perform this reaction in general with high regio- and stereoselectivity (e.g. for steroids as substrates). Furthermore, enzyme engineering may allow to tune the regioselectivity of the enzyme. Regioselective in-chain hydroxylation of shorter or linear molecules (fatty acids), however, remains challenging even with this enzyme class, due to the high similarity of the substrate's backbone carbons and their conformational flexibility. CYPs are well described for hydroxylating fatty acids selectively in the chemically more distinct α- or ω-position. In contrast, selective in-chain hydroxylation of fatty acids lacks precedence. The peroxygenase CYP152A1 (P450Bsβ) is a family member that displays fatty acid hydroxylation at both, the α- and β-position.

Herein we report the influence of hydrophobic active site residues on the hydroxylation pattern of this enzyme. By site directed mutagenesis and combination of the libraries, double and triple variants were identified, which hydroxylated decanoic acid (C_{10}) with improved regioselectivity in the β-position. Variants were identified with 10-fold increase of β-regioselectivity (expressed as β/α ratio) compared to the wild type. In total 102 variants of CYP152A1 (P450Bsβ) were investigated. Initially all variants were evaluated with the electron transfer system CamAB.

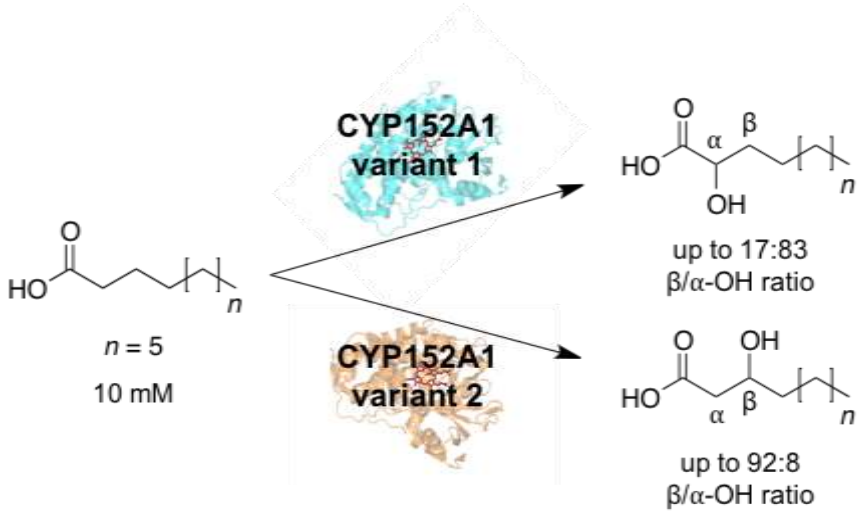

Figure 10 – Hydroxylation selectivity on α- and β-positions of selected CYP152A1 variants

COMPUTATIONAL STUDY OF THE STRUCTURE-FUNCTION RELATIONSHIP OF AN ARTIFICIAL FRIEDEL-CRAFTS ALKYLASE

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Key Words: Artificial Metalloenzymes, Friedel-Crafts, Molecular Modelling, Molecular Dynamics, Quantum Mechanics

Artificial Metalloenzymes (ArMs) have emerged as an attractive approach to perform new-to-nature reactions by combining homogeneous and enzymatic catalysis. Although several advances in the field have been achieved, finding novel or improved activities by chemically modifying the protein host remains a challenge. In this regard, the lack of knowledge about how these changes can alter the structure/dynamics of the artificial enzyme is among the major limitations for obtaining the desired catalytic outcomes.

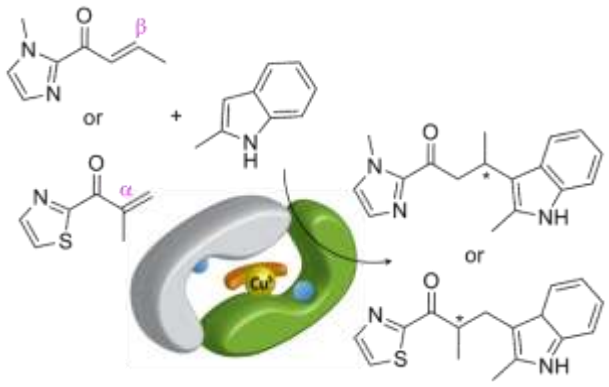

Figure 11 – Artificial Friedel-Crafts alkylase modeled in this work

The work exposed here shows how computational state-of-the-art modelling approaches are able to decipher the structure-function relationship of a new enantioselective Friedel-Craft alkylase, designed at Gerard Roelfes's group, resulting from the supramolecular embedding of a copper(II) containing phenanthroline cofactor at the dimer interface of LmrR, a versatile protein able to host efficiently a large variety of chemical transformations.[1]

Additionally, this study deciphers the quantum basis for which a- and b-substituted substrates lead to different catalytic outcomes in the Friedel-Crafts alkylation reaction, a generalized and unsolved question that also concerns the artificial alkylase focus of this study.

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UNDERSTANDING ENZYMES SPECIFICITIES AS A TOOL FOR COFACTOR ENGINEERING

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Key Words: S-adenosylmethionine, protein engineering, methyltransferases.

S-adenosylmethionine (SAM) is the second most abundant cofactor in nature after adenosyl triphosphate (ATP). SAM is synthesized in a cell by Methionine adenosyltransferase (MAT) using ATP and methionine and it is used as a methyl donor to methylate different substrates (DNA, protein, RNA, small molecules) by methyltransferases (Mtases). The methylation is a key process for cellular regulation and aberrant methylation associated with the disease condition. We are aiming at engineering two-steps pathway for an orthogonal cofactor, for this purpose we first decided to explore the promiscuities of the nucleotide base of ATP for two enzymes (MAT and Mtases). To find good candidates for these engineering we expressed and purified MAT from different organisms. We found MAT from specific organisms are promiscuous for the new nucleotide-based cofactor. It is very interesting that certain MAT is promiscuous for nucleotides-based cofactor but are these newly formed cofactors also promiscuous for the methyltransferase (Mtases) who is the main user of theses cofactors? Further, we have also investigated promiscuity of the DNA methyltransferase (Mtases) from bacteria. Overall these findings lay the foundation for our engineering studies and hint at the evolution of these enzymes.

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A STRUCTURE-BASED APPROACH TO CONTROL THE SIZE AND LINKAGE-TYPE OF SUCROSE-DERIVED ALPHA GLUCANS

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Key Words: Bioderived polymers, Glucansucrases, Structure-function studies, Structure-guided engineering,

The demand for bio-derived and biodegradable polymers and oligomers is impressively increasing due to societal and environmental concerns. Among the bio-sourced polymers, the a-glucans produced from sucrose using the glucansucrases from the family 70 of glycoside-hydrolases are quite attractive. They can display various structures, in terms of linkage type, molar mass and polydispersity. Their specific traits directly govern their physico-chemical and biological properties at the source of their potential usage in pharmaceutical, cosmetic or agro-food industries. The accessible molecular diversity is thus large and of interest to extend a-glucan applications. However, the fine control of the structures still suffers from a lack of basic knowledge in the mechanistic and structural determinants at the source of the GH70 enzyme specificity.

To fill this gap, we have recently selected different glucansucrases, named DSR-OK, DRS-M and ASR. They synthesize polymers with marked structural differences in terms of size or linkage content. We have solved several 3D structures of these enzymes, unliganded or in complex with different substrates and/or products. The structural analysis of these proteins combined to mutagenesis and biochemical characterization enabled us to identify key determinants of specificity and elaborate mechanistic scenarios for both polymer elongation and linkage type formation.

We will first give an overview of the molecular mechanisms involved in polymer formation and will discuss our recent advances with regard to the complexity of the occurring phenomena. We will also show how our findings can rationally serve to construct mutants and chimera leading to a broader range of bioproducts with well-defined structures.

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ENGINEERING THE BIOSYNTHESIS OF NON-RIBOSOMAL PEPTIDES

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Key Words: Non-ribosomal, peptide, pyoverdine

Non-ribosomal peptides are a class of natural product that exhibit diverse properties and function as toxins, antibiotics, siderophores, and pigments. Their range of activity means they have roles in medicine, agriculture and bioremediation. Non-ribosomal peptides are biosynthesised by linking monomers together via peptide bonds. They are assembled from a pool of hundreds of monomers, and often contain cyclisation or other modifications not found in ribosomally-synthesised peptides. Their structural diversity means they can be expensive and/or difficult to synthesise. Consequently, many non-ribosomal peptides are produced using fermentation and then modified to generate compounds suitable for medical or industrial applications. Modifying the biosynthetic pathways could provide a cheap and scalable source of new compounds but attempts to engineer them have previously had a low success rate. Using pyoverdine as a model system, this study investigated how to rationally engineer non-ribosomal peptide biosynthesis and generated modified pyoverdines in 6/9 cases. The results of modifying pyoverdine were then used to engineer a second pathway to make dipeptides with a 3/5 success rate. The high success rate and similar results using two biosynthetic pathways suggest this approach is highly transferable and will be valuable for engineering other pathways.

IDENTIFICATION AND ENGINEERING OF A DYE-DECOLORIZING PEROXIDASE (DYP) FOR C—C-BOND FORMING CARBENE-TRANSFER REACTIONS

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Key Words: Directed evolution, ISM, C-C bond formation, carbene-transfer, Golden Gate

A new enzymatic reaction-type of carbene-transfer reactions has been shown by the seminal work of Arnold and coworkers in 2013.[1] An impressive set of reactions was demonstrated since then predominantly employing the enzymes $P450_{BM3}$, Myoglobin and cytochrome C.[2]

In 2016, we identified the dye-decolorizing peroxidase (DyP) YfeX from *E. coli* to catalyse carbene-transfer reactions. YfeX is an iron-heme enzyme with histidine as proximal ligand and remarkable expression as well as stability levels. Using this DyP, we could show carbonyl olefinating activities in the presence and absence of phosphines.[3]

Recently, we were able to demonstrate the activity of YfeX on C—H functionalisations of indole using the standard carbene-precursor ethyl diazoacetate (EDA) in collaboration with the Koenigs lab (RWTH Aachen).[4] In this study, we could additionally show and investigate the carbene-transfer reaction of diazoacetonitrile (DAN) onto indole. This reaction is of high interest as it provides direct access to important precursors for the synthesis of tryptamines such as serotonin. We could improve both reactions by an initial alanine-scan of the active site residues and deuterium-labelling experiments providing insights into the mechanism, which revealed a different reaction mechanism of the two diazo-compounds.

To further improve these carbene-transfer reactions, we set out to develop several high throughput techniques to allow applying directed evolution protocols. We developed a novel Golden Gate-based mutagenesis method, which allows the saturation of up to five positions simultaneously within one day.[5] Aided by the freely available online tool for the primer design as well as a graphical analysis of the sequencing—accessible at https://msbi.ipb-halle.de/GoldenMutagenesisWeb/ —this Golden Mutagenesis technique provides rapid and facile access to small and large mutant libraries.

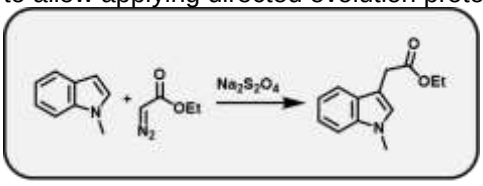

Figure 12 – The carben-transfer reactions with N-methyl-indoles

We used this technique and others to perform five rounds of directed evolution on the carbene-transferring reaction to indole. We applied a combination of single-codon saturation mutagenesis (SCSM)[6] and iterative saturation mutagenesis[7] that resulted in reshaping the active site.

The evolved enzyme shall be applied in microproduction units in the framework of the Leibniz Research Cluster.

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PURIFICATION AND PRESSURE DEPENDENCE OF ALANINE RACEMASE FROM THE PSYCHRO-PIEZOPHILIC BACTERIUM SHEWANELLA VIOLACEA DSS 12

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Key Words: *Shewanella violacea*, alanine racemase, piezophilic bacterium,

Shewanella violacea DSS12 (*S. violacea*) is a psychrophilic and piezophilic bacterium, isolated from mud of the Ryukyu Trench in Japan. The bacterium displays optimal growth at 8°C and 30 MPa. Alanine racemase is an enzyme which catalyses the interconversion of L-alanine and D-alanine, and is responsible for the synthesis of D-alanine contained in the peptidoglycan of bacterial cell wall. In this study, we purified alanine racemase from *S. violacea* and investigated the enzymological characteristics of alanine racemase.

The bacterium was aerobically cultured using marine broth 2216 in a 5-liter medium bottle at 4°C for 3 days. The bacterial cells were lysed by applying of 100 MPa pressure using a French press, and the lysate was centrifuged. The supernatant obtained was ultracentrifuged at 141,000 g, and the supernatant obtained was applied to ammonium sulfate fractionation. The active fraction was dissolved and passed through a butyl-Toyopearl, phenyl-Sepharose, and shodex KW-200 columns to obtain a partially purified enzyme. Consequently, the enzyme was purified 540-fold and showed a specific activity of 2.68 μmol/min/mg. Alanine racemase exhibited high activity against L-Ala and L-Ser as substrates. The optimal pH and temperature of alanine racemase were 9.0 and 25°C, respectively.

The enzyme showed 100% of residual activity after 30-min-incubation at 100-150 MPa and began to be inactivated at greater than 150 MPa for 30 min. In the case of dependency of activity on pressure, interestingly, the optimal pressure depended on the substrates (125 MPa for L-Ala, 0.1 MPa for L-Ser, and 0.1 MPa for L-Phe). To our knowledge, no other enzyme exhibits the pressure dependency for substrate specificity like alanine racemase. There is currently great interest in the mechanism of this observation of alanine racemase activities under high pressure.

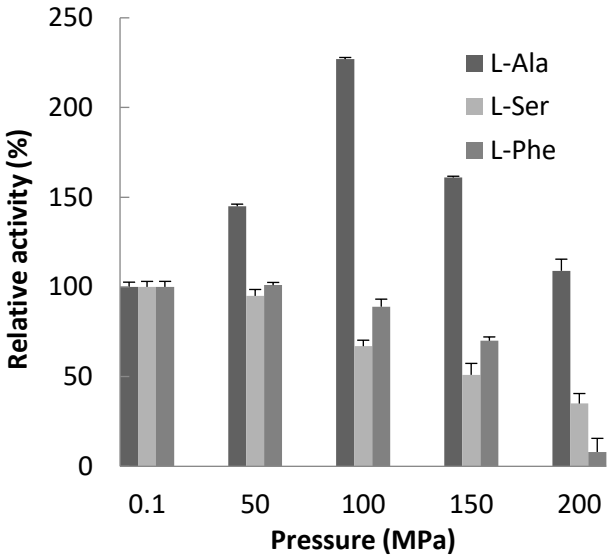

Figure 13 – Effect of pressure on substrate specificity

CLONING AND EXPRESSION OF A SERINE RACEMASE GENE HOMOLOGUE OF THE GREEN ALGA *CHLAMYDOMONAS REINHARDTII* AND CHARACTERIZATION OF THE GENE PRODUCT.

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Key Words: D-amino acids, Enzyme, serine racemase

A unicellular green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) has served as a model system to study many fundamental biological processes. We demonstrated that some D-amino acids have no inhibitory effect on the growth of *C. reinhardtii* and the green alga has alanine racemase and D-threonine aldolase. The homologous gene of serine racemase was found on the genome sequence of *C. reinhardtii.* In this study, a homologous gene of serine racemase on the genome of *C. reinhardtii* was cloned and expressed in *E. coli* cells, and the gene product was purified and characterized.

Total RNA was extracted from *C. reinhardtii* cells. Sense and antisense primers were designed for PCR based on the upstream and downstream regions of the putative gene for serine racemase. First strand cDNA was synthesized from the mRNA and the antisense primer. Amplification of nucleotides between the two primers was performed with the cDNA. The fragment (*ser-h*) was sequenced. The deduced protein consisted of 340 amino acids with a molecular weight of 35,300.

The amino acid sequence of the protein showed similarities to the reported serine racemases; *Oryza sativa*, 55%; *Mus musculus*, 52%; *Schizosaccharomyces pombe*, 39%. A modified serine racemase homologous (*ser-h'*) whose codons were optimized for E. coli was synthesized and used to construct pET24/*ser-h'* and to transform BL21 (DE3). SDS-PAGE of the crude extract revealed that the gene product was overexpressed. The gene product was purified to electrophoretic homogeneity from the recombinant cells using ammonium sulfate fractionation and Column chromatography. Further characterization and crystallization of the enzyme are currently under study.

EXPLORING THE POTENTIAL OF ANCESTRAL PHENYLALANINE/TYROSINE AMMONIA-LYASES FOR THERAPEUTIC APPLICATIONS

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Key Words: Ancestral sequence reconstruction, phenylalanine/tyrosine ammonia-lyase, enzyme evolution, enzyme stability, substrate promiscuity

Phenylalanine/tyrosine ammonia-lyases (PAL/TAL) have been approved by the FDA for treatment of phenylketonuria and may also harbor potential for complimentary treatment of hereditary tyrosinemia type II. Herein, we explore ancestral sequence reconstruction as an enzyme engineering tool to increase stability and alter substrate specificity, which could enhance the therapeutic potential of these enzymes. We used MEGA and PAML to reconstruct putative ancestors of PAL/TAL from fungi and compared them to two modern enzymes that have a relatively low PAL/TAL activity ratio. The majority of ancestors could be functionally expressed in *E. coli* and showed activity towards both phenylalanine and tyrosine. All ancestral enzymes displayed increased thermostability compared to both modern enzymes, however, the increase in thermostability was accompanied by a loss in activity when going back in the phylogenetic tree. One reconstructed ancestral enzyme could be interesting for further development, as its catalytic turnover of tyrosine is slightly higher than one of the modern enzymes and it is significantly more thermostable than both modern enzymes. More detailed characterization of the ancestral variants with a focus on stability is currently ongoing. Our results indicate that ancestral sequence reconstruction programs are robust in terms of stability, whereas activity of ancestral variants seems to vary depending on the reconstruction method. We believe that this approach has great potential for enhancing the properties of therapeutic enzymes and biocatalysts for various applications.

MOLECULAR PACKAGING OF BIOCATALYSTS USING A ROBUST ENCAPSULIN

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Key Words: encapsulin, nanocage, substrate specificity, stabilization.

Bacterial microcompartments serve as metabolic modules (pseudo-organelles) that help to increase the local concentration of enzymes, facilitate substrate transfer between a series of connected reactions and sequester potentially toxic substrates or products. Encapsulation of enzyme(s) might be beneficial for biocatalytic approaches due to efficient transfer of intermediates, protection of packaged proteins against proteolysis, extended lifetime of the enzyme and protection against thermal inactivation.

We have developed a tool for encapsulation of different enzymes through the co-expression of encapsulin and individual guest enzymes. Co-expression was based on a two-plasmids system, one carrying the encapsulin-encoding gene and another carrying the gene encoding the cargo enzyme fused to an encapsulation tag. In this work, we describe structural features of a protein nanocage originating from a thermophilic bacterium, which is a stable scaffold easily overexpressed in *E. coli* with yields that approach 1 g protein per L of culture broth. Solving the structure by electron microscopy and x-ray crystallography provides insight into the details of this unique protein-based nano-capsule. Dynamic light scattering (DLS) measurements in 0.5 - 6M urea indicated that the encapsulin is extremely robust. According to ThermoDLS experiments, the encapsulin is stable up to 55°C.

We have demonstrated the successful encapsulation of peroxidase, oxidase, catalase and a BVMO (Fig. 1). However, activity was confirmed only for some of these cargo enzymes due to restrictive passage of substrates through the encapsulin pores. Encapsulin is permeable for a plethora of substrates ranging from small phenolic compounds to ABTS and cellotetraose. However, NADPH and bulky dye molecules cannot enter the lumen of encapsulin.

Encapsulation of enzymes result in marked increased enzyme stabilities and could be used to alter the substrate scope of encapsulated biocatalysts.

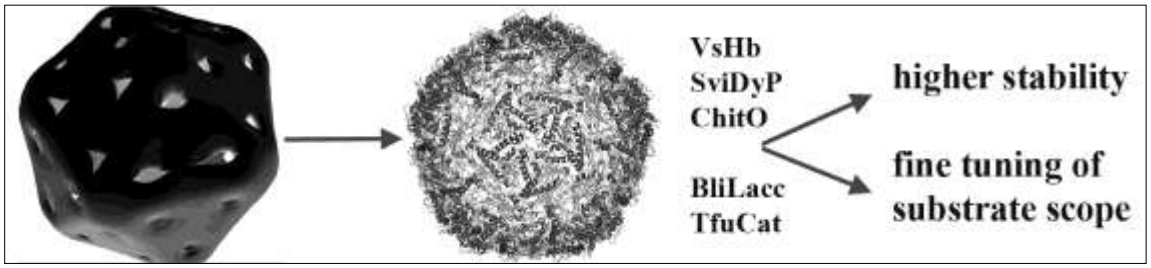

Figure 14 – CryoEM and X-ray crystallography allowed insight into structural features of a novel robust protein nanocage, EncMh. A short signal peptide added to the C-terminus of a variety of enzymes, including metallo-, heme- and flavin-containing enzymes, enabled in vivo loading of EncMh with cargo proteins.

PREPARATION OF PECTATE LYASE/$Cu_3(PO_4)_2$ HYBRID NANOFLOWER AND ITS CATALYTIC PERFORMANCE AS AN IMMOBILIZED ENZYME

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Biocatalysts could substitute conventional chemical reagents in textile industrial process for green-production process as well as lowering the costs. Alkaline pectate lyases (Pels) are enzymes that could be used in scouring and degumming process, in which the biochemical properties of Pels with high activity and stability under process conditions are of great interests. In our previous studies, an alkaline pectate lyase PEL168 derived from *Bacillus subtilis* 168 was engineered with improved enzymatic performance. The obtained Pel3 mutant presented an increased specific activity of 4.3-fold and extended T_{50} to 330 min. Here, we introduce a facile and rapid method of preparing an immobilized enzyme Pel3-inorganic hybrid nanoflower to increase its biocatalytic efficiency. After evaluating four divalent ions, including Mn^{2+}, Ca^{2+}, Zn^{2+} and Cu^{2+} as inorganic part with PBS buffer, Pel3/$Cu_3(PO_4)_2$ hybrid nanoflower was obtained with improved biocatalytic properties. The optimum temperature and pH of Pel3/$Cu_3(PO_4)_2$ hybrid nanoflower were determined to be 55℃ and pH 9, respectively, exhibiting subtle difference from the free Pel3. However, the Pel3/$Cu_3(PO_4)_2$ hybrid nanoflower maintained 33% total activity after treated at 55℃ in 24 h, while the free Pel3 completely lost its activity in 18 h. Furthermore, the residual activity of the Pel3/$Cu_3(PO_4)_2$ hybrid nanoflowers remain over 50% even after four times of repeatitive utilization, demonstrating its promising stability for practical application.

FUNGAL PEROXYGENASE CHIMERA IDENTIFIED BY SUB-DOMAIN SHUFFLING AND SPLIT-GFP REVEAL RETAINED ACTIVITY AND ALTERED SPECIFICITY

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Key Words: Fungal Peroxygenases, C−H oxyfunctionalization, modular expression system, chimeric enzymes, enzyme engineering

Fungal peroxygenases[1] catalyse the hydroxylation of aromatic and aliphatic C-H bonds with high activities, selectivities and thereby relying solely on hydrogen peroxide as a cofactor.[2] Although there are more than 4000 putative fungal peroxygenase genes annotated, only *Agrocybe aegerita* unspecific peroxygenase (*Aae*UPO) could be heterologously expressed in *S. cerevisiae* after five rounds of directed evolution and screening more than 9000 transformants.[3] Four of the identified nine mutations were located in the signal-peptide. However, as "you get what you screen for" in directed evolution, the enzyme has been evolved towards the substrate and not just on the increased expression level. Many fungal genomes carry several dozens of UPO genes suggesting they harbour different specificities and perhaps even catalytic activities. This requires a substrate unspecific screening system for the evolution of these UPOs for heterologous expression.

We therefore established a substrate unspecific high-throughput system to monitor and evolve expression in *S. cerevisiae*. This system is based on a 15 amino acid peptide tag *C*-terminal attached to the protein of interest.[5] Upon recombining with a truncated non-fluorescing-GFP it starts emitting again and allows specific protein quantifications even in crude lysates. Using this system and hypothesising that the *N*-terminal signal-peptide or certain subdomains are pivotal for active expression, we employed a Golden Gate-based modular cloning system to shuffle and reassemble all these different parts.
We first tested the putative UPO gene of *Galerina marginata* (*Gma*UPO) with twelve different signal peptides.

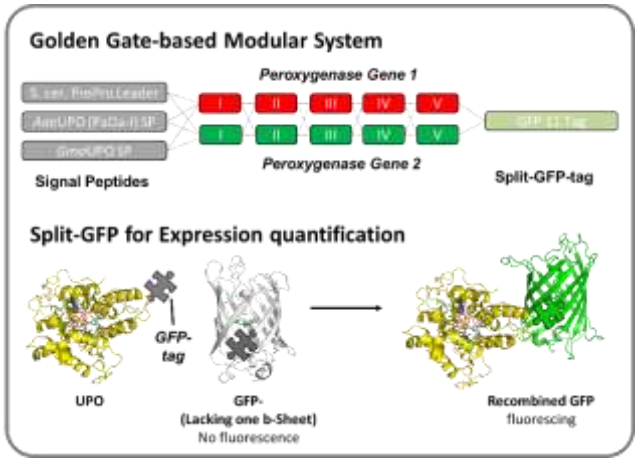

Figure 15 – Illustration of the modular concept and the GFP-tag

We were astonished to notice by split-GFP that more than 50 % of the constructs showed expressions – some of them higher rates than PaDa-I. However, we were not able to detect any activity with the standard peroxidase or peroxygenase assays. By cutting the gene of PaDa-I into five parts and shuffling it with the *Gma*UPO gene, we could identify an active chimera, with a 16-fold higher activity for the peroxygenase reaction over the peroxidase activity (with the utilised substrates ABTS and NBD). The discovered chimera consists of four parts of PaDa-I and one part of *Gma*UPO. It shows comparable activities and stabilities to PaDa-I but shifted selectivities.
By performing iterative saturation mutagenesis using the Golden Mutagenesis protocol[4] we were able to further elucidate the function of the inserted fifth subdomain.
Here, we wish to present our findings as well as the developed methodologies.

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WITHDRAWN

A RETROBIOSYNTHETIC APPROACH TO GENERATE TERPENE-DERIVED POLYMERS

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Key Words: Terpene, Biopolymer, Retrosynthesis, Oxidoreductases, enzyme engineering

With an increasing global population and an enhanced awareness of the impact of human activities on our environment and climate, innovative concepts for resource efficiency are urgently needed.[1] This is especially relevant for synthetic polymers, that today are mainly generated from petroleum. In fact, at present only 1% of all man-made synthetic materials are generated from renewable resources, which is not aligned with the global vision of a circular bioeconomy. Upgrading bio-based molecules from wood and other sustainable raw material into activated monomers to generate biopolymers is thus a contemporary research area.[1-3]

Herein, a novel retrobiosynthetic approach[4] to enable biopolymer generation by chemoenzymatic catalysis is presented (Figure 1). By employing a retrobiosynthetic analysis applied to polyesters (Figure 1A), and capitalizing on synthetic biology in concert with polymer chemistry, my research group has recently successfully generated unprecedented biopolymers with interesting thermal properties (Figure 1B).[4,5]

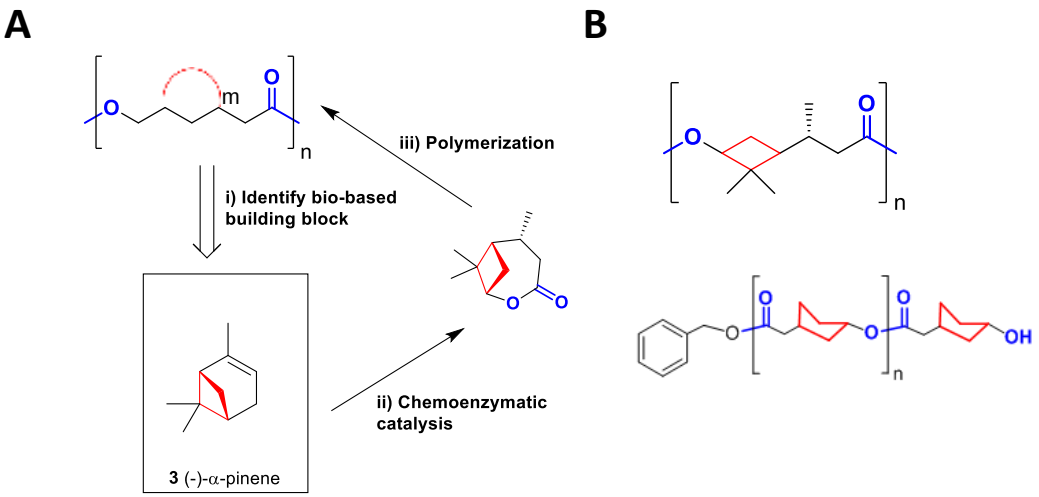

Figure 1. A) Retrobiosynthetic analysis for polyesters harboring rings as backbone motifs enabled biocatalytic formation of green caprolactone-analogues and corresponding terpene-derived polyesters.[4] B) Examples of novel materials generated in my group by polymer retrobiosynthesis (Stamm et al.[4] and Farhat et al.[5]).

The forefront in sustainability science lays in the development of biocatalytic processes based on engineered enzymes, to enable mild generation of biofuels, biomaterials and fine chemical synthons from renewable resources.[2] In particular, terpenoids and other multicyclic scaffolds represent a rich and untapped source of chiral biochemicals and biopolymers.[3] However, the inert backbones towards controlled polymerization found in many terpene-based building blocks hamper their full potential in material science. By merging biocatalysis, enzyme engineering and synthetic biology mild valorization of renewable synthons into desired monomer structure can be achieved with high-precision. Recent results from my research group on design and engineering of oxidoreductases in concert with polymer chemistry to enable access to advanced green functionalized terpene-derived materials by polymer retrobiosynthesis will be discussed.

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ENGINEERING OF A SPECIFIC CYP450 FOR AN INDUSTRIAL PROCESS SHOWS 700-FOLD INCREASE IN ACTIVITY WITH K_{CAT} OF 6.2 S^{-1} – RESIDUES CAUSING HYDROGEN MIGRATION AND DOUBLE HYDROGEN ABSTRACTION AT Δ^X CARBON IDENTIFIED BY QUANTUM MECHANICS REVEALED TO BE THE GAME CHANGER

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Key Words: Enzyme engineering, CYP450, Desaturation, Quantum Mechanics/Molecular Mechanics, Transition States.

A CYP450 catalyzed desaturation reaction for the synthesis of an Active Pharmaceutical Ingredient (API) via biocatalytic route that is estimated to top up the revenue of the company to 8.5 million dollars was identified in this study. Currently this route is being tested for the industrial scale production of the API. This was achieved by engineering a specific membrane bound CYP450 enzyme that was initially inactive towards the substrate. Extensive modelling studies were carried out to obtain an initial promiscuous activity (0.53/min). Here the substrate enters through the transmembrane region of the enzyme that acts as a tunnel and moves to the Heme Binding Site in the CYP450 during which it undergoes rotation of ~180°. The substrate entry was identified as the first rate limiting step in the reaction. Using a grid-based path optimization method this path was engineered to facilitate the easy entry and movement of substrate in to the active site. The second rate limiting step was observed in the formation of two transition states of the reaction which was identified using Quantum Mechanics hybridized with Molecular Mechanics (QM/MM) simulations. The enzyme was engineered using quantum polarized grid technology to reduce the energy of these transition states. The mutations introduced, improved the enzyme activity > 700 folds giving a K_{cat} value of 6.2 s^{-1} which translated to a good yield of the product in the lab scale fermenter. A greater finding in this project is the desaturation reaction mechanisms involving the hydrogen migration and double hydrogen abstracted from the same Δ^X Carbon. This was obtained using extensive QM/MM simulations studies and a high-resolution grid energy evaluation method which revealed transition and intermediate states of the reaction, not reported before in any of the desaturation mechanisms solved so far. Some important aspects of this study include, modelling yeast membrane with different lipids and varying substrate concentrations, enzyme behavior at high substrate concentrations and process optimization. In conclusion, this is a cost effective route with a conversion rate that is ~10 times more than the chemical route. This process can be applied to engineer CYP450 for any chemical routes that involve a desaturation reaction.

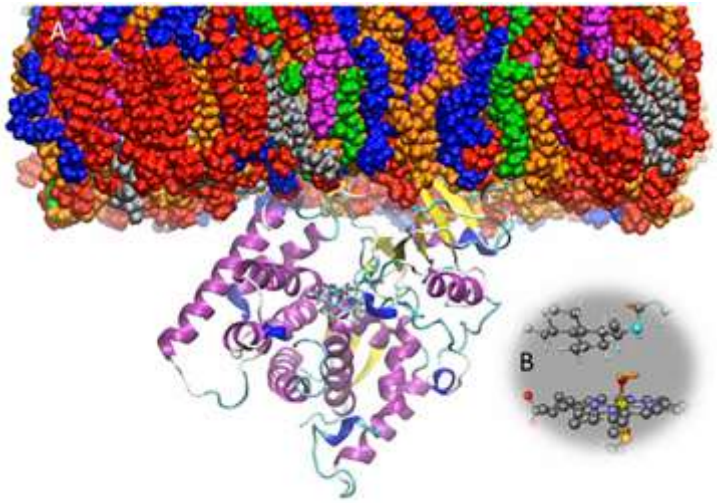

Figure 1A. – Cyp450 complexed with heme and substrate integrated in the yeast hypo membrane B. Shows the rate limiting transitions state of the enzymatic reaction

Variants	Number of mutations	Activation energy of TS1 and TS2 [kcal mol^{-1}]	*invitro* Activity K_{cat} Sec^{-1}
Wild	-	48.3 and 43.6	0.008
V1	3	35.8 and 30.3	6.2±0.8
V2	3	35.6 and 33.2	4.8±1.2
V3	2	32.7 and 33.8	3.5±0.5

Table 16 – Energies of the crucial transition states and the experimental K_{cat} values

ENABLING MEDIUM-CHAIN FATTY ACID PRODUCTION IN YEAST VIA HIGH-THROUGHPUT MALDI MS-BASED ENZYME ENGINEERING

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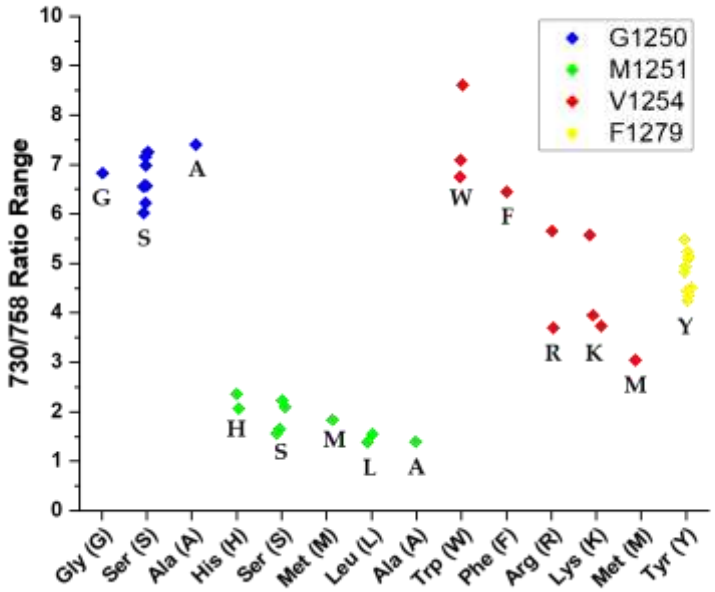
Key Words: Free fatty acids, Fatty acid synthase, *Saccharomyces cerevisiae*, Metabolic engineering, High-throughput screening

Free fatty acids (FFAs) are considered important and valuable products, existing in crucial nutrients, soaps and fuels. Accordingly, the production of FFAs in fungi has been investigated extensively over years due to its metabolic capability. Particularly for controlling the fatty acid chain length compositions that can benefit formation of desired biofuels and chemicals. However, existing methods for production and selection of shorter-chain length FFAs are still time-consuming and labor-intensive. Here, we report a mass spectrometry (MS) based high-throughput screening method for detecting medium-chain fatty acids (MCFAs) in *Saccharomyces cerevisiae* by using lipids as a proxy. Four first shell key residues in the fatty acid synthase (FAS) are chosen to perform site-saturation mutagenesis and 288 colonies are screened to achieve 95% library coverage. The results from the Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) MS indicate improved shorter acyl-chain phosphatidylcholine (PC) production in ~100% of the mutants compare with the wild type. Finally, we identify several mutations among all four libraries of interest that can help with baker's yeast shorter acyl-chain length phospholipids production. Furthermore, we correlate the lipids with MCFAs productions by GC-MS characterization on selected candidates after sequencing check. The presented technology should be generally applicable for controlling fatty acid compositions in a high-throughput manner far beyond baker's yeast.



Hypothesis:

730/758 > 1: potential mutant

730/758 < 1: WT

Figure 17 – Sequencing result of Top 10 730/758 m/z peak ratio colonies of all 4 libraries. Multiple different amino acids can help with increasing shorter acyl chain lipid production.

ENGINEERING ALCOHOL OXIDASES FOR SUBSTRATE SCOPE AND THEIR APPLICATION IN FLOW AND CASCADE BIOCATALYSIS

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Key Words: oxidation, primary alcohol, secondary alcohol, high-throughput screening, thermostability

Alcohol oxidases have significant advantages over alcohol dehydrogenases (ADHs) for biocatalytic oxidation of alcohols: they don't require addition of (expensive) nicotinamide cofactors (or a recycling system for cofactor regeneration) and the catalytic reaction is irreversible. Although alcohol oxidases generate hydrogen peroxide when they turn over, this issue can be alleviated by addition of catalase, which, not only removes the peroxide, but also creates more oxygen for cofactor regeneration. Alcohol oxidases are perceived to have a limited substrate scope preventing their wider use in synthesis. Thus, we present the engineering of two alcohol oxidases for increased substrate scope, one for the selective oxidation of primary alcohols and one for secondary alcohol oxidation.

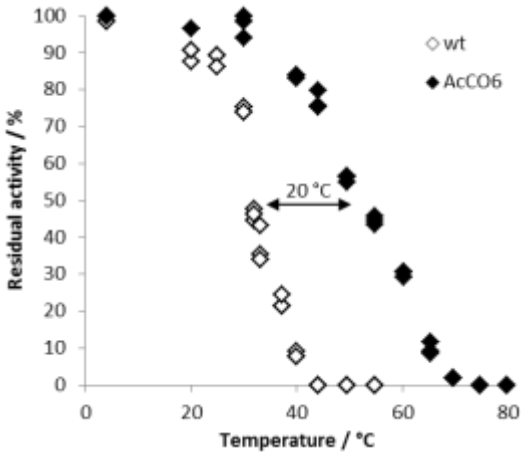

Figure 1. Improved thermostability of choline oxidase six-point variant compared to the wildtype.

Arthrobacter chlorophenolicus choline oxidase and *Streptomyces hygroscopicus* cholesterol oxidase were selected as the primary and secondary alcohol oxidase, respectively. Examination of crystal structures of homologous alcohol oxidases revealed positions in the active site and entrance channel to target for saturation mutagenesis. Libraries were screened using a high-throughput assay based on the detection of hydrogen peroxide using hexanol as the substrate for evolution of choline oxidase and cyclohexanol for evolution of cholesterol oxidase. Mutations from positive hits were combined and, in these cases, resulted in variants with further improvements in both k_{cat} and conversion to the carbonyl in biotransformations. The enzyme variants maintained selectivity for primary or secondary alcohols consistent with the source alcohol oxidase. Choline oxidase variants also showed increased activity towards a variety of other primary alcohols such as terminal diols and benzylic alcohols such as vanillyl alcohol and cinnamyl alcohol. Mutation of residues with high B-factors led to an enzyme with increased thermostability, with a T_{50} 20 °C above that of the wildtype (Figure 1) as well as improved solvent tolerance. For cholesterol oxidase we also gained activity with linear secondary alcohols, and secondary benzylic alcohols such as indanol.

These evolved alcohol oxidases have been immobilized and applied in a flow system. Due to the solvent tolerance of the primary alcohol oxidase we were able to run the reaction in pure cyclohexane.[2] However, modelling showed that, despite the increased solubility of oxygen in cyclohexane compared to water, the reaction was still oxygen limited. Thus, we have applied it in a microreactor with catalase and hydrogen peroxide for increased oxygen supply. The oxidases have also been successfully applied in cascade reactions, in particular with reductive aminases for conversion of alcohols to secondary amines.[3]

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USING PROTEIN ENGINEERING TO ACCELERATE IMPLEMENTATION OF CONTINUOUS BIOCATALYSIS FOR API SYNTHESIS

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Key Words: continuous process, enzyme, biocatalysis, reactor selection, protein engineering

Biocatalysis is an invaluable tool for the synthesis of many active pharmaceutical ingredients[1,2]. Enzymatic reactions are frequently performed in batch, but continuous biocatalysis is gaining interest in industry[3], not least because it would allow better integration of chemical and enzymatic reaction steps[4]. However, selecting a suitable reactor configuration for continuous biocatalysis is often challenging due to the common limitations of enzymes, such as poor kinetic constants towards industrially relevant substrates, substrate/product inhibition, the need for an aqueous reaction environment and/or pH control. For this reason, we have recently developed a tool[5] that presents a variety of reactor configurations, combining continuously stirred tank reactors (CSTRs) and continuous plug-flow reactors (CPFRs), to overcome the aforementioned limitations and facilitate reactor selection. However, the continued development of protein engineering technologies[6,7] has revealed one of the strongest advantages of biocatalysis over chemocatalysis, namely that the properties of the catalyst can be modified to remove limitations and better suit industrial production processes.[8,9] In this presentation we highlight how protein engineering, guided by process-specific targets, can streamline process development. For example, reducing product inhibition by protein engineering could allow operation in a single CPFR where the possibility of complete conversion greatly simplifies downstream processing, improving the tolerance of enzymes towards organic solvents could allow water-free operation without mass transfer limitations between phases and improving pH tolerance could remove the need for acid/base addition to combat pH changes during operation. Such changes to the catalyst could greatly facilitate process development in an industry where reduction of time-to-market is often critical.

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INNOV'SAR: A NEW APPROACH FOR PROTEIN ENGINEERING AND SCREENING

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Key Words: Enzyme engineering, Biocatalyst, Chemical Manufacturing, Artificial Intelligence

We present a strategy that combines wet-lab experimentation and computational protein design for engineering polypeptide chains. The protein sequences were numerically coded and then processed using Fourier Transform (FT). Fourier coefficients were used to calculate the energy spectra called "protein spectrum". We use the protein spectrum to model the biological activity/fitness of protein from sequence data. We assume that the protein fitness (catalytic efficacy, thermostabilty, binding affinity, aggregation, stability…) is not purely local, but globally distributed over the linear sequence of the protein. Our patented method does not require protein 3D structure information and find patterns that correlate with changes in protein activity (or fitness) upon amino acids residue substitutions. A minimal wet lab data set sampled from mutation libraries (single or multiple points mutations) were used as learning data sets in heuristic approaches that were applied to build predictive models. We show the performance of the approach on designed libraries for different examples[1] and discuss how our approach can tackle epistatic phenomena[2]. We can screen up to 1 billion (10^9) protein variants in a very short time.

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A DATA-DRIVEN APPROACH FOR EXPLOITING ENZYME PROMISCUITY AS A MEANS TO PREDICT NOVEL BIOCHEMICAL REACTIONS

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Key Words: machine learning, chemo-informatics, novel reactions, synthetic biology

Systems metabolic engineering has been widely used to produce chemicals of high commercial value from low cost substrates. But this process has challenges for some applications, such as harnessing lignocellulosic biomass for biofuel and biochemical production, due to our limited metabolic knowledgebase. With current advances in protein engineering, it is possible to exploit substrate promiscuity of enzymes to enable novel biochemical reactions. Nevertheless, performing experiments to determine what substrates an enzyme can act on can be time consuming and it is not always clear what potential substrates to test. So, the current work aims to employ machine learning approaches for identifying novel substrates and in turn, predicting novel reactions that are more promising than the putative reactions predicted simply based on compound similarity measures (e.g., Tanimoto coefficient). A highly accurate (up to 88.3%) machine learning model was developed to identify candidate substrates for *alcohol dehydrogenase* (ADH) using a dataset consisting of 23 metabolites (with 8 of them being known positives) and 46 chemo-informatics based molecular descriptors (e.g., topology, stereochemistry, and electronic features). In addition, support vector regression proved to be a useful method for estimating enzyme kinetics (characterized by Michaelis-Menten constants, K_m and V_{max}) for a variety of oxidoreductases that are typically found in biofuel biosynthesis pathways. Such machine learning methods can be applied to other classes of enzymes and hence, used as a tool to expand the knowledgebase of metabolic reactions paving the way for next generation of metabolic/ pathway engineering.

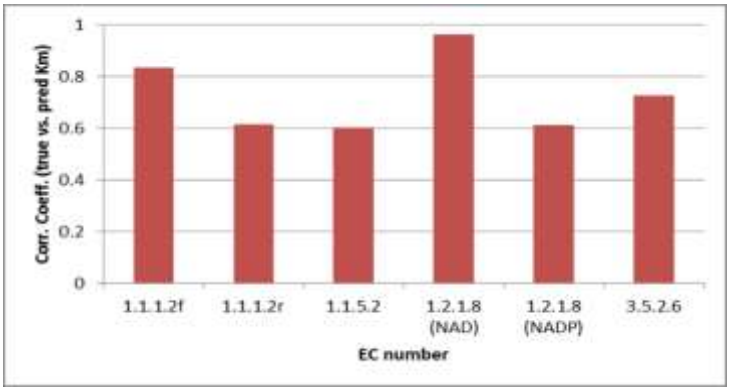

Figure 18 – Correlation coefficient b/w true and model predicted K_M values for different enzyme-substrate datasets [f - forward reaction; r - reverse reaction]

ENGINEERING ENZYMES WITH NON-CANONICAL ACTIVE SITE FUNCTIONALITY

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Key Words: non-canonical amino acids, enzyme design, engineering, mechanisms, stop codon suppression

The combination of computational enzyme design and laboratory evolution provides an attractive platform for the creation of protein catalysts with new function. To date, designed mechanisms have relied upon Nature's alphabet of 20 genetically encoded amino acids, which greatly restricts the range of functionality which can be installed into enzyme active sites. Here, we have exploited engineered components of the cellular translation machinery to create a protein catalyst which operates via a non-canonical catalytic nucleophile. We have subsequently shown that powerful laboratory evolution protocols can be readily adapted to allow optimization of enzymes containing non-canonical active site functionality. Crystal structures obtained along the evolutionary trajectory highlight the origins of improved activity. Thus our approach merges beneficial features of organo- and biocatalysis, by combining the intrinsic reactivities and greater versatility of small molecule catalysts with the rate enhancements, reaction selectivities and evolvability of proteins.

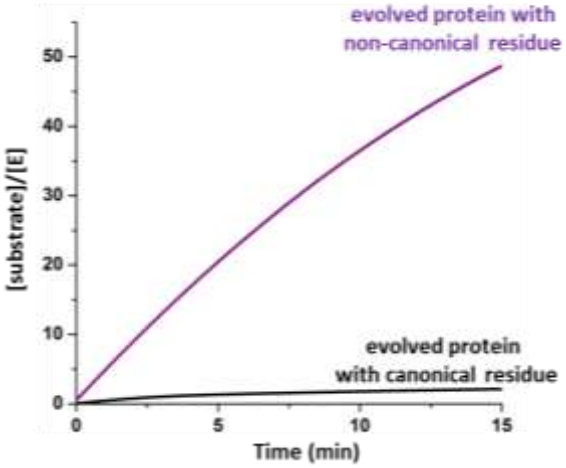

Figure 19 – Reaction profile showing the most evolved variant. Replacement of the non-canonical amino acid with a canonical residue abolishes catalytic activity

ENZYME ENGINEERING OF FUNGAL-DERIVED FAD-GDH BY CIRCULAR PERMUTATION

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Key Words: FAD-GDH, Protein engineering, Substrate preference, Circular permutation

The flavin adenine dinucleotide dependent glucose dehydrogenase (FAD-GDH; EC 1.1.5.9) comprises oxidoreductases that catalyze the initial oxidation of glucose and other sugar molecules, using FAD as the primary electron acceptor. FAD-GDH has received attention as biocatalyst for glucose monitoring, especially self-monitoring of blood glucose.

Narrowing the substrate specificity of FAD-GDH for glucose is desired for future application. In this study, we employed a technique called circular permutation (CP) to explore the effects on enzyme substrate specificity. CP is a method to create protein variants by connecting the native protein termini via a covalent soft linker and introducing new ends through the cleavage of an existing peptide bond (Fig 1) (1, 2).

Starting with FAD-GDH derived from *Aspergillus iizukae* (AiGDH), we explored various the amino acid linker to connect the original termini. Subsequently, 16 CP variants with new termini in selected parts of the protein structure were generated and tested for catalytic activity toward glucose. The activity of wild type AiGDH toward xylose is approximately 10% of that for glucose. Termini relocation in the leading CP variants resulted in a 1.5 to 2-fold reduction of relative activity for xylose over glucose. At the same time, wild type and CP variants exhibited only residual activity for maltose (<1%).

Thermostability of CP variants was measured by the T_{50} values, which is the temperature at which 50% residual activity is maintained following a heat treatment. The CP variants exhibit T_{50} values lower than wild-type. We tried the secondary engineering of CP variants to improve the thermostability. We found the thermostable variants through the introduction of a new disulfide bridge. The combination of CP with the introduction of a disulfide bridge showed the functional benefits, exhibiting T_{50} values higher than original CP variants.

Our results suggest that CP variants display reduced xylose interference and reduced cross-reactivity with a range of sugars. Secondary engineering of CP variants exhibit the functional benefits, which improve the thermostability. As such, they could be useful biosensors for self-monitoring of blood glucose.

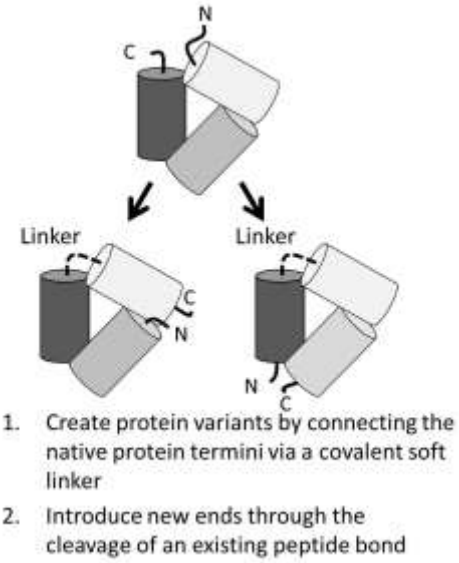

Fig 1. Schematic models of circular permutation.

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APPLICATION OF DIRECTED DIVERGENT EVOLUTION STRATEGY IN NATURAL PRODUCT BIOSYNTHESIS

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Genetic diversity is a result of evolution, enabling multiple ways for one particular physiological activity. Here, we introduce this strategy into bioengineering. We design two hydroxytyrosol biosynthetic pathways using tyrosine as substrate. We show that the synthetic capacity is significantly improved when two pathways work simultaneously comparing to each individual pathway. Next, we engineer flavin-dependent monooxygenase HpaBC for tyrosol hydroxylase, tyramine hydroxylase, and promiscuous hydroxylase active on both tyrosol and tyramine using directed divergent evolution strategy. Then, the mutant HpaBCs are employed to catalyze two missing steps in the hydroxytyrosol biosynthetic pathways designed above. Our results demonstrate that the promiscuous tyrosol/tyramine hydroxylase can minimize the cell metabolic burden induced by protein overexpression and allow the biosynthetic carbon flow to be divided between two pathways. Thus, the efficiency of the hydroxytyrosol biosynthesis is significantly improved by rearranging the metabolic flux among multiple pathways.

A TECHNOLOGY PLATFORM FOR *IN VITRO* TRANSCRIPTION AND TRANSLATION OF ENZYMES IN MICRO COMPARTMENTS

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Key Words: microfluidics, droplet screening, emulsification, function-based metagenomics, synthetic enzyme cascades.

Enzymes are crucial elements of all living cells. As biological catalysts they accelerate chemical reactions without being consumed in the process. The modern bioeconomy strives to identify new enzymes for improved usage in biotechnological applications such as the production of fine chemicals or pharmaceuticals. A promising source for the discovery of new enzymes are metagenomes. As there is no intermediary cultivation step for the extraction of the genetic material necessary, the genetic pool of a metagenome comprises genes of cultivable and non-cultivable microorganisms, which is beneficial for the detection of new enzymes. Here we introduce a new technology platform towards high efficient screening of whole metagenome libraries by combining *in vitro* compartmentation with a cell-free protein synthesis approach (Figure 1). A key element of this platform is a centrifugal microfluidic cartridge which encapsulates the metagenome library in up to 100’000 monodisperse droplets with a volume of 520 pl. The micro droplets are generated by centrifugal step emulsification (1) and are further transported into a standard reaction tube, decoupling the emulsification from the downstream processing. Based on substrate specificity, droplets with active enzymes are selected and a subsequent sequencing analysis allows the identification of the DNA sequence of these enzymes. The high number of generated micro droplets enables a high-throughput of large libraries and the high coverage increases the chance of finding new or rare enzymes. Compared to traditional approaches, the introduced all-in-one metagenome screening platform decreases screening time to a large extend by replacing heterologous expression with *in vitro* protein synthesis and massive screening. Further, the selection process minimizes the sequencing and annotation effort.

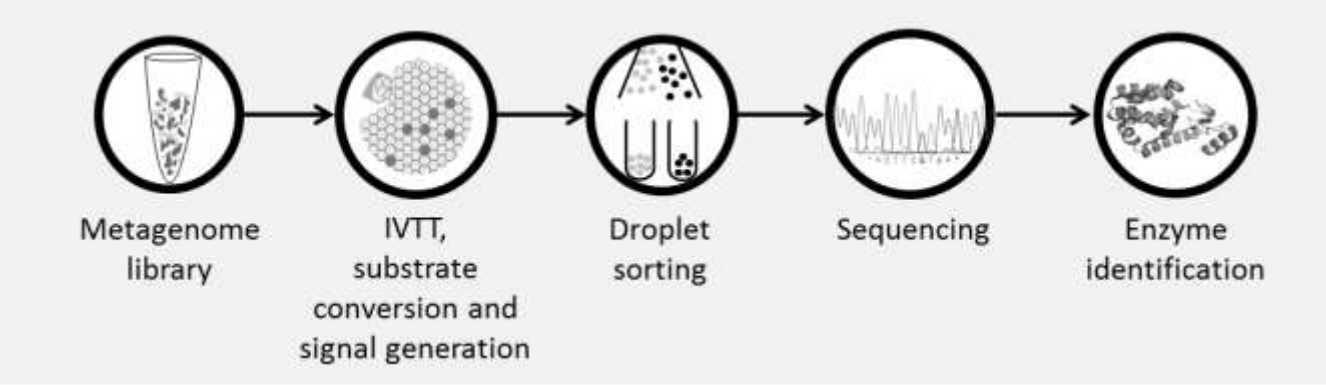

Figure 1 Workflow of the all-in-one metagenome screening platform. DNA fragments of metagenome library are emulsified in micro droplets. Inside the droplet the in vitro transcription and translation (IVTT) takes place. Upon substrate conversion specific enzymes will generate a signal. Following droplet sorting the DNA sequence of active enzymes can be determined.

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DESIGN STRATEGY FOR CREATING CATALYTICALLY ACTIVE METAL BINDING PROTEINS

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Key Words: Enzyme Design, Metalloenzyme, Rosetta, Computational Design

Metalloenzymes catalyze a wide variety of reactions in nature by taking advantage of the versatility and reactivity of transition metals. Despite the diversity of reactions catalyzed by natural proteins, there is still a demand for designer enzymes. In many cases, all that is needed is routine re-engineering of the native enzymes to perform efficiently under the demanded application conditions. In other cases, the reaction or reaction condition desired differs so much from natural conditions that mere redesign of natural proteins is not practical. *De novo* enzymes, which are generated entirely from first principles rather than modified from natural proteins, are ideal for these situations. These *de novo* enzymes would allow us to generate enzymes that can survive at much higher temperatures, work in many different solvents and solutions, or perform completely novel functions.

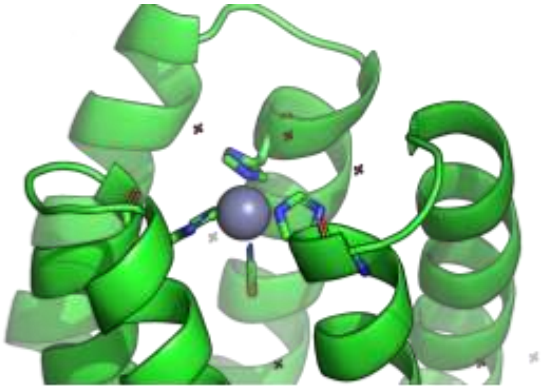

Figure 20 – Metal binding site of designed zinc – binding helical bundle with inverted tetrahedral complex

Currently, most new metalloenzymes are still developed via mutations or evolution of natural proteins. While there are previous examples of designed metalloenzymes in *de novo* scaffolds, these designs are generally limited to incorporation of the metal binding motif into monomeric three and four helix scaffolds. The limitations of this shape greatly limits the functionalities possible for these enzymes. With recent advances in *de novo* design of proteins, it is now possible to generate a wide array of helical bundle oligomers, and create a diverse set active site topologies to enable a variety of novel reactions. Figure 1 shows the crystal structure of a zinc binding protein created using our design methods. Here we present a strategy for design of *de novo* proteins around a desired metal coordination site. This process allows for the generation of myriad topologies custom-designed for the reaction of interest, while still maintaining the stability that designed proteins can afford.

EVOLUTION OF THE THDP DEPENDENT PYRUVATE DEHYDROGENASE E1 SUBUNIT FOR THE CONVERSION OF LONG CHAIN ALIPHATIC KETOACIDS

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Key Words: Thiamine diphosphate, acyloins, kinetic control, decarboxylation

Thiamine diphosphate (ThDP) dependent enzymes can catalyse the synthesis of chiral acyloins from both aldehydes and ketoacids as donor substrates, but the latter are generally preferred as they render the reaction under kinetic control.[1] While a large variety of aromatic aldehydes and polar donor substrates such as hydroxypyruvate and oxoglutarate are accepted by wild-type enzymes, the conversion of linear and branched chain aliphatic ketoacids remains a formidable challenge even after several rounds of directed evolution.[2] Due to its naturally large active site volume, the pyruvate dehydrogenase E1 subunit from *E.coli* (EcPDH E1) is a promising enzyme scaffold for directed evolution towards the conversion of sterically demanding, aliphatic ketoacids. Here we present initial results on the enzyme's kinetic properties and its substrate scope.

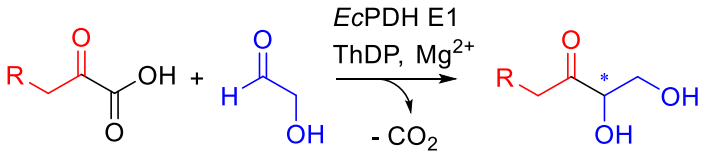

Figure 21 – EcPDH E1 catalysed conversion of aliphatic ketoacids using glycolaldehyde as acceptor.

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ENGINEERING OF HALOHYDRIN HYDROGEN-HALIDE-LYASE (H-LYASE) FOR EFFICIENT L-CARNITINE PRODUCTION

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Key Words: halohydrin hydrogen-halide-lyase, L-carnitine

L-carnitine is a beta-hydroxy carboxylic acid known as a dietary supplement with the effect of fat-burning or anti-fatigue. Although several production processes of L-carnitine are proposed including fermentation, chiral resolution or asymmetric synthesis, there are disadvantages with each from the point of view of yield, side-reactions or productivity. We have engaged in technology development of efficient enzymatic production of various chemicals for years, represented with the world's first industrialized bioprocess for acrylamide. Leveraging our specialty including proprietary nitrile-related enzymes and associated technologies, we attempted to develop a further efficient production process of L-carnitine (Figure 1).

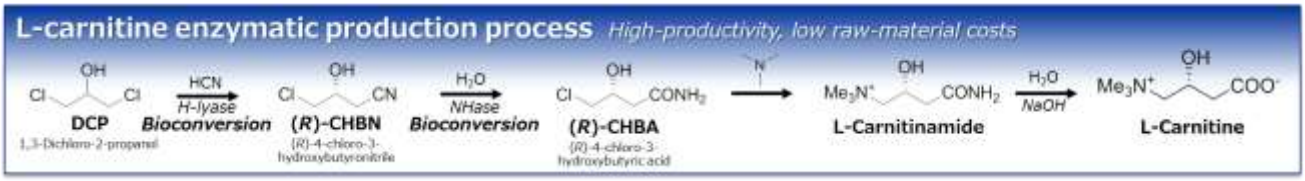

Figure 1 – L-carnitine enzymatic production process

Halohydrin hydrogen-halide-lyase (H-lyase) is the enzyme capable of catalyzing the reversible dehalogenation of vicinal haloalcohols with formation of the corresponding epoxides (Figure 2). We envisioned the enzymatic process for L-carnitine production using HheB, a B-type H-lyase from *Corynebacterium* sp. N-1074, which can convert 1,3-dichloro-2-propanol (DCP) and hydrogen cyanide into 4-chloro-3-hydroxybutyronitrile (CHBN) with *R*-preference [1]. Although its unique feature and enantioselectivity were promising as the biocatalyst for our L-carnitine process, its performance was not adequate from the point of view of industrial use. Thus, we attempted to improve the tolerance for product-inhibition and enantioselectivity of H-lyase by enzyme engineering.

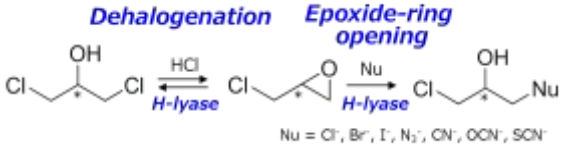

Figure 2 – Reaction catalyzed by H-lyase

Regarding alleviation of product-inhibition, we adopted a random approach. After screening over 20,000 variants by evaluating activities in the presence of the product, that is (*R*)-CHBN, a few variants with alleviated inhibition were successfully obtained. Especially, D199H showed alleviated product inhibition not only by (*R*)-CHBN but also by chloride ion, resulting in increased accumulation of (*R*)-CHBN. As for enantioselectivity improvement, in contrast, a structure-based approach was conducted. The crystal structure of HheB led us to identify some key residues possibly affecting the enantioselectivity. Through saturation mutagenesis on focused residues, several variants with improved enantioselectivity were discovered. Among them, F71-variants showed remarkable improvement of enantioselectivity with increased accumulation of product, in spite of reduced initial activity. Eventually, by combining those useful mutations, we succeeded in evolving wild-type HheB into the practical biocatalyst for L-carnitine production with industrial-level performance [2, 3].

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IMPROVED BIOMASS CONVERSION WITH *TRICHODERMA REESEI* BETA-GLUCOSIDASE CEL3A ENGINEERED FOR BROADER SUBSTRATE SPECIFICITY

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Key Words: Beta-Glucosidase, Substrate Specificity, Protein Engineering, Lignocellulosic Biomass

Fungal cellulolytic enzyme cocktails are widely used for the hydrolysis of biomass into fermentable sugars. Beta-Glucosidases enhance enzymatic biomass conversion by relieving cellobiose inhibition of endoglucanases and cellobiohydrolases. Fungal GH3 beta-glucosidases are often specific for hydrolysis of pyranose glycosides and severely inhibited by monomeric glucose.

Crystal structure models of *T. reesei* beta-glucosidase Cel3A and beta-xylosidase Xyl3A were compared for geometry of the -1 substrate binding subsite. Mutations were introduced into Cel3A to accommodate the -1 subsite for binding of xylosides. Mutation V43L (Figure 1) increased the affinity and activity for beta-xylosides, but had glucoside affinity and activity similar to wild-type Cel3A. Surprisingly, inhibition of Cel3A V43L by both product glucose and substrate cellobiose was reduced by about 3.5-fold compared to wild-type Cel3A.

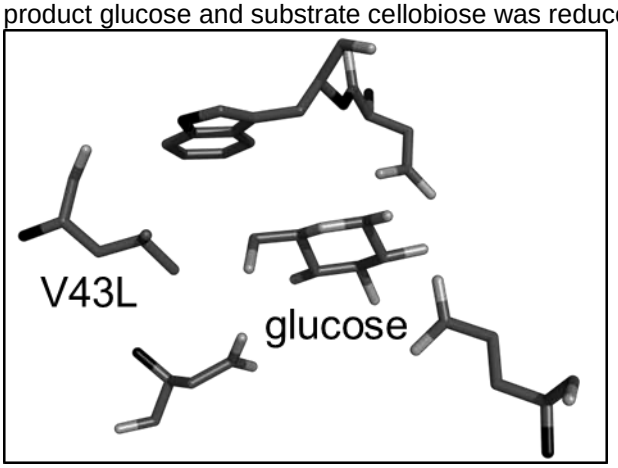

Figure 1 Model of Cel3A variant V43L with glucose bound at -1 substrate binding subsite

Dilute ammonia pretreated corn stover (DACS) contains both glucan and xylan. Cel3A V43L was compared with wild-type Cel3A in the hydrolysis of DACS. Cel3A V43L increased the release of total fermentable sugars over wild-type Cel3A, most notably at elevated substrate loadings where the highest glucose levels were produced. Cel3A V43L resulted in increased titers of both glucose and xylose.

Relaxing the substrate specificity appears to be a promising approach to improve enzymes for biomass saccharification.

SUBSTRATE PROMISCUITY IN EVOLVED ALCOHOL DEHYDROGENASE A (ADH-A)

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Key Words: *Sec*-alcohols, Ketones, Aromatic, Substrate binding, Regio-, and Stereoselectivity.

Alcohol dehydrogenase A (ADH-A) from *Rhodococcus ruber* DSM 44541 tolerates organic solvents, and therefore this became a useful biocatalyst for asymmetric synthesis of organic compounds.[1] ADH-A is capable of catalyzing stereoselective oxidation of phenyl-substituted *sec*-alcohols and reduction of the corresponding ketones. Importantly, these compounds are precursors for the synthesis of a range of biologically active compounds.[1,2] Therefore, we have been studying engineering of ADH-A for the purpose of developing new enzymes with pre-designed catalytic properties regarding substrate scope and selectivity. We have been isolated a number of ADH-A variants which have been isolated from CASTing libraries for different purposes and function. Variants isolated from a library originally generated from random mutagenesis of residues Y294 and W295 (called “A”, clones A1, A2, A2C3 and A2C2B1) represent hits from different generation of directed evolution, selected for improved activity for the non-preferred *R*-enantiomer of 1-phenylethanol.[2] Other mutants that were selected (variants B1 and B1F4) for improved activity with a disubstituted *sec*-alcohol also displayed altered regioselectivity as compared to the wild type.[3] In a third evolution effort, enzyme variants C1 and C1B1 were isolated after selection for improved activity with the vicinal diol (*R*)-1-pheny-1,2-ethanediol.[4]

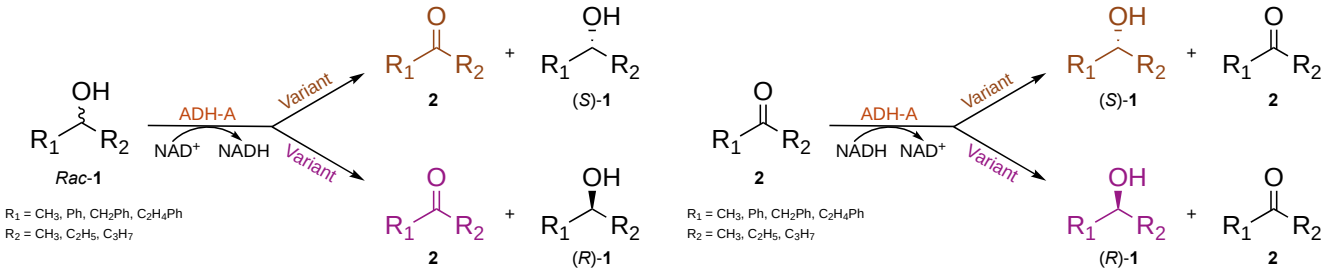

Figure 1. ADH-A wild type and variants oxidation of Rac-1, and reduction of 2 respectively.

The above-mentioned enzyme variants contribute a spectrum of slightly diverse active site structures,[2–5] and are therefore expected to display different substrate-, regio- and stereoselectivities when challenged with substrates that they were not originally selected to accept. Thus, the purpose of the current investigation was to test for possible substrate promiscuity, and to assess the biocatalytic potential in alcohol oxidation, and stereoselective ketone reduction for a spectrum of potentially new substrates.[5]

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UNRAVELLING THE RELATIONSHIP BETWEEN SUBSTRATE SELECTIVITY AND PRIMARY SEQUENCE OF UDP-GLYCOSYLTRANSFERASES

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Key Words: Natural products, selectivity, glycosyltransferase

Plant natural products (NPs) are widely utilized in biotechnology, for example as fragrances, aromas, dyes and medicine. Although nature provides thousands of different NPs, only a small fraction of them is currently used in applications, partly because of problems in solubility and stability. These properties can be enhanced through glycosylation, but synthesis of glycosylated natural products is challenging. Enzymatic route to NP glycosylation is therefore of high interest. In plants, the enzymes responsible for NP glycosylation are called UDP-glycosyltransferases (UGTs) since they use UDP activated sugars as sugar donors. A single plant can have hundreds of UGTs allowing glycosylation of different compound groups. Understanding the bases of substrate selectivity would be important in allowing efficient engineering of UGTs for specific substrates and/or higher catalytic activities. Although UGTs have conserved tertiary structures, the relationship between UGT primary sequence and acceptor substrate is not well understood making enzyme engineering challenging. Main obstacles in creating a predictive model for substrate selectivity is the lag of UGT structures (currently nine plant UGT structures are available through PDB) and the lag of comparable information of UGT selectivity. Interestingly, it has been shown that UGT substrate selectivity is not related to phylogeny. Therefore, we wondered if more insights would be gained from comparing different phylogenetic groups to each other rather than trying to create a common predictive model for the whole enzyme group. By comparing structural information and sequence alignments, we indeed observed differences in substrate binding pocket folding when comparing UGTs from different phylogenetic groups. We hypothesize that this variation has led to difficulties in predicting substrate selectivity from UGT primary sequence, since some residues lining the binding pocket vary from one phylogenetic group to another. Therefore, it might be more feasible to predict substrate selectivity for each UGT phylogenetic group independently instead of the whole enzyme family.

OLIGOPEPTIDES PRODUCTION BY A METHOD INVOLVING AN ENZYMATIC REACTION AND A SUBSEQUENT CHEMICAL REACTION

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Key Words: peptide, amide, thioester bond, adenylation domain, superfamily of adenylate-forming enzymes

We previously reported that an amide bond is unexpectedly formed[1)] by an acyl-CoA synthetase, AcsA, which plays an essential role in acid utilization in the nitrile-degrative pathway[2)]. Although AcsA essentially catalyzes the formation of a carbon-sulfur bond (the ligation of an acid with CoA), it surprisingly synthesized *N*-acyl-L-cysteine when a suitable acid and L-cysteine are used as substrates. Furthermore, this unexpected enzyme activity was also observed for acetyl-CoA synthetase and firefly luciferase, both of which belong to the same superfamily of adenylate-forming enzymes. However, the mechanism underlying the carbon-nitrogen bond synthesis remained unknown.

Next, we succeeded in producing *N*-(D-alanyl)-L-cysteine (a dipeptide) from D-alanine and L-cysteine by using DltA, which is homologous to the adenylation domain of nonribosomal peptide synthetase (NRPS) and belongs to the superfamily of adenylate-forming enzymes. To elucidate the mechanism of these surprising reaction, DltA was used. When cysteine derivatives with a protected amino group *N*-Boc-L-Cys was used instead of L-cysteine, we confirmed the formation of an thioester intermediate. Thereby, we proposed the following unprecedented reaction mechanism underlying these carbon-nitrogen bond synthetic reactions by the thioester-bond-synthesizing enzymes: (i) the formation of *S*-acyl-L-cysteine as an intermediate via its “enzymatic activity” and (ii) subsequent “chemical“ *S*→*N* acyl transfer in the intermediate, resulting in peptide formation[3)]. Step (ii) of this reaction mechanism is identical to the corresponding reaction in native chemical ligation, a method of chemical peptide synthesis, whereas step (i) is not.

We predicted that enzymes belonging to the superfamily of adenylate-forming enzymes can synthesize peptide/amide compounds by the same mechanism. Accordingly, we tried to express and purify DhbE, a stand-alone adebylation domain of NRPS, for production of valuable peptide/amide compounds. The purified DhbE synthesized *N*-aromatic acyl-L-cysteine[4)].

Here, we reported the first demonstration of the *N*-acylation by “internal” adenylation domains in the multidomain enzyme DhbF. The adenylation domain of NRPS originally is responsible for its selective substrate recognition and activation of the substrate. DhbF is an NRPS involved in bacillibactin synthesis and consists of multiple domains (adenylation domain, condensation domain, peptidyl carrier protein domain, and thioesterase domain). DhbFA1 and DhbFA2 (here named) are “internal” adenylation domains in DhbF. Here, we firstly succeeded in expressing and purifying “internal” adenylation domain DhbFA1 or DhbFA2 separately. When glycine and L-cysteine were used as substrates of DhbFA1, the formation of *N*-glycyl-L-cysteine (Gly-Cys) was observed. When L-threonine and L-cysteine were used as substrates of DhbFA2, *N*-L-threonyl-L-cysteine (Thr-Cys) was formed. Furthermore, DhbFA1 or DhbFA2 synthesizes not only dipeptides but also various oligopeptides. Because many adenylation domains that could activate the respective substrates are present in the natural world, we can synthesize various peptides or amides by using adenylation domains or enzymes belonging to the superfamily of adenylate-forming enzymes.

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A NON-NATURAL NICOTINAMIDE COFACTOR FOR BIOTRANSFORMATION AT EXTREME CONDITIONS

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Key Words: nicotinamide cofactor, biomimick, dehydrogenase, stabilization

Redox enzymes are very useful tools for establishing greener routes in organic synthesis, mostly due to their highly selective reduction, oxidation and oxyfunctionalisation reactions. However, the metastable nicotinamide cofactors (NAD/P(H)), required for catalysis, are prone to fast degradation, when utilized under non physiological conditions. Establishing of robust artificial enzymatic cascade reactions and cell free biotransformations at extreme conditions is therefore still a major challenge. We show one $NAD(P)^+$ derivative with highly increased stability and very similar redox potential compared to its natural counterpart that can be used very efficiently with a number of biocatalysts. We have tested more than 50 redox enzymes and found a substantial number of them being able to utilize the nicotinamide derivative. Among them, we also successfully identified those that are commonly used in cofactor regeneration systems. Applying enzyme engineering to a model enzyme, it was possible to develop variants with activities even higher than towards the natural cofactor, allowing efficient biotransformations at 60°C and above for many hours.

In addition further characterization and additional comparative molecular dynamic simulations revealed an improved understanding of the biomimick's recognition on a molecular level, facilitating the transfer to other enzymes.

WITHDRAWN

DESIGNING DE NOVO RETRO-ALDOLASE CATALYSTS

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Key words: computational enzyme design, de novo protein design, retro-aldolase activity, directed evolution

Evolutionary history of native proteins, shaping observed sequences by complex interplay between mutational drift, maintaining stability and developing functionality, often complicates rationalization of protein engineering experiments making it hard to learn even from large datasets available with advent of high throughput screening and deep-sequencing technologies.

Use of de novo protein scaffolds for gain of function design projects should, arguably, allow better understanding of fundamental principles underlying implementation of this function in nature and application of these principles to new protein engineering problems.

Computational design of enzymatic activity in the de novo built idealized protein scaffolds instead of natural proteins from PDB has a promising advantages of avoiding limitations associated with evolutionary history and virtually unlimited number of geometric variants that can be generated for given scaffold to accommodate catalytic machinery.

I am going to present computational strategy used to design de novo proteins with enzymatic activity and experimental data collected using recently identified de novo designed beta-barrels catalyzing retro-aldolase reaction. This information helps to narrow down range of catalytic mechanisms compatible with the structural model, which in turn help to highlight features and interactions potentially important for catalysis.

TAILORED BIOSYNTHESIS OF PSEUDOSUGARS AGAINST DIABETES BY REPURPOSING THE PROMISCUOUS MICROBIAL ENZYMES

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Key Words: enzyme evolution, psedosugars, biosynthesis

Synthetic biology plays an important role in not only revealing the complicated functions of organisms but also providing a promising route for bio-manufactures for medicine. However, we still faced the challenges for building a cost-efficient process due to shortages of suitable enzymes for the desired reactions. Molecular evolution of the promiscuous enzymes based on the knowledge of enzyme catalytic mechanisms provide a promising route for turning from proof-of-concept towards precision function. Here, we give some examples for discovering and evolving the promiscuous enzymes in artificial synthetic pathways, especially for biosynthesis of voglibose against diabetes. The precise control of catalysts enhances the ability to control the biological system, therefore provides a new path for targeting the precision function in synthetic biology.

GENETICALLY ENCODED BIOSENSOR FOR ENGINEERING BRANCHED-CHAIN HIGHER ALCOHOL PRODUCTION PATHWAY IN *SACCHAROMYCES CEREVISIAE*

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Key Words: Biofuel biosensor, Metabolic pathway engineering and optimization, Enzyme engineering, High-throughput screen, Mitochondrial and cytosolic isobutanol pathways

Branched-chain higher alcohols (BCHAs) including isobutanol, isopentanol, and 2-methyl-1-butanol, are promising alternatives to the first-generation biofuel ethanol. These alcohols have better fuel properties than ethanol, such as higher energy density, ease of refining, and better compatibility with existing gasoline engines and infrastructures [1]. We have developed a genetically encoded biosensor to measure the metabolic activity of BCHA biosynthesis in *Saccharomyces cerevisiae*. This biosensor enables high-throughput screens to identify strains with higher metabolic flux to BCHA synthesis. The versatility of this tool has allowed us to use it in several applications, including *in vivo* BCHA metabolic pathway engineering/optimization and enzyme engineering. We have been able to screen for isobutanol hyper-producing stains with optimum combinations of genes from the mitochondrial isobutanol pathway (Mito-IbOH-pathway) [2]. The ability of this biosensor to monitor the activity of both the mitochondrial [2] and cytosolic isobutanol pathways [3], has allowed us to engineer several enzymes and regulatory proteins involved in the isobutanol pathways in either compartment, boosting enzymatic activity by as much as 400%. Thus, we have demonstrated the use of this new technology to accelerate the development of strains and enzymes to boost BCHA production in mitochondria and the cytosol. Future applications include combining the biosensor with optogenetic regulation of BCHA biosynthesis for closed-loop dynamic control of this pathway, and using the biosensor to empower systems biology studies for gene discovery, enzyme evolving, and enzyme engineering to boost BCHA production.

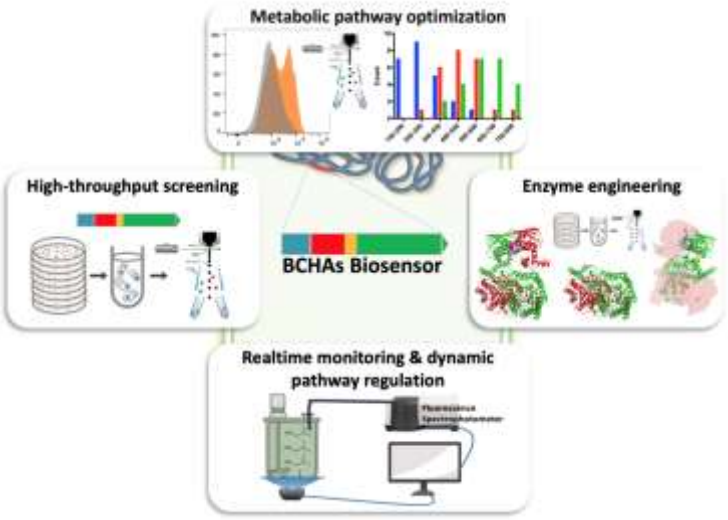

Figure 22 – The versatile applications of the branched-chain higher alcohols (BCHAs)

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NEWLY DISCOVERED ENZYMES AND CASCADES FOR THE DETERMINATION OF AMINO ACIDS

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Key Words: L-amino acid oxidase, L-amino acid dehydrogenase, micro-determination

The high stereo- and substrate specificities of enzymes have been utilized for micro-determination of amino acids. With the successful practical use of oxidoreductases such as the NAD^+-dependent L-Phe dehydrogenase for phenylketonuria among more than 5,400,000 of neonates in Japan [1], many enzymes with higher substrate specificities have been screened and discovered from Nature [2].

In this report, successful screening and uses of selective enzymes for micro-determination of amino acids will be discussed. Focuses on amino acid oxidases that are renewing the common sense of classical biochemistry will be described: L-Thr dehydrogenase [3], L-Trp dehydrogenase [4], L-Lys e-oxidase [5], L-Trp oxidase [6,7], L-Arg oxidase [8], Gly oxidase, Taurine oxygenase [9], etc, all of which show high selectivity.

In addition, novel enzymatic assays with the systematic use of cascades of known enzymes, including assays based on a pyrophosphate detection system using pyrophosphate dikinase for L-Met, L-Cit, L-Arg [10] and a variety of L-amino acids with amino-acyl-tRNA synthetase (AARS) [1,11] will be discussed. The X-ray crystallographic structures of some of the enzymes and the protein engineering are often necessary to use them practically. Together with the use of microbial determination of amino acids with lactic acid bacterium transformed with marker enzymes [12], most of the amino acids can be determined by the enzymatic methods (Table 1). The success and the potential for amino acid determination in biological samples will be shown.

Table 1. Amino acid determination by various enzymes and enzyme system

	Previous study	Our study				Previous study	Our study		
		Oxido-reductase	AARS assay	Bio-assay			Oxido-reductase	AARS assay	Bio-assay
Ala	•		•		Met		•	•	
Cys			•		Asn				
Asp			•		Pro			•	•
Glu	•			•	Gln				
Phe		•	•		Arg		•		•
Gly	•		•		Ser			•	
His	•		•	•	Thr		•		
Ile			•	•	Val			•	•
Lys	•	•	•		Trp		•	•	
Leu	•			•	Tyr		•		

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ENGINEERING AN ALDEHYDE DEHYDROGENASE TOWARD ITS 2 SUBSTRATES FOR ENHANCING THE PRODUCTION OF 3-HYDROXYPROPIONIC ACID

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3-Hydroxypropionic acid (3-HP) can be produced via two enzymatic reactions: dehydration of glycerol to 3-hydroxypropanal (3-HPA) and oxidation to 3-HP. Commercial production of 3-HP has been beset with several problems. Some of these problems are associated with the toxicity of 3-HPA and the efficiency of NAD^+ regeneration. We engineered α-ketoglutaric semialdehyde dehydrogenase (KGSADH) for the second reaction to address these issues. The residues in the putative binding sites for the substrates, 3-HPA and NAD^+, were randomized, and the libraries were screened for higher activity. Isolated KGSADH variants had lower Km values for both substrates. The enzymes showed higher substrate specificities for aldehyde and NAD^+, less inhibition by NADH, and greater resistance to inactivation by 3-HPA than the wild-type enzyme. A recombinant *Pseudomonas denitrificans* strain with one of the engineered KGSADH variants exhibited less accumulation of 3-HPA, decreased levels of inactivation of the enzymes involved in the production of 3-HP. These attributes facilitated sustained production of 3-HP in to the late stages of culture and enhanced the final titer of 3-HP by approximately 40%.

A NOVEL C-TERMINAL PROTEIN DEGRON IDENTIFIED IN BACTERIAL ALDEHYDE DECARBONYLASES USING DIRECTED ENZYME EVOLUTION

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Key words: Alkane, aldehyde decarbonylase, directed evolution, degron, protease

Metabolic engineers have successfully synthesized alkanes, the bulk component of gasoline, using microbial cell factories as a sustainable alternative to petroleum-based fuels. Aldehyde decarbonylases (AD), enzymes which transform acyl aldehydes into alkanes, have been identified as the bottleneck in these alkane producing pathways. Previous studies demonstrated degradation of AD in *E. coli* cells via unknown molecular mechanism. Here, we present the discovery of a degradation tag (degron) in AD from *Prochlorococcus marinus.* AD variants were generated by random mutation using error-prone PCR, transferred into *E. coli*, and grown in chemostat culture with 2g/L hexanal to select for positive mutations. A short C-terminal sequence of AD from *P. marinus* was proven to be an intact degron by fusing to fluorescent proteins. Statistical analysis of C-terminal sequences of 371 non-redundant ADs from bacteria revealed a conserved sequence in this region, which was proven to be an effective degron. We also showed that ATP-dependent proteases clpAP and lon are responsible for the degradation of AD degron tagged protein. Furthermore, our results indicate that the AD degron caused 91.4% of green fluorescent protein (GFP) degradation when fused to its C-terminus, whereas its elimination in AD enhanced alkane production *in vivo*. Thus, our work demonstrated the presence of a protein degron tag in bacterial ADs, thereby facilitating further improvements in AD-based alkane production pathways.

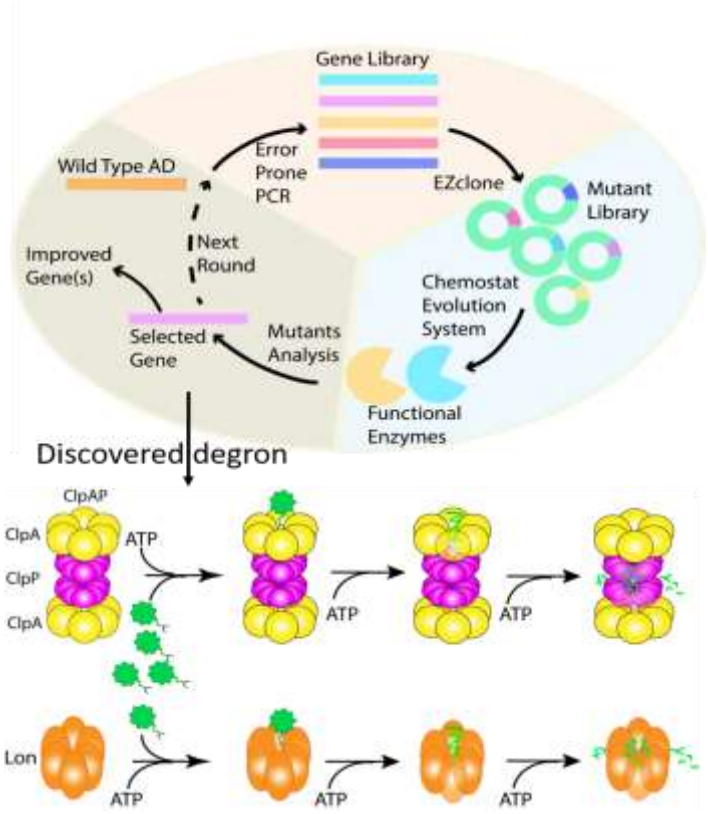

Figure 1- Discovery of ATP-dependent proteases recognized C-terminal degron in AD via directed evolution

ENANTIOCOMPLEMENTARY SYNTHESIS OF CHIRAL ALCOHOLS COMBINING PHOTOCATALYSIS AND WHOLE-CELL BIOCATALYSIS IN A ONE-POT CASCADE PROCESS

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Key Words: photocatalysis; enzyme catalysts; whole-cell process; one-pot cascade; chiral alcohols

As a powerful tool in synthetic organic chemistry, photocatalysis has the features of green, better atom economy, and mild conditions [1-2]. Recently, some cascade reaction protocols have been properly designed by combining photocatalysis and biocatalysis[3-4]. For example, Zhao and Hartwig reported an asymmetric reaction which coupled photocatalysts for E/Z isomerization of alkenes with ene-reductases for the reduction of carbon–carbon double bonds, to generate valuable enantioenriched products [5], which achieved the dual-advantages of both photocatalysis and biocatalysis. We envisioned a photochemo-enzymatic one-pot whole-cell process to convert a series of carboxylic acids into corresponding chiral alcohols with good yields (up to 93%) and excellent stereoselectivity (up to 99% ee). The photocatalysis step was conducted in aqueous phase by using O_2 as oxidant and the following whole cell bioreduction without the addition of the expensive cofactor NADPH was a much milder and more efficient approach to obtain chiral alcohols. All these advantages indicate that the photochemo-enzymatic one-pot transformation may have great potential in green synthetic chemistry.

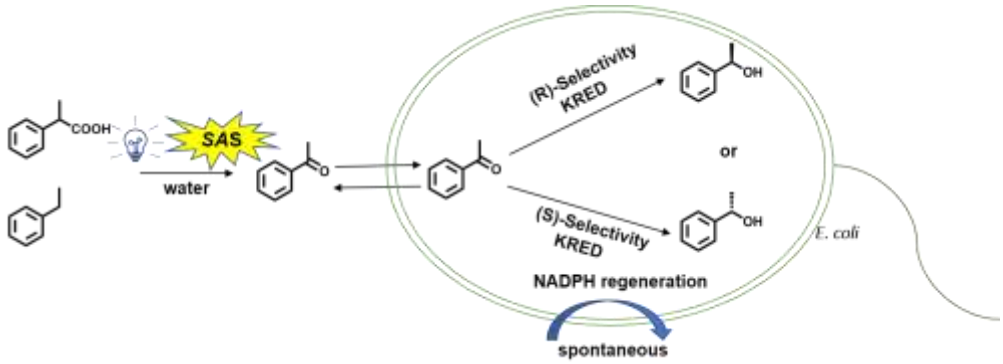

Figure 23 – Photochemo-enzymatic one-pot cascade in the whole-cell reaction system.

This abstract is subjected to the topic of Biocatalysis and enzymology.

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SUBSTRATE SPECIFICITY OF tRNA-DEPENDENT AMIDE-FORMING ENZYME

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Key Words: nonribosomal peptide synthetase, tRNA-dependent, natural product, biosynthesis

Streptothricins (STs) produced by Streptomyces strains are broad-spectrum antibiotics and are characterized by a streptothrisamine core structure with the L-β-lysine (β-Lys) residue and its oligomeric side chains [oligo(β-Lys)]. In addition to the STs, Streptomyces strains has been known to produce ST-related compounds, BD-12, citromycin, glycinothricin, etc., which possess a glycine-derived side chain rather than the β-Lys residue. We have reported that the amide bonds connecting the amino-acid side chains in ST and BD-12 are formed via NRPS (1) and tRNA-dependent (2) pathways, respectively (Figure 1).

Here, we report that a tRNA-dependent amide bond-forming enzyme found by genome mining showed a broad substrate specificity, and generation of new compounds using unnatural aminoacyl-tRNAs as substrates will be presented.

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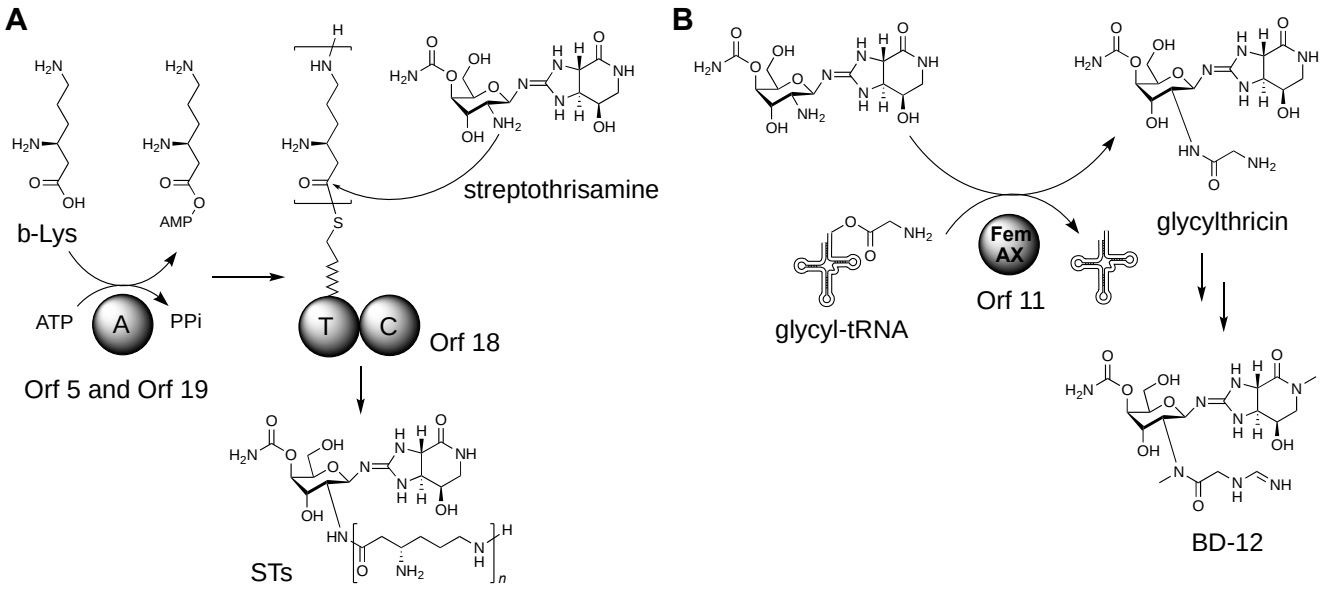

Figure 1. Peptide-bond formation between amino sugar and amino acid in the STs (A) and BD-12 (B) biosynthesis.

ENGINEERING OF PENICILLIN G ACYLASES FOR THE PRODUCTION OF B-LACTAM ANTIBIOTICS ON AN INDUSTRIAL SCALE

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β-lactam antibiotics are the most important class of antibacterial compounds in clinical applications, which have been industrially produced by conventional chemical methods. The chemical synthesis of β-lactam antibiotics is carried out under stringent conditions which results in high downstream process costs and the environmentally-damaging processes. For those reasons, the efforts have been made to replace traditional chemical processes with enzymatic conversion processes for more sustainable production of β-lactam antibiotics. Penicillin G acylase (PenG acylase) has been found to be useful in the synthesis of β-lactam antibiotics. In order to adopt a process advantageous to industrial and economic angles, it is preferred that the enzyme activity, synthesis/hydrolysis ratio, and operational stability are sufficiently high. Using directed evolution in combination with high-throughput screening system, PenG acylase from Achromobacter sp. CCM 4824 was engineered and then immobilized in epoxy or amide type acrylate resin. In the synthesis of cephalexin, cefprozil, cefaclor, cephradine, cefadroxil, or amoxicillin, specific activity was highly increased by 165, 121, 154, 153, 36, 853-fold, respectively, by using 7-ADCA, 7-ACCA, or 6-APA as a β-lactam nucleus, and PGM, HPGM, or DHME as an acyl donor, which also exhibited high synthesis/hydrolysis ratio up to 68-folds, compared with the wild type. All enzymes could be recycled over 200 cycles without any inactivation, making sure the repetitive use for the industrial application. These data indicate that the engineered PenG acylase from Achromobacter can be used to direct the synthesis of various β-lactam antibiotics by the combination of β-lactam nuclei with various acyl groups.

FUSING ENZYMES TO TRANSCRIPTION ACTIVATOR LUXR FOR THE RAPID CREATION OF METABOLITE SENSORS

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Key Words: Metabolite sensors/ Fusion protein/ LuxR/ Random mutagenesis/ Isoprenoids

Metabolite sensors have been applied for high-throughput screening for improved biosynthetic pathways, as well as for dynamic control of the metabolic networks. Obviously, however, current repertoire of natural sensors covers only a small fraction of the known metabolite.

We have been developing the new robust workflow for the rapid creation of metabolite sensors where biosynthetic enzymes can be adopted as the sensory (recognizing) components. Most of the known metabolites act as the direct substrates of some enzymes, and they are recognized and converted by these enzymes in physiologically relevant concentrations. Thus, ever-increasing repertoire of available enzymes is a rich and reliable source of sensory units. We found that the transcription activator LuxR can be fused with various biosynthetic enzymes without losing its function. By adding moderately de-stabilizing mutations, typically by random mutagenesis of the resultant fusion proteins followed by screening a small number (~100) of variants, we could have quickly isolated variants that can activate LuxR-dependent promoter in response to the substrates of the enzymes fused to LuxR. In this presentation, we demonstrate various metabolites can be detected by this manner.

Detailed analysis of the thus-obtained fusion proteins indicated that function of LuxR is dependent on the substrate binding-induced stabilization of the enzymes. The biosensors with this mode of action exhibited various unique features. For instance, we found that the sensitivity (EC50) and dynamic range of these sensors to the target metabolites can be flexibly altered by the concentration of homoserine lactones, the cognate ligand of LuxR, in the media. Also, this provides unique opportunity to indirectly visualizing the substrate-binding to the enzyme in high-throughput manner. Indeed, multi-round mutagenesis and screening of the fusion protein of isopentenyl diphosphate isomerase (IDI) with LuxR variant (IDI-LuxR) revealed that many of the mutations that improved sensory performance of IDI-LuxR also elevated the catalytic performance of IDI. Some of such mutations turned out to elevate IDI activity even without fusion partner LuxR. Altogether, by fusing to LuxR, random mutagenesis, and traditional reporter (fluorescence)-based screening, one can not only adopt a variety of biosynthetic enzymes as sensor components but also laboratory evolve their catalytic functions.

EXPRESSION AND CHARACTERIZATION OF KERATINASE FROM *Deinococcus gobiensis* I-0

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Key Words: *Deinococcus gobiensis* I-0, Keratinase, Keratin, Feather Degradation

Keratin is a nonnutritious hard protein widely distributed in feather, wool, animal hoof, horn, and toenail. The disulfide bond interacts to form a dense structure of keratin, which is difficult to be degraded and utilized. Keratinase is a kind of enzymes that can destroy the dense structure of keratin to achieve the degradation, and has a good application prospect. In order to further tap the important gene resources of keratinase, improve its hydrolytic activity，and provide theoretical basis for industrial production, this experiment cloned a gene encoding keratinase from *Deinococcus gobiensis* I-0 isolated from Gobi desert of Xinjiang and named it as Kerdg. Prokaryotic expression vector pET-22B-Kerdg was constructed and then induced, expressed and purified in vitro, the optimal temperature and pH of the crude enzyme solution were determined through the hydrolysis activity to feathers. Results showed that the first 50 amino acids of N terminal had a great influence on the expression and purification of protein Kerdg. The crude enzyme solution of recombinant strain completely decomposed feathers in three days. The transparent circle on milk powder plate appeared more notable in crude enzyme solution of recombinant strain than that of empty strain. Kerdg adapted to a wide range of temperatures and pH，among which the optimal temperature was 60℃ and the optimal pH was 5.0. The Kerdg can degrade feathers and thus will have great application space in the future industrial production and treatment of waste feathers.

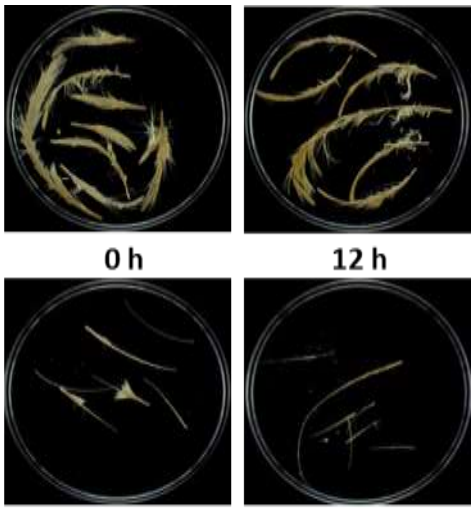

Figure1 Degradation of intact chicken feathers by Kerdg

Figure1 Degradation of intact chicken feathers by Kerdg.

To examine its keratinolytic ability, Kerdg expression strain was cultured at 37 °C in medium with insoluble chicken feathers as the only source of nutrients. After two days of culture, the feather degradation indicated that Kerdg expression strain produced an extracellular keratinase to degrade feather keratin as a nutrient source. These properties made Kerdg an ideal candidate for keratinase purification and identification.

ENGINEERING T1 LIPASE FOR DEGRADATION OF POLY-(*R*)-3-HYDROXYBUTYRATE

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Key Words: Amorphous P(3HB); PhaZ7$_{Pl}$; Structure and function; Substrate specificity; T1 lipase

Enzymes with broad substrate specificities that can act on a wide range of substrates would be valuable for industrial applications. T1 lipase is known to have broad substrate specificity in its native form, with active site residues that are similar to polyhydroxylalkanoate (PHA) depolymerase (PhaZ). PhaZ6 *from Pseudomonas lemoignei* (PhaZ6$_{Pl}$) is one of PhaZs that can degrade semicrystalline poly-(*R*)-3-hydroxybutyrate [P(3HB)]. The objective of this study is to enable T1 lipase to degrade semicrystalline P(3HB) similar to PhaZ6$_{Pl}$ while maintaining its native function. Structural analyses on PhaZ6$_{Pl}$ built structure revealed that it does not contain a lid, as opposed to T1 lipase. Therefore, T1 lipase were designed by removing its lid region. This was performed by using *Bacillus subtilis* lipase A (BSLA) as the reference for T1 lipase modification as the latter does not have a lid region and that its structure fits almost perfectly with T1 lipase based on their superimposed structures. A total of three variants of T1 lipase without lid were successfully designed, namely D1 (without α6–loop–α7), D2 (without α6) and D3 (α6 and loop) in the lid region. All the variants showed PHA depolymerase activity towards P(3HB), with D2 variant exhibiting the highest activity amongst other variants. Further analysis on D2 showed that it was able to maintain its native hydrolytic activity towards olive oil, albeit with decrement in its catalytic efficiency. Results obtained in this study highlighted the fact that native T1 lipase is a versatile hydrolase enzyme which does not only perform triglyceride degradation but also P(3HB) degradation by simply removing the helix 6 which was specifically proven to affect catalytic activity and substrate specificity of the enzyme.

DEVELOPMENT AND APPLICATION OF NOVEL ENGINEERED TRANSAMINASE PANELS ASSISTED BY IN-SILICO RATIONAL DESIGN FOR THE PRODUCTION OF CHIRAL AMINES

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Key Words: biocatalysts; enzyme engineering; transaminase; enantioselectivity; industry.

There is a high demand for the synthesis of chiral amines as building blocks for a large number of industrially valuable compounds. Transaminases (TAm) offer an enzymatic route for the synthesis of chiral amines that avoids complex chemical synthesis [1]. However, their catalytic efficiency towards bulky ketone substrates is greatly limited by steric hinderance [2]. This poster highlights a rational design strategy of combining in silico and in vitro methods to engineer the transaminase enzyme with a minimal number of mutations, achieving high catalytic activity and high enantioselectivity. The wildtype TAm showed no detectable activity towards the ketone 2-acetylbiphenyl but upon introduction of two mutations detectable enzyme activity was observed. The reaction rate was improved a further 1716-fold with the rationally designed variant, that contained a further 5 mutations, producing the corresponding enantiomeric pure (S)-amine (enantiomeric excess (ee) value of >99%)[3].
In addition, screening of in silico designed (R)-TAm mutant panels in resolution mode offered an attractive and efficient route for the preparation of problematic (S)-amines. A mutant was identified from the panels that gave complete resolution of the racemic amine (high substrate loading) to leave the desired enantiomer at a low enzyme loading fit for process development towards an economically viable scale up process.

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WITHDRAWN

PURIFICATION AND CHARACTERIZATION OF A NOVEL ALGINATE LYASE FROM THE MARINE BACTERIUM *BACILLUS* SP. ALG07

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Key Words: alginate lyase; marine bacterium; characterization; alginate oligosaccharides

Alginate is the most abundant carbohydrate in brown algae, accounting for up to 10–45% of the dry weight of brown algae. As a linear homopolymer, alginate is composed of (1–4)-linked α-L-guluronic acid (G) and its C5 epimer β-D-mannuronic acid (M), in blocks of poly-α-L-guluronate (polyG), poly-β-D-mannuronate (polyM), and random heteropolymeric sequences(polyMG). Alginate oligosaccharides with various bioactivities can be prepared through the specific degradation of alginate by alginate lyases. Therefore, alginate lyases which can degrade alginate under mild conditions, have drawn great interest. Although many alginate lyases have been discovered and characterized, few can be applied in industrial production of alginate oligosaccharides.

In this study, a novel marine strain with efficient degradation ability toward brown algae was isolated and classified to *Bacillus* sp. A novel alginate lyase, named as AlgA, with high specific activity was purified from the culture medium of this strain. AlgA had a molecular weight of approximately 60 kDa, wtih the optimal temperature and pH of 40°C and 7.5,respectively. The activity of AlgA was dependent on sodium chloride and could be considerably enhanced by Mg^{2+} or Ca^{2+}. Under optimal conditions, the activity of AlgA reached up to 8306.7 U/mg, which is the highest activity recorded for alginate lyases. Moreover, the enzyme was stable over a broad pH range (5.0–10.0), and its activity negligibly changed after 24 h of incubation at 40 °C. AlgA exhibited high activity and affinity toward poly-β-D-mannuronate (polyM). These characteristics suggested that AlgA is an endolytic polyM-specific alginate lyase (EC 4.2.2.3). The degradation products of alginate and polyM by AlgA were purified and identified through fast protein liquid chromatography and electrospray ionization mass spectrometry, revealing that AlgA mainly produced disaccharides, trisaccharides, and tetrasaccharide from alginate and disaccharides and trisaccharides from polyM. Therefore, the novel alginate lyase AlgA has potential applications in the production of mannuronic oligosaccharides and poly-α-L-guluronate blocks from alginate.

IN SILICO APPROACH TO BETTER UNDERSTAND THE ROLE OF ACTIVE SITE RESIDUE

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Key Words: Imine reductases, Protein engineering, Biocatalysis, Molecular dynamics

Chiral amines are the major building blocks of many life-saving drugs and it has been estimated that nearly 40-45% of the currently used pharmaceuticals contain chiral amine functional groups in their structure. However, the major challenges associated with the chemical synthesis of enantiopure amines are the use of toxic chemicals, formation of many by-products, and multi-step synthesis process. To address these limitations, cost-effective biocatalytic methods are emerging as potential alternatives for the synthesis of chiral amines in enantiomerically pure forms.

Imine reductases (IRED) are the class of enzymes that can synthesize chiral amines by reducing the cyclic prochiral imines in presence of NADPH cofactor. Especially for R-selective IREDs, NADPH acts as a hydride source and an active site aspartate residue acts as a proton donor during catalysis. The role of NADPH in imine reduction is well studied. However, the role of aspartate as a proton donor remains elusive. For example, in R-IRED from *S. kanamyceticus* (Q1EQE0), the mutation of Asp-187 in the active site completely abolished the activity. Though in a similar R-IRED from *Streptomyces sp.* (GF3587), the mutation of the homologous aspartate (Asp-172) reduced the enzyme activity. This essentially points out that there might be other amino acids involved directly or indirectly in proton transfer. In the current study, we employed classical molecular dynamics (MD) simulations to examine the role of several active site residues including the highly conserved aspartate in substrate binding and catalysis of R-IRED from Streptomyces sp. Based on our *in silico* approach, we have identified an additional histidine residue in the active site that maybe critical in catalysis along with Asp-172.

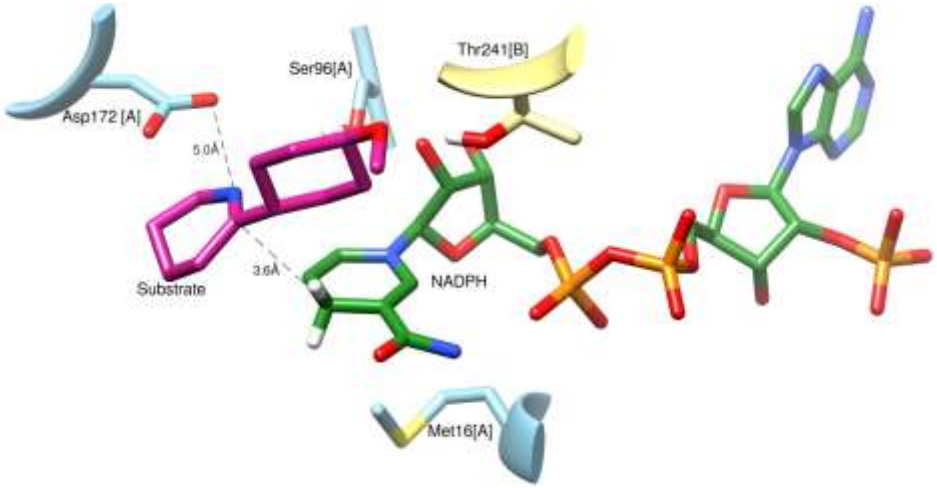

Figure 24 – Active site structure of Imine reductases

WITHDRAWN

DELETION STUDIES FOR ELUCIDATING THE ROLE OF *STREPTOMYCES GRISEUS* CHIC NON-CATALYTIC RESIDUES

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Key Words: Chitinase, rational deletion, *in silico*, molecular dynamics simulation

The soil bacterium, *Streptomyces griseus*, produces an antifungal chitinase (SgChiC) which has a smaller catalytic domain (in addition to a chitin binding domain) when compared with its counterparts from plants. Here, we carried out rational deletion of residues distant from the active site residues in the catalytic domain from 205 to 49 amino acid residues. The truncated residues were reconstructed and its 3-dimendional model predicted by homology modeling. In an *insilico* binding study, tri-N-acetyl glucosamine ($(GlCNAc)_3$) was observed to bind to the active site of the truncated model similarly as in the wild type catalytic domain. This suggests that the variant model of SgChiC with a truncated catalytic domain possibly retains its chitinolytic properties. Further analysis of the simulation results revealed an increase in conformational space and flexibility of the reconstructed model over the less dynamic structure of the wild-type model. This suggests that the deleted residues played a role in the compactness and rigidity of the domain. Experimental assays to investigate the hydrolytic and kinetic properties of this truncated variant are currently been carried out. Outcomes of this study will reveal the relationship between the architecture of the ChiC domain and its function. This will guide future design studies for the enhancement of its functional properties and consequently its efficiency as a biocontrol agent.

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IMPROVING THE CATALYTIC ACTIVITY OF ISOPENTENYL PHOSPHATE KINASE THROUGH PROTEIN COEVOLUTION ANALYSIS

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Protein rational design has become more and more popular for protein engineering with the advantage of biological big-data. In this study, we described a method of rational design that is able to identify desired mutants by analyzing the coevolution of protein sequence. We employed this approach to evolve an archaeal isopentenyl phosphate kinase that can convert dimethylallyl alcohol (DMA) into precursor of isoprenoids. By designing 9 point mutations, we improved the catalytic activities of IPK about 8-fold in vitro. After introducing the optimal mutant of IPK into engineered E. coli strain for β-carotenoids production, we found that β-carotenoids production exhibited 97% increase over the starting strain. The process of enzyme optimization presented here could be used to improve the catalytic activities of other enzymes.

IMPROVING THE THERMOSTABILITY OF GLUTAMATE DECARBOXYLASE BY CONSENSUS MUTAGENESIS

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Key Words: glutamate decarboxylase, GABA, consensus mutagenesis, thermostability

Glutamate decarboxylase (GAD) is a crucial enzyme for the preparation of gamma-aminobutyric acid (GABA) by biological methods. In this study, the Consensus Finder (http://cbs-kazlab.oit.umn.edu/) was used, and 8 sites were screened with the most prevalent amino acid (over 60% threshold) among the homologous family members, and stabilized variants of GAD were performed by site-directed mutagenesis. The results showed that the mutants T383K and A163S showed higher thermostability than that of GAD wild-type. Compared to GAD wild type, the A163S and T383K mutants displayed the shift in thermostability, with 1.74-fold and 1.45-fold increase in half-life ($t_{1/2}$) at 55 °C, 2.9 °C and 1.8 °C increase in T_{50}^{10}. By the differential scanning fluorimetry (DSF) analysis, the best mutant, T383K, showed a melting temperature (Tm) of 41.9 °C, which corresponded to a 1.3 °C increase relative to the wild-type GAD. Molecular dynamics simulation indicated that mutations of A163S and T383K lowered the overall root mean square deviation (RMSD) for the overall residues at 310K and consequently increased the protein rigidity.

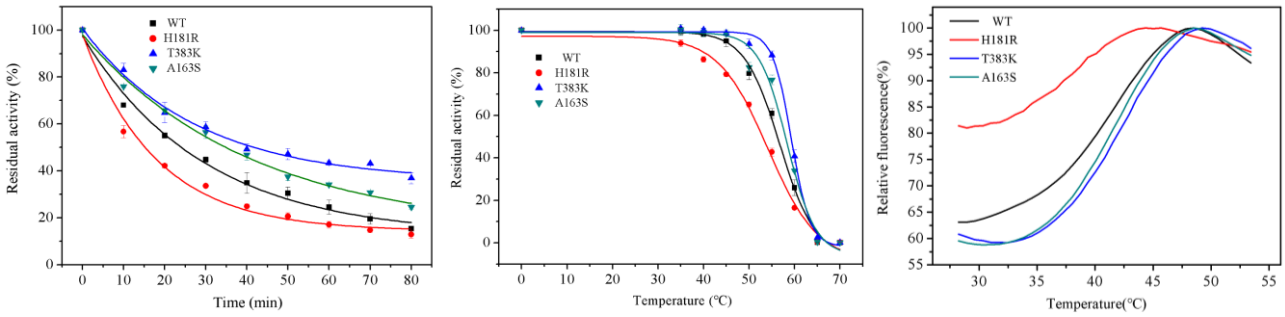

Figure 1 Stability of GAD wild-type and stabilized mutants

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