

October 11- 14, 2016
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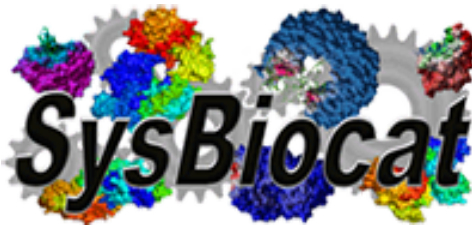


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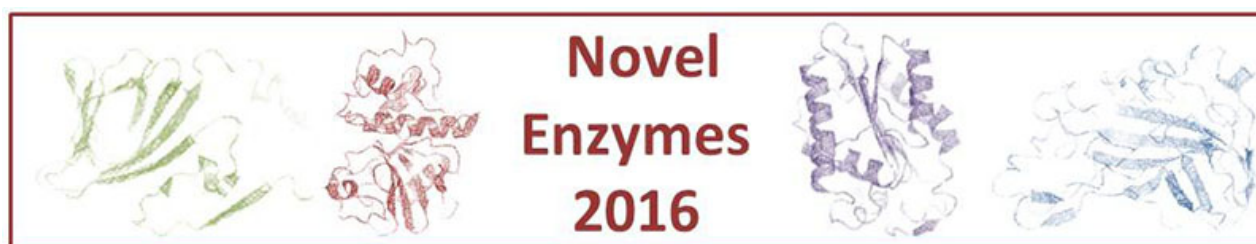
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Rethink Tomorrow



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The conference on Novel Enzymes aims to provide a forum for the presentation of the most exciting advances and new findings concerning enzymes. The conference is a continuation of the conference series on Novel Enzymes, of which the previous was held in 2014 in Ghent, Belgium. The goal of the current conference is to provide an overview on recent developments and future perspectives on enzymology research. Emphasis will be given to discovery of new enzymes, engineering approaches, and newly developed enzyme applications.

The conference is aimed at bringing together researchers, from academia and industry, working in the field of enzymology, and to facilitate stimulating discussions. Keynote lectures are delivered by reputed academic and industrial scientists who will present new developments in diverse areas of molecular and applied enzymology.

Topics

- Novel enzymes by discovery
- Novel enzymes by engineering
- Novel enzymes-based applications

Scientific Committee

Prof. Dr. Isabel W.C.E. Arends, University of Delft, The Netherlands
Prof. Dr. Lubbert Dijkhuizen, University of Groningen, The Netherlands
Dr. Jaap Visser, DuPont, The Netherlands
Dr. Jan-Metske van der Laan, DSM, The Netherlands
Prof. Dr. Jennifer Littlechild, University of Exeter, United Kingdom
Prof. Dr. Tom Desmet, Ghent University, Belgium
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Dr. Johannes Roubroeks, Novozymes, Denmark

Organizational Committee

Prof. Dr. Marco W. Fraaije
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Ms. Tamara Hummel
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WIFI

Free wifi is available at the conference venue.

Program Novel Enzymes 2016

Tuesday, October 11, 2016

12.00	Registration	
13.45	Marco Fraaije (chair) University of Groningen, The Netherlands	Welcome and introduction
Novel Enzymes by Discovery session		
Chair: Roland Wohlgemuth (Sigma-Aldrich Chemie GmbH, Switzerland)		
14.00 IL1	John Gerlt University of Illinois, USA	Transport system solute binding protein (SBP)-guided discovery of novel enzymes in novel metabolic pathways
14.40 L1	Martina Andberg VTT Technical Research Centre of Finland, Finland	A novel aldose-aldose oxidoreductase having dual activities on sugars
15.00 L2	Mirjam Kabel Wageningen University, The Netherlands	Plant phenolics enhance oxidative cleavage of plant polysaccharides
15.20	Break	
15.50 IL2	Daniela Monti Istituto di Chimica del Riconoscimento Molecolare, Italy	Novel "hot" epoxide hydrolases: from discovery in metagenomes to synthetic exploitation
16.30 L3	Adiphol Dilokpimol CBS-KNAW Fungal biodiversity centre, The Netherlands	Fungal glucuronoyl esterases: genome mining based discovery and biochemical characterization
16.50 L4	Elisa Lanfranchi University of Groningen, The Netherlands	Scent of bitter almond and a pinch of <i>-omics</i> : discovering a novel hydroxynitrile lyase
17.10	Break	
17.30	Poster Pitch Talks	
	Afsheen Aman University of Karachi, Pakistan	Immobilization of dextranase on chitosan microspheres: An effective approach for increasing recycling efficiency & stability
	Franziska Birmes University of Münster, Germany	Dioxygenases for inactivation of the virulence-associated <i>Pseudomonas aeruginosa</i> quinolone signal
	Marco Bocola RWTH Aachen, Germany	QM/MM calculations reveal substrate scope and a new thiolate pocket of the unique arylpropionate racemase AMDase G74C
	Régis Fauré LISBP - INRA/CNRS/INSA, France	Design of chromogenic probes for identification and evaluation of heteroxylan active enzymes
	Maximilian Fürst University of Groningen, The Netherlands	Structure inspired use of a thermostable Baeyer-Villiger monooxygenase as biocatalyst
	Vijaya Gupta Panjab University, India	Deletion of domain 3 of a novel laccase by random mutagenesis: Understanding the structure-activity relationship
	Stefanie Hamer RWTH Aachen University, Germany	Efficient phosphate recovery from agro waste streams by enzyme, strain, and process engineering


	Poster Pitch Talks	
	Hamid Kalhor Sharif University of Technology, Iran	Engineering P450 monooxygenase to make a fused Tetrahydrofuran Ring
	Mohammad Khan Université catholique de Louvain, Belgium	Does homologous oligomerisation promote protein evolution?
	Fernando López Gallego CIC biomaGUNE, Spain	Enzymes in radiochemistry; An efficient solution for the point of care synthesis of ¹³ N-radiotracers
	Luuk Mestrom TU Delft, The Netherlands	Aqueous asymmetric oxidation of unprotected β -amino alcohols using alcohol dehydrogenases
	Linda Otten TU Delft, The Netherlands	Enzymatic enantioselective synthesis of α -hydroxy ketones and vicinal diols
	Maria Ribeiro University of Lisboa, Portugal	Lysozyme magnetized fibers: old enzyme to new uses as biocatalyst for cancer therapy
	Albert Schulte Suranaree University of Technology, Thailand	Effective electroanalysis with allosteric enzymes: The reductase unit of an <i>A. baumannii</i> hydroxylase as model
	Elisabeth Streit Biomim Research Center, Austria	Discovery of an enzyme for fumonisin B1 degradation in animal feed
	Lina Zermeño-Cervantes Instituto Politécnico Nacional, Mexico	Modification and application of vibriophage endolysins as new biocontrol agent against pathogenic strains
18.30	Reception	The reception is offered to you by the University of Groningen, the Municipality of Groningen and the Province of Groningen
		



Wednesday, October 12, 2016

Novel Enzymes by Discovery session		
Chair: Jennifer Littlechild (University of Exeter, United Kingdom)		
09.00 IL3	David Leys University of Manchester, United Kingdom	Unravelling the chemistry underpinning reversible decarboxylation in the UbiX-UbiD system
09.40 L5	Willem Dijkman TU Braunschweig, Germany	Forming the bioplastic monomer FDCA using a single enzyme
10.00 L6	Tohru Dairi Hokkaido University, Japan	New enzymes for biosynthesis of ketomemycin, a pseudotripeptide with carbonylmethylene structure
10.20	Break	
10.50 IL4	Kirk Schnorr Novozymes, Denmark	Novel Enzymes in an industrially relevant context: the tale of two xylanases and other stories
11.30 L7	Alexander Pelzer BRAIN Aktiengesellschaft, Germany	Identification of the novel serine protease Aurase as promising candidate for chronic wound treatment
11.50 L8	Gianluca Molla University of Insubria, Italy	A dream come true: structure-function relationships in L-amino acid deaminase
12.10	Lunch	
13.20	Poster session I	

Novel Enzymes by Discovery session		
Chair: Jaap Visser (Wageningen, The Netherlands)		
14.20 IL5	Florian Hollfelder University of Cambridge, United Kingdom	Rules and tools for efficient enzyme evolution, recruitment and discovery based on catalytic promiscuity
15.00 L9	Carine Vergne-Vaxelaire CEA/IG/Genoscope/LCAB, France	Asymmetric reductive amination by a wild-type amine dehydrogenase from the thermophilic bacteria <i>Petrotoga mobilis</i>
15.20	Break	
15.50 IL6	Kohei Oda Kyoto Institute of Technology, Japan	A bacterium that degrades and assimilates poly(ethylene terephthalate) and its enzymes involved in the degradation
16.30 L10	Griet Dewitte Ghent University, Belgium	Enzyme cascade reactions for efficient glycosylation of small molecules
16.50 L11	Wolf-Dieter Fessner TU Darmstadt, Germany	Engineering a thermostable transketolase for carboligation of arylated substrates

Thursday, October 13, 2016

Novel Enzymes by Engineering session		
Chair: Lubbert Dijkhuizen (University of Groningen, The Netherlands)		
		
09.00 IL7	Manfred Reetz Philipps-University Marburg, Germany	Recent methodology developments in directed evolution
09.40 L12	Ayelet Fishman Israel Institute of Technology, Israel	Combining protein engineering strategies for improving lipase stability in methanol
10.00 L13	Ligia Martins Universidade Nova de Lisboa, Portugal	Directed evolution of PpDyP, a bacterial DyP-type peroxidase, for improved oxidation of phenolic compounds
10.20	Break	
10.50 IL8	Magali Remaud-Simeon University of Toulouse, France	Structurally-guided engineering of enzymes and enzymatic pathways for novel product
11.30 L14	Bert van Loo University of Münster, Germany	Functional transitions in enzyme evolution: balancing stability, folding and catalytic specificity
11.50 L15	Anthony Green University of Manchester, United Kingdom	A chemically programmed proximal ligand enhances the catalytic properties of heme enzymes
12.10	Lunch	
13.20	Poster session II	



Novel Enzymes by Engineering session		 cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY
Chair: Tom Desmet (Ghent University, Belgium)		
14.20 IL9	Emma Master University of Toronto, Canada	Polysaccharide utilization loci as sources of unique carbohydrate active enzymes
15.00 L16	Bhuvana Shanbag Monash University, Australia	Engineering carbonic anhydrase with self-assembly peptide as functional nanoparticles
15.20	Break	
15.50 IL10	Dick Janssen University of Groningen, The Netherlands	Computational approaches in enzyme engineering
16.30 L17	Tea Pavkov-Keller University of Graz, Austria	Changing the chemoselectivity of an aldo-keto-reductase to a flavin-free ene-reductase
16.50 L18	Binuraj Menon University of Manchester, United Kingdom	Flavin dependent halogenase enzymes for aromatic regioselective bio-halogenation
17.10	Break	
Oxidative Biocatalysis session		
Special session on the H2020-EU project ROBOX		
Chair: Marco Fraaije (University of Groningen, The Netherlands)		
17.30 IL11	Monika Muller DSM, The Netherlands	Application of P450 monooxygenases on kg scale
17.50 IL12	Rubén Gómez Castellanos University of Pavia, Italy	Insights from the crystal structures of cyclohexanone monooxygenase from <i>thermocrisium municipale</i>
18.10 IL13	Boris Schilling Givaudan, Switzerland	Use of biocatalysis for the production of flavor and fragrance ingredients
19.00	Conference Diner	<i>There will be busses to bring you to the restaurant: Restaurant NiHao, Gedempte Kattendiep 122, Groningen</i>



ROBOX is supported by the EU Framework Programme Horizon 2020



Friday, October 14, 2016

Novel Enzymes - Biocatalysis session		 cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY
Chair: Isabel W.C.E. Arends (University of Delft, The Netherlands)		
09.00 IL14	Thomas Barends Max Planck Institute for Medical Research, Germany	Hydrazine synthase, a bacterial enzyme producing rocket fuel
09.40 L19	John Ward University College London, United Kingdom	Norcochlorine synthase: mechanism and production of novel tetrahydroisoquinoline alkaloids
10.00 L20	Thierry Gefflaut Université Baise Pascal, France	Aldolases and transaminases from biodiversity for new aldolase-transaminase cascades
10.20	Break	
10.50 IL15	Berndt Nidetzky Graz University of Technology, Austria	Novel synthetic glycosylations and phosphorylations in single and multi-enzyme catalyzed transformations
11.30 L21	Laurence Hecquet Institut de Chimie de Clermont Ferrand, France	One-pot, two-step cascade synthesis of naturally rare ketoses by coupling thermostable transaminase and transketolase
11.50 L22	François Stricher Global Bioenergies, France	Artificial metabolic pathways for bio-based isobutene
12.10	Lunch	
Novel Enzymes - Biocatalysis session		 cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY
Chair: Jan-Metske van der Laan (DSM, The Netherlands)		
13.20 IL16	Leandro Helgueira Andrade Universidade de São Paulo, Brazil	From enzyme prospecting to synthetic applications with hetero-compounds
14.00 L23	Claudia Wahl RWTH Aachen University, Germany	Fast optimization of multi-enzyme cascade reactions by analysis with multiplexed capillary electrophoresis
14.20 L24	Thomas Bayer Technische Universität Wien, Austria	'Substrate/redox funneling' as a novel flux optimization tool for synthetic enzyme cascades <i>in vivo</i>
14.40 IL17	Slavko Kralj DuPont, The Netherlands	Efficient enzymatic synthesis of inulooligosaccharides
15.20	Closure	



COST is supported by the EU Framework Programme Horizon 2020

Invited Speakers

Thomas Barends

Max Planck Institute for Medical
Research
Germany

José Rubén Gómez Castellanos

University of Pavia
Italy

Florian Hollfelder

University of Cambridge
United Kingdom

Slavko Kralj

DuPont
The Netherlands

Emma Master

University of Toronto
Canada

Monika Müller

DSM AHEAD Innovative Synthesis
The Netherlands

Kohei Oda

Kyoto Institute of Technology
Japan

Magali Remaud-Simeon

University of Toulouse
France

Kirk Schnorr

Novozymes
Denmark

John Gerlt

University of Illinois
USA

Leandro Helgueira Andrade

University of São Paulo
Brazil

Dick Janssen

University of Groningen
The Netherlands

David Leys

University of Manchester
United Kingdom

Daniela Monti

Istituto di Chimica del Riconoscimento
Molecolare
Italy

Berndt Nidetzky

Graz University of Technology
Austria

Manfred Reetz

Philipps-University Marburg
Germany

Boris Schilling

Givaudan Schweiz AG - Fragrances S&T
Switzerland

Lecture Abstracts

Transport system solute binding protein (SBP)-guided discovery of novel enzymes in novel metabolic pathways

John A. Gerlt

*Institute for Genomic Biology
University of Illinois, Urbana-Champaign
Urbana, USA*

The number of proteins in the UniProt database (>64M in Release 2016_06) is increasing at the rate of 2%/month—perhaps 50% of the proteins have uncertain or unknown functions. Because sequence homology alone is not sufficient to assign *in vitro* activities and *in vivo* metabolic functions to uncharacterized (“unknown”) enzymes, we are devising tools and strategies to facilitate the prediction and subsequent experimental verification of their activities and functions. We have developed “genomic enzymology” web tools for large-scale 1) analysis of sequence-function relationships for entire enzyme families that place restrictions on possible reactions and substrate specificities for uncharacterized members (sequence similarity networks; EFI-EST; efi.igb.illinois.edu/efi-est/) and 2) identification of genome context to provide clues about the identities of the metabolic pathways in which they participate (genome neighborhood networks; EFI-GNT; efi.igb.illinois.edu/efi-gnt/). The insights provided by these tools facilitate experiment-based functional assignment guided by large-scale screening of the ligand specificities of solute binding proteins (SBPs) for bacterial ABC, TRAP, and TCT transport systems. Because the genes that encode the transport system often are co-located with the genes that encode the catabolic pathway for the ligand, this approach identifies 1) the substrate for the first enzyme in the pathway and 2) the subsequent enzymes and intermediates in the pathway. Examples of the use of these tools and strategies will be described.

A novel aldose-aldose oxidoreductase having dual activities on sugars

Andberg M^{1*}, Maaheimo H¹, Taberman H², Toivari M¹, Rouvinen J², Penttilä M¹, Koivula A¹

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² Department of Chemistry, University of Eastern Finland, Joensuu, Finland

Keywords: enzyme catalysis, tightly-bound cofactor, glucose-fructose oxidoreductase NMR

Plant cell wall cellulose and hemicellulose could provide a range of monosaccharides, i.e. D-Glucose, D-xylose and L-arabinose, for further biochemical conversions e.g. for sugar acids or sugar alcohols having a wide set of application potential. We have a long-term interest in enzymes for sugar oxidation reaction pathways and their biotechnical applications, and have also carried out more detailed characterisation of a set of different enzymes involved in these pathways

In the present work an open reading frame CC1225 from the *Caulobacter crescentus* CB15 genome sequence, belonging to the Gfo/Idh/MocA protein family and having 47% amino acid sequence identity with the glucose-fructose oxidoreductase from *Zymomonas mobilis* (*Zm* GFOR), was discovered¹. When the gene was expressed in *Saccharomyces cerevisiae*, the enzyme was shown to produce xylonic acid and xylitol from D-xylose in equimolar amounts. We named the enzyme as aldose-aldose oxidoreductase (*Cc* AAOR). Further characterization of the purified enzyme demonstrated that the enzyme is a dimer and catalyses oxidation and reduction of several monosaccharides at the C1 position to produce the corresponding aldonolactone and alditol, respectively². It uses a non-dissociable nicotinamide cofactor (NADP) which is regenerated in the oxidation-reduction cycle. *Cc* AAOR is a unique enzyme, able to catalyse the oxidation and reduction of a single substrate in the same catalytic cycle, in a redox neutral reaction cycle. It is active on different hexose and pentose sugars, and is interestingly also able to catalyse oxidation of several oligosaccharides. We used in our studies different spectroscopic methods including NMR to study the substrate specificity and details of the reactions carried out by *Cc* AAOR. Furthermore, the 3D structures of *Cc* AAOR, in complex with its cofactor and several saccharides and sugar alcohols, have been solved^{3,4}. These structures demonstrated the molecular basis for substrate binding and provided new insight into the reaction mechanism of this intriguing enzyme.

Acknowledgements: This study was financially supported by the Academy of Finland through the Centre of Excellence in White Biotechnology–Green Chemistry (decision number 118573), and National Docotral Programme in Informational and Structural Biology.

References

1 Wiebe MG, Nygård Y, Oja M, Andberg M, Ruohonen L, Koivula A, Penttilä M, Toivari M. (2015) A novel aldose-aldose oxidoreductase for co-production of D-xylonate and xylitol from D-xylose with *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, 99, 9439-9447.

2 Andberg M, Maaheimo H, Kumpula EP, Boer H, Toivari M, Penttilä M, Koivula A. (2016) Characterization of a unique *Caulobacter crescentus* aldose-aldose oxidoreductase having dual activities. *Appl Microbiol Biotechnol*, 100, 673-685.

3 Taberman H, Andberg M, Koivula A, Hakulinen N, Penttilä M, Rowinen J, Parkkinen T. (2015) Structure and Function of *Caulobacter crescentus* Aldose-aldose Oxidoreductase . *Biochem J*, 472, 297-307.

4 Taberman H, Parkkinen T, Rowinen J. (2016) Structural and functional features of the (NAD(P)) dependent Gfo/Idh/MocA protein family oxidoreductases. *Protein Sci*, 25, 778-786.

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Plant phenolics enhance oxidative cleavage of plant polysaccharides

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Keywords: Oxidase, LPMO, cellulose oxidation

Plant degrading fungi boost the degradation of lignocellulosic plant biomass via oxidation by using lytic polysaccharide monooxygenases (LPMOs). Recently, we described that a new *MtLPMO9A*, obtained from the ascomycete *Myceliophthora thermophila* C1, cleaves β -(1→4)-xylosyl bonds in xylan under formation of oxidized xylo-oligosaccharides, while it simultaneously cleaves β -(1→4)-glucosyl bonds in cellulose under formation of oxidized gluco-oligosaccharides (1). These *MtLPMO9A*-driven cleavages are considered important for loosening the rigid xylan-cellulose polysaccharide matrix in plant biomass, enabling increased accessibility to the matrix for hydrolytic enzymes.

In order to oxidize polysaccharides, however, LPMOs demand electrons to activate molecular oxygen in their copper-containing active site. A direct way is via donation by reducing agents like small molecular weight compounds, of which mainly ascorbic acid is used in LPMO-research. Little is known about the effect of plant-derived reducing agents on LPMOs activity. We show now for in total, 34 reducing agents, mainly plant-derived flavonoids and lignin building blocks, their ability to promote LPMO activity. Reducing agents with a 1,2-benzenediol or 1,2,3-benzenetriol moiety gave the highest release of oxidized and non-oxidized gluco-oligosaccharides from cellulose for three *MtLPMOs* (2).

Our newest findings include the synergy of other oxidative enzymes with LPMO activity for oxidative cellulose degradation.

References

¹ Frommhagen, M., et al. (2015). Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase. *Biotechnol Biofuels* 8:101.

² Frommhagen, M., et al. (2016). Accepted in *Biotechnol Biofuels*. Lytic polysaccharide monooxygenases from *Myceliophthora thermophila* C1 differ in substrate preference and reducing agent specificity.

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Novel "hot" epoxide hydrolases: from discovery in metagenomes to synthetic exploitation

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Keywords: epoxide hydrolases, metagenomics, biocatalysis, stereoselectivity

Epoxide hydrolases (EHs) represent an attractive option for the synthesis of chiral epoxides and 1,2 diols which are valuable building blocks for the synthesis of several pharmaceutical compounds. In the framework of the FP7 Collaborative Project "HotZyme", two new members of the limonene-1,2-epoxide hydrolase (LEH) family, namely Tomsk-LEH and CH55-LEH, have been recently identified from hot environments by applying a metagenomic approach.¹ These two LEHs show EH activity toward different epoxide substrates, differing in most cases from the previously identified *Rhodococcus erythropolis* enzyme (*Re*-LEH) in terms of stereoselectivity. Moreover, they show a markedly thermophilic character, with higher optimal temperatures and apparent melting temperatures than *Re*-LEH. The new LEH enzymes have been crystallized and their structures solved to high resolution. The structural analysis has provided insights into the LEH mechanism and the substrate specificity and stereoselectivity of these new LEH enzymes. Moreover, the complementary stereopreference for the limonene oxide isomers shown by the now available set of different LEHs has been exploited to perform the biocatalytic resolution of *cis/trans* mixtures of (+)- and (-)-limonene oxide, thus allowing the simple and straightforward preparation of enantiomerically pure limonene oxides, as well as the recovery of the (1*S*,2*S*,4*R*)-limonene-1,2-diol and the (1*R*,2*R*,4*S*)-limonene-1,2-diol products.² Remarkably, after some optimization studies, all the preparative-scale reactions were performed under solvent-free conditions by simple addition of neat substrates to the respective enzyme solutions, thus allowing a significant improvement of the processes productivity.

References

1 E. E. Ferrandi, C. Sayer, M. N. Isupov, C. Annovazzi, C. Marchesi, G. Jacobone, X. Peng, E. Bonch-Osmolovskaya, R. Wohlgemuth, J. A. Littlechild, D. Monti (2015) Discovery and characterization of thermophilic limonene-1,2-epoxide hydrolases from hot spring metagenomic libraries, *FEBS Journal* 282:2879-2894.

2 E. E. Ferrandi, C. Marchesi, C. Annovazzi, S. Riva, D. Monti, R. Wohlgemuth (2015) Efficient epoxide hydrolase catalyzed resolutions of (+)- and (-)-*cis/trans*-limonene oxides, *ChemCatChem* 7:3171-3178.

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Fungal glucuronoyl esterases: genome mining based discovery and biochemical characterization

Adiphol Dilokpimol¹, Miia R. Mäkelä², Miaomiao Zhou¹, Kristiina Hilden² and Ronald P. de Vries¹

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² Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland

Keywords: glucuronoyl esterase, plant biomass, fungi, genome mining.

4-*O*-methyl-D-glucuronic acid (MeGlcA) is a side-residue of glucuronoarabinoxylan and can form ester linkages to lignin, contributing significantly to the strength and rigidity of the plant cell wall. Glucuronoyl esterases (4-*O*-methyl-glucuronoyl methylesterases, GEs) can cleave this ester bond, and therefore may play a significant role as auxiliary enzyme in biomass saccharification for the production of biofuels and biochemicals. GE was first discovered in 2006 in a wood-rotting fungus *Schizophyllum commune*¹. It belongs to a separate family of carbohydrate esterases (CE15) in the CAZy database², but so far less than 10 GEs have been characterized. To explore additional GE candidates, we used a genome mining strategy. BLAST analysis with characterized GEs against approximately 250 publicly accessible fungal genomes identified more than 150 putative fungal GEs, which can be classified into 8 phylogenetic groups. Selected GEs from both Ascomycetes and Basidiomycetes were selected for recombinant production in *Pichia pastoris* and further biochemical characterization. Highlights from this study will be presented.

References

¹ Spániková, S, Biely, P (2006), Glucuronoyl esterase--novel carbohydrate esterase produced by *Schizophyllum commune*, *FEBS Lett.* 580: 4597-4601.

² Lombard et al., (2014), The carbohydrate-active enzymes database (CAZy) in 2013, *Nucl. Acids Res.* 42: D490-D495.

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Scent of bitter almond and a pinch of -omics: discovering a novel hydroxynitrile lyase

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Keywords: hydroxynitrile lyase, cyanohydrins, Bet v 1, fern

Discovering enzymes from scratch gives the opportunity to identify novel unpredictable sequences and protein folds, which would not be found by the most popular homology based algorithms. Herein, we show that the interconnection between transcriptomics, proteomics and enzymatic screening enabled the discovery of a new hydroxynitrile lyase (HNL) from white rabbit's foot fern (*Davallia tyermannii*). Structural studies show *DtHNL* belongs to Bet v 1 protein superfamily with a new catalytic center. Finally, enzymatic characterization and substrate scope were investigated. *DtHNL* is a robust enzyme, tolerant to low pH and able to convert various substrates. Obtained results open prospective for a new class of biocatalysts, broadening the toolbox for the stereoselective synthesis of cyanohydrins.

References

¹ Lanfranchi E et al. (2015), Bioprospecting for hydroxynitrile lyases by blue native PAGE coupled HNL detection, *Current Biotechnology*, 4: 111-117.

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Unravelling the chemistry underpinning reversible decarboxylation in the UbiX-UbiD system

David Leys

University of Manchester, United Kingdom.

The UbiX-UbiD enzyme system has been shown to interconvert unsaturated hydrocarbons (often aromatic) with corresponding $\alpha\phi\alpha$ - $\beta\epsilon\tau\alpha$ unsaturated carboxylic acids. A wide range of substrates has been reported, including those having benzene, furan, indole and polyene carbon skeletons. In addition, the reaction catalysed appears readily reversible depending on $[\text{CO}_2]$ level. However, the application of UbiD enzymes has been hampered by a lack of fundamental understanding. Our recent work on these enzymes demonstrates UbiD relies on a novel cofactor, a prenylated flavin (prFMN). The latter is made in a reduced form by the associated UbiX enzyme, and oxidative maturation of the cofactor is proposed to take place within the UbiD active site. This step generates an iminium form (prFMNiminium) that has azomethine ylide character (i.e. the dipole). We propose this is key to a transient 1,3-dipolar cycloaddition with the alkene substrate (i.e. the dipolarophile) that underpins the reversible decarboxylation step. New data completing the UbiX-UbiD mechanistic picture will be presented.

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Forming the bioplastic monomer FDCA using a single enzyme

Willem Dijkman¹, Marco Fraaije^{2*}

1 Institut für Biochemie, Biotechnologie und Bioinformatik, Technische Universität Braunschweig, Braunschweig, Germany

2 Molecular Enzymology Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

Keywords: bioplastics, oxidation, engineering, mechanism

To meet the growing demand for bio-based chemicals, efforts are made to produce plastics based on furandicarboxylic acid (FDCA). In the past few years, numerous chemical and several multi-enzymatic routes have been reported on the synthesis of FDCA by oxidation of 5-hydroxymethylfurfural (HMF).

We identified a bacterial FAD-dependent oxidase which is active towards HMF and related compounds. This oxidase has the remarkable capability of oxidizing HMF completely to FDCA. This involves three consecutive oxidations of both alcohol and aldehyde groups. After structure based engineering, the oxidase can produce FDCA from HMF with high yield at ambient temperature and pressure.¹ Examination of the underlying mechanism shows that the oxidase acts on alcohol and thiol groups only and depends on the hydration of aldehydes for the oxidation reaction required to form FDCA.² An engineered enzyme is also active on secondary alcohols.

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New enzymes for biosynthesis of ketomemycin, a pseudotriptide with carbonylmethylene structure

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Keywords: ATP-grasp ligase, pseudopeptide, carbonylmethylene.

We recently identified a novel peptide ligase (PGM1), an ATP-grasp-ligase, that catalyzes amide bond formation between (*S*)-2-(3,5-dihydroxy-4-hydroxymethyl)phenyl-2-guanidinoacetic acid and ribosomally supplied oligopeptides in pheganomycin biosynthesis. This was the first example of an ATP-grasp-ligase utilizing peptides as nucleophiles ¹). To explore the potential of this type of enzyme, we examined biological functions of orthologs found in actinobacteria.

The orthologs of *Streptomyces mobaraensis*, *Salinispora tropica*, and *Micromonospora* sp. were found in similar gene clusters consisting of six genes. To probe the functions of these genes, we heterologously expressed each of the clusters in *Streptomyces lividans* and detected structurally similar and novel pseudotriptides (ketomemycin) with carbonylmethylene structure in the broth of all transformants. A recombinant PGM1 ortholog of *Micromonospora* sp. was demonstrated to be a novel dipeptide ligase catalyzing amide bond formation between amidino-arginine and dipeptides to yield tripeptides; this is the first report of a peptide ligase utilizing dipeptides as nucleophiles ²).

We also revealed the mechanism of formation of the carbonylmethylene structure (pseudo-phenylalanine-phenylalanine) in ketomemycin. An aldolase catalyzed the formation of bezylmalonyl-CoA from malonyl-CoA and phenylpyruvate, followed by dehydration and reduction to yield 3-benzylsuccinyl-CoA. Finally, an ortholog of glycine-*C*-acetyltransferase formed the pseudo-phenylalanine-phenylalanine structure from 3-benzylsuccinyl-CoA and phenylalanine.

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Novel enzymes in an industrially relevant context: the tale of two xylanases and other stories

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Novozymes A/S

The definition of novelty for enzymes, to be relevant in an industrial context, is often associated with how the enzymes are used in applications and not necessarily a novel enzyme activity. Two cases will be shown where two enzyme families, working on the same substrate, xylan, have been used to improve two separate industrial processes. The two examples, for the paper pulp industry and the baking industry illustrate how mastery of the process is vital to understanding the requirements for a successful plug in enzyme solution. Other examples will also illustrate some of the hurdles for establishing novel enzymes in existing and emerging industries.

Identification of the novel serine protease Aurase as promising candidate for chronic wound treatment

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Keywords: Aurase, Protease, Fibrin, Debridement

Chronic wounds are difficult to treat because they usually evolve into non-healing ulcers containing dead tissue, debris, and bacteria. The removal of debris and contaminated tissues from a wound bed (debridement) is the first step of wound bed preparation. A major component of debris is fibrin that is built as false covering hindering healing. Methods for debridement comprise surgical, mechanical, autolytic and enzymatic debridement as well as maggot therapy. In maggot therapy, live fly larvae from *Lucilia sericata* are applied to patient's wounds. Maggot therapy is an effective form of debridement but many patients find such treatment revolting and the appropriate infrastructure must exist.

This study describes the identification and characterization of the major fibrin-degrading component from medicinal maggots.

A cDNA library from total RNA of medicinal maggots was constructed and transferred into *Escherichia coli* for gene expression. Clones showing proteolytic activity on casein were further analyzed for fibrinolytic activity and clones producing fibrin-degrading proteases were collected. After sequencing of all positive clones, one single gene sequence was identified encoding for a novel trypsin-like serine protease (Aurase). Subsequently, the Aurase gene was cloned into *Pichia pastoris* expression vectors for enzyme production. Aurase was purified *via* affinity chromatography and crucial enzyme properties were characterized. *In vitro* testing of Aurase demonstrated protease activity at pH 7 - 10 and a high activity against fibrin networks. Furthermore, Aurase shows a high degree of compatibility with various wound irrigation solutions, wound dressings, and wound gels. Formulation experiments were performed in order to establish stable Aurase formulations. Based on these results, prototype wound care products were developed that show excellent usability and long term stability.

The novel serine protease Aurase represents a promising enzyme for the development of products for wound bed preparation by offering quick and effective debridement.

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A dream come true: structure-function relationships in L-amino acid deaminase

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Keywords: biocatalysis, L-amino acids, racemic resolution, three-dimensional structure

L-amino acid oxidases (LAAOs) are FAD-containing flavoenzymes of main interest in biotechnological processes for the production of α -keto acids, enantiomerically pure D-amino acids, etc. Above all, D-amino acids are high value-added chemicals for the synthesis of pharmaceutical drugs, antibiotics and insecticides. LAAOs catalyze the enantioselective deamination of L-amino acids into α -keto acids, ammonia and hydrogen peroxide. In recent years, several protocols for the production of optically pure L-amino acids using an enantioselective D-amino acid oxidase have been set up. Unfortunately, these processes cannot be adapted for the production of the opposite enantiomer because of the drawbacks in recombinant production of a LAAO activity¹.

An alternative to LAAO is represented by L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD), a flavoprotein associated to the cellular membrane which catalyzes the oxidative deamination of L-amino acids with no production of H₂O₂. We produced a fully soluble His-tagged PmaLAAD variant that was purified to homogeneity and retained (in the presence of exogenous *E. coli* membranes or of artificial electron acceptors) full enzymatic activity. PmaLAAD is specific for large and hydrophobic L-amino acids (specific activity = 1.4 U/mg protein) and for several natural and unnatural L-amino acid derivatives (L-DOPA, L-tert-Leu, L-Phe-methyl ester, etc.). The reduced FADH₂ cofactor of PmaLAAD did not react directly with molecular oxygen, but electrons are transferred to a cytochrome b-like protein or, *in vitro*, to artificial acceptors. The three-dimensional structure of PmaLAAD resembles the one of known amino acid oxidases but with peculiar features, such as an additional, unusual, α + β subdomain close to the putative transmembrane α -helix and to the active site entrance which is large and accessible².

The detailed structural and functional characterization of PmaLAAD represents the starting point for its *in vitro* evolution to generate improved PmaLAAD variants suitable for biocatalytic processes.

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Rules and tools for efficient enzyme evolution, recruitment and discovery based on catalytic promiscuity

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'Promiscuous' enzymes possess additional activities in addition to their native ones, challenging the textbook adage "one enzyme – one activity". The observation of strong promiscuous activities in the alkaline phosphatase (AP) superfamily - where one active site can catalyse up to six chemically distinct hydrolytic reactions with promiscuous second order rate accelerations between 10^9 and 10^{17} - suggests that even broadly promiscuous catalysis can be rather efficient. We demonstrate by directed evolution and phylogenetic analysis that crosswise promiscuity relationships in the AP superfamily indicates that an enzyme is 'pregnant' with another activity, i.e. has the potential to be mutated or evolved into a new catalyst. These catalysts are multifunctional generalists that have won additional activities, at varying trade-off cost to the other existing activities. The systematic comparative analysis promiscuous relationships in enzyme superfamilies on the level of structure, sequence similarity, specificity and reactivity suggests factors that govern evolutionary adaptation.

To efficiently explore the interconversion of promiscuous enzyme, we use picoliter water-in-oil emulsion droplets produced in microfluidic devices as high-throughput screening reactors. We present new workflows that allow screening of $>10^6$ clones and allows successful selections from single protein and metagenomic libraries, where lower throughput approaches have failed.

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Asymmetric reductive amination by a wild-type amine dehydrogenase from the thermophilic bacteria *Petrotoga mobilis*

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Keywords: Biocatalysis, chiral amine, amine dehydrogenase, reductive amination, thermophile

The biocatalytic NAD(P)H-reductive amination of ketone with ammonia to primary chiral amine is currently one of the most challenging reaction. Hard engineering work was accomplished recently to obtain this amine dehydrogenase (AmdH) activity starting from α -aminoacid dehydrogenases¹ or from imine reductase (IRED).²

Using a genome-mining approach, we found proteins capable of catalyzing the reductive amination of ketones without carboxylic function in α or β position. The synthesis of (4*S*)-4-aminopentanoic acid (*ee* \geq 99.5%) was achieved with the thermoactive AmdH4 from *Petrotoga mobilis* in 88 % yield starting from the corresponding 4-ketopentanoic acid with a formate/formate dehydrogenase cofactor recycling system at high substrate concentration (0.5 M).

The high stability and substrate tolerance make this amine dehydrogenase a very good starting point for further discovery of reductive amination biocatalysts with wider substrate specificity.

This is the first report of wild-type enzymes with related genes having proper NAD(P)H-amine dehydrogenase activity.

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A bacterium that degrades and assimilates poly(ethylene terephthalate) and its enzymes involved in the degradation

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Keywords: Poly(ethylene terephthalate)(PET), *Ideonella sakaiensis*, PET hydrolase (PETase), Mono(2-hydroxyethyl) terephthalic acid hydrolase (MHETase).

Poly(ethylene terephthalate) (PET) is used extensively worldwide in plastic products, and its accumulation in the environment has become a global concern. Because the ability to enzymatically degrade PET for microbial growth has been limited to a few fungal species, biodegradation is not yet a viable remediation or recycling strategy. By screening natural microbial communities exposed to PET in the environment, we isolated a novel bacterium, *Ideonella sakaiensis* 201-F6, that is able to use PET as its major energy and carbon source. When grown on PET, this strain produces two enzymes capable of hydrolyzing PET and the reaction intermediate, mono(2-hydroxyethyl) terephthalic acid (MHET). Both enzymes are required to enzymatically convert PET efficiently into its two environmentally benign monomers, terephthalic acid and ethylene glycol.

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Enzyme cascade reactions for efficient glycosylation of small molecules

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Keywords: glycosyltransferases, glycosylation, stevia rebaudiana, curcumin

Glycosyltransferases (GTs, EC 2.4) are a large class of carbohydrate active enzymes able to catalyze sugar transfer to an acceptor molecule. To date, more than 230,000 GT sequences have been deposited, but less than 1% of those have been characterized.¹ Promising enzymes can be found in family GT-1, which contains a large number of plant GTs that use UDP-sugars as donor substrate (so-called UGTs) and a variety of natural products as acceptor substrate.

Here, five different plant UGTs from GT-1 were recombinantly expressed in *E. coli* to enable their biochemical characterization, with particular emphasis on their acceptor promiscuity and applicability as glycosylating biocatalysts.² The yields of these GTs were stepwise improved by evaluating different promoter strengths, expression systems and strains. In-depth characterization was performed on the two enzymes with the highest expression yields, i.e. the salicylic acid UGT from *Capsella rubella* (UGT-SACr) and the stevia UGT from *Stevia rebaudiana* (UGT-76G1Sr). The latter was found promiscuous with activities on a wide diversity of structures, from aliphatic and branched alcohols, over small phenolics to larger flavonoids, terpenoids and even higher glycosides.

As an example for the potential of UGT-76G1Sr, glycosylation of curcumin was thoroughly evaluated. Curcumin is a yellow substance commonly used as food colorant and known to have pharmaceutical properties such as antioxidant and anti-inflammatory activities. The biggest issue of curcumin is its very poor water solubility, a feature which can significantly be enhanced by glycosylation. Under optimized conditions, 96% of curcumin was converted within 24 h into the corresponding curcumin-glycosides. In addition, the reaction was performed in a coupled system with sucrose synthase, enabling the efficient (re)generation of expensive UDP-glucose from sucrose as abundant and renewable resource. Alternatively, various end-to-end fusion proteins were created and evaluated.

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Engineering a thermostable transketolase for carboligation of arylated substrates

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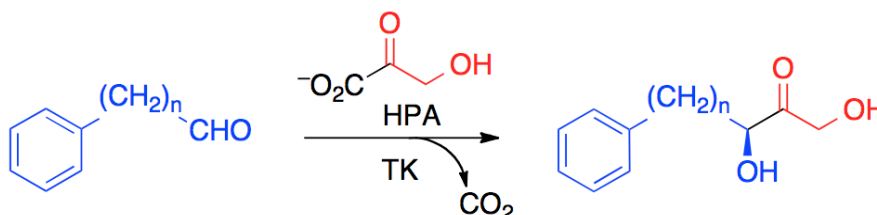
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Keywords: carboligation, protein engineering, substrate promiscuity, stereoselectivity.

Non-covalent interactions involving aromatic rings are key stabilizing elements in both chemical and biological recognition. Because of their hydrophobic nature and low chemical reactivity, aromatic ring systems are of paramount importance as constituents of synthetic building blocks for rational drug design and lead optimization in medicinal chemistry. However, aromatic components are difficult substrates for enzymes catalyzing stereoselective C–C bond forming reactions.

We have engineered the transketolase (EC 2.2.1.1) from *Geobacillus stearothermophilus* by directed evolution¹ to convert arylalkanal and benzaldehyde as the electrophilic substrate with hydroxypyruvate (HPA) as the nucleophile. Variants showing rate accelerations up to 28-fold were discovered that convert 2-phenylethanal, 3-phenylpropanal and related compounds with formation of the corresponding aryl-substituted 1,3-dihydroxyketones in good yields (60-72%) and virtually complete enantioselectivity (>99% *ee*).



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Recent methodology developments in directed evolution

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Directed evolution of enzymes as catalysts in synthetic organic chemistry and biotechnology provides a means to eliminate the limitations traditionally associated with biocatalysis, namely the often observed lack of substrate acceptance, insufficient activity and stability, as well as poor or wrong stereo- and/or regioselectivity (Perspective on biocatalysis in organic chemistry: M. T. Reetz, *J. Am. Chem. Soc.* 2013, *135*, 12480-12496). Essentially any gene mutagenesis method such as epPCR, DNA shuffling or saturation mutagenesis lead to some degree of success, depending upon how much time and effort the researcher is willing to invest. The real challenge is to develop optimal mutagenesis strategies which rapidly and reliably provide highest-quality mutant libraries requiring the minimal amount of screening (the bottleneck of directed evolution). The lecture focuses on recent methodology developments as illustrated by the directed evolution of hydrolases, monooxygenases and reductases, generally with emphasis on stereo- and regioselective transformation which are problematic when attempting to apply synthetic transition metal catalysts or organocatalysts. Selected lessons learned from directed evolution are also featured in this presentation.

Combining protein engineering strategies for improving lipase stability in methanol

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Keywords: Lipase, Methanol, Protein Engineering, Stability

Enzymatic production of biodiesel by transesterification of triglycerides and alcohol, catalyzed by lipases, offers an environmentally-friendly and efficient alternative to the chemically catalyzed process while using low-grade feedstocks. Methanol is utilized frequently as the alcohol in the reaction due to its reactivity and low cost. However, one of the major drawbacks of the enzymatic system is the presence of high methanol concentrations which leads to methanol-induced unfolding and inactivation of the biocatalyst. Therefore, a methanol stable lipase is of great interest for the biodiesel industry.

In this study, different protein engineering approaches were used to develop a methanol stable lipase originating from *Geobacillus stearothermophilus* T6. The best variant of the random mutagenesis library, Q185L, exhibited 23-fold improved stability yet its methanolysis activity was decreased by half compared to the wild type. The best variant from the structure guided consensus library, H86Y/A269T, exhibited 66-fold improved stability in methanol. Rational substitution of charged surface residues with hydrophobic ones resulted in improved variant R374W. The combined triple mutant H86Y/A269T/R374W, had a half-life value at 70% methanol of 324 min which reflects an 87-fold enhanced stability compared to the wild type together with elevated thermostability in buffer and in 50-70% methanol. This variant also exhibited an improved biodiesel yield from waste chicken oil compared to commercial Lipolase 100L® and Novozyme® CALB. Engineering several tunnels within the enzyme resulted in enhanced stability as well. Crystal structures of the wild type and the methanol-stable variants provided insights regarding structure-stability correlations. The most significant features were the extensive formation of new hydrogen bonds between surface residues directly or mediated by structural water molecules, and the stabilization of Zn and Ca binding sites. Combining knowledge-based protein engineering with targeted libraries is highly efficient for the design and tuning of biocatalysts towards enhanced stability while minimizing the screening efforts.

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Directed evolution of PpDyP, a bacterial DyP-type peroxidase, for improved oxidation of phenolic compounds

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Keywords: ligninolytic enzymes, protein expression, enzyme specificity, biorefineries

Dye-decolourising peroxidases (DyPs) are a novel prominent family of heme-containing peroxidases in bacteria, showing activity for a wide number of substrates, including synthetic dyes, phenolic and nonphenolic lignin units, iron and manganese ions. DyPs are very attractive biocatalysts for application in the industrial biotechnology field. In this study a laboratory evolution approach was followed to improve the enzyme specificity of *Pseudomonas putida* MET94 PpDyP¹ for phenolic compounds. Three rounds of random mutagenesis of the *ppDyP*-gene followed by high-throughput screening led to the identification of an evolved variant 6E10, featuring a 200-fold enhanced catalytic efficiency (k_{cat}/K_m) for 2,6-dimethoxyphenol, similar to those exhibited by high redox peroxidases. The evolved variant shows additionally an improved activity for a number of phenolic, aromatic amines and the lignin model phenolic dimer, guaiacylglycerol- β -guaiacyl ether. Importantly, the hit variant 6E10 exhibits optimal pH at 8.5, an upshift of 4 units as compared to the wild-type, shows resistance to hydrogen peroxide inactivation, and is produced at 2-fold higher yields. Biochemical analysis of hit variants from the *in vitro* evolution, and variants constructed using site-directed mutagenesis, unveiled the critical role of the accumulated substitutions, from the structural, catalytic and stability viewpoints. Details of the catalytic cycle were elucidated through transient kinetics to characterize the 6E10 reaction with hydrogen peroxide and guaiacol. This study opens new perspectives for further evolution of these enzymes for new specificities and applications and for a better insight into the structure-function relationships within the DyP-type peroxidase family of enzymes.

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Structurally-guided engineering of enzymes and enzymatic pathways for novel products

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Protein engineering and computational design are extremely powerful technologies that allow the efficient conception of new enzymes for enzyme-based process, chemo-enzymatic cascades or novel metabolic pathways. We propose to give an overview of our recent achievements in this field. A specific focus will be placed on the computational-aided engineering of α -retaining transglucosylases from glycoside-hydrolase family 13 and 70. These enzymes are sucrose-active enzymes. They transfer the glucosyl unit of sucrose onto a large panel of hydroxylated acceptors and can produce a broad range of α -glucans, glucooligosaccharides or glucoconjugates. Engineering strategies were applied to generate novel transglucosylases working on unnatural oligosaccharide acceptors^{1,2}, which were chemically protected to integrate programmed chemo-enzymatic cascades. In this way, new routes for the development of various patterns of antigenic oligosaccharides could be proposed. Similar approaches were also recently investigated to conceive a new and artificial metabolic pathway dedicated to di-hydroxybutyrate production. The strategies and approaches developed for both cases will be described and discussed with regards to enzyme integration in either chemo-enzymatic pathways or living organisms.

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Functional transitions in enzyme evolution: balancing stability, folding and catalytic specificity

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Keywords: ancestral reconstruction, enzyme evolution catalytic promiscuity, phospho/sulfohydrolase

Evolutionary pathways by which proteins have evolved in Nature over billions of years have resulted in an impressive diversity of structures that carry out many functions with unrivalled efficiency. Directed protein evolution in the test tube can emulate natural evolution, but is often limited by low hit rates and small improvements during evolutionary cycles. Furthermore, the combination of mutations that is needed for large improvements cannot always be reached by one-by-one mutational steps due to the occurrence of general loss-of-function or epistatic ratchets. The question then arises how evolutionary dead ends can be avoided. Important parameters that shape these fitness landscapes are e.g. expression level, stability and catalytic activity/specificity. We are currently probing these parameters for ancestral sequences inferred from phylogenetic relationships between members of the catalytically diverse metallo- β -lactamase¹ and alkaline phosphatase²⁻⁴ superfamilies. Mapping of substrate specificity profiles on the genetic relationships allowed the identification of the ancestral nodes between which transitions in primary function most likely occur. The latter is one of the key processes in evolution of new functions. The substrate specificity profiles of the current enzymes suggest that the change in primary function is the result of a shift in substrate preference rather than *de novo* evolutionary invention of a novel activity.

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A chemically programmed proximal ligand enhances the catalytic properties of heme enzymes

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Keywords: non-canonical mutations, heme enzymes, proximal ligand, catalytic mechanism

Biocatalysis is widely recognized as a sustainable technology for the production of high-value chemicals. At present, enzyme production and evolution strategies rely exclusively on a standard amino acid alphabet of twenty canonical residues which contain limited functionality. Here we demonstrate that the incorporation of new chemically programmed amino acids into existing evolutionary strategies *via* genetic code expansion provides a fruitful avenue to probe enzyme mechanism and can lead to modified biocatalysts with significantly enhanced catalytic properties. Specifically, introduction of a modified proximal ligand into heme enzymes can lead to a dramatic improvement in catalytic parameters and reveals crucial insights into the role of proximal pocket hydrogen bonding interactions in the stabilization of high-valent ferryl intermediates.¹ More generally our studies suggest that metallo-enzymes with enhanced properties or novel reactivities can be created by extending the metal co-ordinating ‘ligand set’ beyond those presented by the genetic code or through Nature’s biosynthetic machinery.

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Polysaccharide utilization loci as sources of unique carbohydrate active enzymes

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Hemicelluloses are one of the major components of plant cell walls and can be used for the production of renewable chemicals and materials. Given the structural diversity of corresponding polysaccharides, its broader use requires the concerted action of a large repertoire of different carbohydrate active enzymes (CAZymes). Some members of the dominant gut-associated bacterial phylum, Bacteroidetes, have evolved polysaccharide utilization loci (PULs), which consist of clusters of physically linked genes that are co-regulated to sense, bind, degrade and import specific polysaccharides. Accordingly, the PUL database (PULDB, www.cazy.org/PULDB) is an important resource for discovering new and novel CAZymes capable of modifying complex glycans. PULDB mostly contains predicted CAZymes, however there are some proteins that have no known function. A protein of unknown function (“Unk4”) was chosen for characterization from a PUL containing xylan active CAZymes, such as glycoside hydrolases from family 43 and 115, and carbohydrate esterases from families 1 and 6. Unk4 includes a predicted GDSL-like lipase/acylhydrolase family domain; protein structure modeling also suggests structural similarity with CE2 or CE12 families. Unk4 demonstrated activity on acetylated xylooligosaccharides, released acetyl groups from xylopyranosyl (Xylp) subunits also substituted by methyl glucuronic acid (MeGlcA), and significantly enhanced the activity of a xylan α -1,2-glucuronidase from family GH115, suggesting synergistic action between the two enzymes.

Engineering carbonic anhydrase with self-assembly peptide as functional nanoparticles

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Keywords: Bovine carbonic anhydrase, peptide, self-assembly, enzymatic nanoparticles

Carbonic anhydrase (CA) is an industrially important enzyme for CO₂ capture applications. Enzyme-based process of CO₂ capture is an environmentally friendly alternative to the amine-based process. However, its feasibility at industrial scale is limited by the lack of reusability of the free CA, as enzyme replenishment adds to process cost. Immobilization of CA onto solid supports allows reuse but often results in lower enzyme activities compared to free CA. Hence it is highly desirable to engineer CA that can be easily recovered without compromising activity. To achieve this we have engineered bovine carbonic anhydrase (BCA) fused with a self-assembling peptide that allows non-covalent formation of nanoparticles. The BCA-peptide fusion protein has been produced in high yield in recombinant *Escherichia coli* and the purified enzyme showed 98% of the wild-type hydratase activity. When subjected to a reduced pH, the BCA-peptide forms nanoparticles with a particle size in the range of 50–200 nm which is desirable for their re-use and recovery using existing membrane-based processes¹. The enzyme nanoparticles retained both their self-assembled structure as well as their catalytic activity under CO₂ capture conditions up to 50°C. It is anticipated that the engineered BCA nanoparticles demonstrated in this work offer a new approach to stabilize and reuse CA in a simple and cost-effective manner for application to the CO₂ capture process.

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Computational approaches in enzyme engineering

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Exploring natural and theoretical sequence space for novel biocatalytic activities has a major impact on the development of sustainable enzyme-catalyzed conversions in preparative chemistry. Powerful approaches for the discovery of enzymes with new activities or selectivity include traditional enrichment cultures, functional metagenomics, genome mining, laboratory evolution and computational design. Classical enrichment of microorganisms and screening techniques will remain essential, since it explores part of sequence space that are poorly accessed by laboratory or computational tools.

We have studied the use of computational protein design and in silico screening of mutant enzymes to develop an enzyme engineering strategy in which most of the laboratory screening that is typical for directed evolution is replaced by in silico evaluation. This yielded a framework for rapid enzyme stabilization by computational library design. We applied it to enzymes obtained by classical enrichment, including an epoxide hydrolase, two dehalogenases and a peptide amidase, producing enzyme variants with high thermostability ($\Delta T_{m,app}$ +15-35°C) and high cosolvent resistance (DMSO, DMF, methanol). The stabilized enzymes proved to be good templates for further mutagenesis aimed at selectivity engineering. Computational protocols also replaced most of the laboratory screening required to develop enantiocomplementary enantioselective epoxide hydrolases for the conversion of meso substrates to highly enantioenriched diols.

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Changing the chemoselectivity of an aldo-keto-reductase to a flavin-free ene-reductase

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Keywords: enzyme engineering, aldo-keto-reductase, flavin-free, ene-reductase,

Ene-Reductases are of great interest for industrial biocatalysis due to their ability to generate chiral molecules via an asymmetric reduction of C=C bonds. This is exploited on a broad scope in the synthesis of enantiopure molecules for application in the chemical, pharmaceutical and fragrance industries. Concerning the reduction of activated alkenes, flavin-dependent, NAD(P)H-utilizing proteins from the old yellow enzyme family are applied as state-of-the-art biocatalysts¹.

In contrast, Xylose Reductase (XR) is a flavin-free NADPH/NADH dependent oxidoreductase that belongs to the protein superfamily of aldo-keto reductases (AKR). In the yeast *Candida tenuis*, XR is involved in the metabolic processing of D-xylose by catalyzing its reduction to xylitol.

By using computational tools and structural information on *Candida tenuis* XR², several active-site designs for flavin-free ene-reductase activity of a homologous fungus XR were calculated. The wildtype and several designed XR variants with putative ene-reductase activity were cloned, heterologously expressed in *E.coli*, purified, crystallized and structurally characterized. Furthermore, enzymatic activity assays confirm the presence of designed ene-reductase activity for two of the variants, which is not detected for the wild type. Further mutations for improvement of the activity for these variants are suggested.

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Flavin dependent halogenase enzymes for aromatic regioselective bio-halogenation

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Keywords: Flavin dependent halogenase, Regio-selective halogenation, biosynthetic pathways, chemo-enzymatic reactions.

In recent years, many Flavin dependent halogenase (Fl-Hal) enzymes were discovered from various bacterial and fungal biosynthetic pathways and these enzymes are the versatile biocatalysts for regioselective halogenation of wide range of aromatic compounds. Consequently, Fl-Hal is one of the potential biocatalysts for the chemo-enzymatic synthesis of pharmaceuticals and other valuable products, which are derived from haloaromatic precursors. However, the application of Fl-Hal enzymes, *in vitro*, has been hampered by their poor catalytic activity, lack of stability and desired substrate scope. Our recent advancements in understanding the regioselectivity of Fl-Hals, the methods applied to improve stability and activity of these enzymes, incorporation of Fl-Hal to chemo-enzymatic reactions, identification of other novel Fl-Hal enzymes with different substrate scope and reactivity and incorporation of these enzyme into engineered biosynthetic pathways are discussed.

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Binuraj R. K. Menon, Jonathan Latham, Mark S. Dunstan, Eileen Brandenburger, Ulrike Klemstein, David Leys, Chinnan Karthikeyan, Michael F. Greaney, Sarah A. Shepherd and Jason Micklefield (manuscript submitted)

5) *RadH a versatile halogenase for integration into synthetic and engineered biosynthetic pathways.*

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Application of P450 monooxygenases on kg scale

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Royal DSM N.V. is a global life science and material science company actively developing sustainable processes for production of chemical building blocks and intermediates from renewable resources. As an industry leader in sustainability DSM takes a multidisciplinary approach including chemo- and biocatalysis, organic synthesis and metabolic engineering. In this approach sustainability of process concepts and designs are evaluated early on to focus development on the most cost-efficient and sustainable option.

Oxy-functionalization of non-activated C-H by cytochrome P450 monooxygenases have attracted significant academic as well as industrial interest in the past years. Next to the availability of enzymes with sufficiently broad substrate scope and high activity, efficient production concepts are required for the successful application of these enzymes in biocatalytic processes. Here we will report on the generation of a P450 enzyme platform as well as exemplify the developments of successful process strategies and scale-up for this challenging enzyme class up to kg scale.

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Insights from the crystal structures of cyclohexanone monooxygenase from *Thermocrispum municipale*

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Keywords: Cyclohexanone monooxygenase (CHMO), Baeyer-Villiger monooxygenase (BVMO), flavoenzymes, crystal structure.

Baeyer-Villiger Monooxygenases (BVMOs) are flavoenzymes that catalyze a wide variety of oxidative reactions such as enantioselective Baeyer-Villiger oxidations and sulfoxidations. Cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 is the prototypical BVMO catalysing the transformation of a number of cyclic ketones into lactones, as well as other types of oxidation. CHMOs, therefore, have been targeted in the ROBOX project for the screening and functional evaluation of engineered variants that will target the conversion of cyclohexanone (to ϵ -caprolactone for synthesis of Nylon 6 precursors) and cyclohexanone derivatives (for alternative specialized polymer precursors).

Through the screening of the available wealth of genome sequence information, a CHMO variant has been identified. Here, we present the crystal structures of a thermostable CHMO from *Thermocrispum municipale* (CHMO_{Tm}) bound to FAD, NADP⁺ with and without a ligand to resolutions up to 1.6 Å; native CHMO_{Tm} was crystallized by sitting-drop vapour diffusion. X-ray diffraction data were collected at the Swiss Light Source (SLS) and at the European Synchrotron Radiation Facility (ESRF). The images were integrated and scaled; intensities were merged and converted to amplitudes and the structures were solved by molecular replacement using the coordinates of CHMO from *Rhodococcus* sp. HI-31 (CHMO_{Rsp}).

Similar to the reported structures for CHMO_{Rsp}, we identified two conformations, one with the cofactors and the ligand tightly bound in their respective domains and one without a ligand characterized by a flexible loop between residues 487–504 and a flexible nicotinamide moiety that is not well defined. These structures increase the insight into how CHMOs exploit the NADP⁺ cofactor for multiple purposes during the catalytic cycle. Furthermore, structural analysis of both BVMOs reveals that the thermostability of CHMO_{Tm} versus CHMO_{Rsp} may be explained by a significantly higher amount of salt bridges present in the former (31 vs 16). Overall, this work provides guidance into potential engineering strategies to optimize both the stability and the efficiency of CHMOs.

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Use of biocatalysis for the production of flavor and fragrance ingredients

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Biocatalysis using whole cell microorganisms or isolated enzymes has been successfully used to produce natural flavor ingredients for many years. In contrast, Fragrance materials are either naturals such as essential oils, or they are produced using organic chemistry routes. While traditional chemical synthesis generally produces a mixture of isomers, enzymatic reactions allow to synthesize specific stereoisomers that have preferred sensory properties. A fragrance chemist's current toolbox of enzymes is small, however, the ROBOX program provides a unique chance to demonstrate the feasibility of using biocatalysts in a specialty chemicals environment and to strengthen our capabilities in applying the principles of green chemistry to product development.

Hydrazine synthase, a bacterial enzyme producing rocket fuel

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Over the last decades, our view of the global nitrogen cycle has undergone dramatic changes, mainly because of the discovery of the bacterial "anammox" pathway. This anaerobic ammonium oxidation pathway combines ammonium with nitrite, converting it nitrogen gas. It has been estimated that the anammox process is responsible for up to 50% of biological nitrogen production in some ecosystems.

Interestingly, the anammox pathway relies on the highly reactive intermediate hydrazine, which is unique to biochemistry and known to most people only as rocket fuel. How such a toxic and reactive compound is prepared and handled by cells is now beginning to be elucidated.

We have determined crystal structures of the multienzyme complex hydrazine synthase responsible for biological hydrazine synthesis. The crescent-shaped complex contains two heme-containing active sites connected by a tunnel, as well as a number of metal ions and an interaction site for a redox partner. Solution small-angle X-ray scattering confirms the overall shape and size of the complex, and EPR spectroscopy is consistent with the observed heme coordination. The structural data suggest a mechanism for biological hydrazine synthesis using hydroxylamine as an intermediate.

Norcoclaurine synthase: mechanism and production of novel tetrahydroisoquinoline alkaloids

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Keywords: Alkaloids, Benzyloisoquinoline, Pictet-Spenglerase, Biocatalysis

The benzyloisoquinoline alkaloids (BIA) are a diverse family of bioactive compounds synthesised by plants and there are over 2,500 BIA alkaloid structures known. Many alkaloids and their derivatives are used as pharmaceuticals and they represent a huge repository of functional chemical space.

We have been developing synthetic pathways and cascades¹ to new alkaloid structures centred around the key coupling enzyme (*S*)-norcoclaurine synthase (NCS)^{1,2}. The NCS is a Pictet-Spenglerase enzyme that uses dopamine and an aldehyde and couples these together to make a new ring forming a substituted (*S*)-tetrahydroisoquinoline. Using the *Thalictrum flavum* NCS (*Tf*NCS) we have reassessed the mechanism of the enzyme³ and shown that the dopamine binds first in the active site and using active site mutations of *Tf*NCS we have increased the substrate spectra for non-native aldehydes. We have recently discovered that several ketones are substrates for some of the mutants of *Tf*NCS and cyclic ketones form novel spiro compounds.

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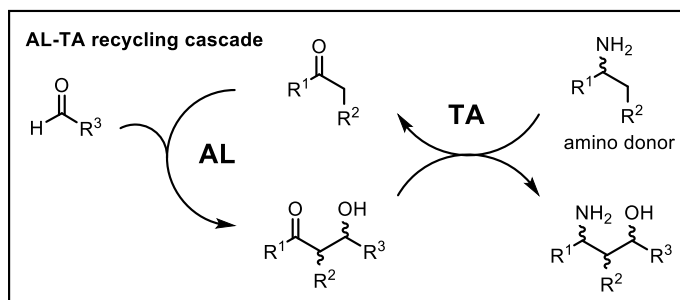
Aldolases and transaminases from biodiversity for new aldolase-transaminase cascades

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Keywords: Aldolase, Transaminase, Genome mining, Enzymatic cascade.

Aldolases (AL) and transaminases (TA) both constitute efficient tools for the development of biocatalytic processes.^{1,2} The discovery of new AL and TA with expanded substrate spectra is of great interest to develop new synthetic applications and thoroughly exploit AL and TA catalytic potential. Moreover, the combination of AL and TA in a bienzymatic cascade constitutes a straightforward approach to prepare γ -amino-alcohols with high stereoselectivity: the ketol selectively obtained from simple carbonyl compounds through AL-catalysis can be converted with high stereoselectivity into γ -amino alcohol by action of a TA in the presence of an amino donor substrate. Nevertheless, few examples of AL-TA cascades have been described so far.³ In the course of a larger project devoted to the discovery of new AL and TA from microbial diversity using a genome mining approach,⁴ we have identified a set of AL and TA potentially suitable for the synthesis of various valuable γ -amino alcohols. To improve the efficiency of AL-TA bienzymatic processes, we have developed a recycling cascade model, in which the nucleophilic substrate of AL is generated from the amino donor substrate of TA (figure opposite). This innovative "AL-TA loop" process thus brings the benefit of optimal atom economy. Moreover, the thermodynamically favoured aldolisation, is expected to shift the transamination equilibrium, thus solving the reversibility problem often encountered with TA-catalysed reactions. We have already validated the AL-TA loop process principle through the synthesis of hydroxylated amino acids ($R^1 = CO_2H$).



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Novel synthetic glycosylations and phosphorylations in single and multi-enzyme catalyzed transformations

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Keywords: Glycobiotechnology, glycosyl and phosphoryl transfer, Leloir glycosyltransferases, cascade biotransformations

Glycosides are molecules of fundamental importance in biology, chemistry and the related technologies. Their synthesis usually involves glycosidic bond formation as the main challenging task. For that, an activated sugar donor is normally used to drive the covalent attachment of the glycosyl residue onto an acceptor molecule. Prime difficulty in doing so is to control the reaction's selectivity while retaining a suitable reactivity at the same time. Enzymes are proficient in managing this dual task and are therefore regarded highly as glycosylation catalysts for synthetic use. However, a large gap in scope exists between the glycosides in demand and the biotransformations available for their synthesis.

Biocatalytic glycosylations typically make use of enzymes from one of two main classes: transglycosidases and nucleotide sugar-dependent (Leloir) glycosyltransferases. General requirements and new applications of transglycosidase-catalyzed glycoside syntheses will be discussed. In virtue of their usually very high acceptor site selectivity, Leloir glycosyltransferases have long been considered potential "game changing" catalysts of glycosylation, enabling glycoside synthesis with high precision in a single reaction step suitable for full-scale production. However, in an industrial arena these enzymes are yet to play a significant role. Examples from the synthesis of nucleotide sugars and natural product glycosides are used to discuss what is needed to unlock the full potential of glycosyltransferases for glycoside synthesis. The development of efficient glycosyltransferase cascades is shown. New approaches of enzymatic phosphorylation are used to synthesize phospho-sugars and nucleotides as substrates of glycosyltransferase conversion.

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One-pot, two-step cascade synthesis of naturally rare ketoses by coupling thermostable transaminase and transketolase

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Keywords: thermostability, ketoses, enzymatic cascade, C-C bond formation

Thermostable enzymes offer many advantages, such as improved solubility of organic substrates at elevated temperatures, increased tolerance toward unconventional media and greater resistance to protein destabilizing factors introduced by mutagenesis.¹

We recently cloned and overexpressed the first thermostable TK from the thermophilic bacterium *Geobacillus stearothermophilus* (TK_{gst}).² TK_{gst}, like other TK sources, preferentially accepts (2*R*)-hydroxylated aldehydes. Remarkably, at high temperature, TK_{gst} was also able to accept (2*S*)-hydroxylated aldehydes yielding (3*S*,4*S*) ketoses stereospecifically.³ None of the mesophilic TKs have been reported to catalyze the conversion of (2*S*)-configured hydroxyaldehydes. For synthetic purposes, the main problem of TK_{gst}-catalyzed reactions at high temperature is the limited stability of the artificial donor substrate Li-HPA.⁴

We will report on the identification and characterization of a novel thermostable serine-glyoxylic acid L- α -TA from the thermophilic bacterium *Thermosinus carboxydivorans* DSM 14886 (TA_{tca}) and its use for the *in situ* biocatalyzed synthesis of HPA at high temperature from natural L-serine and pyruvate. TA_{tca}-catalyzed reaction is shifted towards HPA by coupling to the irreversible TK_{gst}-catalyzed reaction in an efficient one-pot two-step simultaneous cascade at elevated temperature.⁵ This procedure is applied to the synthesis of highly valuable and naturally rare L-erythro (3*S*,4*S*)-ketoses, L-ribulose, 5-deoxy-L-ribulose, D-tagatose and L-psicose obtained with excellent stereoselectivity and good yields. Such a configuration is currently inaccessible with mesophilic TKs. TK_{gst} activities towards the (2*S*)- α -hydroxylated aldehydes, which are generally poor TK_{gst} substrates, were greatly enhanced by performing the reactions at high temperature, leading to excellent conversion rates within a reasonable time (24 h to 96 h).

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Artificial metabolic pathways for bio-based isobutene

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Keywords: Synthetic Biology, light olefins, isobutene, metabolic engineering

As of today, most industrial bio-production processes are based on naturally occurring metabolic pathways, limiting the scope of industrial biology, and preventing the access to many of the chemistry's largest market.

The purpose of Global Bioenergies is to develop innovative metabolic routes for the bio-production by direct fermentation of light olefins, core molecules of the petrochemical industry, such as propylene, linear butylene, butadiene and isobutene. However, these volatile compounds are not naturally produced by microorganisms and no bioprocess to convert renewable resources to these molecules has been industrialized so far.

Global Bioenergies has developed an artificial metabolic pathway including all the necessary enzymatic reactions from feedstock (glucose, sucrose or second generation) to isobutene. The metabolic route leading to isobutene uses natural reactions, from both endogenous and heterologous enzymes, as well as non-naturally occurring reactions to bridge the gap between natural metabolites and the final product, including the decarboxylation of hydroxyisovaleric acid into isobutene. To do so, Global Bioenergies engineered novel artificial biocatalysts, by taking advantages of the natural catalytic and substrate promiscuity of exogenous enzymes, and by applying systematic, random and semi-rational evolution approaches.

Commercialization efforts took a step forward with the announcement of the successful production of isobutene at industrial pilot scale in Pomacle (France), where operations started since November 2014. Samples of bio-based isobutene or of isooctane derived exclusively from bio-isobutene were delivered to Arkema, Audi and to CFBP (an industry organization representing several gas supply distribution companies), and the process has now reached 70% of the commercial yield. Importantly, light olefins are gaseous and therefore spontaneously volatilize from the fermentation medium, making the process less energy intensive and cheaper. Furthermore, a Demo Plant, currently being built in Leuna (Germany), is scheduled to be operational in fall 2016.

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From enzyme prospection to synthetic applications with hetero-compounds

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The current interest in applying enzymatic reactions in organic chemistry is mainly related to the preparation of optically active compounds with high stereoselectivity under environmentally friendly conditions. Many types of enzymes, which are present in bacteria, fungi, yeasts and plants, can be applied for that purpose. We have showed that promising enzymes can be found on bacterial strains isolated from different biomes (Atlantic forest, Amazon forest and Antarctic Peninsula). We also studied enzymatic reactions of organic compounds containing heteroatoms, such as selenium, boron and silicon, and catalyzed by alcohol dehydrogenases, lipases, transaminases or monooxygenases.

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Fast optimization of multi-enzyme cascade reactions by analysis with multiplexed capillary electrophoresis

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Keywords: biocatalysis, multi-enzyme cascades, nucleotide sugars, glycoconjugates.

The translation of glycoconjugate multi-enzyme synthesis into larger scale is hampered by multi-parameter optimization of enzyme-modules. Optimization of product yields in such multi-enzyme modules is governed by a considerable time effort when conventional analytic methods like capillary electrophoresis (CE) or HPLC are applied. For glycan synthesis with Leloir-glycosyltransferases nucleotide sugars are considered as bottleneck and expensive substrates. In our ongoing project “The Golgi Glycan Factory (GGF)” we set up modular enzyme systems for the synthesis of sixteen different nucleotide sugars starting from monosaccharides and sucrose as substrates. We further combine these modules with glycosyltransferase modules for the synthesis of glycan epitopes.

We here introduce for the first time multiplexed CE (MP-CE) as fast analytical tool for the optimization of multi-enzyme cascade reactions for nucleotide sugar and glycan synthesis. We have developed a universal CE separation method for nucleotides, nucleotide sugars, and glycans enabling us to analyze the composition of reaction mixtures in a 96-well high-throughput format. We demonstrate here the optimization of parameters (T, pH, inhibitors, kinetics, cofactors and enzyme amount) for the synthesis of UDP- α -D-glucuronic acid (UDP-GlcA), UDP- α -D-galactose (UDP-Gal), UDP- α -D-N-acetylgalactosamine (UDP-GalNAc), and UDP- α -D-N-acetylglucosamine (UDP-GlcNAc). Optimized reactions in a 96-well format (300 μ L/well) were scaled up to 1 L yielding e.g. 10 mMol UDP-GalNAc in 1.5 h reaction time. In this way we achieve high space-time-yields of 1.8 g/L*h (UDP-GlcA), 4.0 g/L*h (UDP-GalNAc), and 17 g/L*h (UDP-Gal). We further demonstrate the optimization of glycan synthesis by MP-CE analysis yielding the tetrasaccharide di-LacNAc (N-acetyllactosamine, LacNAc, Gal β 4GlcNAc) from GlcNAc monosaccharide in 2 h reaction time. The presented MP-CE methodology has the impact to be used as general analytical tool for fast optimization of multi-enzyme cascade reactions.

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‘Substrate/redox funneling’ as a novel flux optimization tool for synthetic enzyme cascades *in vivo*

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Keywords: flux optimization, substrate/redox funneling, synthetic enzyme cascade, carboxylic acid reductase

The maximization of product titers of artificial enzyme cascades *in vivo* is highly desired.¹ Strategies to enhance the carbon flux through *de novo* pathways such as the introduction of synthetic protein scaffolds² and the knock-out of endogenous enzyme activities³ proved their applicability beyond doubt but were elaborate and time-consuming. Herein, an easy to apply yet conceptually different approach called ‘substrate/redox funneling’ was used for the optimization of an *in vivo* cascade for the preparation of reactive aldehyde intermediates from primary (aromatic) alcohols and their subsequent aldol reaction with dihydroxyacetone (DHA) for the production of valuable polyhydroxylated compounds. ‘Substrate/redox funneling’ economically redirected the carbon flux from undesired carboxylate byproducts toward the target aldehydes solely by the introduction of an enzyme with opposing functional group activity. The potential of the optimized synthetic pathway, combined with a simple solid-phase extraction purification protocol, was demonstrated by the production of the aldol (3*S*,4*R*)-1,3,4-trihydroxy-5-phenylpentan-2-one from 2-phenyl ethanol and DHA on preparative scale yielding 70% pure aldol in short reaction times.

Since ‘substrate/redox funneling’ is fully compatible with other cascade type reactions and can be applied to reverse other byproduct forming reactions, it offers a novel and complementary flux optimization tool for synthetic enzyme cascades *in vivo*.

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Efficient enzymatic synthesis of inulooligosaccharides

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Inulooligosaccharides (IOS) can be found naturally in common foods such as chicory and Jerusalem artichoke. They contain $\beta(2-1)$ linked fructose units, with an average degree of polymerization (DP) of around 30. Plants need at least two distinct enzymatic activities, to catalyze priming and elongation reactions in inulin biosynthesis. Microbial fructansucrases of GH68 polymerize the fructose moiety of sucrose into fructans which possess either levan or inulin structures with $\beta(2-6)$ and $\beta(2-1)$ linkages, respectively. Various inulosucrase enzymes have been described. However, most of them synthesize large inulin polymers.

Inulin-type fructans are of particular interest due to their demonstrated pronounced *in vitro* prebiotic effects. Several other applications of inulin and inulooligosaccharides have been described. Inulin is used in the food industry as fat replacer, and for providing texture and stability in several products, such as desserts, bakery and fermented dairy products, as well as infant formula. Carbamoylated inulin has emulsifying properties, which could be potentially find use as biodegradable surface-active agent. Carboxymethylated inulin has the potential to be used as antiscalants, preventing the scaling of inorganic compounds (e.g., CaCO_3) from aqueous solutions.

Isolating of inulin from plant is difficult because of chain length variation. There is an increasing demand for inulin oligosaccharides and alternative procedures for synthesizing inulin are attractive. We identified a fructansucrase which efficiently synthesizes a broad range of inulooligosaccharides (GF3 – GF30), and no polymeric material, from sucrose very similar to plant derived inulin. Here we report on the molecular and biochemical characterization of this novel inulosucrase enzyme.

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Poster Abstracts
(in alphabetical order)

Probing the catalytic flexibility of HAP phytases

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Keywords: phosphatase, phytase, mutagenesis, inositol phosphates

AppA, the *Escherichia coli* 6-phytase¹ of the histidine acid phosphatase (HAP) family², has been well characterized and successfully engineered for use in animal feed supplementation. Despite this, the molecular mechanisms underlying its rather rigid preference for the initial site of cleavage of phytic acid (IP₆) at the 6'-phosphate is poorly understood. In contrast, multiple inositol polyphosphate phosphatases³ (MINPPs), also members of branch 2 of the HAP superfamily, demonstrate pronounced catalytic flexibility, catalysing mixed 1/3-, 5- and 4/6-phytase activities. To help shed light on this difference, the role of the catalytic proton donor residue in AppA has been investigated in comparison with that found in MINPPs. Three AppA active site mutants were generated by site-directed mutagenesis HDT amino acid sequence motif containing the presumed proton donor aspartic acid found in the wild type enzyme. In so doing, the MINPP-like HAE sequence motif was introduced and the profiles of inositol polyphosphate intermediates (IP) generated from phytic acid hydrolysis by these mutants were analysed by HPLC. Wild type AppA generates as major intermediate the IP₅ 4/6-OH species with a minor IP₅ 1/3-OH intermediate also detected. The HDE mutant (T305E) has a similar IP profile to the wild type but with increased preference for hydrolysis at the 4/6-position. The HAT mutant (D304A) lacks a proton donor and displays only 1% of the phosphate release activity of the wild type. Interestingly, this mutation almost abolishes 6-phytase activity and the mutant displays almost exclusive 1/3-phytase activity. Finally, the HAE double mutant (D304A, T305E) containing the MINPP-like proton donor motif shows an enhanced catalytic flexibility with diminished 4/6-phytase and enhanced 1/3-phytase activities. Taken together, these results provide the first evidence for the involvement of the proton donor motif in determining the initial site of attack of HAP phytases on their IP₆ substrate.

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Immobilization of dextranase on chitosan microspheres: An effective approach for increasing recycling efficiency & stability

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Keywords: Immobilization, dextranase, thermal stability, *Bacillus megaterium*.

Thermophiles are important natural resources for production of countless thermostable enzymes. These special microorganisms are isolated from numerous environmental niches including hydrothermal springs, volcanic eruption vents, subsurface petroleum reservoirs or compost piles. These thermostable biocatalysts are used in biotechnological processes because they can function under hostile environmental conditions. These constraints have diverted attention of investigators to discover novel enzymes. In the current study, local flora of a geothermal hot spring was explored for the detection of dextranase which is widely used in sugar industries to avoid production of dextran. Dextran lowers sugar production due to its gelling property. Hydrothermal spring isolate, *Bacillus megaterium*, was taxonomically and genotypically identified and was found proficient in synthesis of dextranase that hydrolyze dextran. Hydrolytic action was studied using surface topology of dextran after treatment with dextranase using scanning electron microscopy. To maximize dextranase activity and stability, a covalent crosslinking immobilization technique was employed using chitosan hydrogel microspheres. Results suggest that this technique allows the reuse of the catalyst and also facilitated its efficient recovery from reaction media with continuous operation. The technique involves the reduction in size of the carrier that provided a large surface area with better immobilization efficiency. Improvement in recycling efficiency, thermal stability and activation energy also distinctly improved, whereas, the anchoring of substrate at the active site of the native enzyme showed an increase in K_m with no change in V_{max} value after immobilization. Results suggest that higher titers of dextranase and its stability could contribute in the production of multiple isomalto-oligosaccharides that are used as prebiotics and could also prevent dextran contamination in sugar industries using immobilized biocatalyst.

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An engineered pathway using ω -transaminases to produce a novel amine

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Keywords: ω -transaminase, engineered pathways, amines, *Pseudomonas putida*.

The ability to engineer metabolic pathways provides the potential to create novel molecules that are difficult to synthesis chemically and would not otherwise be produced in nature. Here we have taken 3 enzymes from the TOL metacleaveage pathway which originates from *Pseudomonas putida*, the final product of this short truncated pathway is 2-hydroxymuconic-semialdehyde (figure 1). We have screened this aldehyde with a wide selection of omega-transaminases from our in house library of transaminases and observed conversion with several transaminases.

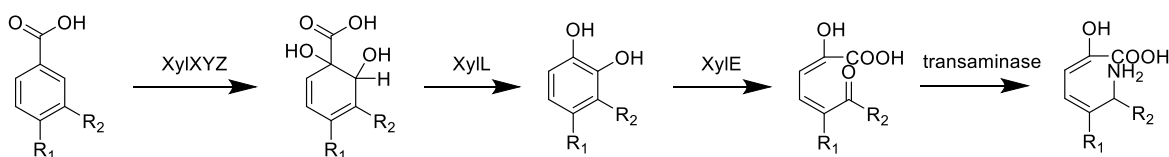


Figure 1. An engineered pathway comprised of a truncate of the TOL metacleaveage pathway and an omega transaminase to produce a novel amine molecule.

The pathway has been expressed with alternative starting substrates and with methylbenzylamine, serine and glutamine as alternative amine donors for the transaminase step.

An increase in the use of biocatalysis, both in research and in industry, will inevitably lead to a requirement of a wider range of host organisms to accommodate a more diverse range of possible reactions. With this in mind, the expression of the above engineered pathway in alternative organisms has been compared. Various strains of *Pseudomonas putida* have been compared to traditional *E. coli* expression and have shown comparable conversion to the 2-hydroxymuconic-semialdehyde.

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Vinyl sulfone-activated silica as a tool for covalent immobilization of alkaline unstable enzymes

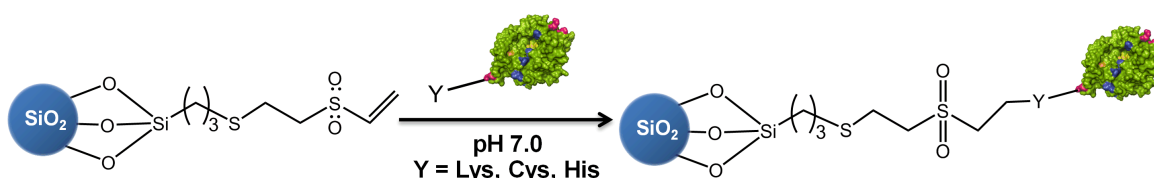
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Keywords: immobilization, enzyme stabilization, bioreactors, fructooligosaccharides.

Most methodologies for covalent immobilization of enzymes usually take place at high pH values to enhance the nucleophilicity of protein reactive residues; however, many enzymes inactivate during the immobilization process due to their intrinsic unstability at alkaline pHs. Vinyl sulfone (VS)-activated carriers may react with several protein side-chains at neutral pHs.¹



In this work, levansucrase -an alkaline unstable enzyme of technological interest because it forms fructooligosaccharides (FOS) and levan from sucrose- was covalently attached to VS-activated silica at pH 7.0 in a short time (5 h).² Theoretical immobilization yields were close to 95% but the apparent activity did not surpass 25%, probably due to diffusional restrictions and random attachment with unproductive orientations.

Due to diffusional hindrance, the immobilized levansucrase was unable to produce levan but synthesized a similar amount of FOS than the free enzyme [95 g/L in 28 h, with a major contribution of FOS of the $\beta(2\rightarrow1)$ type]. The VS-activated biocatalysts showed a notable operational stability in batch reactors. After 17 cycles, the biocatalyst conserved around 50% of its initial activity

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Metagenomics and transaminases: from the sample collection to the synthesis of valuable cinnamylamines

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Keywords: metagenomics, biocatalysis, transaminases, cinnamylamines.

The metagenome contains the DNA from all organisms in an environment, including all uncultivable bacteria and thus enables access to many genes for enzymes inaccessible by classical means. Our aim is to use a metagenomics approach to identify new biocatalysts,¹ in particular transaminases (TAMs) for the synthesis of chiral building blocks or bioactive molecules not easily accessible using classical organic chemistry.^{2,3}

In our approach, the DNA from an environment is extracted, analysed and sequenced. Subsequently a contiguous (contig) read library is generated *in silico* and formatted into a BLAST database. Once the library is created, it can be searched by enzyme type. Here, 11 putative TAMs from a tongue metagenomic sample have been identified, cloned and overexpressed. The 11 TAMs were screened as crude cell lysates using several assays against a set of substrates covering a wide structural diversity (aromatic, cyclic, aliphatic, functionalized ketones). Several TAMs have been identified with activities towards different substrates and the results will be presented. Available TAMs from the current UCL TAMs toolbox have also been screened against several interesting aromatic ketones. These results will also be presented.

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One-pot transaminase and transketolase system for the production of L-*gluco*-heptulose

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Keywords: transaminase, transketolase, HPA, one-pot

The use of biocatalysis for the synthesis of high value added chemical building blocks derived from biomass is becoming an increasingly important application of future sustainable technologies. The production of a high value added chemical [1] from L-arabinose, a major component of sugar beet pulp, using a transketolase enzyme coupled with a transaminase enzyme has been achieved here. The transketolase reaction requires the use of hydroxypyruvate (HPA) as a two-carbon donor and though commercially available, HPA is a relatively expensive compound limiting its usage on an industrial scale. Alternatively, HPA can be synthesised enzymatically using a transaminase enzyme. A sequential cascade of two enzymes (transaminase and transketolase) for the synthesis of HPA and L-*gluco*-heptulose from serine, α -ketoglutaric acid and L-arabinose as substrates was reported. When tested at 50 °C, the transaminase and transketolase enzymes were twice as active than at 25 °C. Once implemented, the two-step enzyme cascade could be performed in a one-pot system for the efficient production of L-*gluco*-heptulose.

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‘Substrate/redox funneling’ as a novel flux optimization tool for synthetic enzyme cascades *in vivo*

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Keywords: flux optimization, substrate/redox funneling, synthetic enzyme cascade, carboxylic acid reductase

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Since ‘substrate/redox funneling’ is fully compatible with other cascade type reactions and can be applied to reverse other byproduct forming reactions, it offers a novel and complementary flux optimization tool for synthetic enzyme cascades *in vivo*.

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Sucrose Synthase as Cost-Effective Mediator of Glycosylation Reactions

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Keywords: glycosyltransferases (GT), sucrose synthase (SuSy), fructansucrase (FS), small molecule glycosylation

Glycosyltransferases (GTs)¹ are extremely efficient biocatalysts that can be used for the synthesis of special carbohydrates and glycoconjugates. Unfortunately, the large-scale application of GT has been hampered by their low operational stability and by the high cost of their glycosyl donor. The FP7-project 'SuSy' aims to solve both problems by a combination of enzyme and process engineering. On the one hand, the production of stable biocatalysts will be facilitated by the development of a suitable expression system, the design of optimized variants and the development of immobilized formulations. On the other hand, the production and recycling of nucleotide-activated sugars will be accomplished by exploiting the reaction of sucrose synthase (SuSy)². With the help of SuSy, UDP-glucose can be produced from sucrose as cheap and abundant substrate. Furthermore, other nucleotide sugars can be obtained when sucrose analogues are employed. These alternative substrates will be produced here with fructansucrase (FS), an enzyme that can couple fructose to various monosaccharides. However, the activity of both FS and SuSy towards sucrose analogues will have to be improved to become economically viable. In that way, the proposed concept can be developed into a generic procedure, in which the nucleotide moiety can be recycled to establish a constant supply of glycosyl donor.

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Testing applicability of Catalytic Fields in enzyme design: artificial enzymes and their mutants

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Keywords: differential transition state stabilisation, catalytic field, de novo enzyme design

The general goal of our research is to develop from first principles new methodology aiding enzyme design, without involving empirical parameters. Following this *ab initio* approach, we base our proposal on Differential Transition State Stabilisation (DTSS) concept^{1,2}, which, contrary to conventional enzyme design techniques based on Transition State Stabilisation (TSS), takes into account interactions between catalytic environment and both transition state and reactants. Taking dominant electrostatic contribution to DTSS as the first approximation we obtain information on the topology of optimal catalyst charge distribution - Catalytic Field^{1,2}, which could be used for quick screening of mutants and possessing an intuitive interpretation. A point charge and cumulative atomic multipole³ representation of catalytic field are used in this work. We test this approach on artificial Kemp eliminase KE07 and its seven mutants⁴. To take into account the dynamic aspect of protein environment, we perform 30 ns molecular dynamics simulation of all of these enzymes, using produced trajectories to average the DTSS energy over an ensemble. Our findings suggest that qualitatively DTSS energy better agrees with experimental catalytic activities than standard transition state stabilisation TSS. These findings provide a clue to find a better *de novo* catalyst design.

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P450_{BM3} fused to phosphite dehydrogenase allows phosphite-driven selective oxidations

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Keywords: drug metabolites, enzyme catalysis, fatty acids, NADPH regeneration, oxidation, protein engineering.

To facilitate the wider application of the NADPH-dependent P450_{BM3} we fused the monooxygenase with a phosphite dehydrogenase (PTDH). The resulting monooxygenase-dehydrogenase fusion enzyme acts as a self-sufficient bifunctional catalyst, accepting phosphite as a cheap electron donor for the regeneration of NADPH.

The well-expressed fusion enzyme was purified and analyzed in comparison to the parent enzymes. Using lauric acid as substrate for P450_{BM3}, it was found that the fusion enzyme had a similar substrate affinity and hydroxylation selectivity while it displayed a significantly higher activity than the non-fused monooxygenase. Phosphite driven conversions of lauric acid at restricted NADPH concentrations confirmed multiple turnovers of the cofactor. Interestingly, both the fusion enzyme and the native P450_{BM3} displayed enzyme concentration dependent activity and the fused enzyme reached optimal activity at a lower enzyme concentration. This suggests that the fusion enzyme has an improved tendency to form functional oligomers.

To explore the constructed phosphite-driven P450_{BM3} as a biocatalyst, conversions of the drug compounds omeprazole and rosiglitazone were performed. PTDH-P450_{BM3} driven by phosphite was found to be more efficient in terms of total turnover when compared with P450_{BM3} driven by NADPH. The results suggest that PTDH-P450_{BM3} is an attractive system for use in biocatalytic and drug metabolism studies.

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Dioxygenases for inactivation of the virulence-associated *Pseudomonas aeruginosa* quinolone signal

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Keywords: *Pseudomonas aeruginosa*, cofactor-less dioxygenases, quorum quenching enzymes, alkylquinolone degradation.

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen often associated with patients suffering from cystic fibrosis or burn wounds. It regulates its virulence factor production via a complex quorum sensing network incorporating the signal molecules PQS (*Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1*H*)-quinolone) and HHQ (2-heptyl-4(1*H*)-quinolone) besides *N*-acylhomoserine lactone signals.

In order to interfere with quorum sensing (quorum quenching) and thereby reduce the virulence of *P. aeruginosa*, we searched for enzymes able to degrade PQS. We now have a pool of four cofactor-less dioxygenases differing in stability and catalytic activity towards PQS. All four enzymes share at least 40 % identical amino acid sequence.

The first dioxygenase found to be capable of cleaving PQS is an enzyme involved in 2-methylquinoline degradation by *Arthrobacter* sp. Rii61a termed Hod. It is very stable toward physical and chemical denaturing agents, however, it catalyzes the ring cleavage of PQS to CO and *N*-octanoylanthranilate with a specific activity of only 0.2 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹. Nevertheless, addition of the enzyme to *P. aeruginosa* cultures quenched the production of key virulence factors¹. In contrast to Hod, AqdC1 and AqdC2 from *Rhodococcus erythropolis* BG43² and AqdC from *Mycobacterium abscessus* (Aqd for alkyl quinolone degradation) show high activity towards PQS, with specific activities in the range of 15 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ (AqdC1) and 60 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ (AqdC). However, AqdC proteins are difficult to isolate and temperature sensitive. Moreover, all enzymes are susceptible to degradation by the *P. aeruginosa* exoprotease LasB.

To be applicable as quorum quenching enzymes, the proteins need to be stabilized by enzyme engineering, immobilization, or encapsulation. The characterization and comparison of the four PQS dioxygenases in terms of structure, catalytic properties, and stability open up new perspectives to develop a novel quorum quenching enzyme for reducing the virulence of *P. aeruginosa*.

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Reduction of C-C double bonds at high substrate loading using “new” old yellow enzymes at Johnson Matthey

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Keywords: ene reductase, reaction scale-up, enzyme characterization

Johnson Matthey has expanded the portfolio of biocatalysts to complement its diverse catalogue of metal based catalysts. Our current collection of wildtype and mutant enzymes covers the areas of ene reductases, alcohol dehydrogenases, transaminases, esterases and others. A sample of the best Johnson Matthey biocatalysts has been commercialised as an enzyme kit. Recently we have added three ene reductases, ENE-101, ENE-102 and ENE-103,¹ to our commercial offering

The substrate scope of these enzymes was characterized by testing the reduction of a number of alkenes activated by ketone, aldehyde, nitro and ester groups. Each enzyme showed a specific substrate scope pattern and many of the reactions proceeded to completion in a short time maintaining good enantioselectivities. Therefore reactions at high substrate loading were investigated.

At 50 mL scale, complete conversion was achieved with substrate loading of 0.75 and 1.5 M using dimethyl itaconate and 1-acetyl-1-cyclohexene as substrates. To readily provide reduced NADH cofactor, glucose/glucose dehydrogenase cofactor regeneration was mediated by the Johnson Matthey enzyme GDH-101.

Biotransformations using ene-reductase at these high substrates concentrations have little precedence in literature and prove the potential of these enzymes for synthesis of API and fine chemicals.

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QM/MM calculations reveal substrate scope and a new thiolate pocket of the unique arylpropionate racemase AMDase G74C

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Keywords: racemase, rational protein design, substrate scope, molecular modeling

Enzymatic racemization allows the smooth interconversion of stereocenters under very mild reaction conditions. Most racemases are restricted to specific natural substrates such as α -hydroxy aryl acetic acids and amino acid derivatives. The limited substrate spectrum and low activity of racemases for non-natural compounds represent obstacles for synthetic applications. Protein engineering can alleviate these issues.

The engineered cofactor-independent racemase AMDase G74C¹ is an interesting object for the mechanistic investigation of racemization of pharmacologically relevant derivatives of 2-phenylpropionic acid (profenes). The mechanism and substrate scope of the unique arylpropionate racemase AMDase G74C² was investigated by a multilevel quantum mechanics/molecular mechanics (QM/MM) approach.

The simulations revealed two thiolate pockets, enabling the reaction via a deprotonated cysteine. While the first plays a role in the natural decarboxylative activity of AMDase, the second stabilizes the artificially introduced thiolate group of C74. The presence of the two structural motifs is a prerequisite for the promiscuous racemization reaction of AMDase G74C. QM/MM simulations show that the deprotonation and reprotonation proceed in a stepwise fashion, in which a planar enedionate intermediate is stabilized by a delocalized π -electron system on a vinylic or aromatic substituent of the substrate.

The presented isomerization mechanism² is in line with the experimentally observed sharp pH-profile and substrate spectra of the designed catalyst AMDase G74C.

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Studies on chemoenzymatic synthesis of enantiomerically enriched δ -lactones

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Keywords: biocatalysis, enzymatic kinetic resolution, δ -lactones.

Lactones are vital class of organic compounds with many essential biological properties, among which the antimicrobial activity has been most studied. They have been found to be cytotoxic, stimulate apoptosis, inhibit HIV protease, play important role in insect world acting as a pheromones and be important flavor compounds of various foods and fruits^{1,2}.

Biological activity of lactones is strictly connected with substituents present in lactone structure as well as with its enantiomeric form. Hence developments of new, efficient and versatile strategies for synthesis of enantiopure lactones are high desirable³. Within many organic chemistry methods biocatalytic use of enzymes becomes a more and more popular. Mainly due to high substrate specificity, enantioselectivity and possibility of using nonconventional solvents like ionic liquids or organic solvents⁴.

In present work, we present studies on chemoenzymatic synthesis of enantiomerically enriched δ -lactones. A set of δ -substituted hydroxy esters has been synthesized and subjected to enzymatically catalyzed kinetic resolution and subsequent lactonization reaction. Possibility of use metal catalyzed racemization of substrates by various racemization catalysts has been tested. Our approach allowed to obtain δ -lactones with good yields and enantiomeric excesses.

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Directed evolution of a type A feruloyl esterase for increased thermostability and organic solvent tolerance.

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Keywords: feruloyl esterase, directed evolution, temperature and solvent resistance

Feruloyl esterases (FAE) are carboxylic esterases (EC 3.1.1) that cleave the bond between ferulic acid and L-arabinofuranosyl residues of arabinoxylans. These enzymes, in addition to helping the deconstruction of lignocellulosic biomass have a natural capacity to synthesize a wide range of bioactive molecules with interesting properties (notably anti-oxidant) in the fields of food, pharmaceuticals or cosmetics.¹

Within the OPTIBIOCAT European project, a methodology to express, generate and screen diversity for *Aspergillus niger* feruloyl esterase A was developed, tested and implemented in a high-throughput fashion, using *ad hoc* chromogenic probes.² A total of ~10,000 mutant clones were generated and screened to isolate 13 mutants with improved thermal and/or solvent stability. The most interesting mutations were individually studied while their random recombination was performed in a second round of evolution through Staggered Extension Process (StEP).

In this poster, we will describe our two-step selection protocol and the best mutants, in terms of thermal and solvent resistance, transfer ability and catalytic properties.

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The re-evaluation and isolation of a novel Michael hydratase from *Rhodococcus rhodochrous* ATCC 17895

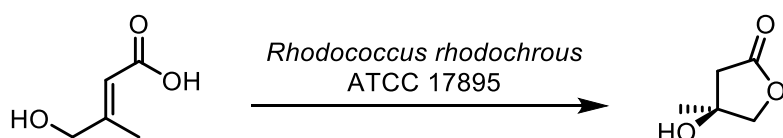
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Keywords: Michael hydratase, *Rhodococcus rhodochrous*, enantioselective water addition, chiral alcohols

The enantioselective addition of water to α,β -unsaturated carbonyl (Michael) acceptors leading to chiral alcohols is rated as a chemically challenging but highly desirable reaction.¹ Based on a short notification in 1998,² it was recently reported that whole-cells of *Rhodococcus rhodochrous* ATCC 17895 convert model substrate 4-methyl-2(5H)-one to give (S)-4-hydroxy-4-methylfuranone in high enantiomeric excess.³

However, present NMR studies revealed the model substrate structure to be (E)-4-hydroxy-3-methylbut-2-enoic acid instead while the described product formation was confirmed.



Due to these new results, a re-evaluation of the enantioselective water addition using whole-cells was carried out. Additionally, the isolation of the Michael hydratase from *Rhodococcus rhodochrous* ATCC 17895 is in progress.

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Artificial metalloenzymes: Towards *in vivo* catalysis

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Keywords: artificial metalloenzymes, directed evolution, whole cell catalysis, enantioselective catalysis.

There is a growing interest in implementing organometallic catalysis in the context of synthetic biology for sustainable production of chemicals. Some of the recent achievements in this field include development of bio-compatible cyclopropanation by Arnold and Balskus groups[1,2]. As a first step towards interfacing microbial metabolism we aim to utilize Artificial metalloenzymes (ArMs) to perform catalysis in the cell to augment cellular bio-synthesis.

ArMs are a blend of homogeneous and enzymatic catalysts. The Roelfes group has focused on Lactococcal multi-drug resistance regulator, a transcription factor with a large hydrophobic pocket, to create a novel class of ArMs by using diverse anchoring strategies including covalent, supramolecular and biosynthetically incorporated unnatural amino acid (UAA) [3,4]. Here we will show you our progress towards achieving catalysis by ArMs in living cells.

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High overexpression of dye decolorizing peroxidase *TfuDyP* leads to the incorporation of heme precursor protoporphyrin IX

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Keywords: dye decolorizing peroxidase, *Thermobifida fusca*, protoporphyrin IX, heme

Abstract

Heterologous overexpression of the dye decolorizing peroxidase from *Thermobifida fusca* (*TfuDyP*) is extremely high (~250 mg per liter culture broth) when expressing it fused to the small ubiquitin-related modifier protein (SUMO). In contrast to the poorly expressed native *TfuDyP*¹, overexpressed SUMO-*TfuDyP* was almost inactive. Analysis of the enzyme by UV-vis absorption spectroscopy and high-resolution mass spectroscopy showed that a large fraction of the overexpressed enzyme contained the iron deficient heme precursor protoporphyrin IX (PPIX) instead of heme. Here we show that the heme to PPIX ratio and thus the activity of the enzyme was dependent on the protein expression level.

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Tapping into the synthetic potential of enolizing enzymes

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Keywords: enolizing enzymes, structural enzymology, coenzyme A

Enolates are exploitable reaction intermediates in the synthesis of chiral compounds. However, the need for strong bases and the difficulty to achieve a stereoselective synthesis deters from their appeal as a synthetic route. Enolizing enzymes achieve the formation of an enolate intermediate under very mild conditions, and stabilize it long enough to make it react with an electrophile in a stereoselective fashion. Enoyl-coenzyme A isomerases (ECIs) are CoA-dependent enolizing enzymes converting a 3*E*- or 3*Z*-enoyl-CoA substrate into its 2*E*- diastereoisomer, an intermediate of the β -oxidation lipid-degrading pathway. The ECI reaction mechanism is a hydrogen-bond assisted enolization. An active site feature described as the oxyanion hole stabilizes the enolate, allowing a weak base such as a carboxylate to perform the proton abstraction. The current project aims to modify well-studied ECI systems, adapting them to accept prochiral substrates. The research work draws on previous structural data obtained from the yeast (ScECI2) and the human (HsECI2) peroxisomal ECIs^{1,2}. The generation of suitable functional variants will be driven by performing MD simulation and residue interaction network analysis on previous and new enzyme-substrate or -ligand complexes. Structure-functional work will be used as the template to further develop a family of organocatalysts mimicking oxyanion holes (synthetic oxyanion holes - 'SOX' - catalysts), which are being synthesized by a collaborating group.

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Oleate hydratase-catalyzed hydration of short-chain fatty acids and alkenes

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Keywords: oleate hydratase, selective water addition, reaction optimization, upscaling

The selective addition of water is a challenging issue in synthetic organic chemistry and requires harsh conditions. In nature, oleate hydratases (EC 4.2.1.53) catalyze the cofactorindependent regioselective hydration of oleic acid to (*R*)-10-hydroxyoctadecanoic acid. In 2009, the oleate hydratase from *Elizabethkingia meningoseptica* (*Em*-OAH) was biochemically characterized¹ and only recently the crystal structure was solved.² Although no net redox change is involved in catalysis, the enzyme does contain a flavin cofactor, whose function remains obscure. From an industrial point of view, the application of cofactor-independent enzymes is highly desirable as it facilitates an easy handling of *in vivo* and *in vitro* systems without the utilization of cofactor recycling systems.³ *Em*-OAH was selected as a promising candidate for the stepwise engineering towards the hydration of smaller molecules including fatty acids and alkenes. The enzyme showed high activity and broad substrate specificity towards the hydration of various fatty acids (C18- C14). Although a minimum chain length of 14 carbon atoms is described to be essential for conversion³, shorter fatty acids (C10-C11) were tested as substrates. Low activity was observed towards (*Z*)-undec-9-enoic acid (C11), which was chosen as a model substrate for the improvement of reaction conditions regarding the hydration of non-natural substrates. Conversion was strongly increased applying MODDE for statistical experiment design.

Optimized conditions were used for an upscaling of (*Z*)-undec-9-enoic acid conversion to 10-hydroxyundecanoic acid that was verified by GC MS and NMR analysis. Moreover, the improved conditions enabled the hydration of 1-decene by *Em*-OAH representing the next step towards the conversion of short-chain molecules.

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Oxygen supply to biooxidation reactions

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Keywords: oxidases, oxygen supply methods, bubble-free aeration.

Oxygen-dependent enzymes are becoming increasingly relevant in the synthesis of fine chemicals, flavors and fragrances as well as pharmaceutical intermediates. Oxidases can use molecular oxygen as an electron acceptor, the most inexpensive and innocuous oxidant available. One of the drawbacks when using isolated enzymes in oxidation reactions, is their insufficient stability under relevant reaction conditions at industrial scale (1). In particular, oxygen-dependent enzymes may deactivate at gas-liquid interfaces due to interactions with the interface of air/oxygen bubbles. Moreover, for biooxidation processes the mass transfer of oxygen from the gas to the liquid phase has proved to be a limiting factor (2,3). In order to develop an industrial process using these enzymes, the oxygen supply to the reaction is a critical factor to be taken into account. Bearing all of this in mind, bubble-free oxygenation must be considered. This contribution presents different bubble-free oxygenation methods for in situ generation of oxygen using membranes or enzymes such as chlorite dismutase (Cld) (4) or catalase, by the supply of chlorite and hydrogen peroxide, respectively. Process considerations of the use of these methods will be addressed together with a comparison with the traditional oxygen supply by bubbling.

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The halohydrin dehalogenase enzyme family – a 2016 update

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Keywords: enzyme discovery, dehalogenation, database mining

Halohydrin dehalogenases are biotechnologically versatile enzymes which provide direct synthetic access to a large number of diverse compounds such as epoxides, β -substituted alcohols, and other pharmaceutical precursors.¹ Consequently, these enzymes have been exploited in various industrially relevant processes. The multitude of explored reactions were developed with only five different enzymes (HheA/A2, HheB/B2, HheC) recombinantly available.

To make more members of this enzyme family available for biotechnological research and application, an in-silico database mining approach resulted in the identification of 37 novel halohydrin dehalogenases.² The data presented will provide a 2016 update of the halohydrin dehalogenase enzyme family and will highlight biochemical properties of selected enzyme representatives.³

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Improving the affinity of a bacterial Sucrose Synthase for UDP by introducing plant residues

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Keywords: Biocatalysis, Carbohydrates, Sucrose Synthase, UDP-glucose recycling

Sucrose Synthase (SuSy) catalyzes the reversible conversion of sucrose and a nucleoside diphosphate (NDP) into NDP-glucose and fructose. Biochemical characterization of several plant and bacterial SuSys has revealed that the eukaryotic enzymes preferentially use UDP whereas prokaryotic SuSys prefer ADP as acceptor. In this study, SuSy from the bacterium *Acidithiobacillus caldus*, which has a higher affinity for ADP as reflected by the 25-fold lower K_m value compared to UDP, was used as a test case to scrutinize the effect of introducing plant residues at positions in a putative nucleotide binding motif surrounding the nucleobase ring of NDP. All eight single to sextuple mutants had similar activities as the wild-type enzyme but significantly reduced K_m values for UDP (up to 60 times). In addition we recognized that substrate inhibition by UDP is introduced by a methionine at position 637. The affinity for ADP also increased for all but one variant, although the improvement was much smaller compared to UDP. Further characterization of a double mutant also revealed more than two-fold reduction in K_m values for CDP and GDP. This demonstrates the general impact of the motif on nucleotide binding. Furthermore, this research also led to the establishment of a bacterial SuSy variant that is suitable for the recycling of UDP during glycosylation reactions. The latter was successfully demonstrated by combining this variant with a glycosyltransferase in a one-pot reaction for the production of the C-glucoside nothofagin, a health-promoting flavonoid naturally found in rooibos (tea).

Expanding the toolbox of the artificial enzymes using p-aminophenylalanine

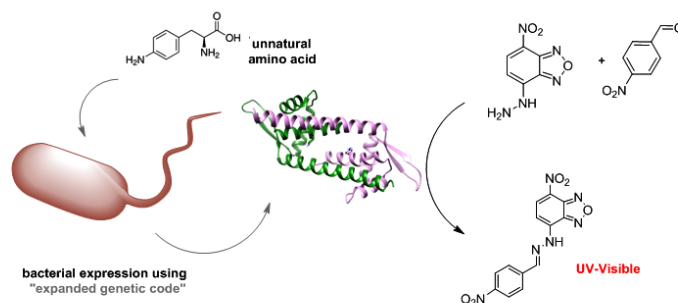
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Keywords: artificial enzymes, novel reactivity, unnatural amino acids, catalysis

Genetically encoded unnatural amino acids offer great possibilities for construction of artificial enzymes. Different novel enzymes containing metal chelators, redox mediators, and click chemistry reagents have been described¹. Recently, the metal-binding unnatural amino acid (2,2'-bipyridin-5yl)alanine has been introduced into the transcription factor LmrR, which contains a hydrophobic pocket at its dimer interface. Upon a Cu(II) binding, this complex was employed successfully as a novel artificial metalloenzyme in catalyzing asymmetric Friedel-Crafts alkylation, giving rise to good *ee*'s and conversions².

Herein, we introduce a novel artificial enzyme based on p-aminophenylalanine (pAF) as active site residue. The side chain of this amino acid, aniline, is known to act as an efficient nucleophilic catalyst in hydrazone or oxime formation³. To date, this unnatural amino acid has not yet been utilized in the catalysis. Several different positions located inside the hydrophobic pocket of LmrR were mutated to incorporate pAF. The resulting artificial enzymes were capable to catalyze formation of hydrazone faster than traditional aniline-based catalysts with significantly lower catalyst loading needed. Differences in reaction rate depending on the position of pAF in the scaffold were observed, with V15pAF being the best. A detailed mechanistic study of the system is currently being performed in order to develop this novel catalyst further.



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Furfurylamines from biomass: transaminase catalyzed upgrading of furfurals

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Keywords: Furfural, transaminase, biocatalysis, biomass

In this work transaminases (TAMs) have been investigated as a mild sustainable method for the amination of furfural and derivatives to access furfurylamines. Furfural is recognised as an attractive platform molecule for the production of solvents, plastics, resins and fuel additives.¹ Furfurylamines have many applications as monomers in biopolymer synthesis and for the preparation of pharmacologically active compounds.² Preliminary screening with a recently reported colorimetric assay highlighted that a range of furfurals were readily accepted by several transaminases and the use of different amine donors was then investigated.³ To demonstrate the potential of using TAMs for the production of furfurals, the amination of selected compounds was then investigated on a preparative scale.

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Directed enzyme evolution using insertions and deletions

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Keywords: insertions and deletions, evolutionary mechanisms, directed evolution, protein engineering.

In Nature, proteins evolve and acquire new functions by accumulating mutations. Substitutions and InDels (Insertions and Deletions), as well as circular permutations and rearrangement of protein domains, account for the majority of evolutionary changes. While the effects of substitutions have been extensively studied and documented, understanding the structural and functional effects of InDels still remains a challenge. InDels are assumed to be highly deleterious mutations because they are more likely to disrupt the structural integrity of proteins than are substitutions. On the other hand, they may induce significant structural changes that substitutions alone cannot cause and thus are believed to be key players in many natural evolutionary processes, such as the modification of active site loops to generate new enzyme functions¹ or the emergence of new protein structures².

We aimed at performing directed protein evolution by randomly incorporating InDels to investigate how they would be tolerated and whether they could be selected for functional improvements. Starting from a previously reported methodology³, we developed a library construction approach to randomly incorporate InDels within a DNA sequence of interest and applied it to generate InDel variant libraries of a promiscuous enzyme. We screened the resulting libraries (i) to study the impact of InDels on the parental enzyme, (ii) to identify adaptive InDels improving a new (or promiscuous) activity and (iii) to investigate the interaction between InDels and substitutions in an adaptive process. Our results show that, while being generally more deleterious than substitutions, InDels can also lead to functional improvements and may allow access to alternative evolutionary trajectories.

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Structure-based reaction mechanism of oleate hydratase from *Elizabethkingia meningoseptica*

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Keywords: Oleate hydratases, protein structure, enzyme mechanism, flavin cofactor.

Hydratases provide access to secondary and tertiary alcohols by regio- and/or stereospecific addition of water to carbon-carbon double bonds. Thereby, hydroxyl groups are selectively introduced without the need for costly co-factor recycling. A number of chemical hydration reactions are impossible currently, for example the regioselective hydration of the *cis*-9 double bond of oleic acid to yield (*R*)-10-hydroxy stearic acid, which is the reaction performed by oleate hydratases¹. Currently, the applicability of hydratases on an industrial scale is limited primarily by their narrow substrate scope and by restricted information on protein structure and mechanism. Here, the recombinant oleate hydratase originating from *Elizabethkingia meningoseptica* was biochemically and structurally characterized in the presence of the flavin cofactor. Remarkably, the redox state of FAD does play a role in the bioconversion of oleic acid to (*R*)-10-hydroxy stearic acid. Deprivation of FAD abolished hydration activity. Reduction of the cofactor to FADH₂ enhanced the turnover rate of the reaction by roughly one order of magnitude. Rational amino acid exchange experiments strongly suggest that E122 acts as base and that Y241 concomitantly provides protons in this concerted reaction. Based on the highly conserved regions among oleate hydratases, we are confident that our findings will pave the way for developing this enzyme class for industrial applications in the near future.

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Design of chromogenic probes for identification and evaluation of heteroxylan active enzymes

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Keywords: hydrolysis, CAZymes, screening, indolyl and nitrocatechol substrates.

Enzyme discovery is being increasingly empowered by high-throughput approaches, such as metagenomics and directed evolution techniques, providing vast reservoirs of new enzymes. Consequently, the bottleneck has gradually been shifted to adequate screening and precise characterization of newly identified biocatalysts. In this respect, chromogenic substrates are particularly appropriate because they provide quick and easy detection of enzyme activity at the microbial colony level and in liquid assays, and are compatible with automated strategies.

Heteroxylan-related biocatalysis is still a challenging topic, requiring the discovery and characterization of a wide range of hemicellulases, which are important enzymes for biomass hydrolysis.¹ Indeed, heteroxylans are chemically and structurally complex polysaccharide that forms up to 35% of the dry weight of hardwoods and cereals, and participate in the cross-linked network of the plant cell walls resulting in its mechanical resistance towards enzymes. A library of indolyl and nitrocatechol substrates has thus been designed as *ad hoc* tools for function-based screenings that can be used for prospecting and evaluating arabinoxylan active enzymes.^{2,3} Herein, we will detail this work concerning the synthesis and the uses of these practical and valuable substrates.

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Structure inspired use of a thermostable Baeyer-Villiger monooxygenase as biocatalyst

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Keywords: Baeyer-Villiger Monooxygenase, crystal structure, steroids

Baeyer-Villiger monooxygenases (BVMOs) are flavoenzymes catalyzing the oxygenation of aldehydes and ketones to the corresponding acids, esters and lactones. Enzymatic catalysis with molecular oxygen as co-substrate represents an attractive alternative to the chemical route using hazardous peroxy acids¹. Reactions performed by BVMOs are usually regio- and stereoselective and reported substrates are aromatic, linear or cyclic ketones and other industrially relevant biomolecules like steroids. Moreover, these enzyme can oxygenate heteroatom containing compounds and perform epoxidations². However, a major drawback can be their limited stability, which hampers their exploitation as industrial biocatalysts. The only exception, the highly thermostable phenylacetone monooxygenase (PAMO; EC 1.14.13.92) from *Thermobifida fusca*, is rather limited in substrate scope. Efforts to engineer activity on compounds yielding high value products as well as the opposite approach – engineering BVMOs with desired catalytic activity towards more stability – showed only limited success so far³.

We screened for BVMO-encoding genes in the sequenced genome of *Myceliophthora thermophila*, a thermophilic fungus synthesizing cellulolytic enzymes that were already commercialized⁴. A candidate gene was selected and the corresponding protein expressed in *Escherichia coli*. Stability assays confirmed the enzyme's tolerance to heat and organic solvents. Elucidation of the crystal structure of the enzyme allowed a structure inspired biocatalytic analysis. This resulted in the identification of bulky carbonyl compounds as substrates. By producing the BVMO fused to a co factor recycling phosphite dehydrogenase, full conversion of large cyclic ketones and several steroids into the corresponding lactones could be achieved.

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Optimization of the iodine assay: Towards a more convenient and fast procedure

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Keywords: amylases, branching enzymes, enzyme assay, method

Abstract

The iodine assay (1) is a frequently used method for determining the activity of starch converting enzymes, such as α - and β -amylases, branching enzymes and other amylolytic enzymes. Its detection is based on the formation of a coloured starch-iodine complex caused by the binding of iodine molecules to linear stretches of α -1,4 linked glucans present in a helix conformation in an approximate ratio of one iodine molecule to six glucose units (2). The length of the glucan chains affects the wavelength of maximum absorption, shifting it from 630 nm for potato amylose to 560 nm and 460 nm for amylopectin and glycogen, respectively (2, 3). The decrease in colour in response to enzymatic degradation of α -1,4 linked glucan chains over time can be used as an indirect measurement of enzyme activity. This assay can be also be used for studying the activity of branching enzymes as the introduction of new branch points due to the transfer of chains upon cleaving α -1,4 linkages followed by formation of α -1,6 bonds decreases the length of the linear stretches of α -1,4 linked glucans (1).

Despite its abundant use as fast activity measurement (4-6), optimizations are sparse. We set out to increase the convenience of the iodine staining assay by adapting it to microtiter plates, making it possible to perform multiple assays simultaneously as well as detection at sequential, short time points (30 sec) to increase the obtained information and accuracy of the method. The use of stable substrate stock solutions (7) and connection to a heating system further increased both speed and convenience. Finally, the low amount of required enzyme ($\sim 2 \mu\text{g}/\text{sample}$) and short length of the assay (15 min) allow a wide array of applications.

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Chasing enzymatic promiscuity by exploring empty space: Identification of enzymatic activity by mining structural databases

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The exploitation of catalytic promiscuity and the application of de novo design have recently opened the access to novel, non-natural enzymatic activities. Here we show a structural bioinformatic method for predicting catalytic activities of enzymes based on three-dimensional constellations of functional groups in active sites ('catalophores')¹ or cavities of active sites represented as point-clouds annotated with various physico-chemical properties. As a proof-of-concept we identify two enzymes with predicted promiscuous ene-reductase activity (reduction of activated C–C double bonds) and compare them with known ene-reductases, that is, members of the Old Yellow Enzyme family. Despite completely different amino acid sequences, overall structures and protein folds, high-resolution crystal structures reveal equivalent binding modes of typical Old Yellow Enzyme substrates and ligands. Biochemical and biocatalytic data show that the two enzymes indeed possess ene-reductase activity and reveal an inverted stereopreference compared with Old Yellow Enzymes for some substrates. This method could thus be a tool for the identification of viable starting points for the development and engineering of novel biocatalysts.

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Deletion of domain 3 of a novel laccase by random mutagenesis: Understanding the structure-activity relationship.

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Keywords: Bacterial laccase, Random mutagenesis, T1 copper center, Deinking

Laccase is a multicopper enzyme, requires oxygen to catalyze its substrates. Publications are getting doubled each year on bacterial laccases due to their advantage over fungal laccases. However, one of the limitations of using bacterial laccases is the requirement of costly mediators. Recycling of waste papers provides low-cost fiber and conserves forest trees. Recently, in our laboratory, waste paper deinking was reported without mediator using a novel laccase from *Rheinheimera* sp. In order to characterize it, the laccase coding gene was amplified (1.8 kb), cloned and over-expressed in *E. coli* BL21 using pET-28a vector. Induced band at position 70kDa (RhLacc) was observed which was further purified using one step purification i. e. Ni-NTA column. Random mutagenesis of RhLacc was done. Sequencing of randomly selected clones was done. Sequence of one clone showed that a stop codon formed after copper domain 2 at position 1101bp thus showing the truncation and absence of domain 3 that contains T1 copper center. It is known that redox potential of T1 center is the key parameter for substrate oxidation. Overexpression of this mutant clone showed induced band at position 40kDa (Δ RhLacc). Comparison of enzyme activities of the expressed RhLacc and Δ RhLacc was done. Δ RhLacc exhibited laccase activity only in the presence of additional copper contrary to RhLacc which showed laccase activity without additional copper ions. Optimum temperature for RhLacc and Δ RhLacc was same i. e. 55°C. However, optimum pH varied for some substrates. Effect of metal ions and organic solvents was also studied. Deinking was performed with RhLacc and Δ RhLacc and both the enzymes were able to deink the paper without mediator. This suggests that the novel property to deink the waste paper without mediator does not depend on the redox potential of T1 but other mechanisms involving domains 1 and 2 may be involved.

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Artificial metalloenzymes by *in vivo* incorporation of metal-binding amino acids

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Keywords: provide four keywords separated by commas.

Artificial metalloenzymes are emerging as an important tool in enantioselective catalysis. They are created by incorporation of catalytically active transition metal complexes into a biomolecular scaffold. The chiral second coordination sphere provided by the scaffold is a key contributor to the rate acceleration and enantioselectivity achieved in a variety of catalytic asymmetric reactions.

Three different approaches are typically used for the insertion of the metal into the scaffold: covalent, supramolecular and dative approach. Recently our group has introduced a new methodology, the *in vivo* incorporation of unnatural amino acids that are capable to bind transition metal ions, by means of the expanded genetic code methodology.

The development of the artificial metalloenzyme requires:

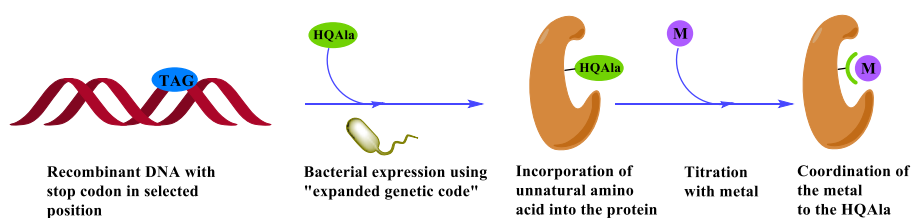
The synthesis of the unnatural amino acid (hydroxyquinolinalanine, HQAla)

The optimization of the sequence of the biological scaffold (the dimeric protein LmrR) by site directed mutagenesis at the selected amino acids of study

Bacterial expression of LmrR with the incorporated HQAla

Titration of the protein with Iron

Catalytic studies



Here we show the recent developments towards an artificial oxygenase, using this novel methodology.

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Towards large scale production of vanillyl alcohol oxidase biocatalysts

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Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is a covalent flavoprotein that has emerged as a promising biocatalyst for the production of aromatic fine chemicals such as vanillin, coniferyl alcohol and enantiopure 1-(4'-hydroxyphenyl) alcohols. The large-scale production of this eukaryotic enzyme in *Escherichia coli* has remained challenging thus far. For that reason an alternative, eukaryotic expression system, *Komagataella phaffii* (formerly known as *Pichia pastoris* or *Komagataella pastoris*) was tested. Additionally, to produce novel VAO biocatalysts, we screened fungal genomes for VAO homologs. Expression of the putative *vao* genes in *K. phaffii* was successful, however expression levels were low (1 mg per litre of culture). Surprisingly, all purified enzymes were inactive and found to contain a non-covalently bound anionic FAD semiquinone. Re-oxidation of the flavin cofactor could only be achieved upon protein denaturation. Expression of a codon optimised, his-tagged VAO in *E. coli* was more successful, reaching levels of about 26 mg of fully active enzyme per litre of culture.

Efficient phosphate recovery from agro waste streams by enzyme, strain, and process engineering

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Keywords: phytase, phosphate recycling, glycosylation, *Pichia pastoris*

Phosphate has been essential to feeding the world since the Green Revolution, due to its phosphate-hungry high yielding crop varieties. Currently phosphate is produced by phosphate rock mining and brought to plants in form of fertilizer, but phosphate rock is an irreplaceable and finite resource. Therefore stewardship and recycling of phosphate has to be tackled.

We plan to implement a new value chain to recover phosphate from plant waste material, to convert it to polyphosphates of industrial value and bring it back to the plants in form of polyphosphates. The process is divided into three steps. In the first step phytate – the insoluble storage form of phosphorus in plants – is degraded by phytases to soluble phosphate, which is then in the second step taken up by yeast and stored in form of polyphosphate. In the third step the synthesized polyphosphate is recovered from yeast strains. Thereby, the current conventional value chain of phosphate rock mining, production of phosphoric acid, chemical synthesis of polyphosphates, can be replaced by a novel value chain, which is sustainable.

For the proposed bioprocess, optimized phytases are needed, since naturally occurring phytases do not fulfill the requirements. One important requirement is a high thermal stability, since elevated temperatures enhance solubility and hydrolase activity. Thermal resistance can be positively influenced by the degree of glycosylation¹. Unfortunately, a detailed understanding of glycosylation on a metabolic and molecular level is missing. Here, we compare different positions and numbers of glycosylation sites via the example of the phytase AppA from *Escherichia coli* produced in *Pichia pastoris* to obtain a deeper molecular understanding of interactions between glycosylation, thermal stability and activity of a phytase.

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Engineering a set of hybrid NRPS using a Golden Gate-based subdomain swap approach

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Keywords: NRPS, bioengineering, natural products, Golden Gate shuffling

Nonribosomal peptide synthetases (NRPS) are large multi-modular enzymes that produce a very broad class of secondary metabolites: nonribosomal peptides (NRPs). These natural products show a wide spectrum of biological and pharmaceutical activities (antibiotic, immunosuppressant, anticancer, etc.), thus they are of great interest to biotech and pharmaceutical companies. Given their modular organization and their mechanism of action, NRPS are considered a high-potential target for engineering experiments, with the aim of generating hybrid enzymes able to synthesize novel (or modified) compounds. Unfortunately, attempts made in the past decades have been rarely successful.

Recently, a new engineering strategy has been developed, which targets at a small region (“subdomain”) that defines the substrate specificity in a single-module NRPS [1]. This strategy proved to be quite successful, resulting in the generation of several hybrid NRPS capable to activate their “new” substrate. Furthermore, one of these hybrids was actually able to interact with the following module of the NRPS system, and incorporate the substrate into the final predicted NRP. Therefore, the potential benefit of this method is that the substrate specificity can be changed without affecting the interdomain interactions.

In this work we adapted the subdomain swapping method and developed a Golden Gate-based system which we successfully used to build six hybrid NRPS genes. Remarkably, the hybrid NRPS were overexpressed in *E. coli* with a high yield. The hybrids were subsequently subjected into *in vitro* peptide production assays, followed by Mass-Spectrometry analysis to assess their ability to produce the predicted compounds.

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Optimization of transfructosylation reaction for production of fructooligosaccharides catalyzed by recombinant levansucrase from *Leuconostoc mesenteroides* Lm 17

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Keywords: levansucrase, fructooligosaccharides, prebiotics, *Leuconostoc mesenteroides*.

Levan-type fructooligosaccharides are of increasing interest because of their potential health benefits to selectively support the intestinal health. Levansucrase (EC 2.4.1.10), which belongs to the glycoside hydrolase family 68 (GH68), catalyzes the synthesis of different types of fructooligosaccharides using sucrose as a donor and different monosaccharides and disaccharides as acceptors.

Leuconostoc mesenteroides Lm 17 produces levansucrase of about 120 kDa and dextransucrase of about 180 kDa. The encoding levansucrase gene from this strain was cloned and expressed in *Escherichia coli* BL21. The recombinant enzyme, called L17, was shown to be 1022 amino acids-long protein with high homology to levansucrase LevS from *L. mesenteroides* NRRL B-512F strain. *In situ* analysis showed an active band of 120 kDa, similar to the one produced by native strain. The optimal conditions for action of enzyme were determined at pH 5.5 and 35 °C. K_m and V_{max} of L17 were at 64 mM of sucrose and 12 U/mg of enzyme. The synthesized fructan fraction was identified as levan. Recombinant levansucrase was evaluated in acceptor reactions with different sugar acceptors. Additionally the effects of acceptor concentration, enzyme concentration and the presence of a co-solvent in the fructosylation efficiency of sugar acceptors were evaluated. It was demonstrated that this reaction is kinetically control, producing the best yield of kestose and nystose when 10% of acceptor and 2u/ml of enzyme were employed. Increased amount of DMSO as co-solvent up to 20% (v/v) reduce enzyme activity more than 30%.

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Co-expression analyses of *P. carnosus* to identify new proteins relevant to biomass conversion

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Keywords: *Phanerochaete carnosus*, co-expression, genes, biomass

Phanerochaete carnosus has been isolated from softwood, and as such, may express unique gene products for lignocellulose conversion.

P. carnosus was thus cultivated on white spruce (*Picea glauca*) and trembling aspen (*Populus tremuloides*); corresponding RNA was extracted from five time points and then sequenced. Predicted gene models were successfully assembled by Novoalign, annotated, and then clustered according to expression profile.

The cultivation of *P. carnosus* on both aspen and spruce led to comparatively high levels of transcript sequences corresponding to three manganese peroxidases (MnP) implicated in lignin transformation. For all three MnP's, expression levels increased over time on both substrates; however, earlier and higher expression was observed on spruce. Transcript sequences encoding two AA3 and one AA5 followed the MnP's in terms of relative abundance of CAZyme sequences. Notably, for both aspen and spruce cultivations, transcripts corresponding to only one lytic polysaccharide monooxygenases (AA9) were highly expressed, and levels remained steady over time. Overall, transcripts predicted to encode hydrolytic enzymes were lower in abundance than those encoding the activities described above. Moreover, the expression profiles of sequences encoding GH3, GH5, GH7, GH10, and GH16 activities were similar in *P. carnosus* cultivated on both substrates. Main differences were observed for sequences encoding GH6 and GH2 enzymes, which were more abundant in cultivations grown on aspen and spruce, respectively. All in all, similar subsets of genes from a given CAZyme family were expressed on both substrates. Time course analyses, however, clarified which those sequences responded most to changes in the substrate composition, and will be used to identify sequences encoding proteins with unknown function that are co-expressed with known lignocellulose-active CAZymes. Protein production and characterization of carefully selected candidates of aforementioned genes that co-express with predicted lignocellulose active enzymes could lead to discovery of new enzyme families relevant to lignocellulose conversion.

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Engineering P450 monooxygenase to make a fused Tetrahydrofuran Ring

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Keywords: Biotransformation, Biocatalyst, P450, Aspidoalbine, Tetrahydrofuran

In our search for a natural compound to inhibit protein misfolding, we recently came across a polycyclic alkaloid, a derivative of aspidoalbine, extracted from a plant, which showed a promising bioactivity towards protein aggregation. In these polycyclic compounds, a fused tetrahydrofuran (THF) ring makes up the sixth ring. Similar compounds of aspidospermine origin recently have been under spot light as antimalaria drugs. We have become interested to use biotransformation and engineering for synthesis of the fused THF ring. In selecting for proper biocatalyst, P450 BM 3, as a versatile catalyst and P450 AurH monooxygenase which catalyzes sequential hydroxylation and hetrocyclization from deoxaureothin, were initially considered. After performing molecular docking to examine which enzyme can best bind the precursors in its active site, P450 AurH was chosen as the candidate enzyme. Initially, the recombinant enzyme P450 AurH was expressed and its activity was assessed in the whole cell extract using various polycyclic compounds as substrates. In order to alter the substrate specificity for the enzyme, site directed mutagenesis is being used to create novel mutations that enable the enzyme to recognize polycyclic compounds. In this rational design, amino acids in the vicinity of heme binding domain of the enzyme are converted to Ile, Trp, and Tyr residues. The rationale behind these specific changes is somewhat similarity of the aspidoalbine to the cholesterol backbone, and that the cholesterol binding motif is enriched in the aforementioned amino acid residue. Each mutant enzyme will be purified and its activity and kinetic parameters will be measured using GC mass spectrometry. Since there has not been much information on the exact nature of enzymes involved in the biosynthesis of aspidoalbine derivatives, creating novel enzyme that perform fused THF on these polycyclic compound would be of great importance.

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A bifunctional plant enzyme: an isopentenyl diphosphate hydrolase fused to a dipeptidyl peptidase III

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Keywords: plant biochemistry, Nudix hydrolase, dipeptidyl-peptidase III, isopentenyl diphosphate

A novel enzyme with dual activity - an isopentenyl diphosphate (IPP) hydrolase from the Nudix family fused to a dipeptidyl peptidase III (DPP III) domain has been discovered in *Physcomitrella patens* and *Arabidopsis thaliana* and biochemically characterized.¹

Heterologously expressed *Physcomitrella patens* and *Arabidopsis thaliana* Nudix-DPP III (PpND and AtND) proteins showed peptidase activity against the preferred artificial substrate of DPPs III. The Nudix domain of these proteins was presumed to be a Nudix hydrolase (phosphatase), since it contains a functional Nudix box motif. Based on sequence similarity with isopentenyl diphosphate isomerase (also a Nudix fold protein), we recognized IPP as a possible substrate. Indeed, in a screen of 73 potential Nudix hydrolase substrates, both PpND and AtND showed preference for IPP, producing isopentenyl phosphate (IP) as a final product. With both phosphatase and peptidase activity confirmed by enzyme assays, we used site-directed mutagenesis to investigate structure-activity relationships. In PpND, Glu92 and Glu592 were identified as putative catalytic residues. Point mutations of each Glu92 and Glu592 to Ala caused an absence of phosphatase and peptidase activity, respectively. Our results confirm the presence of two separate active sites, although both domains are needed for proper folding and activity of this enzyme. Separate domains produced as recombinant proteins were either insoluble or unstable.

The exclusive presence of this fusion protein in plants is still unclear and may have an adaptive role for life on land. The physiological role of the phosphatase might be in the regulation of the pool of IPP, the isoprenoid building block, in plant cell cytoplasm, while DPP III is supposed to be involved in protein catabolism. Functional role of this dual enzyme is being investigated on *Physcomitrella patens* mutants. From a biotechnological point of view, this enzyme might be useful in microbial isopentenol biofuel production.

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Does homologous oligomerisation promote protein evolution?

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Keywords: Evolution, Protein, Oligomerization, Homologous

In this project, the potential role of homologous oligomerization on the evolutionary origins of proteins will be studied by simulating an evolutionary scenario starting by the duplication of a gene encoding the D-malate dehydrogenase (DmlA) of *Escherichia coli*. We will study the potential of the system to evolve towards isocitrate dehydrogenase (IDH) or isopropylmalate dehydrogenase (IPMDH) activity with the directed evolution approach in monogenic and digenic scenarios in an engineered strain of *E. coli*, B3 (HB101 Δ *idh::kan(Kmr)* Δ *ipmdh* Δ *dmlA*). Two catalytic mutant versions of DmlA (D224A and D248A) are constructed in compatible vectors and introduced (single vector or both vectors) in the B3 strain which is not able to grow in a media when only D malate is supplied as sole carbon source. In monogenic system, only inactive homodimeric DmlA is produced but in digenic system, heterodimers with one active site restored due to random assembly of DmlA subunits allow B3 to grow on D-malate. About 20 % of the proteins form heteromers in vivo as depicted from the lysate activity compared with the wild type DmlA. Purified heterodimer is 12 fold less active (k_{cat} 0.37s⁻¹) than wild type DmlA. To demonstrate the advantage of heteromerization and chaperone effect, the DmlA mutant library will be produced by error prone PCR and will be introduced in B3 strain either alone or together with the wild type and colonies will be selected for IDH or IPMDH activity. Higher frequency of clones in digenic scenario will support that the heteromerization is favoured and there is chaperone effect involved to stabilize the new protein complex. Next, both plasmid and phagemid library of DmlA will be introduced in B3 either alone or both libraries together. We are expecting higher frequency of colonies in digenic scenario referring to the combinatorial advantages of oligomerization.

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Enzyme mediated kinetic resolution of δ -hydroxy- α,β -unsaturated esters as a route to optically active δ -lactones

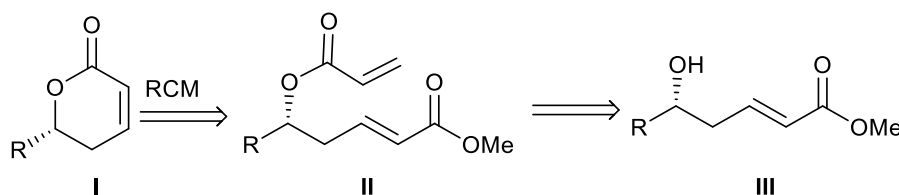
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Keywords: enzymatic kinetic resolution, metathesis, hydrolases, δ -lactones.

The unsaturated chiral δ -lactone ring is an important structural motif of many natural products like; massoia lactone, jasmine lactone, mellein, altholactone or goniodiol.

Derivatives of various lactones play an important role as sex attraction pheromones of different insects and plant-growth regulators [1]. The beneficial or adverse effects of these compounds depend on their absolute configuration making separation of two enantiomers from racemic mixture an important issue. In view of these application importance, synthetic studies of δ -lactones have attracted considerable interest. We propose a new approach to chiral non-racemic α,β -unsaturated δ -lactones (I) which retrosynthetic analysis is given in Figure [2].



Our strategy involves disconnection of the target α,β -unsaturated δ -lactones I to the key synthon δ -acryloyloxy- α,β -unsaturated esters II, which can be prepared from the corresponding δ -hydroxy- α,β -unsaturated esters III via enzymatic kinetic resolution. The influence of temperature, co-solvent, organic additives and the substrate structure on the catalytic behavior of selected hydrolases were studied. Established protocol combining enzymatic kinetic resolution with ring closing metathesis was successfully applied in the synthesis of the enantiomerically pure (6*R*)-phenyl-5,6-dihydro-2H-pyran-2-one which plays crucial role in the synthesis of the number of bioactive compounds

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On the Way to subjugate FMO-dependent human metabolism: two proven concepts

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About ten percent of oxidative human metabolism is catalysed by NADPH-dependent flavin monooxygenase 3 (hFMO₃). This microsomal enzyme converts xenobiotics containing nucleophilic heteroatoms like sulphur or nitrogen into easily secreted metabolites. It uses NADPH as a natural electron donor to perform oxidations [1]. *In vitro*, external use of NADPH is related to very high costs and in cells NADPH is produced in limited quantities. At the moment, there are no efficient and cheap systems to mimic this part of human metabolism. The isolated hFMO₃ enzyme has low activity, is unstable, and as membrane-associated protein is poorly expressed in bacteria. Moreover, use of detergents can influence lipophilic drugs conversions.

The poster presents two solutions for preparation hFMO₃-generated metabolites, which we supported by experimental data. First, the construct optimisation for active enzyme expression in *E. coli* has shown that hFMO₃ fused at the N-terminus of phosphite dehydrogenase (PTDH) [2] can be used in whole cells to convert albendazole.

The second approach is to design a collection of alternative, thermostable flavoprotein monooxygenases from microbial origin, that can be expressed in huge quantities on a large scale. For those enzymes we also prepared fusions with the PTDH expression-tag. Several monooxygenases from *Rhodococcus jostii*, *Acinetobacter calcoaceticus* and *Myceliophthora thermophila* heterogeneously expressed in *E. coli*, were active in the crude extracts toward drugs containing both heteroatoms, sulphur (albendazole, fenbendazole) and nitrogen (nicotine, lidocaine) [3]. Importantly, metabolites were formed in an enantioselective manner. Conversions were run with phosphite as cheap cosubstrate. The PTDH and phosphite play a crucial role as it efficiently regenerates the expensive NADPH cofactor.

In summary, we have shown two different approaches for hFMO₃-dependent metabolite production on a large scale. The first approach is to use whole cells (*E. coli*) optimised for expression of the PTDH-hFMO₃ bifunctional fusion enzyme. The second approach involves the use of various microbial and stable flavoenzymes that form a monooxygenase library by which hFMO activities can be mimicked.

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Enzymatic Baeyer-Villiger oxidation of furanoid aldehydes

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Keywords: Baeyer-Villiger monooxygenases, Furanoid aldehydes, Hydroxymethyl furfural, Biodegradable plastic

Baeyer-Villiger monooxygenases (BVMOs) catalyze the insertion of an oxygen atom adjacent to a carbonyl carbon in a selective manner forming an ester or lactone. Due to their typical high chemo-, regio-, and/or enantioselectivity BVMOs are interesting candidates for biocatalytic applications. Here, we present the oxidation of biomass-derived furanoid aldehydes using BVMOs fused to phosphite dehydrogenase (PTDH), which represent so-called self-sufficient BVMOs [1]. A set of available PTDH-BVMOs were tested for their activity on furanoid compounds. Screening revealed that most of the BVMOs accept furfural as substrate. Phenylacetone monooxygenase (PTDH-PAMO) and a mutant thereof (PTDH-PAMOM446G) were selected for studying their biocatalytic potential in converting furanoids. PTDH-PAMOM446G oxidized the aldehyde group of furanoid compounds mainly into the respective furanoid acid form rather than the expected products, the formate esters. PTDH-PAMO converted 5-hydroxymethylfurfural (HMF) into HMF acid and the formate ester, in almost equal amounts. Our results show that PAMO and PAMOM446G efficiently oxidize the aldehyde group of furanoid aldehydes. Clearly, the ratio of normal to abnormal product produced by BVMOs can be tuned by subtle changes in the active site. This study shows that BVMOs might be useful for the synthesis of useful furanoid compounds.

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Utilising synthetic biology to navigate fitness landscapes effectively for improved biocatalysts

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Keywords: MAO-N, directed evolution, biocatalysis, secondary structure

Diversified methods are developed for searching for the global optimum in a fitness landscape within biocatalysts. Mainstream strategies involved screening the biocatalyst via random mutagenesis (e.g. error prone PCR, DNA shuffling) and active site point mutagenesis, which makes the sequence space difficult to control and is only subjected to active site residues respectively.

Here, we are trying to use monoamine oxidase (MAO-N) as a reference biocatalyst, to develop a systematic directed evolution strategy to derive essential as well as distal residues that improve *k_{cat}*, hence achieving an optimized biocatalyst for the substrates. The designed methodology included the creation of MAO-N library by mutating consecutive residues within every part of the secondary structure, solid phase activity assay, DNA sequencing, liquid phase activity assay and the implementation of genetic algorithm for predicting the upcoming generations.

More than half of the secondary structure residues are conserved. Interestingly, activity assays on MAO-N variants I356V, A289V, F128L showed that there are more than 1.5X improvements in *k_{cat}*. These residues are located on the surface of MAO-N with no direct interactions to the active sites.

Currently, genetic algorithm development is in progress and high throughput robotics is introduced to increase the efficiency of the screening process in order to enable the succeeding of the global optimum in MAO-N and other biocatalysts in the future.

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Novel hydroxynitrile lyase

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Keywords: hydroxynitrile lyase, cyanohydrins, Bet v 1, fern

Discovering enzymes from scratch gives the opportunity to identify novel unpredictable sequences and protein folds, which would not be found by the most popular homology based algorithms. Herein, we show that the interconnection between transcriptomics, proteomics and enzymatic screening enabled the discovery of a new hydroxynitrile lyase (HNL) from white rabbit's foot fern (*Davallia tyermannii*). Structural studies show *DtHNL* belongs to Bet v 1 protein superfamily with a new catalytic center. Finally, enzymatic characterization and substrate scope were investigated. *DtHNL* is a robust enzyme, tolerant to low pH and able to convert various substrates. Obtained results open prospective for a new class of biocatalysts, broadening the toolbox for the stereoselective synthesis of cyanohydrins.

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Novel transaminases from metagenomic sources and their application in biocatalysis

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Keywords: biocatalysis, metagenomics, transaminases, enzymes.

With less than 1-10% of microbial diversity accessible through culturable techniques,¹ up until recently, the realm of uncultured microorganisms was a huge untapped resource. At UCL we have developed a strategy for accessing functional enzymes from metagenomic libraries. In this approach, the DNA from an environmental sample is extracted, sequenced and subsequently used to create an *in silico* contiguous library. Enzymes can then be identified and retrieved from the original DNA through PCR and screened for functionality.²

Our aim is to identify novel enzymes, while improving the low hit rate of conventional metagenomic screening, and increasing the substrate specificity of various enzyme classes. In this work, a number of transaminases from a drain metagenome have been identified, cloned and overexpressed. For comparison, three different assays were used to screen the transaminases against linear, cyclic and aromatic substrates. Active enzymes were then shown to be active with a variety of other substrates, including heterocycles, aldehydes and keto-esters, and these results will be presented.

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Fructose-6-phosphate aldolase (FSA): stereoselective C-C bond formation with multiple nucleophiles

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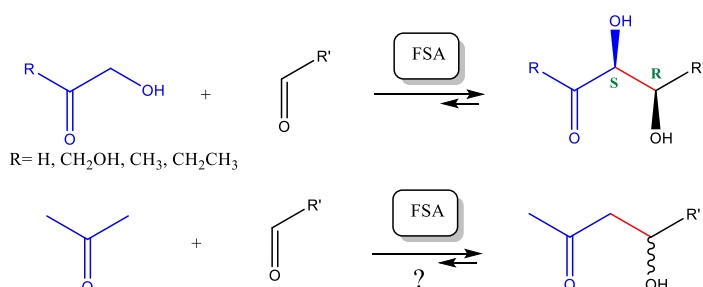
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Keywords: aldolase, aliphatic nucleophiles, promiscuity, stereoselectivity.

Since its discovery in 2001 by Sprenger's group,¹ fructose-6-phosphate aldolase (FSA) never ceased to amaze by its high nucleophile substrate tolerance. Aldolases are well established enzymes, stereoselectively catalyzing C-C bond formation between nucleophile and electrophile substrates². They always show a large tolerance towards various aldehydes as electrophiles, however their strict dependency towards one nucleophile substrate rather limits their applications in organic chemistry. In this context FSA finds its originality, accepting to date a variety of nucleophile substrates.¹ As another particularity, one of them is an aldehyde (glycolaldehyde),³ opening an access to the preparation of aldoses in addition to the usual ketoses.

All these nucleophiles share a hydroxymethyl group next to the carbonyl as illustrated below.



This communication will present a breakthrough in FSA synthetic potential by exploring reactions with non-hydroxylated nucleophiles such as acetone (see scheme above). Screening of new nucleophiles, recent kinetic results on aldolase enantioselectivity as well as some synthetic examples will be discussed with wild-type and variant FSA.

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Expanding the Reaction Scope of Imine Reductases

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Keywords: Imine Reductase, Asymmetric Hydrogenation, Promiscuity, Electron Effects

The synthesis of chiral molecules by asymmetric hydrogenation is an important technology in the pharmaceutical and fine-chemical industries. Chemo-catalytic methods applying rhodium and ruthenium-based metal catalysts and molecular H₂ as hydride donor are established on industrial scale.¹ Due to the tremendous progress in enzyme discovery, enzyme engineering and process development, these technologies are complemented by biocatalytic alternatives. Recently novel natural NADPH-dependent imine reductases were discovered catalyzing the chemoselective reduction of various C=N bonds.²⁻⁴ Compared to C=C or C=O double bonds, the C=N bond provides a greater challenge for enzymatic reduction due to the immediate hydrolysis in aqueous solutions.

To assess the currently proposed catalytic mechanism of imine reductases consisting of hydride- and proton-transfer similar to that of carbonyl reductases, we examined the reduction of aldehydes and ketones. We will demonstrate that the *R*-selective imine reductase from *Streptosporangium roseum* and the *S*-selective from *Paenibacillus elgii* display promiscuous activities for highly reactive carbonyl compounds. Chemoselectivity driven by the reactivity of substituents in α -position to the C=O function was determined. To gain further insights into the catalytic mechanism of imine reductases theoretical calculations of energy barriers for the hydride transfer from NAD(P)H to imines, iminium ions and carbonyl compounds possessing electron-withdrawing substituents will be presented.

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THERMOGENE - Novel Thermostable Enzymes for Industrial Biotechnology

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There is an increasing demand for new thermostable enzymes with enhanced performance and/or novel functionalities that provide savings in time, money and energy for industrial processes in the areas of high value chemical production and other "white" biotechnology applications.

The THERMOGENE project has identified transferase enzymes from genomes and metagenomes isolated from terrestrial and marine hot environments. The project has employed microbiology, large-scale genomics, bioinformatics, biochemistry and structural biology. THERMOGENE has focused on the discovery of four types of transferase enzymes with known and potentially new commercial applications. These include the transketolases which transfer 2-carbon units; two classes of relatively understudied transferase enzymes which transfer amine groups; prenyltransferases which transfer isoprenyl or prenyl groups and hydroxymethyl transferases which transfer hydroxymethyl groups.

The THERMOGENE project has identified and studied a range of thermophilic Class IV BCAT transaminases - 10 of the most soluble enzymes have been characterised and three crystal structures are available to high resolution. Also a range of sugar transaminase class VI enzymes have been identified and three crystallographic structures determined. Partners in the THERMOGENE consortium have cloned, over-expressed and characterised novel transketolase enzymes. The structures of one full length bacterial *Thermovirgo* enzyme has been determined and one novel 'split' archaeal *Carboxythermus* transketolase has been determined at Exeter. The 'split' transketolase has been reconstituted to an active enzyme. These enzymes have been compared with the commonly used *Escherichia coli* transketolase and other members of the transketolase family. A thermophilic hydroxymethyl transferase enzyme has been identified, cloned and crystallised in the Bergen partner and its structure determined at Exeter. Two thermostable archaeal prenyl geranyl transferases been identified and cloned and one has been studied structurally by the Moscow partners.

The robust nature of these new transferase enzymes to both temperature and organic solvents makes them interesting candidates for new industrial applications.

Enzymatic cyclization initiated by protonation

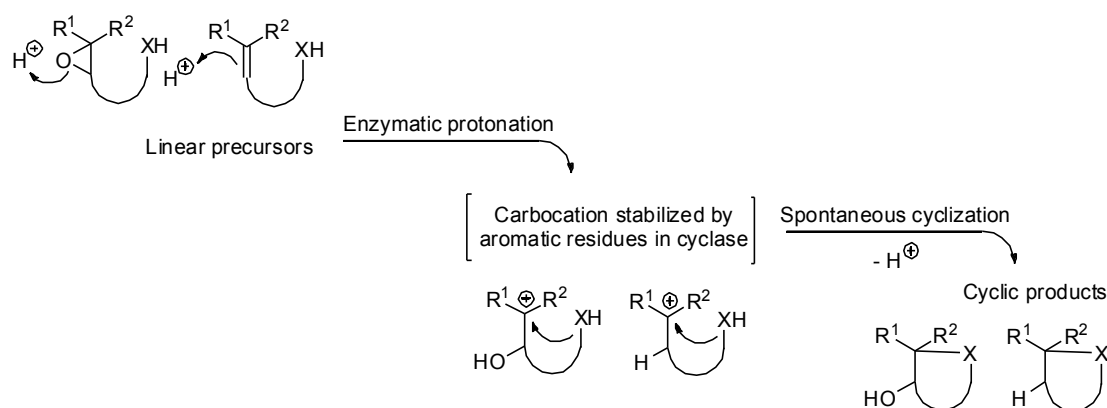
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Keywords: Cyclase, Enzymatic protonation, π -Cation interaction, Stereospecific cyclization

Electrophilic enzymatic cyclization reactions catalyzed by cyclases are initiated by protonation of a carbon-carbon double bond or an epoxide ring, which is carried out by acidic residues, such as Asp or Glu.¹ Within the active site of the cyclase, aromatic residues (e.g. Trp) not only serve as template to bind the substrate in a productive 'bent' conformation to promote cyclization, but also stabilize carbocation intermediates by the aid of their delocalized π -electrons.² Here the reaction mechanism, involving enzymatic protonation followed by spontaneous cyclization³ has been summarized along examples in biosynthesis of prominent cyclases.



Schematic representation of electrophilic enzymatic cyclizations catalysed by cyclases.

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Engineering thermostability of bacterial flavin-monooxygenase for efficient indigo production

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Keywords: indigo, FMO, enzyme engineering, thermostability, cofactor recycling

Currently, industrial indigo dyeing processes involve the chemical synthesis of insoluble indigoid dyes that need to be pre-reduced to become water soluble forms (leuco-indigo) after which it is applied onto a textile. During the dyeing process the cotton yarns are skied to ensure the complete oxidation of the indigoid dye, turning the soluble leuco-indigo into its insoluble form, hence achieving the typical blue color. While indigo blue is currently produced by chemical processes, it would be highly attractive to develop a biotechnological process for the production of this popular dye.

Recently, a microbial flavin-containing monooxygenase (mFMO) was discovered that is able to oxidize indole, resulting in the formation of indigo blue. Although mFMO oxidizes indole and indole derivatives, the catalytic efficiency is rather poor. The enzyme is highly active on small aliphatic amines while aromatic sulfides and amines (indoles) are poorly accepted as substrate. Another bottleneck for mFMO-based applications is its moderate stability. In this project, enzyme engineering approaches will be used to generate a robust mFMO variant that is efficient in indigoid dye synthesis. The crystal structure of mFMO has been elucidated and by applying the recently in-house developed FRESCO methodology, we anticipate a significant improvement of the enzyme stability. The optimized mFMO will be explored for large scale production of indigo blue by using it in a fermentative process. For this approach, the work will build on the concept of producing the monooxygenase fused with dehydrogenases that will enable a cost-effective cofactor recycling.

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Technology platform for expression and secretion of hydrolases

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Keywords: gene expression, protein secretion, flow cytometer screening, signal sequence

The establishment of an efficient recombinant protein production system is essential for many industrial and therapeutic applications. Several challenges in the production of recombinant proteins can be overcome by employing secretion pathways.

In the BioSC-funded project BIOExpresSPro, a novel technology platform consisting of a bacterial system enabling high-throughput cloning, high-level expression and secretion for the detection and production of enzymes will be established. The enzyme-free and sequence-independent cloning method PLICing¹ was employed to generate a signal peptide library consisting of 120 signal sequences from *Bacillus subtilis* (Brockmeier, U et al., 2006). The library was successfully generated for two vector/strain systems for hydrolases and was screened towards increased activity and secretion. For selected hydrolase variants, protein production is further optimized by vector backbone evolution using epMEGAWHOP² (a directed evolution based approach to increase protein production by randomly introducing mutations in the vector backbone) and screened with the colorimetric para-nitrophenyl palmitate MTP-assay (Winkler and Stuckman, 1979). In order to accelerate the directed evolution of the signal peptide library towards higher protein secretion, a flow cytometer high throughput screening assay based on the formation of fluorescent hydrogel around the hydrolase-producing cells (Fur Shell technology³) will be employed with the aim to identify activity- and expression-improved hydrolases.

The presented technology platform has an immense potential to be employed as an important toolbox for the efficient engineering, detection and production of enzymes for industrial and molecular biotechnology.

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Functional transitions in enzyme evolution: balancing stability, folding and catalytic specificity

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Keywords: ancestral reconstruction, enzyme evolution catalytic promiscuity, phospho/sulfohydrolase

Evolutionary pathways by which proteins have evolved in Nature over billions of years have resulted in an impressive diversity of structures that carry out many functions with unrivalled efficiency. Directed protein evolution in the test tube can emulate natural evolution, but is often limited by low hit rates and small improvements during evolutionary cycles. Furthermore, the combination of mutations that is needed for large improvements cannot always be reached by one-by-one mutational steps due to the occurrence of general loss-of-function or epistatic ratchets. The question then arises how evolutionary dead ends can be avoided. Important parameters that shape these fitness landscapes are e.g. expression level, stability and catalytic activity/specificity. We are currently probing these parameters for ancestral sequences inferred from phylogenetic relationships between members of the catalytically diverse metallo- β -lactamase¹ and alkaline phosphatase²⁻⁴ superfamilies. Mapping of substrate specificity profiles on the genetic relationships allowed the identification of the ancestral nodes between which transitions in primary function most likely occur. The latter is one of the key processes in evolution of new functions. The substrate specificity profiles of the current enzymes suggest that the change in primary function is the result of a shift in substrate preference rather than *de novo* evolutionary invention of a novel activity.

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Enzymes in radiochemistry; An efficient solution for the point of care synthesis of ^{13}N -radiotracers

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Keywords: Positron emission tomography (PET), Alanine dehydrogenase, Nitrate reductase, multi-enzyme systems, immobilization

Recent advances in Positron Emission Tomography (PET) have encouraged chemists to synthesize novel radiotracers to enable the non-invasive diagnosis of a larger variety of diseases and the investigation of their molecular basis. The inclusion of ^{13}N in the toolbox of PET chemists might become a valuable alternative to ^{11}C and ^{18}F , either for the preparation of new labelled compounds or the incorporation of the label in different positions. However, the short half-life of ^{13}N ($T_{1/2} = 9.97$ min) demands for the development of simple, fast, and efficient synthetic processes. In this context, biocatalysis can offer attractive solutions because enzymes present an exquisite selectivity and high turnover numbers, enabling fast chemical conversions and yielding highly pure products under extremely mild conditions. In this contribution, we present two representative examples for the success of the biocatalysis in the ^{13}N -radiochemistry: 1) The design and preparation of an immobilized nitrate reductase suitable for the reduction of $[^{13}\text{N}]\text{NO}_3^-$ into $[^{13}\text{N}]\text{NO}_2^-$. Such heterogeneous biocatalyst could be re-used up to 7 reaction cycles preserving its initial activity and readily integrated to the chemo-enzymatic radiosynthesis of *S*- $[^{13}\text{N}]$ nitrosoglutathione starting from $[^{13}\text{N}]\text{NO}_3^-$ as labelling agent. 2) The one-pot, and non-carrier added synthesis of the ^{13}N -labelled amino acids such as *L*- $[^{13}\text{N}]$ alanine, $[^{13}\text{N}]$ glycine, and *L*- $[^{13}\text{N}]$ serine catalyzed by a *L*-Alanine dehydrogenase from *Bacillus subtilis*. In addition *L*-alanine dehydrogenase was coupled with formate dehydrogenase from *Candida boidinii* to *in situ* regenerate NADH during the radiochemical synthesis of the amino acids, which allowed a 50-fold decrease in the concentration of the cofactor without compromising reaction yields. This synthetic strategy resulted in ready-to-inject ^{13}N -labelled amino acids in sufficient amount to approach *in vivo* studies in small rodents, and paves the way towards future solid-supported, multi-purpose, in flow synthetic processes. Based on these enzymatic systems, we envision the implementation of point of care synthetic platforms to produce a plethora of radiotracers based on ^{13}N -Nitrogen.

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Binding pose and affinity prediction for engineered cytochrome P450 mutants

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Keywords: Cytochrome P450, binding affinity calculation, binding pose prediction, free energy calculation

The large flexibility of Cytochrome P450 enzymes (CYPs) make it challenging to accurately predict binding orientations and binding affinities or binding *free energies* of small molecules. Here we show results for free energy calculations on substrate and product binding to engineered Cytochrome P450 BM3 mutants, to study the basis for increased biocatalytic activity of a 437E mutant. The applicability of a recent method to identify binding sites and predict binding poses and direct binding free energies is evaluated for another engineered CYP mutant. The complexes of this CYP BM3 mutant M01 A82W with two different ligands, R- and S- α -ionone, were chosen as model systems. The first results show that the method is able to identify the binding site and predict the difference in binding free energy.

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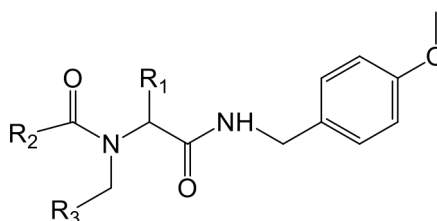
The studies on preparation of peptidomimetics via chemoenzymatic cascade reaction

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Keywords: Ugi four-component reaction (Ugi-4CR), peptidomimetics, cascade, oxidation

We present a new environmental friendly way for the synthesis of the α -aminoacyl amides. We have developed a “one-pot” chemoenzymatic cascade based on enzymatic oxidation followed by Ugi-multicomponent reaction leading to desired α -aminoacyl amides. A first reaction step is oxidation of an alcohol to the corresponding aldehyde using *Trametes versicolor* laccase/TEMPO system¹. The influence of surfactant type on the reaction yield was studied.²



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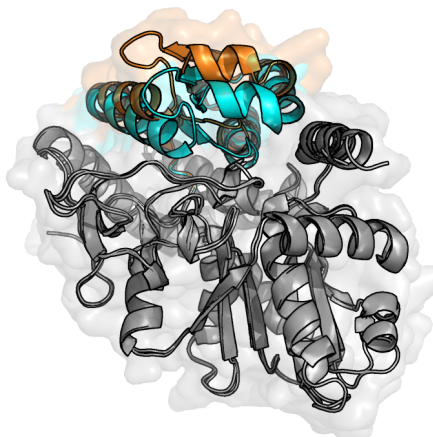
Molecular dynamics studies of tryptophan synthase: Insights into engineered β -subunit and intermediates effects

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Keywords: Tryptophan synthase, Allostery, Molecular Dynamics, and catalysis.

Tryptophan synthase (TrpS) catalyzes the formation of L-Tryptophan from L-Serine and indole glycerol phosphate (IGP). TrpS works as a dimer where allosteric networks between α -subunit (TrpA) and β -subunit (TrpB) are necessary to keep the proper conformations along the catalytic mechanism and to prevent the loss of indole, which moves from TrpA to TrpB through a tunnel.¹ Previous studies have reported different states exhibiting open and closed active site conformations in both subunits, which were found to be key for catalysis.¹ Directed Evolution (DE) was applied to turn TrpB into a catalytically efficient stand-alone unit.² In this study, we use computational tools to investigate how mutations affect catalysis and free TrpB from TrpA allosteric regulation. In particular, we have simulated each variant along the DE process at different reaction intermediates, pyridoxal 5'-phosphate (PLP), external aldimine (Aex_i) and α -aminoacylate (A-A) through nanosecond time-scale molecular dynamics (MD). The simulations bring to light information about how mutations and the different intermediates affect the open and closed states, key interactions, stabilization of IGP, and the gate-keeper states of the tunnel. This study contributes to our current understanding of how distal mutations from the active site affect catalysis, and the enzyme allosteric network, which is of interest for engineering isolated enzyme subunits for biosynthetic applications.



TrpB showed in open state (orange) and closed state (cyan)

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Identification of novel thermostable ω -transaminase and its application for enzymatic synthesis of chiral amines at high temperature

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Keywords: ω -transaminases, chiral amines, thermostable enzyme, biocatalysis, novel enzyme.

Enantiomerically pure amines are frequently used as precursors in pharmaceutical drugs, fine chemicals and many other natural products. Among the various enzymatic routes to synthesize amines, ω -transaminases (ω -TA) have emerged as a potent class of enzyme to generate a wide range of optically pure amines and unnatural amino acids. Despite many advancements in synthesizing amines using ω -TAs, the number of ω -TAs used in industries remain rather modest. Good operational stability in the presence of high temperature, organic solvents and other substrates can make ω -TAs ideal for industrial applications. Thermophiles which have an optimal growth above 60°C constitute an excellent source for identifying thermostable ω -TAs. In this work, a novel thermostable ω -TA from *Thermomicrobium roseum* (ω -TATR) which showed broad substrate specificity and high enantioselectivity was identified, expressed and biochemically characterized. This is the first reported amine-TA from a thermophilic organism and the enzyme's stability at very high temperature was effectively used to remove volatile by-product without employing any co-enzymes or by-product removal system.

Novel substrates for screening and assay of polysaccharide endo-hydrolases

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Keywords: AZCL-polysaccharides, crosslinked substrates, tablet tests, colorimetric oligosaccharides.

Dyed, cross-linked polysaccharides (AZCL-polysaccharides) find widespread use in screening for polysaccharide *endo*-hydrolases. The utility of these substrates has been improved by decreasing the particle size to simplify uniform suspension in agar plates, and by producing them in a range of colours. Uniform dispensing of substrate into wells of plate readers is facilitated by suspending the substrates in dilute solutions (0.05-0.1 %) of xanthan gum. In 0.05 % xanthan gum, the substrate particles remain suspended for several hours. In tablet form, these cross-linked, dyed substrates form the basis of highly specific, quantitative and sensitive assays for β -xylanase, β -glucanase, α -amylase, β -mannanase and several other polysaccharide *endo*-hydrolases and proteases. However, these assays are not readily adapted to automation, so we have developed specific and highly sensitive assays employing oligosaccharide-based chromogenic and fluorogenic substrates. These substrates are used in the presence of saturating levels of specific glycosidases such as thermostable α - and β -glucosidases and β -xylosidase. For the measurement of pullulanase and limit-dextrinase, the substrates synthesised were 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl- β -maltotriosyl (1-6) α -maltotriose (BzCNPG3G3) and 4,6-*O*-benzylidene-methylumbelliferyl- β -maltotriosyl (1-6) α -maltotriose (BzMUG3G3). For the assay of α -amylase, assays are based on 4,6-*O*-benzylidene-4-nitrophenyl- α -maltoheptaose (BzNPG7) and 4,6-*O*-ethylidene-4-nitrophenyl- α -maltoheptaose (EtNPG7). For *endo*-cellulase assay, the substrate synthesised was 4,6-*O*-(3-ketobutylidene-4-nitrophenyl- α -cellopentaose (CellG5), and for assay of lichenase and 1,3:1,4- β -glucanase (malt β -glucanase) the substrate was 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl- β -cellotriosyl (1,3)-D-glucose (BzClNPG443). For assay of *endo*-xylanase the substrate we have prepared is 4,6-*O*-(3-ketobutylidene-4-nitrophenyl- β -xylohexaose (XylX6). On hydrolysis of each of these substrates by the particular *endo*-hydrolase, 4-nitrophenyl or 2-chloro-4-nitrophenyl oligosaccharide is released and this is immediately hydrolysed by the relevant glycosidase in the reagent mixture, to release 4-nitrophenol or 2-chloro-4-nitrophenyl that produce a yellow color in the presence of an alkaline solution. Assay procedures using these modified oligosaccharides are simple to use, specific, accurate, robust and readily adapted to automation.

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Solid phase biocatalytic Baeyer-Villiger oxidations.

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Keywords: Baeyer-Villiger monooxygenases, solid phase biocatalysis, *in situ* substrate supply and product removal

The broadening of the industrial scope of biooxidation processes beyond pharma applications to new fine chemicals, nutrition, feed and materials markets is an important target. One such group of flavin-dependent monooxygenases, Baeyer-Villiger monooxygenases (BVMOs), represents a promising route of performing Baeyer-Villiger type oxidation reactions to yield numerous products from non-natural substrates^{1,2}. The biocatalytic route allows the substitution of conventional, unsafe and unstable oxidants with molecular oxygen, affords superior regio-, chemo-, and enantioselectivities³, and the fulfilment of newly imposed, legislative 'green chemistry' restrictions in industry.

The target reaction of this work is the BVMO-catalysed reaction of the macrocyclic ketone, cyclopentadecanone, to its corresponding lactone. The poor aqueous solubility of both substrate and product leads to a solid phase in the reaction environment. Ultimately it is desired to minimise the water fraction of these two-phase reactions in order to operate at high, industrially-relevant substrate loadings and reduce the cost of downstream processing. Further, it is preferred to accomplish solid phase reactions without the addition of extra process elements (e.g. solvents or sorbents) unless they are essential to overcome substrate or product inhibition^{4,5}. This presentation will detail considerations for how solid phase biocatalytic reactions may be performed, with focus towards the methodology for kinetic characterisations (also involving whole-cells) and reactor design.

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Chemoenzymatic synthesis of amines to oximes. Cascade transformation involving lipase-catalyzed perhydrolysis and chemical oxidation reactions

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Keywords: Cascade reactions, Chemoenzymatic synthesis, Lipases, Oxidation, Oxime

Oximes are valuable organic compounds serving as versatile synthetic precursors of commodity chemicals, pharmaceuticals, polymers, heterocycles, and fine chemicals. Their synthetic importance resided in their easy transformation into a wide range of functionalities such as carbonyl groups, carboxylic acid derivatives and nitrile oxides among others, which opens-up a plethora of possibilities for further modification.

Aerobic oxidation of amines into oximes allows the production of oximes normally under heterogenous catalysis and drastic conditions.¹ More recently, a metal-free oxidation has been reported using *meta*-chloroperbenzoic acid at room temperature,² although a mixture of undesired side products such as aldehyde, nitrile and imine derivatives is observed in some cases, which leads to a loss of selectivity in the entire process.

Biocatalysis offers multiple possibilities for oxidative process by using redox enzymes,³ although hydrolases have been found as ideal catalysts for global chemoenzymatic oxidative processes.⁴ Herein, we describe a chemoenzymatic cascade for the production of oximes consisting in two steps that occurs in one-pot. Initially, a perhydrolysis reaction of a carboxylic acid leads to the formation of a reactive peracid intermediate, which is the responsible for the chemical oxidation of amines into oximes.

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Structure-function relationships of family GH70 glucansucrase enzymes

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Keywords: glucansucrase; linkage specificity; GH70; α -glucan.

Lactic acid bacteria (LAB) are known to produce large amounts of α -glucan exopolysaccharides, which are widely explored for applications in the food, medicine and cosmetic industries. In the biological environment, the sticky nature of α -glucan polysaccharides plays a key role in biofilm formation, enabling microorganisms to adhere to surfaces. Mutans produced by *Streptococcus* strains, especially *S. mutans*, facilitate microorganisms to adhere to teeth enamel and have been recognized as the major pathogenic factor for dental caries. α -Glucan polysaccharides with various linkage composition, degree of branching and size are synthesized by GH70 glucansucrase enzymes with the successive transfer of glucosyl units of sucrose to growing glucan chains. GH70 glucansucrase enzymes contain a circularly permuted (β/α)₈ catalytic barrel compared to the closely related GH13 family enzymes. The elucidation of the crystal structures of representative GS enzymes has advanced our understanding of their reaction mechanism, especially structural features determining their linkage specificity. Crystallography studies showed that the polypeptide chain of GH70 glucansucrase enzymes take a U shape course to form 5 different domains (domains A, B, C, IV and V). We investigated the linkage specificity determinants of GH70 glucansucrase enzymes using random mutagenesis guided by the crystal structures. We showed that residues from both domain A and domain B at acceptor binding subsites are critical determinants for the linkage specificity and formation branching. The specific interactions between the acceptor substrate and its acceptor binding sites determines which hydroxyl group of the non-reducing end glucosyl moiety of an acceptor substrate is capable of attacking the glucosyl-enzyme intermediate to form the next α -glycosidic linkage. A large number of α -glucan polysaccharide variants were produced by our engineered glucansucrase enzymes and hold potential for industrial applications.

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Flavin dependent halogenase enzymes for aromatic regioselective bio-halogenation

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Keywords: Flavin dependent halogenase, Regio-selective halogenation, biosynthetic pathways, chemo-enzymatic reactions.

In recent years, many Flavin dependent halogenase (Fl-Hal) enzymes were discovered from various bacterial and fungal biosynthetic pathways and these enzymes are the versatile biocatalysts for regioselective halogenation of wide range of aromatic compounds. Consequently, Fl-Hal is one of the potential biocatalysts for the chemo-enzymatic synthesis of pharmaceuticals and other valuable products, which are derived from haloaromatic precursors. However, the application of Fl-Hal enzymes, *in vitro*, has been hampered by their poor catalytic activity, lack of stability and desired substrate scope. Our recent advancements in understanding the regioselectivity of Fl-Hals, the methods applied to improve stability and activity of these enzymes, incorporation of Fl-Hal to chemo-enzymatic reactions, identification of other novel Fl-Hal enzymes with different substrate scope and reactivity and incorporation of these enzyme into engineered biosynthetic pathways are discussed.

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4) *Structure and biocatalytic scope of thermophilic flavin-dependent halogenase and flavin reductase enzymes*

Binuraj R. K. Menon, Jonathan Latham, Mark S. Dunstan, Eileen Brandenburger, Ulrike Klemstein, David Leys, Chinnan Karthikeyan, Michael F. Greaney, Sarah A. Shepherd and Jason Micklefield (manuscript submitted)

5) *RadH a versatile halogenase for integration into synthetic and engineered biosynthetic pathways.*

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Aqueous asymmetric oxidation of unprotected β -amino alcohols using alcohol dehydrogenases

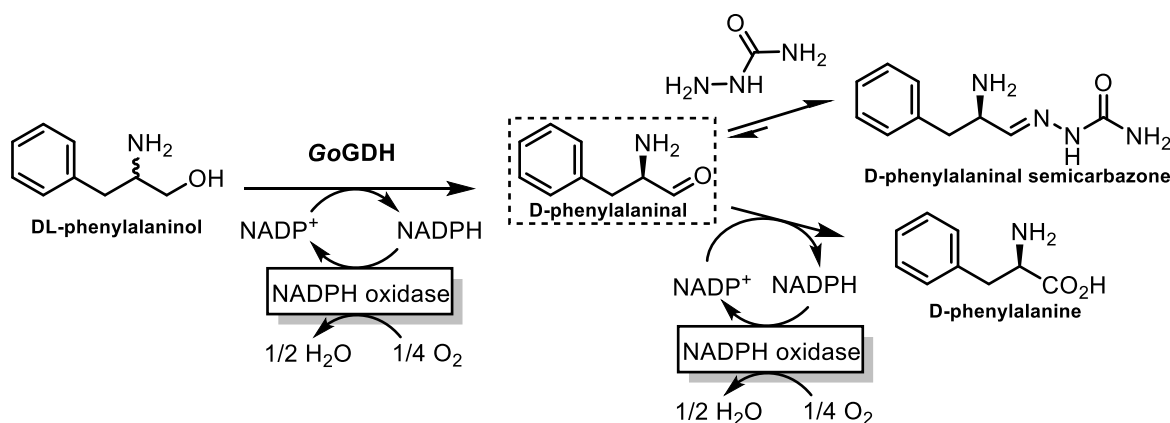
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Keywords: Asymmetric, unprotected, α -amino aldehydes, biocatalysis

The synthesis of oriented derivatives of unprotected α -amino aldehydes is perceived as a formidable challenge in organic chemistry, due to their chemical and optical instability. Previously, others shown the enzymatic oxidation of enantiopure L-phenylalaninol by crude extract from *Equus caballus* (HLADH)¹.

Intrigued by chiral unprotected α -amino aldehydes as promising pharmaceutical building blocks, 18 alcohol dehydrogenases were screened to oxidize DL-phenylalaninol in the presence of an aldehyde scavenging agent semicarbazide. Strikingly, glycerol dehydrogenase from *Gluconobacter oxydans* (GoGDH) shows the promiscuous and enantioselective oxidation of D-phenylalaninol to D-phenylalaninal with observed enantiomeric excess above 96 %.



To the best of our knowledge, no oxidative kinetic resolution using a heterogeneous, homogeneous, or biocatalytic catalyst has been reported to produce D-phenylalaninal and D-phenylalaninal semicarbazone with high enantioselectivity. The general notion that asymmetric α -amino aldehydes are impractical has been challenged², demonstrating the potential of biocatalysis in the synthesis of novel chiral building blocks.

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Biocatalytic properties and structural analysis of eugenol oxidase from *Rhodococcus jostii* RHA1—a versatile oxidative biocatalyst

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Keywords: Biocatalysis, selective oxidation, oxidases, crystal structures

Eugenol oxidase (EUGO) from *Rhodococcus* sp. strain RHA1 is a member of the vanillyl-alcohol oxidase (VAO) family and was previously shown to be active on a limited set of phenolic compounds. In this study, we have explored the biocatalytic potential of this flavin-containing oxidase resulting in a broadened substrate scope and a deeper insight into its structural properties. In addition to the oxidation of vanillyl alcohol and hydroxylation of eugenol, EUGO can efficiently perform the dehydrogenation of various phenolic ketones yielding the corresponding α,β -unsaturated ketones. EUGO was also found to perform the kinetic resolution of a racemic secondary alcohol. Crystal structures of the enzyme in complex with isoeugenol, coniferyl alcohol, vanillin, and benzoate have been determined and refined to 1.7–2.6 Å resolution. The catalytic center is a remarkable solvent-inaccessible cavity on the *si* side of the flavin cofactor. Structural comparison with VAO from *Penicillium simplicissimum* highlights a few localized amino acid replacements that explain their partly non-overlapping substrate preferences.

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A chemically programmed proximal ligand enhances the catalytic properties of heme enzymes

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Keywords: non-canonical mutations, heme enzymes, proximal ligand, catalytic mechanism

Biocatalysis is widely recognized as a sustainable technology for the production of high-value chemicals. At present, enzyme production and evolution strategies rely exclusively on a standard amino acid alphabet of twenty canonical residues which contain limited functionality. Here we demonstrate that the incorporation of new chemically programmed amino acids into existing evolutionary strategies *via* genetic code expansion provides a fruitful avenue to probe enzyme mechanism and can lead to modified biocatalysts with significantly enhanced catalytic properties. Specifically, introduction of a modified proximal ligand into heme enzymes can lead to a dramatic improvement in catalytic parameters and reveals crucial insights into the role of proximal pocket hydrogen bonding interactions in the stabilization of high-valent ferryl intermediates.¹ More generally our studies suggest that metallo-enzymes with enhanced properties or novel reactivities can be created by extending the metal co-ordinating ‘ligand set’ beyond those presented by the genetic code or through Nature’s biosynthetic machinery.

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Enzymatic enantioselective synthesis of α -hydroxy ketones and vicinal diols

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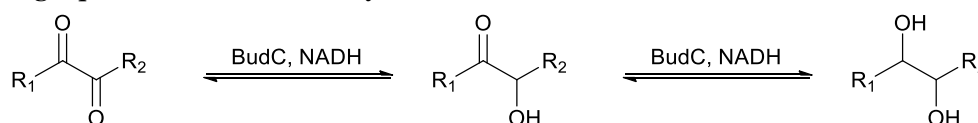
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Keywords: 2,3-butanediol dehydrogenase, BudC, α -hydroxy ketones

α -Hydroxy ketones and vicinal diols are well-known building blocks in organic synthesis. While excellent approaches towards the chiral diols exist, the synthesis of α -hydroxy ketones still is a major challenge. Many chemical and enzymatic synthetic strategies have been devised so far to address higher regio- and enantioselectivities and -purities¹.

A less explored group of enzymes for this task are the acetoin reductases/2,3-butanediol dehydrogenases (EC 1.1.1.4 and 1.1.1.76). They belong to the NADH-dependent metal-independent short-chain dehydrogenase/reductase family (SDR) and are responsible for the accumulation of different stereoisomers of 2,3-butanediol in high titres (>100 g L⁻¹) during the cultivation of species of *Klebsiella*, *Enterobacter*, *Serratia* and *Bacillus* among others².

In this study, we describe a new 2,3-butanediol dehydrogenase (BudC) from *Serratia marcescens* CECT 977. BudC catalyses the selective asymmetric reduction of prochiral diketones to the corresponding (*S*)- α -hydroxy ketones and (*S,S*)-diols. The enzyme can straightforwardly be produced in large quantities and is readily available.



Scheme 1. Regio- and stereoselective reduction of vicinal diketones and the corresponding α -hydroxy ketones using BudC and NADH as nicotinamide cofactor.

We have probed this enzyme using different substrates. As expected, BudC is highly active towards (*rac*)-acetoin and diacetyl (k_{cat} : 465 and 780 s⁻¹, respectively) producing meso-2,3-butanediol and *S*-acetoin in good yields at neutral pH. Structurally diverse diketones were assessed to analyse the substrate specificity of the enzyme using whole-cells and purified enzyme coupled to NADH/NAD⁺ regeneration systems. Vicinal diketones were reduced to their corresponding α -hydroxy ketones using whole-cell preparations, whereas diols were produced with the purified enzyme (Scheme 1). Aliphatic diketones (2,3-pentanedione and 3,4-hexanedione), cyclic diketones (1,2-cyclohexanedione) and alkyl phenyl diketones (1-phenyl-1,2-propanedione) are well accepted, whereas bulky-bulky substrates (benzil and benzoin) are not converted. In the reverse reaction vicinal diols are preferred over other substrates with hydroxy/keto groups in non-vicinal positions.

The results could be rationalised taking into account different substrate-binding modes in the active site of the enzyme. This can help us to improve the activity of this highly interesting enzyme towards desired products.

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Novel cyanide degrading enzymes from metagenomic analysis of a cassava fermentation tank

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Keywords: cassava, metagenomics, enzymes, cyanide degradation

Fermented food produced by traditional methods represents a large part of the diet in certain cultures worldwide. One commonly fermented food in developing countries is cassava. Because of its high carbohydrate content and ability to grow in soils with low nutrients, cassava is considered a major agricultural commodity in these countries. This work presents

the high-throughput metagenomic sequencing analysis of a cassava sour starch tank after 30 days of fermentation. Taxonomic analysis of replicated samples revealed the dominance of lactate-producing *Lactobacillus* (28% of total), followed by *Acetobacter* (22.2%), *Prevotella* (18%), *Gluconacetobacter* (3%), and *Bifidobacterium* (2%) genera. Binning analysis, based on differential contig coverage, resulted in the recovery of eleven near-complete population genomes. Metabolic reconstruction revealed lactate fermentation as the dominant type of fermentation (45.5% of the total reads assigned to fermentation pathways), together with fermentation pathways for the production of ethanol (30%), acetate (17%), and butyrate (7%). In addition, our sequencing efforts allowed us to identify genes involved in the metabolism of the cassava-derived cyanide. Novel genes with putative cyanide dihydratase, and nitrilase activity were recovered from the metagenome. Sequence alignments confirmed the relationship to previously discovered enzymes bearing these enzymatic activities. However, our sequences were incomplete and we had to implement a methodology to construct complete genes based on sequence alignments. Currently, we are testing the recombinant expression of the cyanide dihydratase gene in *E.coli*. The purified enzyme will be characterized kinetically in order to compare its capacity to degrade cyanide with other known enzymes.

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The studies on preparation of chiral δ -valerolactone derivatives via enzymatic desymmetrization of diols.

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Keywords: prochiraldiols, δ -valerolactone, dioldesymmetrization, biocatalysis.

Chiral δ -valerolactone derivatives are useful building blocks for synthesis of many biologically active compounds¹. Unfortunately efficient synthesis of derivatives of this type compounds require multistep synthesis and usually usage of chiral substrates^{1,2}.

We propose to use enzyme-catalyzed desymmetization of diols as a crucial step for synthesis δ -valerolactone derivatives including unsaturated lactones (see Fig. 1). Commonly used enzymatic methods are based on kinetic resolution (KR), however this methods can provide product with maximum 50% yield. Theoretically, preparation of enantiomerically enriched compound via desymmetrization of diol can provide one enantiomer with 100% yield. Lipase catalyzed asymmetric esterification of diol have been shown to be efficient method for obtaining pure enantiomers of compounds with e.e. > 99% and 100% conversion³. The studies on lipase catalyzed desymmetrization of diol, followed by chemical reactions necessary for obtaining chiral δ -valerolactone will be presented.

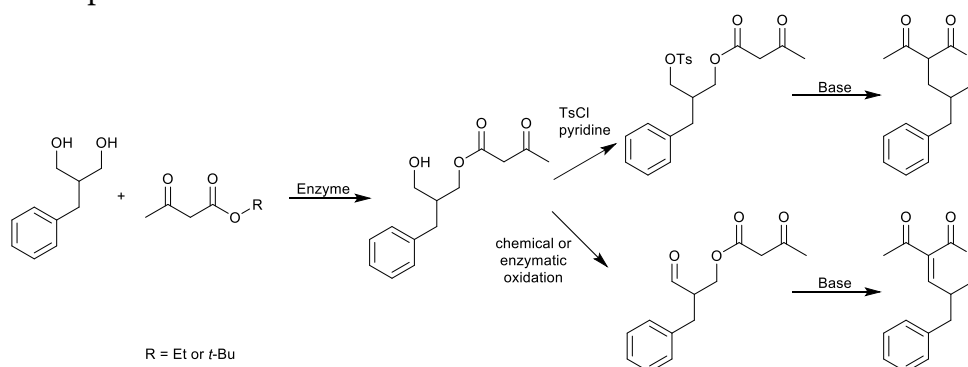


Fig. 1 Synthetic pathway for enantiomerically enriched δ -valerolactone derivatives.

Acknowledgment

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Comparative study of extremophilic beta-glycosidases: environmental adaptation and biocatalytic properties

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Keywords: extremophilic glycosyl hydrolases, biocatalysis, solvents, substrate-scope

Six beta-glycosyl hydrolases, all classified as GH1, have been selected to investigate how environmental adaptation affects biocatalytic properties, as well as stability at different temperatures, pHs and in the presence of solvent. Furthermore, we analyzed whether the subtle differences in the active site of the proteins may indicate a pattern for a preferential substrate recognition.

GH1 from *Thermobaculum terrenum* (*Tte*) and *Thermus Nonproteolyticus* (*Tno*) have been chosen as thermophilic examples, *Halothermothrix orenii* (*Hor*) and *Halobacillus halophilus* (*Hha*) as two halophilic proteins (with *Hor* being also thermophilic), and finally *Colwellia psychrerythraea* (*Cps*) and *Marinomonas profundimaris* (*Mpr*) as psychrophilic ones.

Results show a very interesting behavior specially in the presence of miscible organic solvents; thermophilic proteins markedly favor DMSO vs CH₃CN, while the opposite is true for the cold-adapted enzymes. Halophilic proteins have a more uniform behavior with all tested solvents including alcohols (which are well tolerated also by thermo- and psychrophilic GH1). As expected, thermal stability and activity of the different enzymes reflects the environmental adaptation of the original organisms with thermophilic proteins being significantly more stable (as well as active) at 50-60 degrees than the cold adapted.

Kinetic parameters, measured with four different sugar substrates (PNP-Glu, PNP-Gal, PNP-Fuc, PNP-Xyl) also highlight significant differences among the GH1s specially for what concerns the affinity for the various substrates. A superimposed model of the active sites of all the enzymes reveals only very minor amino acidic alteration (thought the overall sequence identity is low) which appear not to rationalize the differences observed experimentally, however once again a pattern is apparent among cold adapted vs salt adapted vs thermophilic GH1s indicating that environmental adaptation may be a relevant factor to consider for biocatalytic activity.

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Trans-Glycosylation of lactose and galacto-oligosaccharides (GOS) by glucansucrase enzymes

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Keywords: Glucansucrase, Glucosylation, Lactose, Galacto-oligosaccharides

Glucansucrases from *Lactobacillus reuteri* have received increasing interest because of their wide range of acceptor substrates, such as various disaccharides and oligosaccharides. However, except for glucose based oligosaccharides, there is little knowledge about their activity on other acceptors and their transfer products in terms of their size and structure. The ability of the glucansucrases GTFA and GTF180 from *Lactobacillus reuteri* to decorate lactose and various galacto-oligosaccharides (GOS) was studied. Transfer products with various types of linkage and sizes were obtained, isolated by HPEAC-PAD and characterized by NMR spectroscopy. Remarkably a new type of linkage, namely (α 1 \rightarrow 2)-linked to the reducing glucosyl unit of lactose, was introduced. Both studied glucansucrases formed the same branched trisaccharides and tetrasaccharides although they display different glycosidic linkage specificity with sucrose in oligo- and polysaccharide synthesis. The *Lactobacillus reuteri* 180 glucansucrase GTF180 showed capacity to add more than two glucose units to lactose but GTFA was unable to do that. Under optimal biosynthetic conditions the total conversion yield of lactose into these DP3 compounds was 40%. With galactosyl(β 1 \rightarrow 4)lactose (β 4'-GL) and galactosyl(β 1 \rightarrow 6)lactose (β 6'-GL) as acceptor substrate, the glucansucrases GTFA and GTF180 both added one glucosyl residue with different types of linkages. Galactosyl(β 1 \rightarrow 3)lactose (β 3'-GL) was not glucosylated by these glucansucrase enzymes. The prebiotic potential of these newly synthesized products is currently under investigation.

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Stereoselectivity of hydride transfer by a flavoprotein oxidase

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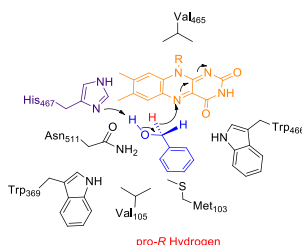
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Keywords: oxidase, oxidation, flavin, mechanism.

The regime of enzymes which are capable of oxidizing prim- and sec-alcohols to their carbonyl analogues at the expense of O₂ is broadly distributed in nature.¹ Most of them rely on catalytic metals (e.g. Cu²⁺), or on flavin cofactors, such as FAD. In alcohol oxidases, FAD serves as electron shuttle between the substrate and molecular oxygen, which acts as final electron acceptor. The catalytic cycle consists of two half reactions: First, a hydride is transferred from the alcohol substrate to the oxidized flavin cofactor. In the second half reaction, the reduced FAD is reoxidized by an electron transfer to molecular oxygen, which is subsequently reduced to hydrogen peroxide.^{2,3}

For several flavoprotein oxidases the mechanism of alcohol oxidation is well investigated. Recently, a 5-(hydroxymethyl)furfural oxidase (HMFO) from *Methylovorus* sp. strain MP688 has been discovered and characterized.⁴ This enzyme shows significant activity not only on the canonical 5-(hydroxymethyl)furfural, but also on benzyl alcohol. Here we present the results of kinetic studies conducted with the latter substrate and (R)- α -mono- and di-deuterated analogues, with emphasis on the stereoselectivity of the hydride transfer step. This was done by determining the primary kinetic isotope effect in pre-steady state measurements. Substrate docking studies supported the mechanistic proposal that the pro-R hydrogen of benzylic alcohol is transferred to N5 of the isoalloxazine ring from FAD. His467 assists the transfer by acting as catalytic base in the proton abstraction from the alcohol functionality.



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Stereoelectronic effects in the reaction of aromatic substrates catalysed by *Halomonas elongata* transaminase and its mutants

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Keywords: transaminase, amines biocatalysis, site directed mutagenesis.

A transaminase from *Halomonas elongata* and four mutants generated by an in silico-based design, were recombinantly produced in *E. coli*, purified and applied to the amination of mono-substituted aromatic carbonyl-derivatives. While benzaldehyde derivatives resulted excellent substrates, only NO₂-acetophenones were transformed into the (S)-amine with high enantioselectivity. The different behaviour of wild-type and mutated transaminases was assessed by in silico substrate binding mode studies.

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Theoretical and experimental study of enzyme kinetics in a microreactor system with surface-immobilized biocatalyst

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Keywords: microreactor, enzyme kinetic, surface-immobilization, biotransformation.

Microreactor system with surface-immobilized enzyme ω -transaminase was developed and used for conducting enzyme-catalysed surface biotransformation. Enzyme immobilization employs Zbasic2-tag appended to the ω -transaminase and is based on ionic interactions between the tag and the microchannel surface.

The Ping-Pong Bi-Bi mechanism was followed to describe enzymatic transamination of (S)-(-)- α -methylbenzylamine and pyruvate to acetophenone and L-alanine. For estimation of enzyme kinetic parameters, batch experiments with free biocatalyst were performed. Time scale analysis based on a detailed mathematical model of transport phenomena and surface enzyme-catalysed process in microreactor system was applied to characterize studied continuous bioprocess with surface kinetics.

The proposed simplified 1D model, which includes transport of the species and the enzymatic surface kinetics, was then used to define the surface concentration of active enzyme. Very good agreement between experimental data and predictions of continuous biotransformations is observed for various inlet substrates concentrations and different flow rates. Furthermore, the verified model was validated based on addition experiments of two consecutively-connected microreactors with surface-immobilized ω -transaminase. developed

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Characterization of *Pseudomonas putida* KT2440 ω -transaminase-alcohol dehydrogenase natural fusion

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Keywords: natural fusion enzymes, transaminase, biocatalysis, alkaloid biosynthesis

Naturally occurring multifunctional enzymes have evolved to effect consecutive catalytic or binding reactions, locking reactive intermediates in the production line between domains. *In vivo*, the presence of multifunctional enzymes in metabolic pathways is thought to confer selective advantage via increased reaction rates and chemical stability or prevention of toxicity from reactive intermediates.

Pseudomonas putida KT2440 is a non-pathogenic soil bacterium that has a diverse range of amino and fatty acid metabolic pathways. We have made an initial attempt to characterise a *P. putida* KT2440 ω -transaminase-alcohol dehydrogenase natural fusion protein PP_2782 (“KT fusion”) in order to explore its properties and biosynthetic abilities for subsequent novel enzyme engineering, so as to enable synthesis of bioactive compounds that are challenging to produce chemically.

In our preliminary results, following KT fusion expression in *E. coli*, as well as the expected monomer size (959 amino acids, estimated at 104 kDa) we observed a dimer band of approximately 200 kDa on SDS-PAGE. We also determined individual catalytic domain boundaries using the enzyme’s primary sequence and a predicted 3D structure homology search. Further analysis of the operon (PP_2788-PP_2777) orthology and synteny have proposed a range of possible substrates for this natural fusion.

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Lysozyme magnetized fibers: old enzyme to new uses as biocatalyst for cancer therapy

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Keywords: Lysozyme, chitosan-PVA, electrospinning, magnetized fibers

Lysozyme is a highly cationic mucolytic enzyme present in nearly all living beings, capable of hydrolyzing the peptoglycan of bacterial cell walls, acting as a defense mechanism against bacteria. In the past, lysozyme was studied as a possible anti-cancer agent. Nano and micro materials have unique characteristics like a higher area/mass ratio and higher reactivity, among others. These properties have been exploited in controlled release of drugs, site specific targeting of biomolecules for different diseases treatments, as cancer.

The main goal of this work was the development of a lysozyme nanofibrous system for the application in the treatment of colon cancer. Research was directed for creating a containment system which will eliminate metastatic cells within the tumor mass region, and any altered cells in the system's vicinity.

The system of lysozyme in polyvinyl-alcohol (PVA) and chitosan (CS)-PVA, was developed using the technique of electrospinning. Electrospun CS-PVA loaded with lysozyme and functionalized with magnetic nanobeads, was developed by emulsion technique. The polymers were dissolved and the solution pumped through a tube system, which ends on a thin nozzle wide enough to allow a single droplet to form, an electric field of about 10-30kV was applied. Core loading of lysozyme in the nanofibers was evaluated by extracting the protein content from fibers, using sodium dodecyl sulfate, a powerful negatively charged detergent. The lysozyme content of the extracted solution was determined by UV-vis spectrophotometry. Functionalization of electrospun nanofibers was performed with magnetic beads of Fe³⁺/Fe²⁺ (molar ratio 2/1). Evaluation of the morphology of nanofibers was carried out by SEM, thermal behaviour by FTIR.

The biological activities of the lysozyme loaded CS/PVA nanofibers were measured with an EnzChek lysozyme assay kit. To evaluate the success of the encapsulation process, in vitro lysozyme release, the ratio of adsorbed lysozyme on the fibers, and lysozyme activity were determined in different pH and buffer, mimetizing the environment of cancer cells. Viability of cancer cells was evaluated, after being exposed to electrospun lysozyme CS-PVA.

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Engineering Cyclohexanone Monooxygenase for the Production of Methyl Propanoate

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Keywords: Baeyer-Villiger oxidation, biotransformation, 2-butanone, cyclohexanone monooxygenase, enzyme engineering, ethyl acetate, methyl propanoate, regioselectivity.

Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO; EC 1.14.13.22) is a FAD- and NADPH-dependent Baeyer-Villiger monooxygenase. 2-Butanone was chosen as a substrate for the present study among the wide variety of ketones converted by this enzyme into esters or lactones. Two regioisomeric products, methyl propanoate and ethyl acetate, are detected in the reactions of AcCHMO with this small aliphatic ketone.¹ Methyl propanoate is of industrial interest as a precursor of acrylic plastic.² Various residues near the substrate and NADP⁺ binding sites in AcCHMO were subjected to saturation mutagenesis to enhance both the activity on 2-butanone and the regioselectivity toward methyl propanoate. Whole cell biotransformations of 2-butanone were carried out with the resulting libraries, which were analyzed using headspace gas chromatography-mass spectrometry.

Two interesting single mutants were discovered as a result of this study. The I491A AcCHMO mutant exhibits a significant improvement over the wild type enzyme in the desired regioselectivity using 2-butanone as a substrate (40% and 26% of the total product is methyl propanoate, respectively). The T56S AcCHMO mutant exhibits a higher conversion yield (92%) and k_{cat} value (0.5 s^{-1}) than wild type AcCHMO (52% and 0.3 s^{-1} , respectively). In addition, the uncoupling rate (i.e., NADPH oxidation rate without a ketone substrate) for the T56S AcCHMO mutant is significantly lower than that for the wild type enzyme. The T56S/I491A double mutant combined the beneficial effects of both mutations leading to higher conversion and improved regioselectivity. This study shows that even for a relatively small aliphatic substrate, catalytic efficiency and regioselectivity can be tuned by structure-inspired enzyme engineering.

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Demethylation of vanillic acid by recombinant LigM: a one-pot cofactor regeneration system

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Keywords: biocatalysis, cofactor recycling, demethylase, lignin.

Lignin is the most abundant renewable source of aromatic polymers on Earth: its degradation is mandatory for carbon recycling. The bacterium *Sphingomonas paucimobilis* SYK-6 is an intriguing microorganism, producing several lignin-degrading enzymes¹. The microorganism produces 2-pyrone-4,6-dicarboxylate from vanillic and syringic acids, a starting compound for biodegradable and high-functional polymers: in this pathway, the tetrahydrofolate (THF)-dependent O-demethylase LigM converts vanillic acid to protocatechuic acid (PCA).

In this work we optimized the recombinant expression of LigM in *E. coli*, and gained deep insight into its biochemical characterization, and its use in demethylation of vanillic acid and lignin model compounds². Based on comparative modeling, a three-dimensional structure of LigM in complex with THF was built: this allowed to rationalize the substrate preference of the enzyme and to propose a putative reaction mechanism. LigM efficiently converts vanillic acid into PCA but the reaction required a 10-fold molar excess of THF cofactor. In order to limit cofactor consumption, the plant methionine synthase MetE enzyme was also overexpressed in *E. coli* and used in combination with LigM. Under optimized conditions, the bi-enzymatic system produced 5 mM PCA using 0.1 mM THF only, a 500-fold decrease in cofactor:substrate molar ratio compared to single-enzyme process. By using the bi-enzymatic one-pot system, the overall costs could be drastically reduced: also owing to the inexpensive enzyme production process, the cost decreased from 20 to 0.2 €/mg of converted vanillic acid. This represents the first regeneration method for THF in a biocatalytic process, an issue of most relevance to expand the use of THF-dependent enzymes in the chemical and pharmaceutical industries.

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Protein crystallography for biocatalysis

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Keywords: X-ray structure determination, peptide amidase, xylose isomerase, xylooligosaccharide oxidase, ω -transaminase.

A few examples of recently determined enzyme crystal structures in the Biotransformation and Biocatalysis group will be discussed. All data of the determined structures is measured with the in-house Cu-K α radiation source, equipped with a MarDTB Goniostat System and a Mar345 image plate detector.

Peptide amidase from *Stenotrophomonas maltophilia* is a versatile catalyst for diverse carboxy-terminal peptide modification reactions. Using computational methods, twelve mutations were discovered and combined to yield a highly thermostable ($\Delta T_m = 23$ °C) and solvent-compatible enzyme. Comparison of the crystal structure with structures predicted by computational design indicates good agreement, validating the design methodology¹.

Xylose isomerase from the fungus *Piromyces* sp. E2 belongs to class II enzymes of xylose/glucose isomerases. The enzyme needs two metal ions in the active site for activity. Several structures of the enzyme without and with various metal ions, and with inhibitors, substrates and product were determined. The structures are in agreement with the promiscuity of the enzyme with regards to metal activation and serve as a basis for protein engineering aimed at improving enzyme performance². Xylooligosaccharide oxidase (XylO) from *Myceliophthora thermophila* C1 is a novel oligosaccharide oxidase with a strong substrate preference towards xylooligosaccharides. XylO crystal structures in complex with a substrate mimic, xylose and xylobiose have been solved. The residues that tune the unique substrate specificity and regioselectivity could be identified. Future studies will reveal the true potential of XylO as biocatalyst³.

ω -Transaminase from *Pseudomonas jessenii* is a pyridoxal-5'-phosphate (PLP) dependent enzyme which can be used for the synthesis of (chiral) amines. Its structure has been determined in 2 apo forms, the PLP-, the PMP- and the PLP/product-bound forms. The structure explains the acceptance of caprolactam-derived 6- α -aminocaproic acid as a preferred substrate for the novel aminotransferase, and provides the basis for improvement of stability by computational design⁴.

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Opposite enantioselectivity in the bioreduction of (Z)-beta-aryl-beta-cyanoacrylates mediated by the Trp 116 mutants of Old Yellow Enzyme

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Keywords: enantioselectivity, opposite binding mode, OYE mutants

Wild type OYE1-2-3 was found not to be able to convert (Z)-β-aryl-β-cyanoacrylates, interesting difunctionalised alkenes because of their possibility to afford different chiral building blocks where the two functional groups can be easily manipulated. It has been established that the Trp 116 residue in *S. pastorianus* OYE1 plays a critical role in the stereochemistry of OYE-mediated alkene reductions¹.

The Trp 116 mutants of Old Yellow Enzyme 1 were found able to catalyse the reduction of this class of substrates, giving opposite enantioselectivity according to the nature of the amino acid in position 116. Small amino acids (*e.g.* alanine) make the substrate bind to the enzyme active site in a “classical” orientation, affording the (*S*)-enantiomer of the reduced product. When the size of the amino acid increases (*e.g.* leucine), a “flipped” binding mode is adopted by the substrate, which is converted into the corresponding (*R*)-derivative. With bulky amino acids (*e.g.* tryptophan in the wild type) the reduction does not occur. The enantiomerically enriched cyanopropanoates thus prepared can be converted into the corresponding (*S*)- and (*R*)-β-aryl-γ-lactams, precursors of inhibitory neurotransmitters belonging to the class of γ-aminobutyric acids, by a simple functional group interconversion in a sequential one-pot procedure.

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High-throughput screening of CAZymes using novel chromogenic substrates

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Keywords: enzyme screening, polysaccharide degradation, carbohydrate active enzymes, high-throughput

Enzymes that degrade or modify polysaccharides are widespread in pro- and eukaryotes and have multiple biological roles as well as biotechnological applications. Recent advances in genome and secretome sequencing, together with associated bioinformatics tools have enabled large numbers of putative carbohydrate-acting enzymes to be identified. However, a serious bottleneck in the development of enzyme-reliant bio-refining processes is a paucity of methods for rapidly screening the activities of these enzymes.

We have developed a new generation of multi-coloured chromogenic polysaccharide and protein substrates that can be used in cheap, convenient and high-throughput multiplexed assays. In addition, we have produced substrates of native biomass materials in which the architecture of plant cell walls is partially maintained to study the availability and accessibility of various polysaccharides within a complex biomass substrate. Chromogenic substrates can be used in a 96 well plate, agar plate or test tube format to screen the activities of glycosyl hydrolases, lyases, lytic polysaccharide monooxygenases (LPMOs) and proteases. We have validated the technique using purified enzymes and enzyme mixtures. Here we show that these new assays enable rapid analysis of endogenous enzymes as well as secreted enzymes from various hosts¹.

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Effective electroanalysis with allosteric enzymes: The reductase unit of an *A. baumannii* hydroxylase as model

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Keywords: Allosteric enzymes, amperometry, biomarker, p-hydroxy-phenylacetate (HPA)

p-Hydroxyphenylacetate (HPA) hydroxylase (HPAH) from *Acinetobacter baumannii* is using cooperative action between reductase (C1) and hydroxylase (C2) enzyme subunits for conversion of HPA into 3,4-dihydroxyphenylacetate [1]. Previous biochemical studies have shown that on its own C1 is capable of enzymatic NADH oxidation, with the change of native cofactor oxygen into detectable hydrogen peroxide (H₂O₂) responsible for nonstop protein redox recycling. An extra observation of the early work was that C1 is molecularly designed to perform cyclic NADH transformation in allosteric fashion, with significant cascade amplification inducible via sensitive HPA affinity binding to a surface receptor entity that is distinct from the enzyme's actual catalytic site.

Reported here will be the exploration of C1 allostery for the creation of a substrate (NADH) and effector (HPA) detection scheme with simple electrochemical H₂O₂ readout. As NADH and HPA are both oxidizable at a practical anodic H₂O₂ detection potential, the amperometry of the signaling target had to use a cathodic platform to be interference-free and suitable as screen for ongoing C1 activity. Sensors were thus screen-printed carbon electrodes with Prussian blue surface modification (PB/SPEs) at which H₂O₂ evolution from C1 action on NADH could be monitored cathodically in amperometric recordings, at -100 mV vs. Ag/AgCl. In typical calibration trials the cathodic H₂O₂ assay was linear till 2 mM ($R^2 = 0.997$), with a sensitivity of $\sim 0.3 \mu\text{A } \mu\text{M}^{-1} \text{ cm}^{-2}$ and a detection limit of $\sim 1 \mu\text{M}$. As desired, interference was not observed for NADH/HPA.

Cathodic PB/SPE-H₂O₂ amperometry well sensed enzyme activity in C1/NADH solutions with and without allosteric effector HPA. In either case, H₂O₂ currents evolved gradually over time, however, the observed current rises, expressive of increasing H₂O₂ levels, were reproducibly much faster in HPA presence. This was perfect experimental proof of the accelerating HPA impact on the biocatalytic reaction of C1 with NADH. The opportunity of an efficient electroanalysis of C1 allostery allowed creation of a novel competitive analytical practice for NADH quantification and, the potential of the involved allosteric effector as disease biomarker in mind, more importantly for HPA valuations, too.

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Computational exploration of epoxide hydrolases enantioselectivity towards substrate control

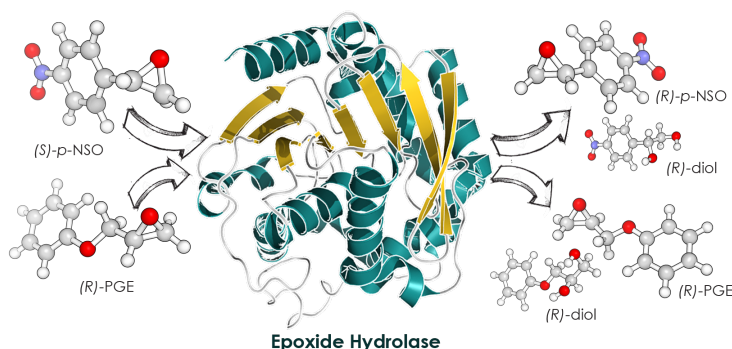
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Keywords: epoxide hydrolases, molecular dynamics simulations, quantum mechanics, enantioselective hydrolysis of epoxides

Epoxide hydrolases (EHs) have been widely studied because of its potential application for manufacturing purposes as biocatalysts for the asymmetric hydration of epoxides racemic mixtures. Most epoxides are recognized as important precursors of add-valuable pharmaceuticals, such as propranolol or alprenolol β -blockers drugs.¹ EHs can catalyze the enantioselective hydrolysis of racemic mixtures of epoxides through the formation of a covalent intermediate between the enzyme and a particular enantiomer, leading to the formation of the corresponding diol and the unreacted enantiomer. However, it is still poorly understood which factors are governing the regio- and enantioselectivities of these processes. For the particular case of the EH from *Bacillus megaterium* (BmEH), its natural (*R*)-selectivity towards aryl glycidyl ethers is reversed when the *para* nitro styrene oxide (*p*-NSO) substrate is considered.² In this study, we evaluate through nanosecond time-scale Molecular Dynamics (MD) simulations and Quantum Mechanics (QM) calculations how the substituents on the substrate aryl group affect the natural BmEH enantioselectivity. MD simulations were done considering different reaction stages, i.e. in the substrate-bound state, the alkyl-enzyme and tetrahedral intermediate states for each possible isomer. Results obtained from MD simulations in combination with QM calculations provide a detailed description of the enzyme conformational dynamics, active site preorganization, and the effect of the substrate substituent on the binding and activation energies, that allow us to rationalize which factors govern the intrinsic regio- and enantioselectivities in BmEH.



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Bioreductions as the key step for the synthesis of biologically active natural products

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Keywords: biocatalysis, ketoreductases, chiral synthons, chemoenzymatic.

Biocatalysis has contributed significantly in the development of simple and straightforward methodology for the stereoselective synthesis of pharmaceutically interesting compounds. More specifically, ketoreductase-catalyzed reductions of various carbonyl compounds have resulted in the synthesis of chiral synthons in high optical purities (>99% de, >99% ee) and chemical yields. These molecules are key intermediates for the synthesis of many natural products¹, pharmaceuticals² and other valuable compounds.

We will present here a chemoenzymatic methodology toward the synthesis of Stereocalpin A³ and Rugulactone⁴. Stereocalpin A is a metabolite of the antarctic lichen *Stereocaulon alpinum*. Lichenous metabolites are potential sources of pharmacological agents. Rugulactone belongs to a family of Cryptocarya apyrone containing natural products isolated from *Citrus rugulosa* extract that exhibit great inhibitory properties for the nuclear factor kB (NF-kB), which is involved in many diseases, such as cancer and chronic inflammatory diseases. It is an optically active d-lactone. Our methodology involves the application of enzyme-catalyzed reductions as the key steps for introduction of the right stereochemistry of the target molecules

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CASoX – analyze, annotate and visualize protein cavity information

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Keywords: CASoX, cavity analysis, cavity calculation, cavity visualization

CASoX is a plug-in for the visualization tool PyMOL¹ and provides a convenient and flexible representation of cavity information of protein structures.

The shape and size of a protein cavity, the physico-chemical properties and the solvent accessibility are often relevant for binding and conversion of substrates of the enzyme. Therefore cavity and cleft information is very useful to understand important protein behaviors, substrate bindings and substrate scope. A convenient visualization, calculation and interaction tool for cavities and clefts will ultimately help to analyze, interpret and present cavity information more easily.

Most of the available cavity detection programs provide and show only output for their best predictions and therefore discard the rest of the cavity information. Also many settings for the cavity detection algorithms cannot be changed easily. This makes it more likely to miss some cavity features, or to interpret cavity or cleft information differently. Additionally, in special cases, the part of a protein which is of interest can be missed completely.

CASoX keeps all calculated cavities and clefts of the protein structure. Therefore, it is easier to identify water or substrate tunnels which are not directly visible or calculable (because e.g. an amino acid blocks the entry). Additionally, it is possible to calculate and map hydrophobicity and accessibility values onto each cavity point. Important cavities can be selected by size or manually, merely by picking cavities based on their overall shape, location and/or property. All settings for the cavity calculation can be changed for special cases. By utilizing the capabilities of PyMOL, every cavity and cavity point can be shown and selected separately.

CASoX is a visual cavity inspection and analysis tool which is fast and easy to use. It can be seen as an “every day” supplement to other existing cavity programs.

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Discovery of an enzyme for fumonisin B₁ degradation in animal feed

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Keywords: mycotoxin detoxification, fumonisin B₁, enzymatic degradation, feed

Enzymes that improve the nutritive value and digestibility of the feed, e.g. phytase, β -glucanase or proteases, have become important feed additives in animal husbandry. Here, we describe the discovery of a feed enzyme for a novel application, the enzymatic detoxification of mycotoxins in the gastrointestinal tract of the animals. The new enzyme, FumD, targets fumonisins, a group of structurally related mycotoxins that are encountered worldwide as frequent contaminants of corn products with fumonisin B₁ (FB₁) being their most abundant representative. Fumonisins interfere with the sphingolipid metabolism by inhibiting a key enzyme, ceramide synthase. This leads to an imbalance of sphingolipids and sphingoid bases causing the toxic effects of fumonisins that manifest for example as porcine pulmonary edema or equine leucoencephalomalacia in domestic animals. The alphaproteobacterium *Sphingopyxis* sp. MTA144, isolated from a soil sample was found to efficiently degrade FB₁. A gene cluster, named *fum* cluster, containing eleven open reading frames (*fumA-K*) was identified as associated with FB₁ degradation and protein predictions were performed for all genes in this cluster. The predicted functions of FumD, FumI, FumH and FumK have been verified experimentally. The type-B carboxylesterase FumD catalyzes the first step of the degradation converting FB₁ into hydrolyzed FB₁ (HFB₁) by cleaving the two tricarballic acid (TCA) side chains. *In vivo* studies with purified HFB₁ typically show a significant reduction in toxicity as compared to FB₁. Therefore, FumD was considered a promising enzyme for the enzymatic detoxification of FB₁ in the gastrointestinal tract of livestock. An expression host strain for large scale production and analytical methods for the detection of FumD activity have been developed. Last but not least, the efficacy of FumD for gastrointestinal detoxification of FB₁ was evaluated in feeding trials.

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Surface charge engineering of a *Bacillus gibsonii* subtilisin protease

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Keywords: Directed evolution, Deamidation, pH optimum, Serine protease, Surface charge engineering

Bacterial subtilisins are the subgroup of serine proteases of major industrial significance and have been studied extensively. Due to their large scale production and outstanding properties subtilisins have a leading application in industries. The development of subtilisins with an emphasis on improved performance/cost ratios, increased activity, improved resistance toward oxidative agents, understanding of protease activity, pH optimum and stability dependent on net charge are the main focus of enzyme engineering field in the recent years. In this work the effect of posttranslational deamidation resulting in change of net protein charge was investigated. Three criteria for selecting amino acid substitutions of the deamidation type in the *Bacillus gibsonii* alkaline protease (BgAP) are proposed and systematically studied. Deamidation change from asparagine (Asn) and glutamine (Gln) residues into negatively charged aspartic (Asp) and glutamic acid (Glu) yielded a protease variant with dramatically increased activity at higher pH (8-10). The general principle regarding the effect of surface changes on enzymes validated on protease can be applied in tailoring any protein/enzyme class.

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Recombinant expression and biochemical characterization of a polyphenol oxidase from *Marinomonas mediterranea*

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Keywords: laccase, membrane-bound, SDS, His-tagged

Laccases are blue multi-copper oxidases which couple the four electron reduction of dioxygen to water with the oxidation of a broad range of substrates (including phenols, polyphenols, arylamines, anilines and thiols): these features make laccases “green enzymes” suitable in several industrial sectors, from food industry to conversion of plant biomass in integrated lignocellulose biorefineries.

Marinomonas mediterranea is a melanogenic marine bacterium that synthesizes a polyphenol oxidase (MmPPOA) with laccase activity. This enzyme was one of the first prokaryotic laccases sequenced, shows peculiar properties such as an high specificity for phenolic compounds and high tolerance towards chloride, making it an interesting candidate for specific biotechnological applications.

Two enzymatic variants of MmPPOA (membrane-associated vs. a soluble form lacking of the N-terminal targeting signal) were expressed in *E. coli* cells as His-tagged proteins and were purified¹.

The presence of the C-terminal His-tag facilitates the protein purification and positively affects the kinetic parameters of MmPPOA. The kinetic parameters of recombinant MmPPOA variants were determined on the non-phenolic ABTS, and the phenolic substrates catechol and 2,6-DMP. The highest affinity and kinetic efficiency was observed for catechol: the values for MmPPOA were higher than those measured for a number of laccases². On the other hand, the soluble variant shows a weak affinity for all the tested substrates (suggesting an alteration in protein conformation). Recombinant MmPPOA variants show interesting biochemical features as a strong tolerance to NaCl, DMSO and surfactants (SDS, Tween-80) and high thermostability.

This work demonstrates that MmPPOA is an interesting biocatalyst for industrial applications requiring high-tolerance to strong environmental conditions and paves the way to its structural characterization.

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Enhancement on the biocatalytic synthesis of glycerol carbonate using cyclohexane as co-solvent

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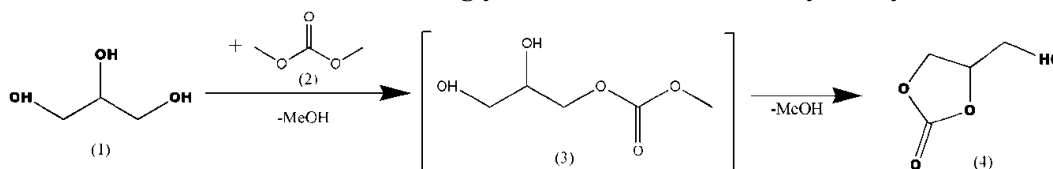
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Keywords: glycerol, glycerol carbonate, lipase, cyclohexane.

Glycerol carbonate (GlyC, 4-hydroxymethyl-1,3-dioxolan-2-one) is one of the most relevant examples of added-value products derived from glycerol (biodiesel industry) with promising valences, e.g. "green solvent" due to its properties, valuable intermediate for the production of coatings/ paints/ detergents, and source of polycarbonates and polyurethane.

Previously, we report a biocatalytic route for the GlyC synthesis involving the reaction of glycerol with dimethyl carbonate (DMC) assisted by a lipase from *Aspergillus niger* source (Scheme 1) under solvent-free conditions¹⁻³. The developed biocatalytic system allowed to synthesized GlyC with maximum 73 % conversion of glycerol and 77 % selectivity in GlyC.



Scheme 1. Reaction of glycerol with DMC for GlyC synthesis. (1) glycerol; (2) DMC; (3) unstable intermediate; (4) GlyC.

Now, we report a new study on the previous described process. Effects of the co-solvents such as cyclohexane and tetrahydrofuran (THF) have been investigated on the process performance.

Liquid cultures of fungal cells (supernatants) were the biocatalysts (extracellular enzymes such as lipase and pyruvate decarboxylase were preponderantly). The cells were cultivated on YPG culture media supplemented with various type of waste glycerol (1% and 3% glycerol).

All tested biocatalysts allowed the total conversion of glycerol (100 % conversion of glycerol) into GlyC (95 % selectivity in GlyC). The mechanism of the process was set up based on the additional tests. All of the experimental aspects related to this study will be detailed during the presentation.

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Guideline of α -pinene bio-conversion through the biocatalyst design

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Keywords: biocatalysis, α -pinene, enzyme immobilization, α -pinene derivatives.

α -pinene is the main component of the monoterpene fraction in essential oils (e.g. mastic oil) and turpentine (i.e. paper and pulp industry residue available in bulk quantities at a low price). Commonly, its applicability is limited to fuel of the recovery boilers. However, α -pinene is considered a platform molecule with a great potential for the production of pharmaceuticals, agrochemicals and fine chemicals.

In this context, we developed several biocatalytic model-systems for the conversion of α -pinene into value-added derivatives (e.g. α -pinene oxide, campholenal, camphene, carveol, verbenol, verbenone, etc) using lipase, H₂O₂ and ethyl acetate as biocatalyst, oxidation reagent and co-reagent/organic solvent, respectively. One of the models involved the lipase- based cross-linked aggregates design (cross-linked enzyme aggregates (CLEA) and cross-linked enzyme aggregates onto magnetic particles (CLEMPA)), which were compared to covalent design (e.g. covalent immobilized enzyme (CIE) on magnetic particles (MP) supports). Both CLEA and CLEMPA designs afforded better epoxidation yields of α -pinene (around 30 % for both biocatalysts) compared to CIE (14 %). Also, the investigated biocatalysts allowed the production of α -pinene oxide (40 %) and derivatives such as camphene (15 %) and campholenal (20 %).

Second model investigated the biotransformation α -pinene using a bifunctional biocatalysts designed as carbohydrate biopolymers entrapping lipase enzyme. Lipase assisted the epoxidation of α -pinene using H₂O₂ as oxidation reagent and ethyl acetate as both acetate-supplier and solvent affording α -pinene oxide as the main product. Further, the biopolymer promoted the izomerization of α -pinene oxide to campholenic aldehyde and trans-careenol. The presence of biopolymers enhanced the catalytic activity of the biocomposites as compared to the free enzyme (ie 13.39×10^3 , 19.76×10^3 and 26.46×10^3 for the free lipase, lipase-carrageenan and lipase-alginate, respectively). The biocatalysts stability and reusability was confirmed in six consecutively reaction runs.

Thus, we offers different alternatives for α -pinene valorization into value-added products related to the biocatalyst design involved in the biochemical process.

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Efficient synthesis of specialty carbohydrates through industrial biotechnology

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Keywords: specialty carbohydrates, strain engineering, production method, biochemical pathway.

Rare sugars such as L-fucose, L-ribose and sialic acid, or human milk oligosaccharides such as fucosyllactose and sialyllactose are very difficult to synthesize. These products are consequently very expensive and not available in sufficient quantities. For the synthesis of such highly complex carbohydrates, organic synthesis is not an efficient production method because of the high chirality and excessive presence of hydroxyl groups in the carbohydrate building blocks. Also extraction of these compounds from natural sources is often hampered by the substrate availability and extraction cost.

To solve this problem, a highly efficient production method for specialty carbohydrates has been developed by the start-up company Inbiose. The method is based on the use of cell factories in which a natural pathway has been expressed for the synthesis of the target specialty carbohydrate. Base strains that are engineered to generate the carbohydrate building blocks are equipped with a functional pathway to produce the target specialty carbohydrate.

Using the Inbiose technology, any naturally occurring specialty carbohydrate can be produced. Depending on the target, a biochemical pathway will be designed and expressed in one of the Inbiose base strains. Through synthetic biology and metabolic engineering, the production strains are optimized. The target carbohydrate is then produced by fermentation and is efficiently excreted in the culture medium. After fermentation, the target carbohydrate is recovered from the fermentation broth in high yield and purity (generally > 99 %) using a simple down-stream processing method.

The production method is generic and has already been proven on an industrial scale. The process is also perfectly scalable so that the availability of these specialty carbohydrates is no longer an issue. Inbiose has access to pilot and production facilities and is able to produce over 1.000 t/a of specialty carbohydrates.

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Fine-tuning of an artificial “mini”- pathway for the synthesis of polyhydroxylated compounds

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Keywords: Artificial pathway, aldol reaction, alcohol dehydrogenase, process optimization

Over the last years, stereo-chemically controlled C-C bond formation reactions promoted by the fructose-6-phosphate aldolase variant (Fsa1-A129S from *Escherichia coli* (*E. coli*)) have been intensively studied to provide biologically relevant molecules.^{1,2} Herein, we present an artificial *in vivo* pathway consisting of the alcohol dehydrogenase (ADH) AlkJ from *Pseudomonas putida* (*P. putida*) for oxidation of a primary alcohols to cytotoxic aldehydes, which are *in situ* converted by the DHA (dihydroxyacetone) dependent Fsa1-A129S to the corresponding chiral aldol products.

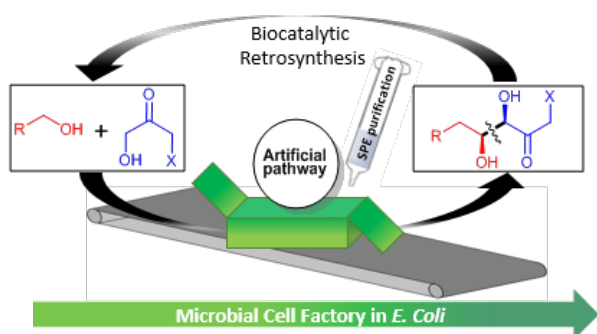


Figure 2 Illustration of the designed biocatalytic process (*AlkJ* and *Fsa1 S129A*) for the synthesis of polyhydroxylated compounds. (R= Ph-, BnO-, Cbz-; X= H, OH)

To improve the overall performance, different strategies including genetic modifications by assembling both genes of interest (*AlkJ* & *Fsa1-A129S*) on a single plasmid (operon, pseudo operon & monocistronic configuration)³ and optimization of process parameters (e.g. Aldol donor concentration and cellular transport) were applied. The engineered strain combined with a refined solid-phase extraction (SPE) purification protocol provides an efficient process for the synthesis of carbohydrate derivatives.

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Metabolic enzymes for integrated synthesis of metabolites

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Keywords: Kinase, Lyase, Synthase, Metabolite.

The overlay of classical retrosynthetic analysis with biochemical pathway analysis often reveals large differences in the number of reaction steps which are involved in the different routes for the synthesis of metabolites utilized by synthetic chemists or by nature. As the synthesis of a large number of pure metabolites is highly important¹⁻², but requires also much work, significant efforts in simplifying the work are needed in order to make it feasible, e.g. in reducing the number of reaction steps, eliminating the introduction and removal of protecting groups and simplifying extensive purification steps in lengthy multi-step routes. Navigating from the target metabolite backwards through the known metabolic pathways and comparing it with classical retrosynthetic analysis has been a valuable approach to integrate high numbers of classical synthetic steps into a single reaction step.

Various new ATP-dependent kinases, a new argininosuccinate lyase and a new DAHP-synthase have been identified and prepared as highly active recombinant enzymes. Their applications in the biocatalytic synthesis of the corresponding metabolites were developed using direct kinetic reaction analysis by ¹H-NMR and ³¹P-NMR. Complete conversions have been achieved for all kinase-catalyzed phosphorylations, whereby ATP was regenerated using phosphoenolpyruvate and pyruvate-kinase. The product recovery of all the phosphorylated metabolites was performed by standard workup. The arginino-succinate lyase-catalyzed addition reaction of L-arginine to fumaric acid has enabled the one-step synthesis of L-argininosuccinate. DAHP has been synthesized in a one-step condensation reaction of phosphoenolpyruvate and D-erythrose-4-phosphate catalyzed by DAHP-synthase.

The success of this approach using new metabolic enzymes has been demonstrated for the synthesis of a number of metabolites with high step economy and inspires a lot of new simplified routes to metabolites.

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Mutations related to amino acid metabolism for enhanced penicillin production in *Penicillium chrysogenum*

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Keywords: Mutations, L-Threonine/L-serine deaminase, L-cysteine biosynthesis, Penicillin production

Based on classical strain improvement, many industrial strains of *P.chrysogenum* have been developed through a complex process of random mutagenesis and further screening in the past 70 years. Throughout this process, *P. chrysogenum* strains accumulated lots of beneficial unknown mutations for increased penicillin production. The relation between the mutations in genes associated with amino acid metabolism and penicillin production in fungus, was studied in three *Penicillium* strains (NRRL1951, Wisconsin 54-1255, and DS17690) that are in a lineage of strain improvement. One unknown protein encoded by Pc20g08350 gene was studied, we found that this protein catalyzes the formation of L-cysteine from O-acetyl-L-serine and H₂S, which proves that the direct sulfhydrylation pathway also existed in *P.chrysogenum* NRRL1951 and Wisconsin 54-1255, besides the transsulfuration pathway that has already been demonstrated before. Our results also show that in mutated strain(DS17690) the branch pathways of L-threonine degradation and L-serine degradation were removed by mutating Pc16g03260 and Pc13g07730 genes, which make more precursors available for L-cysteine biosynthesis compared to the wild-type(Wisconsin 54-1255). Thus, our results demonstrated that the direct L-cysteine biosynthesis from O-acetyl-L-serine exists in *P.chrysogenum* strains and has been improved by random mutagenesis in order to produce more L-cysteine, which is the most important one among three precursors for penicillin production. In conclusion, our finding on the three enzymes give some explanation on how classical strain development has improved *P. chrysogenum* strains to become an excellent cell factory for the production of β -lactam antibiotics.

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Reaction kinetics and GOS product profiles of the β -galactosidases from *Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae*

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Keywords: galactooligosaccharides, β -galactosidase, transgalactosylation, prebiotics

The β -galactosidases from *Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae* are able to convert lactose into a mixture of galactooligosaccharides (GOS), based on their transgalactosylation activity. Here we report a detailed analysis of (changes in) the GOS profiles of these 3 β -galactosidase enzymes in time. The GOS yields of these enzymes were clearly different; the highest yield was observed for the β -galactosidase from *B. circulans* (48.3% GOS at 88.4% lactose consumption), followed by the *K. lactis* (34.9% GOS yield at 91.8% lactose consumption), and *A. oryzae* (19.5% GOS yield at 69.6% lactose consumption) enzymes. Incubations of these enzymes with lactose (30 % w/w) plus the monosaccharides Gal or Glc (20 % w/w) resulted in altered GOS profiles. Experiments with ¹³C₆ labeled Gal and Glc showed that both monosaccharides act as acceptor substrates in the transgalactosylation reactions of all 3 enzymes. The data shows that the lactose isomers β -D-Galp-(1→2)-D-Glcp, β -D-Galp-(1→3)-D-Glcp and β -D-Galp-(1→6)-D-Glcp are formed from acceptor reactions with free Glc and not by rearrangement of the Glc component of lactose in the active site.

The enzyme driven enantioselective Passerini reaction

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Keywords: Passerini reaction, enantioselective, enzymatic, cascade reaction

Isocyanide-based multicomponent reactions (MCRs), such as Ugi and Passerini reactions, have been of particular interest because of the large number of applications in medicinal chemistry and drug discovery.¹ The Passerini three component reaction (P-3CR) involves the condensation of a carbonyl compound, a carboxylic acid and an isocyanide leading to an α -acyloxy carboxamide.² However P-3CR has been widely applied, the challenge of stereocontrol on a newly generated stereogenic center is currently very limited. As an alternative to the commonly employed metalocatalyst and organocatalysts, a biocatalytic approach can be expected to be beneficial due to high selectivity of enzymes and mild reaction conditions. We developed an efficient enantioselective enzyme-driven Passerini type reaction with a broad substrate scope in the presence of enzyme catalysts (Fig 1.). It combines the diversity offered by multicomponent reactions with the selectivity of biocatalysts. We investigated a wide range of enzymes, various organic solvents and water content. With the optimal conditions in hand, the generality of the reaction was next examined. Most reactions gave products in excellent enantioselectivities (up to 99%). The reaction is applicable to a wide range of vinyl esters and isocyanides.

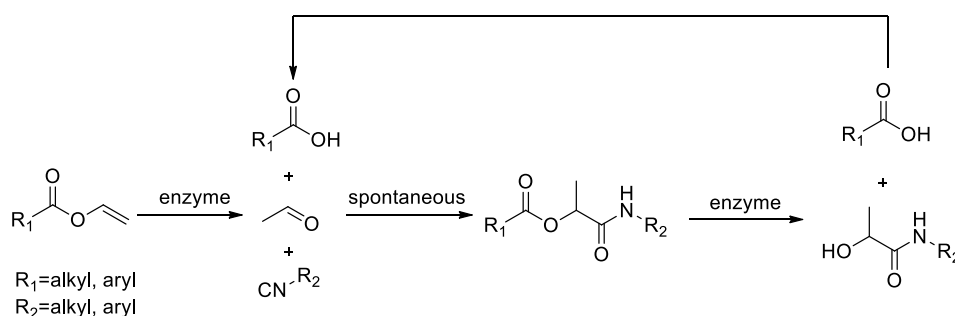


Figure 1. Cascade reaction.

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Modification and application of vibriophage endolysins as new biocontrol agent against pathogenic strains

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Keywords: Endolysins, *Vibrio parahaemolyticus*, chimeric enzyme, antimicrobial peptides

Endolysins are enzymes coded by bacteriophages at the end of their replication cycle to degrade the peptidoglycan of the bacterial host, resulting in cell lysis. In Gram-positive bacteria due to the absence of an outer membrane, endolysins can access the peptidoglycan and destroy these microorganisms when applied externally¹. However the expansion of endolysins as antibacterials against Gram-negative pathogens is hindered by the outer membrane. This physical barrier poses a highly effective permeability for the passage of harmful compounds². In this study we evaluated a recombinant endolysin against Gram-negative *Vibrio parahaemolyticus* strain (*Vp*) called *VpEndI*. Vibriosis is the most prevalent diseases responsible for mortality in aquaculture systems worldwide. Particularly in white shrimp *Litopenaeus vannamei* (*Lv*) farms. In spite of the conserved biological function, endolysins are very diverse in sequence and structure. Particularly *VpEndI* no resemble any reported endolysin. Recombinant expression was performed in *E. coli* Rosetta II strain using pCold as expression vector. Purification was conducted by IMAC standard protocols. The SDS-PAGE analysis revealed a single band of 25kDa, consistent with its theoretical molecular weight. The enzymatic activity showed lytic effect against the reference and pathogenic *Vp* strains. Furthermore, tests were performed to discard its action spectrum against probiotic bacteria. To solve the *Vp* outer membrane, translational fusions in the N-terminal was performed using a peptide with known antimicrobial activity. The *in vivo* test of both wild-type and chimeric enzyme will be directly on *Lv*. Endolysins represent a novel promising class of antibacterial based on its selective cell wall hydrolysis.

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Characterization of highly branched maltodextrins produced by the GH57 glycogen branching Enzymes

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Keywords: glycogen branching enzyme, amylose V, branching degree, degree of polymerization

Glycogen branching enzymes (GBE) are enzymes involved in the synthesis of glycogen, a storage carbohydrate of many microorganisms. They are members of the Glycoside Hydrolase families 13 (GH13) and 57 (GH57), catalyzing the formation of $\alpha(1\rightarrow6)$ linkages by rearrangement of preexisting α -glucans through cleavage of $\alpha(1\rightarrow4)$ linkages and transfer to $\alpha(1\rightarrow6)$ positions. GBE modified starch can be used e.g. as slow release glucose food ingredient (1) or in paper production (2). GH13 GBEs mainly produce high molecular weight maltodextrins (3). However, much less is known about the properties of GH57 GBE and the products (4,5). Here we modified starch with the GH57 GBEs of the hyperthermophilic bacterium *Thermus thermophilus* and the archaeon *Thermococcus kodakarensis* KOD1. Codon optimized GBE genes were overexpressed in *Escherichia coli* and the enzymes were purified by heat treatment and nickel affinity chromatography. Both enzymes showed a relatively high hydrolysis activity next to branching activity. They both produce relatively small, highly branched polysaccharides (11%) with an average degree of polymerization of 60 and 70 respectively, and the most abundant side chains of both products were DP6 and DP7.

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Continuous biocatalysis with an aminotransferase immobilized in a microreactor coated with functionalized electrospun mat

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Keywords: aminotransferase, immobilization, enzymatic microreactor, nanofiber mat

Microscale technology presents great opportunity for establishment of continuous biocatalytic processes¹. Very high surface to volume ratio and small dimensions enabling efficient mass transfer in microreactors present a huge potential for surface enzyme immobilization, which is often prerequisite for its competent use. In order to achieve high enzyme loads and stable, but also readily reversible surface immobilization to allow re-use of the microstructured element, functionalization of biocatalysts and inner surfaces is employed.²

In this work, an aminotransferase ATA-wt (c-LEcta, Germany) was genetically modified with poly(His)-tag to obtain His-ATA-wt. Moreover, commercially available resealable flow cell platform (Micronit, The Netherlands) was coated with electrospinning-based nanofiber mat Tiss[®]-NH₂ (NanoMyP[®], Spain) functionalized with tris(carboxymethyl)ethylene diamine and loaded with Cu²⁺. After integration of a membrane in the microreactor, the evaluation of reactor volume and efficiency of His-ATA-wt retention after the exposure to enzyme aqueous solution was performed using high pressure syringe pumps for entering the fluids. Moreover, microreactors with immobilized enzyme were further tested for selected transamination reaction, where an in-line HPLC analysis was used to evaluate reactor performance at various flow rates and substrate concentrations. The stability of a microreactor with immobilized enzyme was tested by performing the continuous biotransformation 24 and 48h after the immobilization.

Based on a rather simple and short immobilization procedure, we were able to immobilize up to 6.6 mg/mL of protein in the microreactor of 68.4 μL internal volume. Up to 90 % conversions of 40 mM (S)-(-)-α-methylbenzylamine and 40 mM sodium pyruvate to L-alanine and acetophenone were achieved at 30 °C in the presence of pyridoxal 5'-phosphate within the residence time of 7 min, which shows very high volumetric productivity of the developed enzymatic microreactor. Furthermore, above 90 % of initial productivity could be retained within the tested period.

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Protein engineering of a polyphenol oxidase towards enhancement of its monophenolase activity

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Keywords: site-directed mutagenesis, polyphenol oxidase, tyrosinase, *Myceliophthora thermophila*

Type-III copper proteins include, among others, the enzymes tyrosinase, catechol oxidase (CO) and aureusidin synthase. These proteins are able to catalyze oxidation reactions and are therefore called polyphenol oxidases (PPOs). The difference between tyrosinases and COs is found in their substrate specificity and catalytic abilities. Tyrosinases catalyze the *o*-hydroxylation of *p*-monosubstituted phenolics, like tyrosine and tyramine, to *o*-disubstituted phenolics and further oxidase these to *o*-quinones (monophenolase/diphenolase activity), whereas COs seem to possess only the second activity. Only on very limited occasions do some COs catalyze the *o*-hydroxylation of a few monosubstituted substrates. Regardless of their different function, both of these enzymes show a similar, highly conserved, structure. The active site of the proteins consists of two copper ions coordinated by three His-residues each.

The *o*-disubstituted phenols can act as antioxidants and are quite valuable, but hard to produce. As a result, the ability of a biocatalyst to convert monosubstituted phenols to these antioxidants, that is to show monophenolase activity, has industrial applications. Due to the highly similar structure of tyrosinases and catechol oxidases, many attempts have been carried out to introduce the monophenolase activity to COs through protein engineering.

In the displaying project, a PPO from the thermophilic fungus *Myceliophthora thermophila* has been engineered, through site-directed mutagenesis, in order to enhance its monophenolase activity. The mutations G292N, L306A and Y296V have been introduced to the gene encoding the fungal PPO, and the corresponding mutated enzymes, as well as the wild type, were heterologously expressed in the methylotrophic yeast *Pichia pastoris*. The mutants were thoroughly examined against a wide variety of phenolic compounds to discriminate their biological activity, while they were also characterized in terms of their thermostability, optimum temperature and pH conditions.

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