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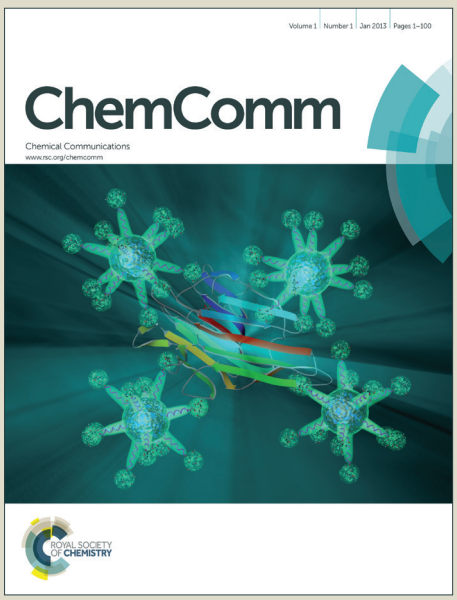

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Feature article

Cascade catalysis – strategies and challenges en route to preparative synthetic biology

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Nature's smartness and efficiency assembling cascade type reactions inspired biologists and chemists all around the world. Tremendous effort has been put in the understanding and mimicking of such networks. In recent years considerable progress has been made in developing multistep one-pot reactions combining either advantage of chemo-, regio-, and stereoselectivity of biocatalysts or promiscuity and productivity of chemocatalysts. In this context several concepts, inspired by different disciplines (biocatalysis, metabolic engineering, synthetic chemistry, material science), have been evolved. This review will focus on major contributions in the field of cascade reactions over the last three years.

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A Introduction

Nature has evolved a highly efficient system in form of cascade reactions which assemble the metabolic networks that ensure life (growth and survival).[1] In a living cell, basically a one-pot system, these multistep reactions are catalysed by numerous enzymes fine-tuned by evolution in an aqueous environment. Mimicking this natural system in chemical synthesis and performing catalytic cascade reactions in a one-pot manner was intensively investigated over the past decades.[2]

Multistep cascades in living organisms commonly function without separation of intermediates; concentrations of all reactants are kept low, which allows high selectivity and avoids by-product formation. Taking nature as a model, application of cascade reactions in organic synthesis offers a lot of advantages over the classical step-by-step approach. As there is no need for purification, operating time, costs and waste are reduced, atom economy and overall yields are improved. Additionally, the problem of unstable or toxic intermediates can be overcome and reactivity and selectivity can be enhanced by evading equilibrium reactions by the cooperative effect of multiple catalyst.[3-5]

Although significant research progress was achieved over the past decades, one-pot multistep reactions are still not of general applicability in chemocatalysis due to problems with compatibility of reaction conditions.[2] Biocatalytic cascades are easier to realize as most enzymes reach their catalytic optimum at similar temperature and pH conditions in aqueous buffers (of compatible salt activities). Additionally, biocatalysis is nontoxic and enzymes are highly chemo-, regio-, and stereoselective.[6]

Bio- as well as chemo-catalytic cascades, generally described as a consecutive series of chemical reactions proceeding in concurrent fashion,[7] are often classified in groups. In domino reactions one catalyst triggers the formation of a reactive intermediate, which undergoes spontaneous transformation. Cascades with two or more catalysts can be performed in sequential or simultaneous mode. Whereas in simultaneous (or relay) cascades all the catalysts and reactants are present from the outset, in the sequential approach the next catalyst is added only after the completion of the previous step.[4]

Whereas enzymatic cascade reactions often benefit from a similar reaction milieu, chemo-enzymatic combinations encounter compatibility problems as either the biocatalyst is unstable in organic solvents or the organo- or metallo-catalyst is inactivated in aqueous environment.[8] Several approaches have been investigated like the stabilisation of enzymes or chemo-catalyst[9] via different protein engineering approaches, compartmentalisation by nanoparticles,[7] protein scaffolds[10] or immobilisation.[8]

Nevertheless, a successful cascade type reaction requires optimized reaction parameters and a perfect process control. Despite compatible temperature, solvent, and pH, a central issue in such reactions is the imbalance due to different enzyme/catalyst properties. Inspired by the systems biology community a rather new approach to maximize the efficiency of multi-enzyme processes by building kinetic models to optimize stoichiometry was developed.[11] For another strategy to enhance synthetic multi-enzyme cascade reactions nature provided the blue-prints with substrate channelling along spatially organized multi-enzyme structures. Efficient through-put is achieved by facilitated transfer of a reaction intermediate from the active site of one enzyme to the active site of a downstream enzyme without first diffusing into the bulk solution. Due to new developments in nucleic acid nanotechnology this system can be mimicked through protein scaffolding.[12]

Recent research also offers possibilities for environmentally benign developments. With a multi-enzyme cascade system based on ultrathin, hybrid microcapsules carbon dioxide conversion to methanol could be achieved.[13] Protein and metabolic engineering in line with a systematic study of pathway bottlenecks accomplished the biodegradation of highly toxic halogenated compounds to glycerol in *E. coli*.[14]

It becomes clear that a multidisciplinary approach is aspiring at the process design of efficient one-pot multistep reaction cascades.[2] Most recent developments highlighting different concepts in the academic area shall be presented here.

B *In vitro* cascades/ Protein engineering

Biocatalytic cascade reactions have recently attracted significant attention enabling multistep transformations in one-pot thereby circumventing the isolation of (unstable or toxic) intermediates. Generally, *in vitro* cascades were performed either with purified enzymes, crude cell extracts (CCE), resting cells or lyophilized recombinant cells. The scope of cascade reactions so far known in literature last from simple combinations of one enzyme with a cofactor recycling system to coupling of thirteen different enzymes.[15] Alcohol dehydrogenases are widely-used in cascade reactions, because of their favourably biocatalyst stability, excellent enantiodivergence and simple cofactor recycling methods.

One prominent example discussed here is a cascade for the amination of primary alcohols by Sattler et al. They combined a thermostable alcohol dehydrogenase (ADH-hT) from *Bacillus stearothermophilus* with an ω-transaminase (ω-TA) from

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Chromobacterium violaceum and an L-alanine dehydrogenase from *Bacillus subtilis* for recycling of the amine donor (Fig. 1A) in a strictly "non" buffered system.[16] The authors demonstrated a redox neutral multi-enzyme network by applying the proper cofactor recycling system. Hence, the equilibrium of the cascade was shifted towards the product side by addition of the cheap amine-donor ammonia. Finally, the authors renounced the use of buffers in their cascade reaction system to avoid any salt waste and make the process environment friendly.

With this system they reached nearly full conversion of 50 mM 1-hexanol to 1-hexylamine and 3-phenyl-1-propanol to 3-phenyl-1-propylamine. After the first successful proof of this concept, the authors investigated the production of 1,ω-diamines from 1,ω-diols (Fig. 1B). By optimizing the reaction conditions (co-solvent, temperature) 99% conversion of 50 mM 1,8-octanediol or 1,10-decanediol to the desired diamines was observed. The best results were obtained by the addition of 10% (v v^{-1}) 1,2-dimethoxyethane at 20°C.

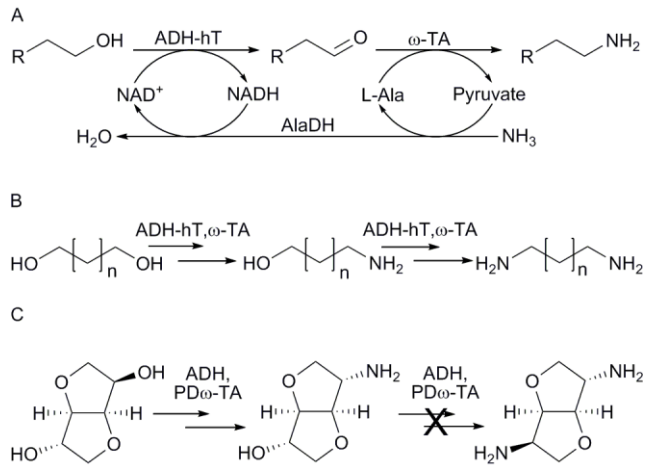

Fig. 1 Redox-neutral multi-enzyme networks for the bioamination of alcohols (ADH-hT, thermostable alcohol dehydrogenase; ω-TA, ω-transaminase; PD, *Paracoccus denitrificans*).

Later on, Lerchner et al. applied the same concept for the synthesis of a more complex diamine starting from the readily available isosorbide (Fig. 1C).[17] Since the activity of most of the known ω-TAs is restricted to aldehydes or sterically non demanding α-substituted ketones, an engineered ω-TA from *Paracoccus denitrificans* (PDω-TA) was used. Site-directed mutagenesis at the active site in position L417 led to two active mutants: L417M and L417R. In contrast to the wild-type enzyme, variant L417M exhibited the same K_M and k_{cat} in the presence or absence of excess PLP, which indicated tight binding of the cofactor. Additionally, this variant showed the highest catalytic activity against all stereoisomers of the substrate and intermediate products. Nevertheless, the cascade stopped at the amino alcohol, because the employed ADH (levodione reductase from *Leifsonia aquatia*) did not accept the intermediate (2*S*,5*S*)-5-amino-2-alcohol (Fig. 1C). Another protein engineering step or a different ADH would be necessary to complete the cascade as proposed by the authors.

The direct oxidation of cycloalkanes to cycloalkanones via a P450 monooxygenase and an ADH (Fig. 2A) represents another successful enzyme cascade.[18] Again, protein engineering of suitable P450 monooxygenases identified the BM3 variants 19A12 and F87V (originating from *Bacillus megaterium* BM3) as the best candidates for cycloalkane hydroxylation. Combination of the ADH from *Lactobacillus kefir* and the BM3 mutants enabled production of cyclooctanone (6.3 mM) and cyclohexanone (4.1 mM) starting from the corresponding cycloalkane (100 mM). Although the sequence gave the desired products, the low activity of the P450 monooxygenase turned out as bottleneck of the cascade. Protein engineering of the P450 monooxygenase with respect to activity, stability, and improvement of the "coupling efficiency" is still an ongoing challenge.

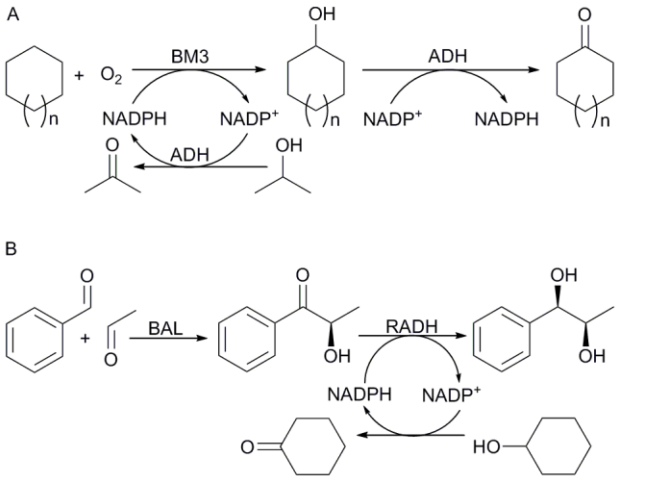

Fig. 2 *In vitro* cascade reaction combining ADHs with other enzymes (BM3, P450 monooxygenase BM3; RADH, *Ralstonia* sp. alcohol dehydrogenase; BAL, benzaldehyde lyase).

A totally different approach as mentioned *vide supra* was published by the group of Rother. Instead of performing an enzymatic cascade in water, they applied a very efficient carboligation/reduction cascade in the presence of organic solvents, catalytic amount of water and lyophilized cells. [19] As introduced before, co-solvents or organic phases often play an important role in enzyme cascades for *in situ* product removal or increased substrate solubility. This particular cascade reaction consisted of an asymmetric carboligation by a benzaldehyde lyase (BAL) from *Pseudomonas fluorescens*, followed by stereoselective reduction of the intermediary α-hydroxy ketone by an alcohol dehydrogenase from *Ralstonia* sp. (RADH) (Fig. 2B). First, the reaction was performed in pure organic solvent without any success. The addition of one equivalent of water to the dry catalyst resulted in a micro-aqueous environment ultimately activating the cascade. Out of nine tested organic solvents, MTBE was identified as most suitable choice for both enzymes. Also substrate concentration, enzyme concentration, enzyme ratio and buffer systems with

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different pH values were improved. Subsuming, the cascade reaction was carried out in MTBE with 0.5 M benzaldehyde, 0.18 M acetaldehyde, 2.5 M cyclohexanol, 25 g L^{-1} BAL catalyst, 33 g L^{-1} RADH catalyst. Acetaldehyde in Et_3N buffer (1 M, pH 10) was pulsed several times in low volumetric amounts (5.8% (v v^{-1})), to maintain the acetaldehyde level constantly above 90 mM. With this substantially low amount of buffer the simultaneous cascade gave 73% enantiopure (1*R*,2*R*)-phenyl-1-propanediol within 6 h, whereas the sequential cascade led to 88% of the desired product within 9 h upon addition of RADH after 5 h. Additionally, the E-factor (describing the mass of waste per mass of product[20]) of the optimized cascade was 21.3 $kg_{waste}\,kg_{product}^{-1}$ rendering this process very eco-friendly. However, if product work-up by laborious and waste-generating solvent extraction were included in the E-factor calculation, the E-value increased to 1927 $kg_{waste}\,kg_{product}^{-1}$, which is beyond any desirable E-value. Furthermore, the cascade was performed in "teabags" to improve the recycling of the lyophilized cells[21], where the RADH could be recycled for more than five batches and the BAL two times.

Within a study by Siirola et al. solvent effects were similarly crucial when employing a three-enzyme cascade for the synthesis of 2-(3-aminocyclohexyl)acetic acid methyl ester derivatives bearing two chiral centers starting from prochiral diketones (Fig. 3).[22] In this sequence, the stereoselective hydrolysis of a C-C bond was catalysed by a β-diketone hydrolase, 6-oxocamphor hydrolase (OCH) from *Rhodococcus* sp. as the first step, followed by an esterification employing the lipase B from *Candida antarctica.* Finally, a chiral amine moiety was introduced by an (*S*)-selective ω-transaminase from *Vibrio fluvialis* or by an (*R*)-selective ω-transaminase (obtained in 11 rounds of mutation) from *Arthrobacter* sp. (ArRmut11-ω-T). Here, the cascade was performed in a mixture of DIPE/H_2O/MeOH in the ratio 97.5:2.5:1 (v v^{-1}), because the esterification could not be carried out in water alone. Furthermore, the OCH was inactivated by MeOH, so that this solvent ratio had to be balanced meticulously.

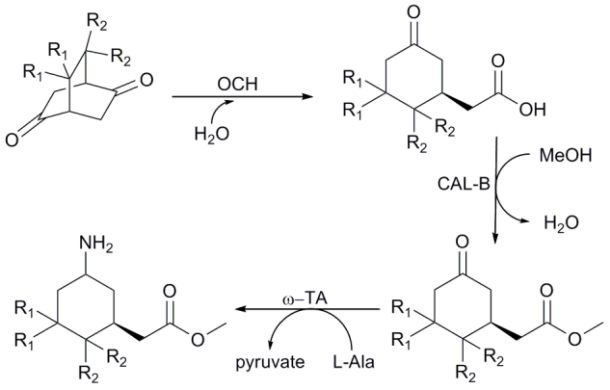

Fig. 3 Three-enzyme sequence for the synthesis of 2-(3-aminocyclohexyl) acetic acid methyl ester derivatives (OCH, 6-oxocamphor hydrolase; CAL-B, *Candida antarctica* lipase B; ω-TA, ω-transaminase).

Apart from strong co-solvent effects for some cascades, the availability of gaseous co-substrate is crucial for oxygen-dependent cascades as well. It was shown, that in the one-pot simultaneous multi enzyme-cascade for the synthesis of enantiomerically pure (*R*)- or (*S*)-2-hydroxy acids starting from L-amino acids, an O_2 pressure of 1 bar was best suited employing L-amino acid deaminase (L-AAD) and L- or D-isocaproate reductase (D-/L-Hic) (Fig. 4).[20] Although the deamination step proceeded best at 2 bar of O_2, the decreased pressure led to >80% isolated product yield for the whole system. The conversion of five different substrates (50 - 100 mM) was completed in 7 - 14 h. The cascade enabled access to the different enantiomers in optically pure form upon utilization of D- or L-isocaproate reductase, respectively.

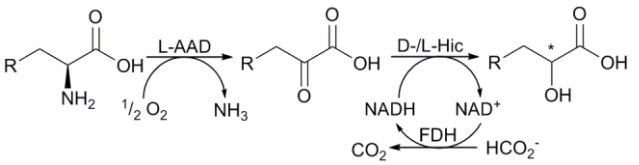

Fig. 4 Enzyme cascade for the synthesis of enantiomerically pure (*R*)- or (*S*)-2-hydroxy acids starting from L-amino acids (L-AAD, L-amino acid deaminase; D-/L-Hic, D-/L-isocaproate reductase; FDH, formate dehydrogenase).

The importance of the right secondary recycling enzymes was demonstrated for the direct conversion of benzylic and cinnamic primary alcohols to the corresponding amines based on a galactose oxidase from *Fusarium austroamericanum* NRRL 2903 (GalOx) and an ω-transaminase (Fig. 5A).[23] An alanine dehydrogenase and a formate dehydrogenase (FDH) were tested initially as secondary enzymes. Only the change from FDH to glucose dehydrogenase (GDH) gave an improvement from 81% to >99% conversion. This was attributed to the inhibitory effect of ammonium formate on GalOx, which was then replaced by NH_4Cl with GDH. To demonstrate the applicability of this enzymatic oxidation–amination cascade, 3-phenyl-allylamine was used as the starting material for the preparation of the potent antifungal agent Naftifine. Thus, the combination of the enzyme cascade and chemical reactions resulted in 51% overall yield of Naftifine via four steps.

Dynamic kinetic resolution of racemic compounds towards one optically pure enantiomer represents another valuable application of enzyme cascades.[24] Chiral benzylic amines were deracemized to the (*R*)-enantiomer by selective monoamine oxidase (MAO-N) mediated oxidation of the (*S*)-amine enantiomer to the imine, which undergoes spontaneous hydrolysis to the corresponding ketone (Fig. 5B). Subsequent reductive amination mediated by an ω-TA provided access to eight different optically pure (*R*)-amines with 80 – 99% conversion. The application of this one-pot MAO-N/ω-TA cascade was also described with a protein engineered variant of MAO-N for the selective N-dealkylation of secondary amines. In order to prevent ω-TA inhibition, catalase was added to eliminate H_2O_2 generated in the MAO-N mediated oxidation.

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When using *in vitro* cascades there is always the choice to select CCE, lyophilized cells, immobilized cells/enzymes or purified enzymes as catalytic entities, in some cases affecting the resulting enantiomeric excess and conversion rates. For example, Sehl et al. developed cascades with enzymes from different toolboxes, which can be efficiently combined, yielding all stereoisomers of the desired Nor-(pseudo)-ephedrine. Here, employing lyophilized whole cells or purified enzymes showed a significant difference with respect to enantiopurity.[25, 26] The combination of a purified (*R*)-selective thiamine diphosphate (ThDP)-dependent carboligase (AHAS-I) and a purified (*S*)- or (*R*)-selective ω-transaminase resulted in the formation of (1*R*,2*S*)-Norephedrine or (1*R*,2*R*)-Norpseudo-ephedrine in excellent optical purities (>99% ee and >98% de, Fig. 5C). As an (*S*)-selective carboligase is currently not available, the authors used a combination of an (*S*)-selective transaminase with an (*S*)- or (*R*)-selective alcohol dehydrogenase to afford (1*S*,2*S*)-Norpseudoephedrine or (1*R*,2*S*)-Norephedrine with >98% ee and >95% de (Fig. 5D). The use of the cheaper lyophilized whole cells decreased the diastereomeric excess for (1*R*,2*S*)-Norephedrine from >95% de to a diastereomeric ratio of approx. 8:2 due to the *E. coli* induced isomerization of the intermediate.

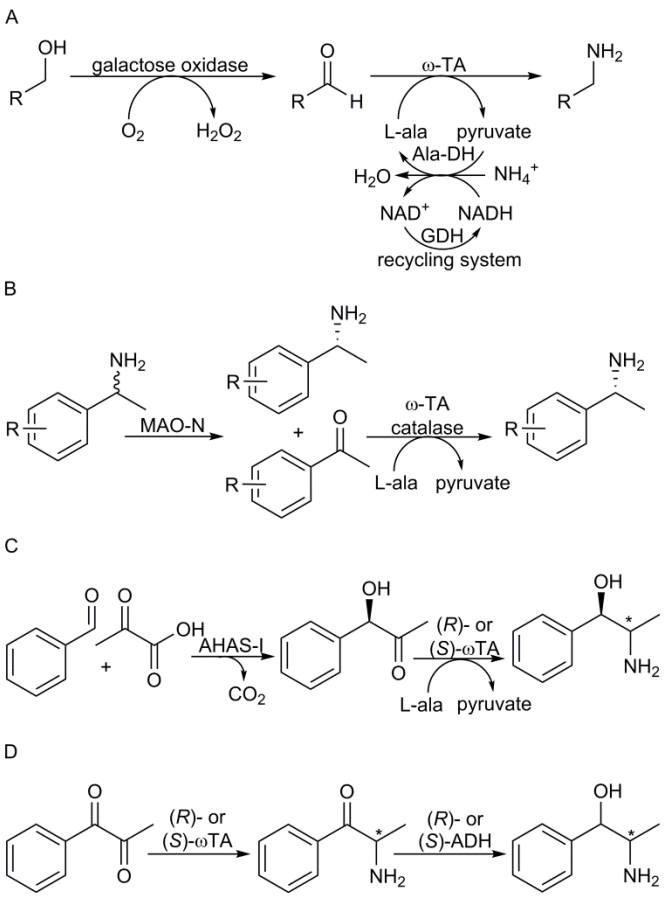

Fig. 5 Enzyme cascades with ω-transaminases (ω-TA, ω-transaminase; Ala-DH, alanine dehydrogenase; GDH, glucose dehydrogenase; MAO-N, monoamine oxidase; AHAS-I, carboligase; ADH, alcohol dehydrogenase).

The challenge to design cascades in a way to circumvent possible side reaction or to favourably shift the equilibrium was addressed by Oberleitner et al. for a three step cascade.[27] Here, an ADH was combined with an enoate reductase and a Baeyer-Villiger-monooxygenase (BVMO). A problem was encountered by the additional activity of the ADH towards the intermediate ketone, the substrate for the BVMO reaction, in a reversible manner. Due to the fact, that the last oxygenation step of the investigated reaction sequence was an irreversible process, a continuous shift of equilibrium to the desired products took place and conversions up to >99% could be achieved. The *in vivo* version of this cascade[28] will be discussed in more detail in the next section.

Application of enzymatic cascade processes in bulk or sugar chemistry represents another challenging field for this particular strategy. You et al. developed a cascade to convert cellulose to amylose via endoglucanases, cellobiohydrolases and an engineered potato α-glucan phosphorylase (PGP).[29] To convert all different connected celluloses, a cocktail of two endoglucanases and three cellobiohydrolases had to be used to synthesise 3.82 g L^{-1} amylose from 20 g L^{-1} insoluble regenerated amorphous cellulose. Linkage of glucose-1-phosphate by PGP turned out as a critical factor for the successful operation of this cascade. Later, the same group established a one-pot reaction composed of sucrose phosphorylase and the PGP or a three enzyme cocktail (*i.e.*, glucose isomerase, glucose oxidase, and catalase) to convert cheap sucrose to synthetic amylose.[30] Applying the two enzyme cascades, they reached an amylose concentration of 32.3 mM at 25 h starting from 100 mM sucrose. The five enzyme system yielded a comparable amount of the product (34.7 mM).

Biofuel cells currently represent another cutting edge application of sugar cascades. Here, glucose was converted to the final product CO_2 by a six-enzyme cascade in 12 steps with 24 electron oxidations.[31] This cascade was composed of PQQ-dependent glucose-, gluconate-, alcohol- and aldehyde dehydrogenases, oxalate oxidase and aldolase, all immobilized on carbon fibre electrodes to perform the complete oxidation of glucose (44% conversion) to CO_2.

A very interesting, smart and completely different approach towards cascade optimization by the use of a classic ink-jet printer was developed by Zhang et al.[32] This ink-jet printing device was used for the construction of multi-enzyme systems, in which a precisely defined enzyme ratio and two-dimensional distribution was obtained by the pre-set 'colour' values. A colour ink-jet printer uses the CMYK colour model, in which CMYK refers to the four inks: cyan, magenta, yellow and key (black) to generate a colour space theoretically containing 1,030,402 different colours by tuning the output of these four primary colours (between 0–100% for each colour in steps of 1%). Upon filling the colour cartridges with different enzyme solutions and substrates a fast screening of variable conditions could be carried out under the premise that a coloured product is formed.

Immobilization of (bio)catalysts is not claimed as conceptually new, but still a very powerful strategy, which often contributes

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positively to enzyme stability and facile product isolation as well as recyclability of the catalyst. Furthermore, in theory it should be possible to immobilize any combination of catalyst (enzymes, cells, chemocatalysts). Besides the classical immobilization techniques[33-35] novel methods were developed especially for cascade reactions.[36-42] Among these, artificial or natural microcompartments (MCPs) have drawn the interest of synthetic biologists and chemists. Due to their novelty most of these methods were until now only applied for model reactions (*e.g.* glucose oxidase/horse radish peroxidase). Here, attention shall be focussed on a few recent examples featuring synthetically more relevant reactions (for detailed reviews on cascade immobilization see references[7, 43, 44]).

The production of Cbz-protected aminopolyols could be improved by covalent immobilization of a chloroperoxidase from *Caldariomyces fumago* and His-tag immobilized rhamnulose-1-phosphate aldolase from *E. coli*.[45] The enzymes were applied in a stirred tank reactor (STR) setup; after optimization of the reaction medium and additional process engineering 100 mM Cbz-protected ethanolamine was converted to Cbz-protected aminopolyol after 72 h in a yield of 87%. To prevent damage of the particles the cascade was then applied in a recirculated packed bed reactor, but this required a significantly elongated reaction time of 192 h. To overcome this obstacle, the authors would like to suggest the use of a novel reactor setup made of a rotating flow cell, which was demonstrated to protect the immobilization matrix while retaining activities comparable to STR.[46]

The group of Jiang combined biomimetic mineralization and bioadhesion to encapsulate a CO_2-fixing cascade in multi-layered MCPs.[13] First, formate dehydrogenase from *Candida boidinii* was immobilized in poly(allylamine hydrochloride), a hydrophilic polymer, doped $CaCO_3$ particles, which were subsequently coated with catechol modified gelatine. Formaldehyde dehydrogenase from *Pseudomonas* sp. was attached to this layer. The second layer contained a yeast alcohol dehydrogenase and Na_2SiO_3. Finally, the $CaCO_3$ core was dissolved by EDTA. The methanol yield after 9 h was increased by two-fold using the MCPs compared to the free enzymes. Furthermore, the immobilized enzymes could be recycled up to nine times with a retained activity of 74% and showed an increased storage stability over 20 d with a retained activity of 85%.

Very recently, van Rantwijk & Stolz reviewed their work on the biocatalytic production of (*S*)-mandelic acid and (*S*)-2-hydroxycarboxylic amides.[47] In general, there are two established chemo-enzymatic routes of hydroxycarboxylic acid production (Fig. 6A). However, both routes have obvious drawbacks. Although the route featuring the (*R*)-specific nitrilase is used in multiton-scale[48-50], it is restricted to the production of only one enantiomer. In the second route, equimolar amounts of salt are produced upon applying hydroxynitrile lyase; moreover it is not compatible with sensitive functional groups due to its harsh reaction conditions. To circumvent these problems a bi-enzymatic cascade combining both routes was designed (Fig. 6B) and applied in two different approaches: (i) the combi-CLEA concept[51] and (ii) the whole-cell strategy.[52]

An (*S*)-specific hydroxynitrile lyase from *Manihot esculenta* (MeHnL) and the unspecific nitrilase from *Pseudomonas fluorescens* EBC191 (PfNLase) were used for the production of (*S*)-mandelic acid. Cross-linked enzyme aggregates (CLEA) of the enzymes were used for immobilization in a molar ratio of approx. 4:1. The reaction medium was contained 30% buffer and 70% total organic phase (DIPE, diisopropyl ether). Using these CLEAs 0.25 M benzaldehyde yielded in 44% (*S*)-mandelic acid (96% ee) within 2 h. A significant amount of

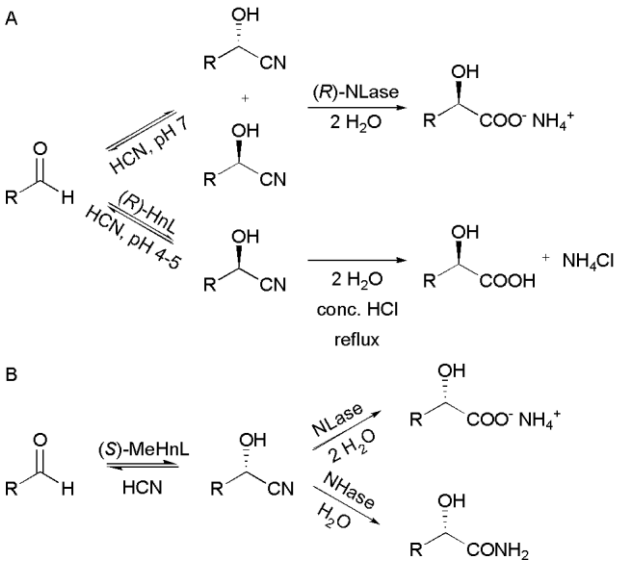

Fig. 6 Chemo-enzymatic routes (A), and the bi-enzymatic approach (B) for the production of hydroxycarboxylic acids or amides (NLase, nitrilase; HnL, hydroxynitrile lyase; NHase, nitrile hydratase).

(*S*)-mandelic amide was observed, which was also converted to the product by the addition of an amidase from *Rhodococcus erythropolis* MP50 (RheAMase) as third enzyme. For the preparation of triple-CLEA, the enzymes were used in a molar ratio of approx. 2:1:2 and indeed, this triple-CLEA construct was able to produce (*S*)-mandelic acid without the formation of the unwanted amide.

In comparison to the CLEA strategy applied, also a whole cell biocatalyst was used without any immobilization. In this case, DIPE was not suitable as organic phase; alternatively ionic liquids (ILs) were used as second phase. Applying this system, a conversion of up to 700 mM benzaldehyde was obtained with combined yields of (*S*)-mandelic acid and amide of 82 – 96% and >94% ee.[53] Furthermore, the formation of acid or amide could be controlled by rational design of the NLase.[54] Whereas the C163A mutant produced less than 5% of the amide, the construction of an amide-producing mutant was more complicated. The formation of amide could then be enhanced to an amide:acid ratio of 9:1 by combining a C-terminal truncation and the C163N mutant.

In summary, the CLEA were highly active in organic solvent, stabilizing especially the NLase. The immobilized catalysts

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were superior in terms of recycling due to the ease of separation, but the whole cell catalyst was easier and cheaper to prepare. Conclusively, both approaches were declared as equivalent and this work represents one of the few examples, for which the use of synthetically relevant concentrations could be realized.

B *In vivo* cascades

The use of *in vivo* cascades might be classified as the interface of chemistry, synthetic biology, metabolic, and protein engineering. In these examples the enzymes applied for cascade reactions are used in a more natural environment, the cytoplasm of a host organism. In contrast to classical metabolic engineering, where only the cell's metabolism is engineered towards a certain metabolite, synthetic biology aims to introduce reactions not occurring in nature, respectively naturally not-connected enzymes into host organisms like *E. coli*. In addition to the already described general challenges of cascade reactions, scientists are facing further problems when using whole cells for cascade reactions:

1. Host background: The desired reaction might suffer from unwanted side-reactions caused by naturally occurring enzymes of the used host. There are only two solutions to solve this problem: One is to change the cascade host, the other one is to knock-out the gene encoding the unwanted enzyme. Furthermore, if performing the latter solution, one might be faced with the next problem:
2. Growth deficiency: This might be a consequence of the mentioned knock-out or by the introduction of the cascade itself, due to competition with the cells metabolism for cascade precursors or the production of toxic intermediates. In this case, intracellular scaffolding or compartmentalization of the cascade might improve the viability of the host.
3. Expression levels: Whereas it is obviously quite easy to balance the enzyme ratios of *in vitro* cascade systems, this is an issue, which cannot be resolved easily for *in vivo* systems. But there are some tools to attribute this problem, *e.g.* based on the BioBricks principles[55, 56] with expression vectors featuring different architectural elements like promoters and origins of replication, and which allow different modes of expression.
4. Additional diffusion barrier: Another obvious drawback of cells as catalyst is the cell membrane, which can impair substrate uptake and product release. On the other hand, the membrane itself forms the beneficial compartment for cascade reactions. To solve substrate uptake or product release problems, enzymes or transport systems for uptake and release could be introduced. Also the permeabilization of the cell membrane using surfactants or other organic molecules could enhance the substance transport.[57]

In the following section recent examples are covered, which had to cope with one or more of the described difficulties.

Based on their previous work[58] the group of Park extended the established cascade for the production of long-chain α,ω-dicarboxylic and ω-hydroxycarboxylic acids towards the formation of ω-aminocarboxylic acids by applying an additional ADH (AlkJ from *Pseudomonas putida* GPo1) and an ω-TA from *Silicibacter pomeroyi* (Fig. 7).[59] In general, 5 mM of saturated or unsaturated fatty acids were used. The corresponding products were obtained in 21 – 51% isolated yield. For two of the substrates used (ricinoleic and lesquerolic acid) a reduction of the *cis*-double bond was observed probably caused by the *E. coli* background. But in this case, it was not necessary to retain the double bond for the production of polyester precursors, although unsaturated building blocks also have certain interesting features.[60]

The group of Li encountered the same problem in their mixed-culture approach for the production of enantiopure δ-lactones using a non-engineered wild-type *Acinetobacter* sp. RS1 for unspecific reduction of a C=C bond, followed sequentially by Baeyer-Villiger oxidation carried out by *E. coli* cells harbouring the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* and the glucose dehydrogenase from *Bacillus subtilis* for co-factor recycling.[61] Subsequently, hydrolysis of the (*S*)-lactone by the *Acinetobacter* cells led to enantiopure (*R*)-lactone. The cascade had to be carried out in a sequential mode due to *E. coli* background reaction on the C=C bond. Cyclopentanone derivatives were used in a concentration of 6 mM as substrates and were converted with an isolated yield of 41 – 56% and an optical purity of 98% ee.

Probably the same *E. coli* background reaction as described for the two preceding examples was observed by us in a tri-enzymatic redox cascade involving two enantiocomplementary alcohol dehydrogenases, two different enoate reductases and the CHMO from *Acinetobacter calcoaceticus*.[28] We resolved this issue by performing the knock-out of the *E. coli* N-ethylmaleimide reductase A (*nemA*) gene. This way, we succeeded in producing highly enantiopure chiral lactones (>99% ee/de) from four different cyclohexenol derivatives and three carveol diastereomers in high yields from 63 – 99%.

In parallel, Agudo & Reetz avoided the *E. coli* background reaction on C=C bonds by exchanging the *nemA* gene with the gene encoding for their used enoate reductase YqjM from *Bacillus subtilis*.[62] This enzyme was used in combination with a P450-BM3 triple mutant for the production of the enantiopure advanced pharmaceutical intermediate precursor methyl-3-oxocyclohexane carboxylate in engineered *E. coli* designer cells carrying an additional endogeneous glucose dehydrogenase. Three approaches were carried out to optimize the overall yield: (1) a mixed-cell approach by mixing cells harbouring either the P450-BM3 or the YqjM, (2) use of *E. coli* cells harbouring both enzyme encoding plasmids, and (3) use of the above described *E. coli* cells with genome-integrated YqjM and plasmid-encoded P450-BM3. Comparison of all three approaches in a 1.5 mM scale showed that using approach (1) a yield of 85% of the desired product was reached for both enantiomers in high

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purity (99% ee) after 75 min. In contrast, the other approaches gave a yield of approx. 50% after 60 min, but also in high purity. An upscaling of the first setup to 7.3 mM substrate concentration resulted in a yield of 69% for both enantiomers with an optical purity of 99% ee.

To overcome limited substrate uptake, Bühler and co-workers identified a protein of the *Pseudomonas putida* GPo1 Alk-operon, which promotes uptake of hydrophobic substrates as exemplified by fatty acid methyl esters (FAMEs)[63] and limonene.[64] This principle was applied for their described alternative route for the production of ω-aminocarboxylic acid methyl esters using the AlkBGT monooxygenase-reductase system from *Ps. putida* GPo1 and the transaminase from *Chromobacterium violaceum*.[65] Here, 1.4 – 2.9 mM substrate were used and resulted in approximately

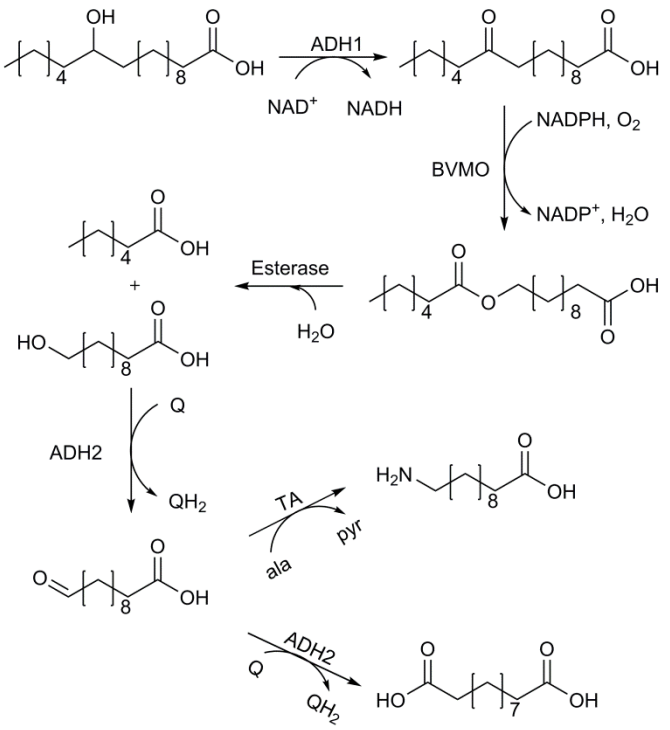

Fig. 7 Production of long-chain α,ω-dicarboxylic and ω-aminocarboxylic acids (ADH, alcohol dehydrogenase; BVMO, Baeyer-Villiger monooxygenase; TA, transaminase; Q, ubiquinone).

5 – 16% yield. Furthermore, the overoxidation of the intermediary terminal aldehyde to the carboxylic acid by AlkBGT was observed. In their parallel work concerning the integral membrane porin AlkL, the co-expression of this protein led to a 62-fold increase of the activity towards FAMEs.[63] Furthermore, in a recent contribution the co-expression of AlkBGT, AlkL and AlkJ (an alcohol dehydrogenase) led to an enhanced production of dodecanedioic acid monomethyl ester (DDAME);[66] the application of AlkL for the production of perillyl alcohol from limonene gave a two-fold improvement of activity.[64] In both cases it was necessary to apply a two-phase system using bis(2-ethylhexyl)phthalate (BEHP) as substrate reservoir and product sink; this strategy is compromised by teratogenic and environmentally harmful properties of this solvent. By increasing the substrate uptake due to the expression of AlkL the intracellular level of DDAME was enhanced to toxic concentrations for the cell and BEHP was necessary to regulate the concentration level below the toxicity threshold. In summary, the application of AlkL significantly enhanced the uptake of hydrophobic substrates.

The Li-group also transferred their previously described *in vitro* cascade[67] for the enantioselective dihydroxylation of aryl olefins into one host cell.[6] The genes of the styrene monooxygenase from *Pseudomonas* sp. strain VLB120 and two enantiocomplementary epoxide hydrolases from *Sphingomonas* sp. HXN-200 respectively from *Solanum tuberosum* were cloned and expressed in *E. coli*. For the characterisation of this cascade and its substrate spectrum also a two-phase system (*n*-hexadecane) was used as substrate reservoir and product sink. It was shown that this cascade was capable to convert 32 out of 44 styrene derivatives with analytical yields >50% and 30 out of 44 substrates with >90% ee. Furthermore, the dihydroxylation of styrene was also carried out with growing cells in a fermenter yielding 120 mM (*R*)-1-phenyl-1,2-ethanediol (96% ee) after 5 h with a productivity of 3.3 g L^{-1} h^{-1}.

Another tri-enzymatic cascade – again in a two-phase system with cyclohexane – featuring the lipoxygenase from *Solanum tuberosum*, the hydroperoxide lyase from *Cucumis melo*, and an endogenous aldehyde dehydrogenase from *E. coli* for the production of azelaic acid was described by Hauer and colleagues.[68] Using up to 5 mM linoleic acid as substrate 200 µM of azelaic acid could be produced after 32 h. However, this cascade suffered from certain problems related to enzyme compatibility. First, the lipoxygenase needs to be activated by its own product. But this intermediate is depleted quickly by the hydroperoxide lyase, which, secondly, shows high substrate and product inhibition. To address this problem the balancing of protein expression was conducted in this work, but also protein engineering was envisaged by the group for future research.

An alternative production route for 1,2-propanediol from lactic acid avoiding toxic intermediates was developed by Niu & Guo.[23] The designed artificial pathway involved the lactoyl-CoA transferase from *Megasphaera elsdenii*, the CoA-dependent lactaldehyde dehydrogenase from *Salmonella enterica*, and the lactaldehyde reductase from *E. coli*. Either D- or L-lactate (56 mM) were converted to approximately 20 mM 1,2-propanediol (35 – 39% yield). Compared to other established 1,2-propanediol production routes, which reached titers up to 5.6 g L^{-1},[69, 70] a rather low titer of 1.7 g L^{-1} was achieved probably due to competition of the introduced enzymes with the host enzymes for the acetyl-CoA pool and the promiscuity of the lactoyl-CoA transferase. One possible solution to this would be the use of the propanediol utilization microcompartment from *Salmonella enterica*, which was shown to be a facile tool for substrate channelling and protein scaffolding.[71-73] Although several knock-outs were performed,

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it was not possible to prevent the formation of ethanol as a main side-product.

A coenzyme-independent alternative route for the production of vanillin was developed by Kino and co-workers.[74] In this cascade, an already described decarboxylase from *Bacillus pumilus* ATCC 14884 was applied in combination with a novel type of oxygenase from *Caulobacter segnis* ATCC 21756, which belongs to the not yet fully understood class of carotenoid cleavage oxygenases.[75, 76] There are several examples for the production of vanillin from ferulic acid via CoA-dependent enzymes,[77-83] but this is the first example, to the best of our knowledge, that solely depends on molecular oxygen as co-substrate. After a reaction time of 32 h 8 mM vanillin was produced from ferulic acid (10 mM). Of course this reaction was far too slow to compete with established processes, probably due to the fact that a compromise concerning the used pH had to be found. But the low activity and also the pH-spectra of both enzymes will be subject to protein engineering.

unsaturated C_{18} fatty acids and benzoic acid. Co-expression of the mentioned enzymes in *E. coli* led to the formation of >350 mg L^{-1} fatty alcohol after 24 h. The production of aliphatic alkanes by changing the AHR to a cyanobacterial aldehyde decarbonylase (ADC) gave only poor yields due to the low activity of this enzyme. Using this pathway for the production of alkanes is still limited due to the low activity of the ADC, so that here again either protein engineering of the known ADCs or the discovery of novel biocatalysts seem to be the only solutions.

In cooperation with Symrise AG we recently described a bacteria derived pathway for the conversion of flavonoids.[85, 86] By genome mining of the anaerobic gut bacterium *Eubacterium ramulus* two enzymes, a chalcone isomerase (CHI) and an enoate reductase (ERED), were identified and cloned into *E. coli*. After optimization of expression (especially the ERED needed anaerobic conditions due to a FeS-cluster), three different flavanones were converted by 63 – 93% into the corresponding dihydrochalcones. Due to the insolubility of the

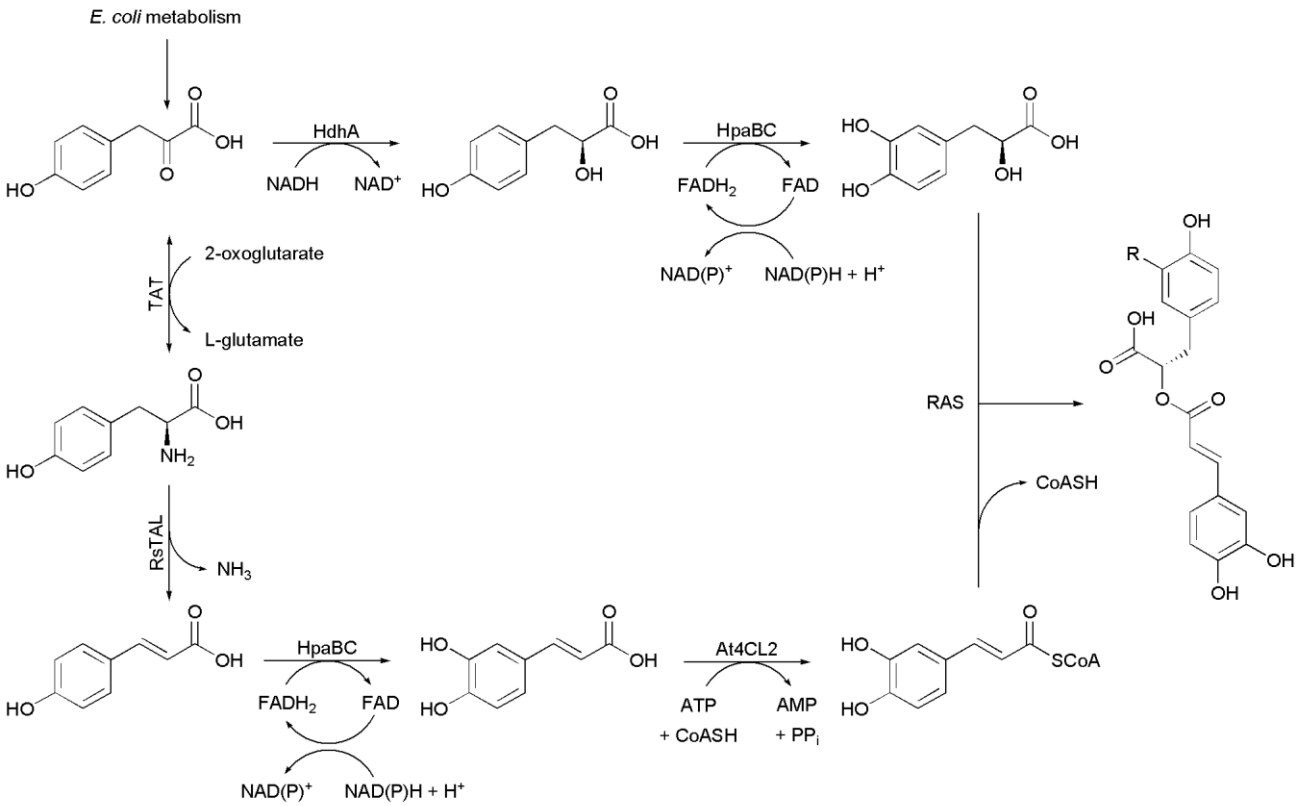

Fig. 8 Complex chimeric cascade for the production of rosmarinic and isorinic acid (HdhA, D-hydroxyisocaproate dehydrogenase; HpaBC, 4-hydroxyphenylacetate 3-hydroxylase complex; RAS, rosmarinic acid synthase; TAT, tyrosine aminotransferase; RsTAL, tyrosine ammonia lyase; At4CL2, 2-coumarate-CoA ligase).

Jones and colleagues identified a new carboxylic acid reductase (CAR) from *Mycobacterium marinum* in cooperation with Turner, which was coupled to thioesterase and aldehyde reductase (AHR) for the production of fatty alcohols.[84] This novel CAR exhibited a broad substrate spectrum from short chain (C_6) to long-chain (C_{18}) aliphatic fatty acids, as well as substrates only 150 µM of each was used, but the product also was highly insoluble, which led to direct precipitation and therefore inhibitory effects could be circumvented for these enzymes.

Based on the BioBrick principles[55], Bloch & Schmidt-Dannert described a complex chimeric cascade for the synthesis of

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rosmarinic (RA) and isorinic acid (IA).[87] This pathway involved six enzymes: D-hydroxyisocaproate dehydrogenase (HdhA) from *Lactobacillus delbrueckii* subsp. *bulgaricus*, 4-hydroxyphenylacetate 3-hydroxylase complex (HpaBC) from *E. coli*, rosmarinic acid synthase (RAS) from *Melissa officinalis*, the endogenous tyrosine aminotransferase (TAT), tyrosine ammonia lyase (RsTAL) from *Rhodobacter sphaeroides*, and 2-coumarate-CoA ligase (At4CL2) from *Arabidopsis thaliana* (Fig. 8). As the product levels achieved (1.8 µM RA and 5.3 µM IA) were quite low after application of precursor feeding and changing the RAS, several optimization approaches were suggested. Intracellular tyrosine availability was identified to be one bottleneck, so that the use of a tyrosine overproducing strain was recommended. Also the use of an already described HdhA-mutant could increase the final RA/IA yield.[88] Furthermore, the formation of a dark pigment was observed, which was probably due to the further oxidation of 3,4-dihydroxy-phenyllactic acid to 3,4-dihydroxy-phenyllactoquinone and subsequent non-enzymatic polymerization of the quinone. In summary, this is an outstanding example how synthetic biology, metabolic engineering and protein engineering can interact in the field of multi-enzymatic cascade reactions.

C Chemo-Enzymatic Cascade Processes

Whereas major attention has been put on the development of enzymatic cascade reactions *in vitro* as well as *in vivo*, the combination of two disciplines, chemocatalysis and biocatalysis, is surprisingly underrepresented in the literature,[8] although both research fields cover a significantly different chemical space in terms of reactivity, selectivity and productivity. In the last decade intensive investigations for the introduction of bioorthogonal functionalities were made to gain deeper insights into cellular mechanisms. Therefore different types of reactions, so far unknown in nature, such as metal assisted C-C couplings (*e.g.* Suzuki,[89] Negishi[90]), cross metathesis or copper-catalysed [2+3] dipolar cycloaddition (Huisgen-reaction[91]), were explored.

On the other hand, biocatalysis offers the possibility of chemical transformations either unknown or poorly understood to chemists like the C-H activation of unactivated C-H bonds, or yield in improved regio,- stereo,- and chemoselectivity in already known reactions. Prominent examples are the use of cytochrome P450 enzymes for the selective hydroxylation of simple cyclic alkanes[18] or complex steroids and Baeyer-Villiger monooxygenases for the regio- and chemoselective insertion of oxygen into substituted cyclic ketones. Having said that, the next logical step would be to combine the two worlds of bio- and chemocatalysis and take advantage of their individual assets. Cascade type reactions involving both disciplines suffer from incompatibilities of their totally different environmental window of operation. Whereas enzymes mainly work in aqueous conditions at ambient temperature, many metal- or organocatalytic systems require water-free conditions in the absence of oxygen. Additionally, inactivation of the catalytic system by either the enzyme or the chemo-catalyst has a severe effect on the overall reaction performance. Nevertheless, recent advances in protein engineering provided the community with tailor-made enzymes with improved stability, substrate scope and selectivity. Hence, chemists improved the stability of chemocatalysts towards water and oxygen significantly. However, combination of both catalytic systems in a cascade fashion is still a challenging task. In the following we describe recent progress in this field and will present the most promising concepts for future applications.

Recently, the dynamic kinetic resolution of a primary amine using a combination of Pd nanoparticles and *Candida antarctica* lipase B (Cal-B) immobilized on siliceous mesocellular foams was described by the group of Bäckvall.[92] Using ethyl methoxy acetate as donor in two-fold excess the lipase produced the (*R*)-amide and the not converted (*S*)-enantiomer was racemized by the Pd nanoparticles. After 16 h, 99% yield and 99% ee was reached. The reaction was performed in small scale (2 mL reaction volume, 0.6 mmol substrate), so that a large scale application still needs to be elucidated.

Schrittwieser *et al.* presented the interesting concept of a chemo-enzymatic stereoinversion combined with a kinetic resolution for the production of optically pure (*S*)-berberines (Fig. 9).[93] They converted racemic benzyl-isoquinolines by an (*R*)-selective monoamine oxidase variant (MAO-D11) into a prochiral imine intermediate, which underwent a reduction by a chemical achiral ammonia-borane complex and afforded the (*S*)-enantiomer in high optical purity (>97% ee) from the racemate. Hence the berberine bridge enzyme (BBE) was used to produce (*S*)-berberines by aerobic C-H activation of the N-methyl group. First, they identified a MAO variant with an extended substrate scope and a perfect (*R*)-selectivity towards different benzyl-isoquinoline derivatives. Second, they modified the chemical reagent by changing to more bulky or less water-soluble boranes, which avoided entrance to the active site of the enzyme and made the system hence more compatible with BBE. Finally, they were able to synthesize (*S*)-berberine derivatives in very good yields (up to 88%) and high optical

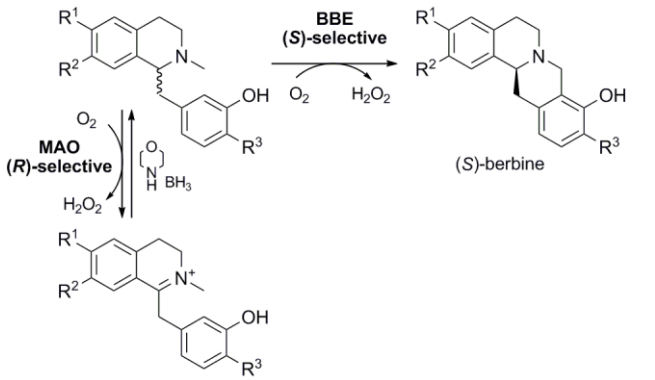

Fig. 9 Chemo-enzymatic stereoinversion and kinetic resolution for the production of optically pure (*S*)-berberines (BBE, berberine bridge enzyme; MAO, monoamine oxidase).

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purity (>97% ee) in an one pot cascade without the addition of catalase.

Another interesting approach for the combination of chemo- and biocatalysis in a single-operation process was published by Fink et al.[94] They demonstrated the power of a stereoselective catalytic reduction conducted in a flow system (optional epimerization with an acidic ion exchange resin) and a subsequent fed-batch BVMO-catalysed oxidation for the synthesis of optically pure aroma compounds. Overall they were able to obtain the desired lactone with >99% ee and >99% de due to the *cis*-selectivity of the BVMO (cyclododecane monooxygenase). They could also monitor the reaction by implementation of an inline IR probe and were able to produce the lactone with a space-time yield of 3.4 g L^{-1} d^{-1}.

Very recently the group of Pietruszka presented the synthesis of arylated dihydrocoumarins by a full chemo-enzymatic continuous flow approach (Fig. 10).[95] Starting from coumarins they performed a catalytic hydrogenation in a flow reactor followed by a laccase-mediated oxidation/Michael addition sequence to afford the desired 3-arylated 3,4-dihydrocoumarins in good to very good yields (40 – 82%). The consecutive approach was performed in acetonitrile for the hydrogenation without work up of the intermediate and a mixture of buffer, enzyme and acetonitrile for the subsequent laccase catalysed arylation. Overall they presented a fully automated and efficient chemo-enzymatic redox-process.

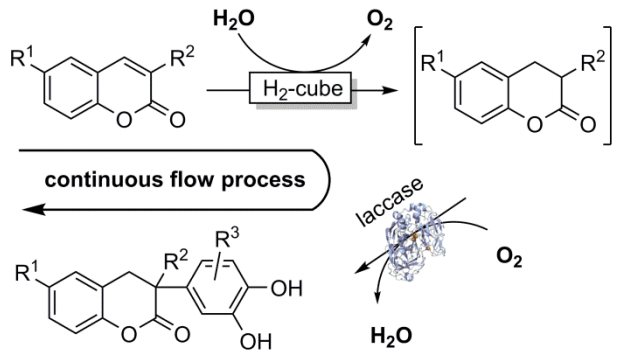

Fig. 10 Self-sustaining and continuous flow chemo-enzymatic redox cascade for the production of 3-arylated 3,4-dihydrocoumarins.

The synthesis of 1,3-diols based on the immobilization of the organo- and biocatalyst as reported by the group of Gröger serves as another impressive study of a chemo-enzymatic cascade (Fig. 11).[8] They performed a stereoselective organocatalysed aldol addition of benzaldehyde-derivatives with acetone in the presence of a lipophilic organic solvent and obtained the desired 1,3-hydroxyketone in up to 95% yield having 95% ee. Subsequent enzymatic reduction with a co-immobilized (*S*)-specific ADH from *Rhodococcus* sp. afforded the desired (1*R*,3*S*)-diols at 89% overall conversion having >99% ee and >35:1 dr. Conceptually, they applied two different immobilization techniques for the organo- and the biocatalyst, in which the organocatalyst was attached covalently to acrylic polymer beads and the biocatalyst including cofactors (NADH) was absorbed to an acrylate-based super-absorber material. The latter material was soaked with buffer, the enzyme and all required cofactors retained in a "second phase", which protects the enzyme from the influence of any organic solvent of the first reaction step. This immobilization strategy circumvented the covalent binding of the enzyme, which often leads to a loss of activity and keeps all required additives (*e.g.* NADH) in close proximity to the enzyme. This technique is an easy to handle approach for the immobilization of especially redox-enzymes and could be the basis for further chemo-enzymatic cascade reactions.

Besides the presented strategies, artificial metalloenzymes are a very promising approach for the design of chemo-enzymatic one-pot reactions. Illustrated by the groups of Ward and Rovis, incorporation of a Cp*Rh(III) pianostool complex within a chirality-inducing protein scaffold was achieved on the basis of the biotin-streptavidin technology, ultimately creating a bifunctional hybrid catalyst.[96] This system catalyses the asymmetric benzannulation reaction of activated benzhydroxamic acid and acrylates. Hence, both selectivity and activity (100-fold increased reaction rate) was improved by site directed mutagenesis. Köhler et al. extended this concept to different cascade reactions including an artificial metalloenzyme containing a transferhydrogenase (ATHase) and different NADH-, FAD- and heme-dependent enzymes.[10] Due to the incorporation of the metal-catalyst into the streptavidin scaffold, mutual inhibition of the latter one was circumvented. Based on this novel concept complex chemo-enzymatic reaction cascades can be designed by homogenous separation of several interacting and interfering catalytic species in a one-pot fashion. Hence, the optimization by simple mutagenesis techniques of the protein scaffold can be achieved.

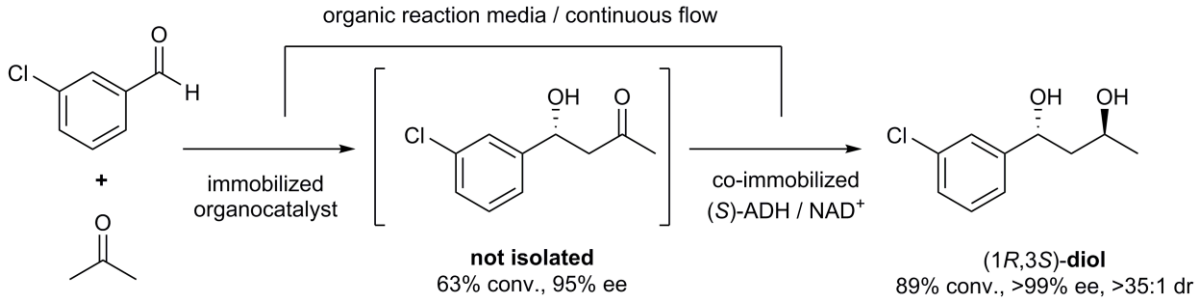

Fig. 11 Continuous flow process combining an asymmetric organo- and biocatalytic reaction in organic media by immobilized catalysts (ADH, alcohol dehydrogenase).

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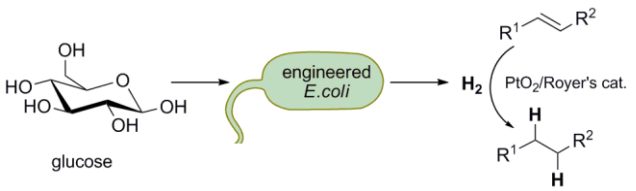

Fig. 12 Design of biocompatible alkene hydrogenation by hydrogen gas producing engineered *E. coli* and a suitable metal catalyst.

A completely different approach for the combination of a metal assisted and an enzymatic reaction was published by the group of Balskus.[97] They applied different techniques derived from metabolic engineering and biocatalysis for the synthesis of small molecules. They envisioned interacting between these two disciplines and investigated a biocompatible activated alkene hydrogenation. Starting from glucose – in a fermentative approach – they engineered the microbial metabolism to produce hydrogen (Fig. 12). In a subsequent step, catalytic hydrogenation was performed in the presence of a PtO_2 catalyst in a one pot cascade. In this particular case, the separation of the biocatalyst and the chemocatalyst was performed by the chassis of the living cell. Suitable conditions for the hydrogenation in water were found and ultimately full integration of both 'reactions' with cellular metabolism provided access to chemical reactivity that otherwise would not be possible in a solely cellular setting.

D Evaluation and modelling of cascade processes

Modelling of chemical processes and synthetic routes is a standard procedure for chemical engineers. Hence, several computer aided tools are available. These methodologies were also applied for biocatalytical processes and cascade reactions.[98, 99]

Very recently, the group of López-Isunza and Woodley developed a kinetic model for a transaminase reaction coupled to a chemical Oppenauer oxidation by shifting the equilibrium towards maximum product formation (Fig. 13).[100] The amination of acetophenone to (*S*)-1-phenylethylamine was chosen as benchmark reaction using isopropylamine as amine donor. The transaminase by-product acetone was removed by the Oppenauer reaction with 1-phenylethanol. This resulted in the formation of the transaminase substrate acetophenone and iso-propanol, which was used as bulk solvent. Due to the water-incompatibility of the Oppenauer catalyst (aluminium isopropoxide), the process was designed using a two-reactor system interconnected by a hydrophobic semipermeable membrane. Several parameter predictions and simulation studies of the developed model were in general agreement with reported experiments, but full experimental validation is still pending. Three years ago, the group of Faulon described a retrosynthetic biology approach and established the RetroPath webserver for the design of metabolic pathways.[101] This computer aided design tool was very recently updated[102, 103] and validated for the production of the flavonoid pinocembrin.[104, 105]

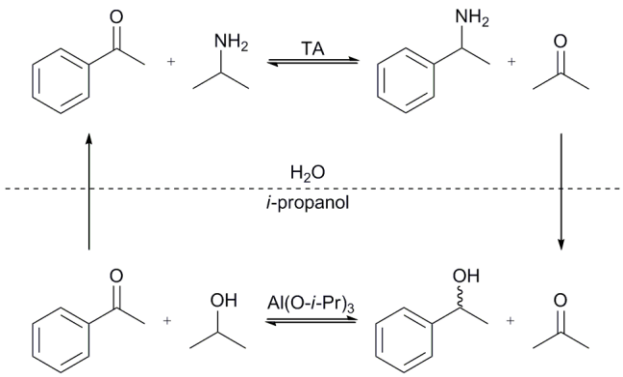

Fig. 13 Equilibrium shift in the TA-catalyzed reaction by combination with Oppenauer oxidation (TA, transaminase; ---------, semipermeable membrane).

The RetroPath tool predicted eleven heterologous pathways connected to endogenous *E. coli* metabolites for the production of the desired substance. These pathways were ranked and the top candidate included a set of 283 enzymes, which would require the assembly of over 8.8 million constructs to test all possible combinations. To reduce these numbers, the model ranked the genes for each reaction based on gene compatibility, enzymatic performance, toxicity of products and steady state fluxes. Finally a set of eight enzymes was considered for pathway construction and subsequent experimental studies led to the accumulation of the first intermediate (*trans*-cinnamate) up to 53.6 mg L^{-1}. By performing flux balance analysis[106] (genome-wide model) the limited availability of malonyl-CoA as co-substrate could be identified as potential bottleneck. Hence, malonyl-CoA production was also subjected to the RetroPath tool to enhance the production of this limiting metabolite. In this case, four heterologous pathways were found. Combination of these four pathways with two of the previous routes led to a significant increase in the pinocembrim titer, which was even increased to 24.1 mg L^{-1} by blocking the fatty acid production using the antibiotic cerulenin, which binds irreversibly to β-keto-acyl-ACP synthase. In summary, the prediction of metabolic pathways in a retrosynthetic manner (not yet comparable to the web-based synthesis planning tool Reaxys®) has become feasible and, consequently, more attractive. The system is, however limited to the available metabolic data, which still is not complete.[107]

Flux balancing by optimization of enzyme expression and ratios was not covered by the above mentioned work. Therefore, the group of Damborsky recently developed a workflow modelling program for optimizing multi-enzyme processes by applying simple steady-state kinetic data to predict the effects of varying enzyme stoichiometry.[11] The well-studied detoxification of 1,2,3-trichloropropane (TCP) to glycerol by a haloalkane dehalogenase (DhaA), a haloalcohol dehalogenase (HheC) and an epoxide hydrolase (EchA) was used as model reaction. Three variants of DhaA were analysed. As predicted, the best result was achieved with an optimized pathway using the engineered variant DhaA31. Thus the catalyst load could be

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reduced from 7.2 mg enzyme (wild-type) to only 3.2 mg enzyme required for 95% conversion.

Based on their established and validated model for *in vitro* cascades,[11] they increased complexity and extended it towards *in vivo* systems.[14] In addition to the steady state enzyme kinetics, parameters like toxicity levels of the substrate and intermediates, information about plasmid copy number and promoter strength were required as input for the model calculations. With these input parameters the distribution of all involved substances during the reaction time was calculated and an optimal gene expression system was predicted. Subsequent evaluation of several calculated models in the lab showed a high agreement of the model with all experimental data. When using the engineered DhaA31, an optimized theoretical ratio of DhaA:HheC:EchA 0.50:0.25:0.25 was predicted to detoxify 2 mM TCP faster. By expression of the enzymes using Novagen's Duet vectors (DhaA31 on pCDFDuet-1, EchA and HheC on pACYCDuet-1) an experimental ratio of 0.60:0.16:0.24 was achieved. The degradation profile over 5 h showed high similarity to the calculated one. Although improved variants of DhaA were already applied for the optimization, this enzyme was still identified by the model as bottleneck of the whole detoxification pathway suggesting that further engineering is required. The predictive model is until now limited to the use of cofactor-independent enzymes, because the competition between recombinantly expressed heterologous enzymes with cell metabolites and the cell's own enzymes represent particular challenges. Also substrate uptake and product release are further parameters, which need to be addressed for the proper prediction of *in vivo* systems. Nevertheless, the Damborsky group provided a computer-based model for fast prediction of optimal enzyme ratios and identification of bottlenecks to the cascade biocatalysis community, which can then be addressed further by protein engineering.

Finally, we wish to comment on the evaluation of cascade processes. In fact, there is already a number of publications dealing with the evaluation of chemo- and multi-enzymatic cascades, and processes in general,[98, 108-112] but the importance of applying simple process- and green chemistry metrics as initiated by Ni, Holtmann and Hollmann in a recent review are somehow often neglected.[113] These authors suggested to calculate at least the E-factor (environmental factor), which is defined as the amount of waste generated per product equivalent, for the comparison of biocatalytic processes with chemical ones. Furthermore, frequently used "facts" and phrases for describing biocatalysis as green alternative to chemical processes (*e.g.* water as green solvent) were questioned and the numbers to compare certain examples from biocatalysis with chemical catalysis were calculated. Along that line, proper calculations of all influencing reaction parameters in a single or cascade type process should be considered and carefully estimated.

Conclusions

In summary, substantial progress in understanding and applying cascade type reactions combining bio/bio- or bio/chemo-catalysts were made in the past few years. Nature and its well evolved biosynthetic pathways serve as model for the construction of artificial mini cascades. In doing so, different concepts like spatial organisation to influence kinetic parameters, compartmentalisation to separate mutual influencing catalysts and catalyst optimization (bio- and chemo-catalyst) were successfully applied and showed the potential for future applications. Another very appealing methodology would be the implementation of enzymatic cascades in a whole-cell biofilm flow-reactor system.[114] The group of Bühler already presented the applicability of this concept on single enzyme processes like the production of *n*-octanol and (*S*)-styrene oxide.[115] A proper combination of these concepts and continued advances in all described research areas will certainly play a role for future developments of cascade type reactions, as well as those based on bio/bio- or bio/chemo-catalyst systems.

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Notes and references

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