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Whole-cell based synthetic enzyme cascades—light and shadow of a promising technology

Florian Rudroff

Mimicking Nature by biocatalytic cascade reactions in a whole-cell environment is a revolutionary development in multistep synthesis for the production of bulk and fine chemicals. In the past decade, several proof of concept success stories demonstrated the power of those synthetic cascades and paved the road for future industrial applications. Although enzymes and their promiscuity are best suited to construct such artificial pathways, the complexity and the lack of understanding of the cellular machinery slowed down this progress significantly. In this review, recent achievements in the field of whole-cell biocatalysis are described, challenges and hidden traps that have to be overcome are depicted, and strategies are illustrated how to increase overall cascade productivity.

Address
TU Wien, Institute of Applied Synthetic Chemistry, Getreidemarkt 9, 163-OC, 1060 Vienna, Austria

Corresponding author: Rudroff, Florian (florian.rudroff@tuwien.ac.at)

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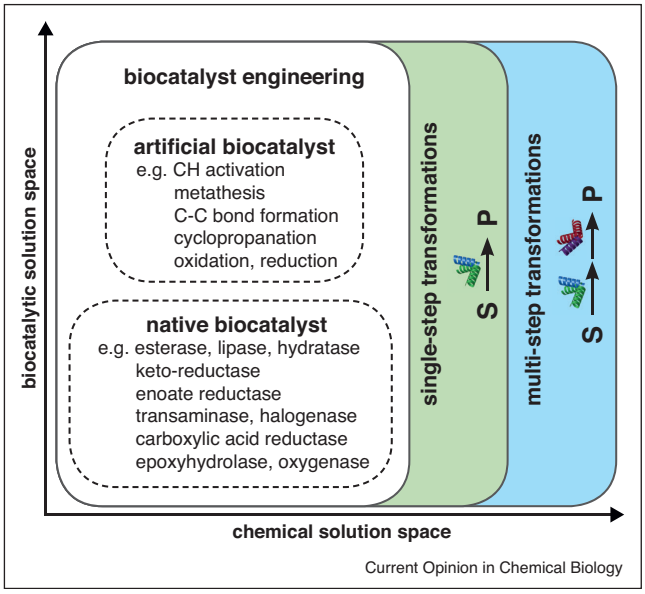
Introduction

The field of biocatalysis has evolved to an extent over the last 30 years, turning it from a curiosity-driven, Nature-inspired exploratory science developing novel bio-based catalysts, into a pillar in the synthesis of bulk,- and fine chemicals. One of the main driving forces of the field was the development of environmentally friendly and efficient catalytic systems, which are petroleum-free alternatives to metal- and organocatalysts. Enzyme-assisted reactions include simple hydrolytic reactions (e.g. esterases, amidases, lipases) as well as complex stereoselective reductions, oxidations, and C-C/C-N/C-P/C-S/C-X-bond-forming reactions. Many of the above-mentioned enzyme classes naturally have a very broad substrate spectrum and often show a strong stereopreference. For further development of these catalysts, for example in the case of problems of too low activity, stability (pH, temperature, solvent) and enantioselectivity, numerous engineering methods [1] have been developed. Meanwhile, more than 15 different classes of enzymes are commercially available and are regularly used in new synthetic strategies. Only recently, biocatalysis was awarded the accolade in the field of synthetic chemistry, since all three fields of catalysis (metal catalysis, organocatalysis, and biocatalysis) should be used in rethinking of retro-synthetic considerations for the construction of (complex) organic molecules. Some excellent reviews from experts in synthetic chemistry as well as biocatalysis have been published lately [2,3•,4]. Moreover, new, non-naturally occurring protein-based catalysts have been developed that have greatly expanded the spectrum of possible transformations towards C-H activation, metathesis, C-C cross coupling, cyclopropanation, oxidation and reduction [5]. With this toolbox of bio-based catalysts and protein engineering methods [6,7] in hand, biocatalysis provided valuable tools for the synthesis of chiral compounds, bioactive substances and bulk as well as fine chemicals. The increasing demand for sustainably produced compounds with enhanced complexity has led to scientists to use these tools and to imitate Nature by connecting several reaction steps in a one-pot fashion, which was inspired by the cellular metabolism (Figure 1).

Thereby operational time would be reduced, waste production would be minimized, unfavorable intermediate reaction equilibria could be shifted to the desired product side and therefore productivity should be increased. With this goal in mind, scientists investigated numerous bio-based cascade reactions by exploiting the whole biocatalytic toolbox, in the last 10 years [8–13]. Different strategies were followed. On the one hand, isolated enzymes were used. On the other hand, it was resorted to microorganisms in which the desired enzymes were overproduced. In both cases most of the examples were simple proof-of-concept studies to show the principle feasibility instead of aiming for a bio-based industrial applicable process. Thereby different substrates (e.g. alkanes, aromatics, fatty acid, terpenes) were converted into bulk chemicals (e.g. cyclooxtanones [14], octylamine [15••], 12-aminododecanoic acid methyl ester [16], terephthalic acid [17], carvolactone [18], ω-amino acids [19]) and fine chemicals (e.g. unnatural amino acids like phenylglycine [20••], phenylethanolamine [21], α- hydroxy acids [22] or substituted chiral piperidines [11]).

Figure 1



Fundamental developments in the field of biocatalysis, namely enzyme discovery, *de novo* enzyme design and tools for biocatalyst engineering, led to a stepwise exploration of the full chemical solution space. Nowadays, even complex molecules can be synthesized in a purely bio-based fashion by combining biocatalysts in cascade type reactions.

A closer look at a fairly related area, namely that of metabolic engineering [23–25], where scientists plan to modulate natural metabolic pathways and redirect carbon fluxes for the production of natural or novel unnatural compounds, shows that success stories are also sparse. In this case, the complexity of the microbial metabolism and its tight regulation to maintain cell viability are the biggest challenges for the construction of an industrial applicable cell-factory. Some success stories have been the synthesis of artemisinic acid [26–28], opioids [29], 1,4-butandiol [30], resveratrol [31], and biofuels [32,33].

These observations raised the question if the combination of different catalytic species, regardless of *in vitro* or *in vivo* is more complex and problematic than expected. Why is it so difficult to improve such synthetic pathways in a way that industrial demands are sufficiently fulfilled?

In this review, different aspects why enzymatic cascade reactions are not as productive as they should, why fundamental knowledge of the system is crucial and how such challenges can be tackled in the future, will be discussed.

Optimal carbon flux

The productivity of a biochemical cell-factory rely on the optimal carbon flux through the cascade. Prerequisites are an unimpeded transport of the substrate into and the final product out of the cell. All pathway enzymes should be actively expressible in the host of choice, the metabolic burden should be minimal and the cross talk with the cellular metabolism should be as little as possible. Furthermore all required pathway components like cofactors (NAD(P)H), energy (e.g. ATP) and reagents (e.g. oxygen, NH_3) should be sufficiently available. Additionally substrate, intermediates and the product should not be toxic to the host. In a perfect world, one would construct enzyme cascades with tools of synthetic biology, genetics, and biocatalysis, the cell-factory can be grown, the substrate can be fed and the product can be isolated in high yield and purity (Figure 2a).

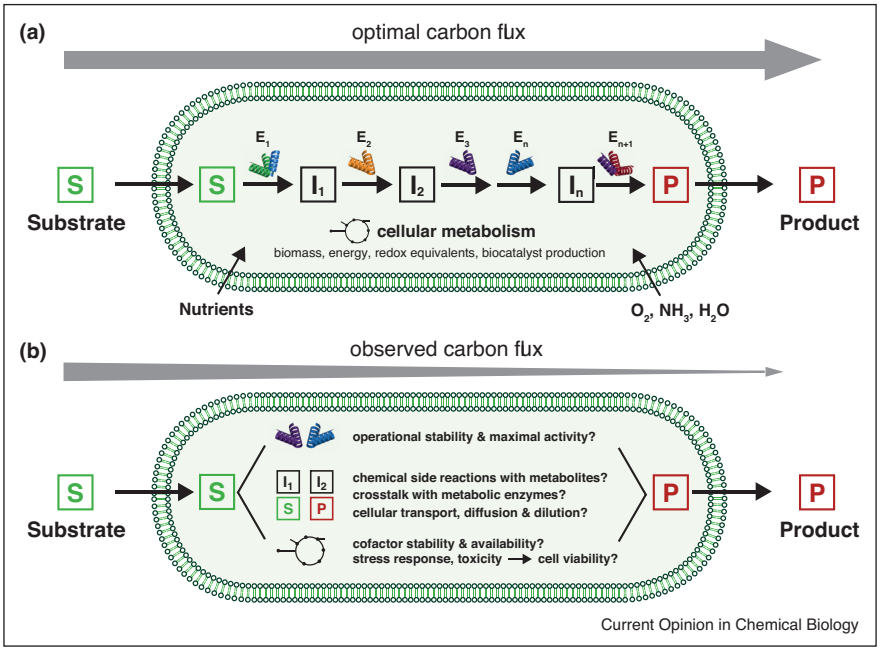
Having a closer look into such an optimal cascade several problems might occur (Figure 2b). First, the operational stability and the maximal activity of the pathway enzymes might be very different. Although all enzymes work in similar reaction regimes, differences in activity and stability are heavily-dependent on pH, temperature and the availability of coenzymes (e.g. NAD(P)H, FMN, FAD). Coenzymes are highly conserved in evolution and are predominantly used by enzymes of the central carbon metabolism, for the biosynthesis of amino acids and nucleotides. Therefore, they are in permanent competition between metabolic enzymes and pathway enzymes. Expression of pathway enzymes within a non-native host are often problematic because enzymes in the cell have to cope with metabolites to which they were never exposed in their metabolic context before. This could lead to cross-inhibition, site-specific mutagenesis, aggregation and cross-linking of protein domains, altered electric charge or damaging of other enzymes (Figure 3 right). Another, often neglected phenomenon is that pathway intermediates are not the only relevant chemical species in chemical space of a cell and continuously undergo damaging chemical side-reactions, and enzymatic side-reactions. These reaction products so called dead-end metabolites are often toxic to the cell, always wasteful and decrease overall pathway productivity (Figure 3 left) [34–37].

Also crucial for the design and an optimal flux of a biochemical cell factory is the cellular transport. In biocatalysis, non-natural substrates rely on simple diffusion processes, whereas in metabolic engineering active transporters can be used (e.g. glucose, xylose, galactose). Intracellular concentrations of pathway metabolites are also important for enzyme activity (e.g. K_M, K_i). Finally, toxicity of pathway metabolites and the stress response of the host are important parameters for the cell viability.

Mitigation strategies

To cope with all these challenges, several mitigation strategies have been developed, which have great potential to bring whole-cell cascade biotransformations to the next level and deliver considerable amount of high-value chemicals in the future (Figure 4).

Figure 2



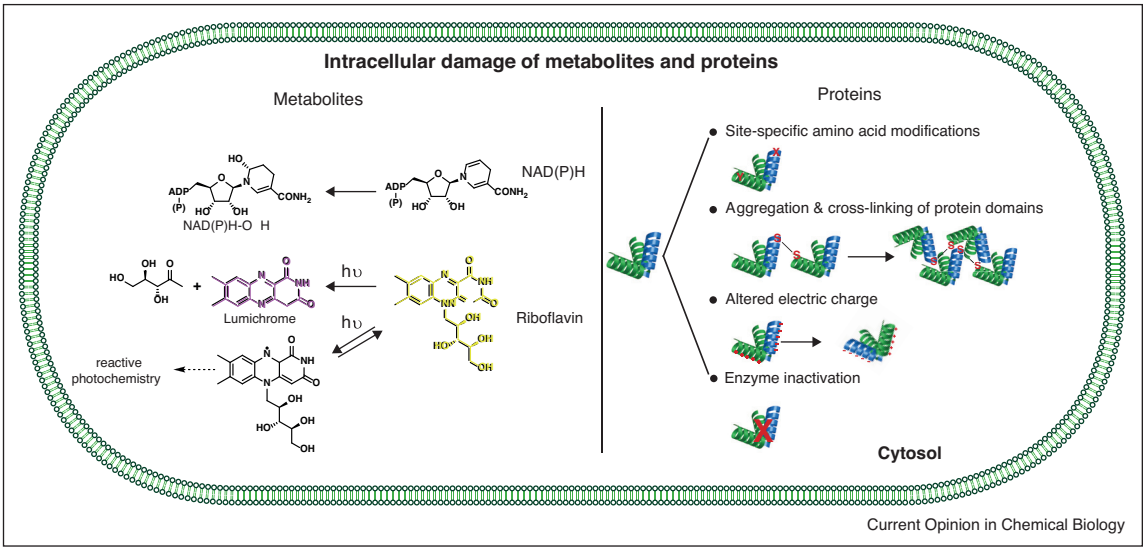
(a) A biochemical cell-factory for cascade biotransformation under optimal conditions. The carbon flux through the synthetic pathway is optimal and the productivity is 100%. **(b)** Most of the synthetic cascade suffer from a reduced carbon flux. Herein the most important challenges are depicted.

Nature provides some strategies coping with the problem of damaged coenzymes and cofactors. For instance, coenzymes like biotin, folates, nicotinamid cofactors (NAD(P)H) or diamin diphosphate have essential roles in metabolic reactions as well as in artificial metabolic pathways. Especially in redox biocatalysis the deactivation of NAD(P)H cofactors by hydration would lead to a reduced availability of these redox equivalents and result in a decreased cascade productivity (Figure 3, left). The cellular repair mechanism is based on an ATP-dependent NAD(P)H-OH dehydratase that recovers the unwanted hydrated NAD(P)H-OH [38,39]. Another prominent example is the protective effect of dodecins that prevent the photochemical breakdown of riboflavin [40,41]. They bind to the riboflavin and stabilize it by mediating ultrafast quenching of its exciting states (Figure 3, left). If photo degradation takes place, dodecins promote the formation of the less reactive lumichrome and sequester it into its active site [42]. Both rescue mechanisms could be useful in optimizing novel cascades in living cells.

Furthermore, pathway enzymes should be fully biochemically characterized, to elucidate their optimal reaction conditions. If possible, enzymes should have comparable activity at similar pH (slightly acidic in most of the microbial hosts use to date) and temperature. It is fundamental to know the intracellular reaction conditions to either select an expression host, which is suitable to the non-native pathway enzymes or *vice versa*. If this is impossible to achieve protein engineering might be a solution or the *de novo* pathway could be spatially organized. Several methods, based on DNA, RNA or protein scaffold were already successfully applied to improve overall flux through a cascade. Dueber *et al.* demonstrated the power of DNA-scaffolding by the production of resveratrol, 1,2-propandiol and mevalonate. They spatially organized the pathways on plasmid DNA within the cytoplasm and were able to increase productivity by approximately 5-fold for all cascades [43]. Although, depending on the enzymes and their kinetics used, proximity is not always the reason for an enhanced flux though the cascade. Recently, Zhang *et al.* investigated an enzymatic cascade composed of glucose oxidase and horseradish peroxidase localized on a DNA scaffold. Improved activity resulted by the change of the local pH at the negatively charged DNA backbone and not by the close proximity of the pathway enzymes [44•]. This can be an interesting tool to change pH value locally within a cell. The group of Silver investigated RNA-assemblies for the production of H_2 *in vivo*. The could optimize the cascade yield as a function of scaffold architecture up to 48-fold compared to the non-organized pathway [45].

In addition to spatial organisation, a general separation from the cellular environment into micro compartments like peroxisomes, carboxysomes or other compartments (mitochondria) can be advantage. These compartments can be designed *de novo*, the microenvironment can be

Figure 3

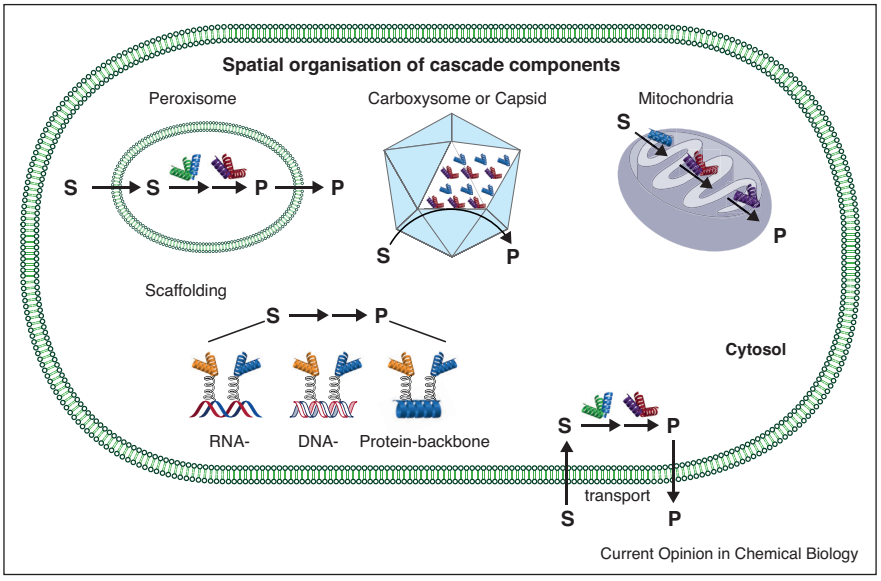


Examples of metabolite and protein damage reactions within a cell. Left: Unwanted side reaction of redox cofactor NAD(P)H into the unreactive hydroxy-derivative (NAD(P)H-OH. Light-induced degradation of riboflavin into the phototoxic product lumichrome. Right: Different protein damaging mechanism that alter pathway productivity and cell viability.

adjusted (e.g. different local pH value) and it can be expressed in the host organism. Peroxisomes are eukaryotic cellular organelles where fatty acids are degraded. Recently, these compartments were exploited for the production of fatty acid-derived alcohols, alkanes and olefins in yeast. The advantage of peroxisomes lies in an increased NADPH concentration provided by isocitrate dehydrogenase isoenzyme and the spatial separation from the cytosol preventing unwanted side-reactions. Indeed, Zhou *et al.* were able to increase the production of fatty acid-based biofuels up to 7-fold by localizing the pathway into the peroxisome [46••]. Recently, the group of Dueber published a similar study for the production of the microbial dye prodeoxyviolacein. The relocation of the pathway into the peroxisome led to an 35% increase in productivity and a 61% reduction of the unwanted chromopyrrolic acid side product [47]. The prokaryotic equivalent to peroxisomes are protein-based microbial microcompartments so called carboxysomes. The first packaging of a non-native enzyme within a carboxysome was demonstrated by loading ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) for the fixation of CO_2 [48••]. The group of Atsumi combined the CO_2 fixation and the valine biosynthetic pathway for the synthesis of isobutylaldehyde in *E. coli* [48••,49]. The group of Stephanopoulos applied another compartmentalization strategy, to increase cascade productivity in yeast. They investigated the production of isobutanol starting from glucose. Instead of overexpression of pathway enzymes, they targeted the complete isobutanol pathway into the mitochondria and could increase the isobutanol production by 260% [50]. The group of Prof. Hilvert investigated so-called ferritins, small protein nanocages with a negatively charged interior, for the encapsulation of four different enzymes. First the encapsulated the highly positively charged green fluorescent protein variant GFP (+36) and confirmed the host–guest interaction by transmission electron microscopy. Furthermore, they scavenged human carbonic anhydrase II, the Kemp eliminase and an artificial (retro) aldolase and were able to maintain the catalytic performance in comparison to the free enzymes [51]. The encapsulated enzymes showed an increased thermostability and resistance against proteolysis. The same group could also demonstrate that even *de novo* designed protein cages can be used for encapsulation of e.g. small interfering RNA (siRNA) for gene regulation. They could modulate the gene expression of GFP protein in HeLa cells by ~70% [52••,53].

As already mentioned in the previous chapter pathway intermediates can undergo side-reactions, they can induce cellular stress and become toxic to the host. Aldehydes represent a compounds class that is highly reactive, often very lipophilic and therefore toxic for the cell. Nevertheless, they are important products for the fragrance and flavor industry. To circumvent such a problem scientists investigated different strategies: first, by genetic engineering or second, by reaction engineering. Kunjapur *et al.* engineered an *E. coli* strain for the accumulation of aromatic aldehydes by knock out of six ketoreductases. Furthermore, they investigated their engineered strain for the production of vanillin and could increase the flux up to 55-fold [54]. The group of Schmid applied a biphasic system to remove toxic pathway intermediates (styrene and styrene oxide) from the reaction

Figure 4



Mitigation strategies for optimized flux through a whole-cell enzymatic cascade. Spatial organization by RNA-, DNA-, or protein backbones and separation by compartmentalization (e.g. peroxisome, carboxysome, mitochondria).

medium [55]. The same group also addressed the transport problem of lipophilic compounds into the cell. For the oxyfunctionalization of limonene, they introduced an alkane-transport protein (AlkL) into the host organism *E. coli*, applied a biphasic system and could increase productivity significantly (5-fold) [56].

This pool of mitigation strategies should lead to a more systematic approach for the development of future whole-cell cascades to fulfil industrial needs.

Perspective of whole-cell cascade catalysis

The development of applied microbial cell-factories that are capable of producing cost-efficiently bulk- and fine chemicals, flavor- and fragrance compounds as well as pharmaceuticals rely on multiple rounds of the so called 'design-construction-evaluation-optimization' principle (DCEO) [24]. Thereby several concepts of cascade design, cascade construction, cascade evaluation and optimization on a systemic level have to be combined, to create and optimize new synthetic enzymatic cascades. This design principle derives from the field of metabolic engineering, where already existing metabolic pathways are modulated for the sustainable production of chemicals. The robustness of the cellular metabolism remains one of the biggest challenges in the field and often counteracts the engineering efforts. Keasling *et al.* recently reported a similar concept — the design-build-test-learn cycle (DBTL) and introduced another facet, which emphasized on the 'learning' part [23]. The obtained knowledge of one particular metabolic engineering effort should be used for upcoming projects to increase the learning curve, gain deeper insights into the pathway and the metabolic network of the host, shorten investigation times and therefore become more cost-efficient. It is necessary to increase the fundamental knowledge of the microbial host, its intracellular environment, the operational stability and efficiency of the applied pathway enzymes and about the cellular response towards the heterologously expressed pathway and its intermediates.

Very recently, the group of Vorholt reported a fundamental piece of work on the longevity of major coenzymes in microorganisms. They investigated the half-life of organic coenzymes like FAD and NAD in different microbial hosts (*Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Sacharomyces cerevisiae* (*S. cerevisiae*)). The novo biosynthesis of such coenzymes is highly complex and is solely used to match the requirements for the dilution due to cell growth [57••]. This is especially interesting for redox enzyme-mediated cascade reactions. Many of them are performed under resting-cell (non-growing) conditions, where no new coenzymes are produced efficiently, which can result in a decreased productivity. In addition, stability of enzymes under reaction conditions is also important for whole-cell systems. Milker *et al.* elucidated the operational stability of a Baeyer-Villiger monooxygenase incorporated into a three-step cascade *in vivo* [58,59]. This class of enzymes is dependent on the availability of NADPH and FAD for its activity and ultimately also for its stability. After careful evaluation of the cascade by intracellular metabolomics, enzyme kinetics and

mathematical modelling [60], they were able to identify the lack of coenzymes as bottleneck. The microbial host (*E. coli*) is simply not capable of providing enough coenzymes for an efficient pathway flux.

Whole-cell cascade catalysis has been established successfully as an alternative to the classical metabolic engineering. In the past years many proof of concept studies have been published and now it is time to tackle the challenges and bring this field to the next level and enter the door towards industrial applications. By combination of the presented tools and methodologies, I am confident that the development of synthetic whole-cell cascades will advance rapidly.

Acknowledgements

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