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Review

Getting the Most Out of Enzyme Cascades: Strategies to Optimize *In Vitro* Multi-Enzymatic Reactions

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Abstract: *In vitro* enzyme cascades possess great benefits, such as their synthetic capabilities for complex molecules, no need for intermediate isolation, and the shift of unfavorable equilibria towards the products. Their performance, however, can be impaired by, for example, destabilizing or inhibitory interactions between the cascade components or incongruous reaction conditions. The optimization of such systems is therefore often inevitable but not an easy task. Many parameters such as the design of the synthesis route, the choice of enzymes, reaction conditions, or process design can alter the performance of an *in vitro* enzymatic cascade. Many strategies to tackle this complex task exist, ranging from experimental to *in silico* approaches and combinations of both. This review collates examples of various optimization strategies and their success. The feasibility of optimization goals, the influence of certain parameters and the usage of algorithm-based optimizations are discussed.

Keywords: biocatalysis; *in vitro* biotransformation; one-pot process; multi-enzymatic reactions; enzyme cascade; cascade optimization; kinetic modelling

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1. Introduction

In recent decades, *in vitro* biocatalysis has emerged as a valuable alternative to classical chemical synthesis approaches, especially for products with complex stereochemistries such as building blocks for active pharmaceutical ingredients (APIs) [1,2]. Compared to classical chemical synthesis, enzymatic reactions typically offer high substrate specificities and both regio- and stereoselectivities [3–5] often bypassing costly and time-consuming separation of intermediates and byproducts [6]. Ongoing research provides a large number of well-characterized enzymes that catalyze reactions ranging from asymmetric ketone reduction and reductive aminations to the complex synthesis of divers natural compounds, some of which are extremely challenging to achieve with classical chemical syntheses [7,8]. Recent breakthroughs in digitalization with respect to cascade construction and protein structure prediction provide tools to further accelerate the process of enzyme cascade design and optimization [9,10].

Within the field of biocatalysis, the combination of two or more catalytic steps has emerged as its own field within the biocatalysis community since the 1990s, as represented by the increasing publication numbers referring to enzyme cascades or multi-step enzymatic reactions (see Figure 1). It is important to note that the combination of two or more enzyme-catalyzed steps is often referred to as enzyme cascade regardless whether product isolation takes place between the different reaction steps [11]. Each additional reaction step further increases the accessible product range, while the overall complexity also increases. Especially when combining enzymes into one-pot reactions that do not occur conjunct

in nature, major challenges can arise, e.g., from side reactions due to substrate promiscuity [12–16] or different optimal reaction conditions (e.g., temperature, pH) [17,18] of the enzymes involved.

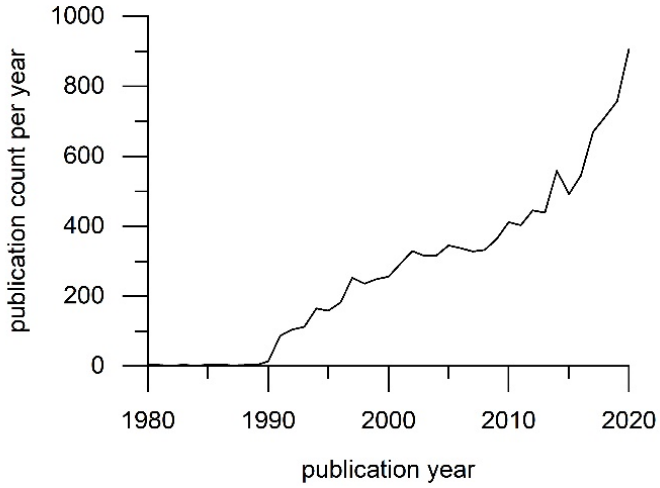

Figure 1. Publications referring to enzyme cascades or multi-step enzyme reactions in their title, abstract or keywords according to web of science. These two keywords were chosen as examples among others such as multi-enzymatic cascades/reactions or biocatalytic cascades, to show the growing total number of publications in this field.

Many cascades are set up to demonstrate a proof of concept in terms of reactivity and feasibility. Nevertheless, these cascades do not *per se* have high conversions, high yields, high reaction rates, stabilities or good atomic yields upon first assembly. When starting to tune enzyme cascades for higher performance, one has to ask eventually: What concepts are there to optimize cascades? What does it mean to optimize a cascade? When is optimization worthwhile? What performance parameter do we optimize for? The literature provides many approaches ranging from experimental-based optimizations [19–21] to in silico approaches [22,23] as well as combinations of both [24,25]. This review aims to give an overview on the concepts for optimizing enzyme cascade reactions, and covers both experimental as well as *in silico* approaches. It discusses the different optimization strategies with a special focus on the interplay of initial situation, 1optimization goals and parameters to be adjusted. The review includes mainly *in vitro* enzyme cascade reactions with isolated biocatalysts. It does not give an all-encompassing overview, but focuses on different enzyme cascade optimization concepts and presents selected examples.

2. Optimization Goals

Working with *in vitro* enzyme cascades and trying to optimize them is associated in most cases with the overall goal of making them more attractive for application. Economic and, increasingly, ecologic criteria play a dominant role when working with enzyme cascades. This includes processes in which few investments have to be made but the highest possible output is produced, ideally in compliance with environmental protection [26]. In this context, a rapid adjustment to market desires is beneficial [27]. Characteristic parameters for the performance of a biotransformation and thus an *in vitro* enzyme cascade are product concentration, (isolated) yield, space–time yield, reaction rate, and step and atom economy (Figure 2) [28]. These values are a measure for the productivity of the system and are predominantly influenced by the enzyme activities and the equilibrium constants, the composition of the reaction mixture (e.g., solvent system, concentration of substrates, enzymes, salts), but also the reaction condition (e.g., temperature, pH) or interactions of the components with each other. It has to be noted that the actually observed, apparent enzyme activity in the reaction mixture, therefore, might be quite different from the activity observed in the standard activity assay. The apparent reaction rate of the

enzymes, however, finally determines the amount of catalyst required. High catalytic activities directly result in the need to use less enzyme and thus a reduction in enzyme production and purification, which has a significant contribution on the environmental impact and costs of biotransformation [29]. Increasing the stabilities of the individual biocatalysts results in a more robust cascade, with higher catalyst life times and total turnover numbers (TTN). This simultaneously leads to fewer batches of protein needing to be produced, which in turn reduces the costs of the overall cascade. In addition to the reaction rate and stability of a cascade, the yield, i.e., the conversion of substrates into high-value products, is of course also crucial for the efficiency of a cascade, and is likewise relevant for economic and ecological considerations [25]. Even though many publications consider a single metric, it should be noted that these metrics can show different trends during optimization. Focusing on just one metric might achieve an optimum for this particular metric, but lead to even inferior outcomes for the other metrics than the starting point. A global optimum, however, is difficult to define, as the objective function, i.e., defining the impact of the different metrics on the global optimum, has to be performed on a case by case basis. This contrary development of performance metrics was shown for a cascade to convert 2,5-furandicarboxylic acid from 5-methoxymethylfufural [30]. Here, among other things, it was shown that increased stability does not necessarily result in a higher TTN, when the enzyme activity becomes lower. This means that a single parameter, such as TTN, cannot be considered as the sole measure of catalyst performance [28]. Another example for competing optimization goals was demonstrated for the production of (*S*)-hydroxy-ketones by a pyruvate carboxylase [31]. Optimal specific space–time-yield and stereoselectivity excluded each other during the optimization of reaction conditions such as temperature, pH, substrate or enzyme concentrations. The product requirements defined the reaction conditions for the synthesis of (*S*)-hydroxy-ketones.

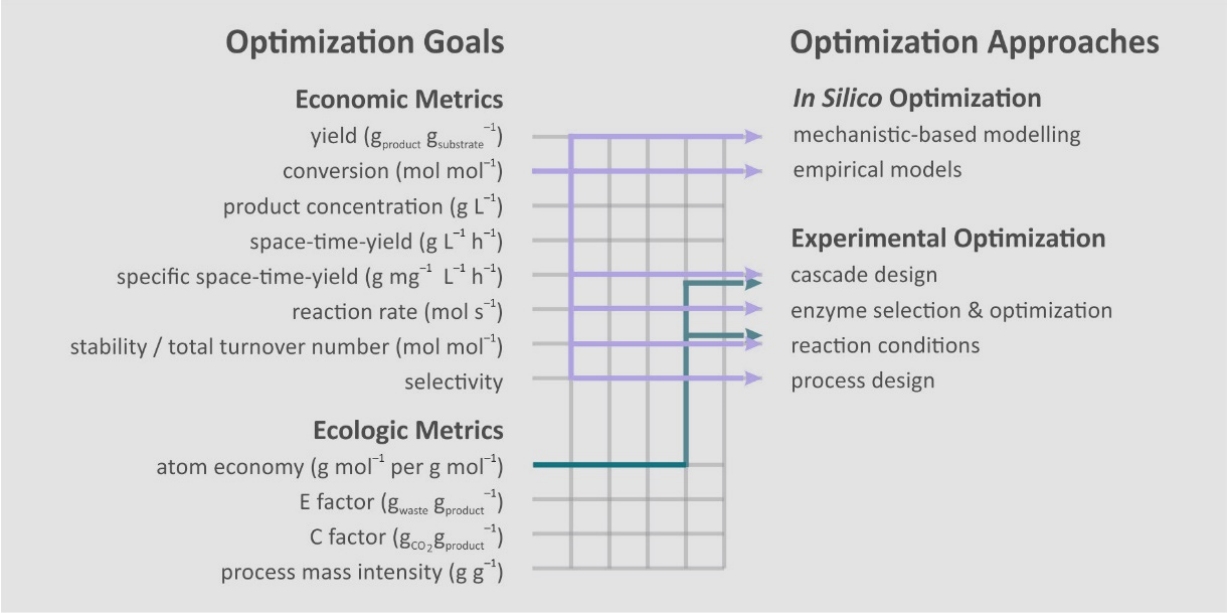

Figure 2. To reach optimization goals [8,32], many computational and experimental approaches are available. The correlations between them are complex and often a certain goal can be addressed by every approach presented here, while others are influenced by only a few. Two examples for these dependencies are highlighted in purple and green for the conversion and atom economy. E factor = environmental factor to calculate mass of waste per mass of product, C factor = cover-management factor to calculate soil erosion.

Often, several objectives are pursued at the same time. Ideally, a cascade is highly productive, results in high yields and has a high operational stability. However, seldom all parameters can be fulfilled at the same time as not all goals are complementary to each other. For example, high product concentrations are not always in compliance with

high reaction rates, as shown for a 27-enzyme cascade for the conversion of glucose into monoterpenes [33]. The optimization resulted in a high stability of more than 5 days with yields above 95% and titers above 15 $g \cdot L^{-1}$ demonstrating the potential for producing chemicals with an enzyme cascade. However, the reaction rate adaptation was neglected in these experiments, resulting in reaction rates that were an order of magnitude lower than those that would have been optimal for industrial use (0.1 versus 1–2 $g \cdot L^{-1} \cdot h^{-1}$).

In addition to the observation that product concentration and reaction rate of a cascade are not automatically dependent on each other, the stability and activity of enzymes do not necessarily correlate. This was demonstrated for a reaction cascade for the synthesis of L-alanine, in which one enzyme of the five-enzyme cascade was exchanged with a 40-fold more active enzyme, but at the expense of lower thermostability and simultaneously reduced TTNs [21]. Therefore, a careful selection of requirements and a ranking of the optimization goals must be made, which need to be adjusted during the process.

3. Optimization Approaches

Various approaches are available to achieve the optimization of the performance of an enzyme cascade. A general categorization can be made by dividing them into experimental-based and model-based approaches. Experimental optimizations are performed in the wet lab, while model-based attempts use algorithms to further investigate the systems. In an optimal scenario, these model-based optimizations are validated with experiments or an iterative approach is chosen.

3.1. Experimental-Based Optimization of Enzyme Cascades

The experimental optimization of enzyme cascades (Figure 3) can be divided into cascade design, enzyme optimization, optimization of reaction conditions and process design. General approaches and specific examples of those are explained in more detail in the following subchapters (Table 1).

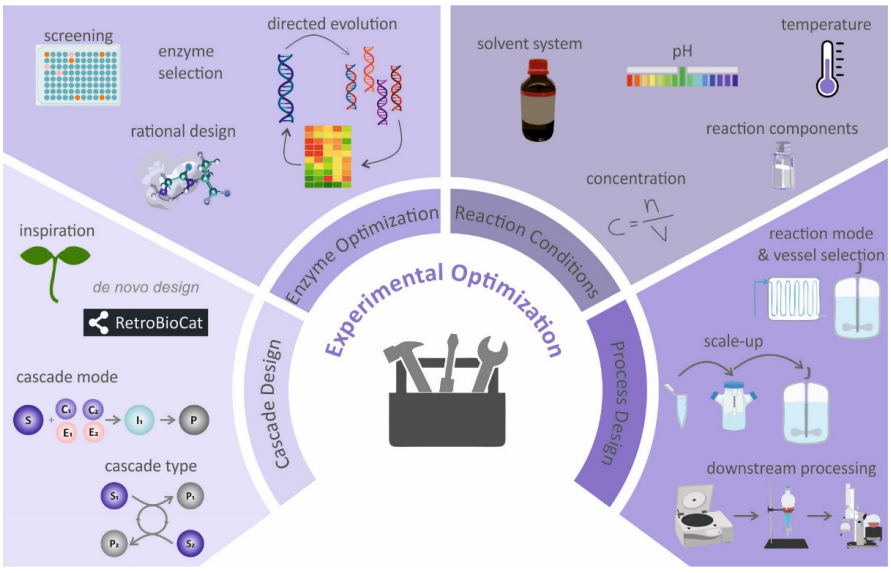

Figure 3. Experimental optimizations can be divided into cascade design, enzyme optimization, optimization of reaction conditions and process design. Each of these approaches have their own toolbox for the alteration of the cascade's performance. Cascade design can be inspired by natural pathways or designed *de novo* while several cascade modes and cascade types are available. Enzymes properties can be adjusted by rational design or directed evolution if screening does not result in the identification of a suitable catalyst. Optimization of the reaction conditions include the adjustment of the solvent system, the concentrations of reaction components as well as reaction parameters (such as pH, temperature amongst others). Finally, the scale-up, reaction mode and downstream processing of the system can be addressed to provide cascade products in larger quantities and purified form.

Table 1. Experimental based approaches for enzyme cascade optimizations.

Number of Involved Reaction Steps	Substrate	Product	Main Optimization Strategy	Optimization Target	Optimization Result	Reference
13	CO_2	Malate	Pathway design, metabolic proofreading, enzyme engineering	CO_2 fixation efficiency, malate production rate	5 nmol CO_2 $L^{-1}\cdot mg^{-1}$, 1080 µM fixated CO_2; 530 µM malate (20-fold improvement)	[19]
5	Mevalonate	Isoprene	Optimization of reaction conditions and balancing of enzyme levels	Flux, conversion efficiency, production rate	6323.5 µmol$\cdot L^{-1}\cdot h^{-1}$ (430 mg$\cdot L^{-1}\cdot h^{-1}$) isoprene in 2 mL; 302 mg$\cdot L^{-1}$ isoprene in 40 h in 50 mL (83-fold improvement)	[34]
12	Chitin	Pyruvate	Flux tuning (enzyme concentrations), cofactor regeneration	Pyruvate production (titer, rate)	2.1 mM pyruvate in 5 h; 0.42 µmol$\cdot mL^{-1}\cdot h^{-1}$ pyruvate (3-fold improvement)	[35]
6	D-Glucose	L-Alanine	Kinetic analysis for enzyme ratios, cosubstrate and buffer optimization	L-Alanine yield	0.17 g L-alanine $L^{-1}\cdot h^{-1}$ in 12 h, yield of >95%	[21]
3	2-Ethynylglycerol	Islatravir	Retrosynthetic pathway design, directed evolution	Islatravir yield	Yield of 51% islatravir	[36]
4	Glycerol	Chiral carbohydrate precursor	Optimization of reaction conditions	Conversion	Conversion of 100% in 8 h (Starting with 42%)	[37]
7	D-Xylose	*(R)*-Acetoin, ethylene glycol	Optimization of reaction conditions	*(R)*-Acetoin production	2.03 mM and 1.02 mM$\cdot h^{-1}$ *(R)*-acetoin, 3.45 mM and 1.73 mM$\cdot h^{-1}$ ethylene glycol	[38]
2	Benzaldehyde	(*R*)-Phenylacetyl-carbinol, (*1R*,*2S*)-norephedrine	Switch from simultaneous to sequential mode and recycling of by-product (cascade design)	Conversion, atom economy	Increase from 2% to 78% conversion	[39]
3	Benzaldehyde	Tetrahydrosiochinolines	Switch from simultaneous to multi-step reaction mode (cascade design)	Conversion	Conversion of 88%	[13]
4	Starch	Fructose	Equilibrium shift by implementation of irreversible step (cascade design)	Yield	Yield of 62% (previous work: 42% [40])	[41]
2	Acetophenone derivatives	(*S*)-1-Phenylethylamine derivatives	Equilibrium shift by implementation of irreversible consumption of by-product (cascade design)	Conversion	Increase from 63% to 99% conversion	[42]
2	Racemic sec-alcohol, prochiral ketone	Enantiopure sec-alcohols	Switch to orthogonal cascade (cascade design)	Cofactor demand, atom efficiency	No additional cofactor recycling necessary	[43]
2	Acetaldehyde, benzaldehyde	1-Phenylpropane-1,2-diol	Switch to unconventional media	Product concentration, downstream processing	Space–time yield 327 g$\cdot L^{-1}\cdot d^{-1}$; E factor 21.3 $kg_{waste}\cdot kg_{product}{}^{-1}$ (1600-fold increase)	[44]
2	4-Methoxy benzaldehyde, acetaldehyde	4-Methoxyphenyl-1,2-propanediol isomers	Setting up self-sufficient recycling cascade (cascade design)	Atom economy, E factor, downstream processing	Atom economy 99.9%, E factor close to 1 $kg_{waste}\cdot kg_{product}{}^{-1}$, space–time yield 165 g$\cdot L^{-1}\cdot d^{-1}$, isolated yield 38%, product purity 99.9%	[45]

In short, cascade design covers the organization of reaction steps in regard to the spatial and temporal addition of the reaction components (cascade mode) as well as possible interconnections between the reaction steps (cascade type). Optimization of the enzymes can be performed, e.g., by directed evolution or rational design, while the main parameters for optimization of reaction conditions are defining solvent system, reaction components, their concentrations, pH and temperature. The topic of process design is a very broad field that covers aspects from selection of vessel/reactor, compartmentalization, stability under process conditions, reusability of reaction components and many more. In addition, the reaction mode (batch, continuous, feeding strategy), scale-up and (integrated) downstream processing are further parameters that should be taken into account when setting up an ecologically and economically advantageous cascade. In this review, we want to focus on the latter, as we believe that these are not always considered in the optimization of enzyme cascades, but are crucial when transferred to an industrially relevant process.

3.1.1. Cascade Design

Reaction Pathway

Enzyme cascades can be designed either from existing natural reaction pathways or by retrosynthetic experiments during which possible reactions are selected to synthesize the desired product. Metabolic pathways already existing in organisms can be used as templates and transferred to *in vitro* multi-enzymatic reactions. These can be parts of the primary metabolism, e.g., glycolysis [46] or of the secondary metabolism such as the mevalonate pathway [47] or chitin degradation [35]. Combinations of primary and secondary pathways were developed to *in vitro* cascades as well, which produces high value products with the benefit of energy regeneration and the usage of bulk chemicals as substrates. Korman et al. created an *in vitro* enzyme cascade consisting of 27 enzymes of various organisms that produces monoterpenes from glucose [33]. By utilizing the glycolysis and mevalonate pathway, limonene and pinene were produced within 5 days with titers of 12.5 $g \cdot L^{-1}$ and 14.9 $g \cdot L^{-1}$, respectively, corresponding to a yield of more than 88% [33]. This significantly exceeds the highest limonene titers achieved with whole-cell biocatalysts [48], demonstrating the potential of such a complex cascade with enzymes of various origins.

The combination of reactions from multiple organisms can be referred to as chimeric synthetic pathways, which can either give access to new compounds or to alter synthesis routes in a beneficial way [35,49]. Ye et al., for example, shortened the *in vitro* Embden-Meyerhof (EM) pathway by replacing the two enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) with the archaeal non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) [49]. This replacement by only one enzyme led to a balanced adenosine triphosphate (ATP) consumption and regeneration, and lactate was produced from glucose with a yield of 100% after 10 h.

Another approach to develop *in vitro* enzyme cascades is to build non-existing synthetic pathways *de novo* [50]. This creates tailored synthetic pathways that can lead to novel chemical compounds. However, designing reaction cascades for the synthesis of a target molecule without an existing blueprint can be challenging. In chemical organic synthesis, retrosynthesis is commonly used to plan the synthesis of a compound. Retrosynthesis, therefore, means to start from the target molecules to identify bonds to be formed and define precursors and intermediates accordingly [51]. This backward planning can also be applied to biocatalytic problems, as comprehensively reviewed by Schrittwieser et al. as well as Turner and Humphreys [4,15]. Computational tools such as RetroBioCat, Selenzymes, or the Metabolic Module (MEMO) algorithm help to gain access to all available enzyme-catalyzed reactions known or to find suitable metabolic modules [10,52,53].

An example for such an artificial route is the CO_2-fixation by the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle by Erb and coworkers [19]. By selecting suitable enzymes, considering all biochemically possible reactions and a subsequent evaluation of their thermodynamic feasibility, a cycle consisting of 12 enzymes

was designed. After optimization, the final CETCH cycle converted CO_2 with a rate of 5 nmol CO_2 $L^{-1} \cdot mg^{-1}$ protein into malate using 17 enzymes in total. Enzymes for cofactor recycling, H_2O_2 degradation and to prevent side reactions were included in addition to the CO_2 fixating cycle [19]. Next to the CETCH cycle, there are various examples of retrosynthesis, such as the synthesis of didanosine or islatravir [36,54]. Both molecules are nucleoside analogs and inhibitors of human immunodeficiency viruses (HIV) reverse transcriptase previously synthesized by organic synthesis. Novel synthetic accesses were developed by retrobiosynthetic analysis, resulting in more efficient enzymatic routes including engineered biocatalysts.

Cascade Mode

When two or more enzyme-catalyzed steps are combined, it is important to distinguish between different cascade modes (see Figure 4), meaning how the reaction steps are organized: sequential or parallel/simultaneously to each other, with or without isolation steps in between. This is important in the context of this review, as choosing a suitable cascade mode can substantially increase the product formation within the enzymatic cascade, e.g., by reducing undesired byproduct formation or implementing an equilibrium shift, as described in the following.

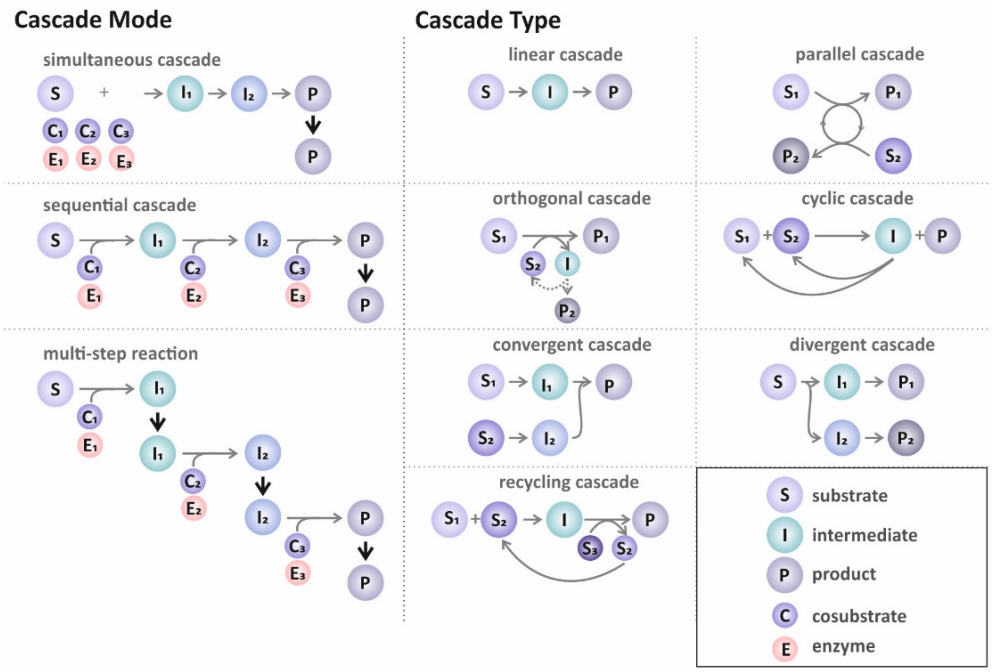

Figure 4. *Cascade Mode:* defines if all reaction components are added at the same time (simultaneous cascade), in a temporal manner (sequential cascade), or if more isolations are needed in between the reaction steps (multi-step reaction). *Cascade Type:* defines how the reaction steps are organized, and if interconnections occur between the reactions. Linear cascade—a single product is synthetized via one or more catalytic steps; parallel cascade—two enzymatic reactions are coupled through cofactors/cosubstrates; orthogonal cascade—product formation is coupled with cofactor/cosubstrate regeneration or with the destructive removal of by-products; cyclic cascade—a combination of substrates is transformed into product and intermediate, while the intermediate is converted back to the substrates; convergent cascade—two intermediates are formed in a linear way which are subsequently combined to one product; divergent cascade—a reaction forms two intermediates which are converted into two different products; recycling cascade—special inter form of linear and orthogonal cascade, where a by-product can be used as substrate for a previous reaction step. S: substrate, I: intermediate, P: product, C: cosubstrate, E: enzyme. Compiled from [11,55–58].

There are three general modes of cascades: all reaction components are added to the same reaction vessel (one-pot system) at the same time and after completion, the final product is isolated (Figure 4 left top). This is referred to as simultaneous cascade in the course of this review. Other publications also call this cascade mode concurrent cascade [11] or multi-component reaction [58]. An example for such a simultaneous cascade from Molla et al. is using a combination of three enzymes of different origins (rabbit muscle aldolase, *sn*-glycerol 3-phosphate dehydrogenase and formate dehydrogenase from *Candida boidinii*) to produce D-glyceraldehyde 3-phosphate and L-glycerol 3-phosphate in one pot [46]. The simultaneous cascade is the most straightforward when thinking of reactions in one pot. However, it is often not possible to add all reaction components at the same time, as, e.g., by-product formation might occur due to enzyme–substrate promiscuity [16].

In the next mode of reaction, the reaction steps are separated either in time or space but only with one isolation step at the end. For separation in time, for example, the enzymes catalyzing the next reaction step are added in a timely fashion (Figure 4 left middle). These cascades are referred to as sequential cascades [11,39]. An example for a sequential cascade is the formation of nor(pseudo)ephedrine reported by Sehl et al., wherein a first step pyruvate is decarboxylated and subsequently ligated to benzaldehyde by the thiamine diphosphate-dependent acetohydroxyacid synthase I yielding (*R*)-phenylacetylcarbinol ((*R*)-PAC) [39]. Then, an amination step yields the final amino alcohol. Here, a timely separation of the two steps is necessary for (1*R*,2*S*)-norephedrine production, as the (*S*)-selective amine transaminase from *Chromobacterium violaceum* would preferably accept the benzaldehyde as substrate over the (*R)*-PAC, resulting in formation of benzyl amine as main product [39]. With the subsequent addition of the amine transaminase after completion of the carboligation step, conversion to norephedrine was increased from 2% to 78%. A comparison of simultaneous and sequential modes for a two-step cascade was performed by Núñez-López et al. with varying substrate concentrations [59]. The flavonoid puerarin polyfructoside is synthesized by two levansucrases with puerarin and sucrose as substrates. Maximum puerarin conversion was reached in a simultaneous mode with 92.4% that was slightly higher compared to the best conversion of 88.9% with sequential addition of the second enzyme [59].

When more than one isolation step is needed, the reaction can be referred to as multi-step reaction (Figure 4 left bottom). An example for this is the three-step reaction towards tetrahydroisoquinolines published by Erdmann et al., where the amine transaminase had to be removed via ultrafiltration to avoid by-product formation at a later step in the cascade, resulting in a total conversion of 88% [13].

Of course, there are also mixed forms between these reaction variants, especially when a particularly large number of reaction steps are combined. Additionally, the catalytic steps do not necessarily have to be catalyzed by different enzymes, sometimes referred to as tandem catalysis [11,60,61] but can also be catalyzed by the same enzyme, sometimes referred to as domino reaction [11,54,62]. Admittedly, the terms enzyme cascade, multi-enzymatic cascade/reaction and multi-step reaction are often used interchangeably in the literature, and the term cascade is used for any reaction sequence consisting of more than one step.

Cascade Type

Furthermore, enzyme cascades can be divided into different types (see Figure 4) referring to possible interconnections between the catalytic steps [11,56,57]. Still, the division in the different cascade types is only a loose framework, as different cascade types may as well be combined into larger more complex cascades. The various options for combining enzymes and reactions in the cascade are particularly interesting in the context of this review as, by clever combination, the overall performance of the cascade can be improved in terms of conversion [41,42,63] or cofactor recycling can be bypassed [43].

When a cascade reaction suffers from unfavorable reaction equilibria within the first reaction steps, this can be overcome, for example by implementing an irreversible

reaction step consuming either the intermediate or a by-product. Thereby, the reaction is shifted to the desired product [63]. Moradian and Benner developed a fructose-producing cascade with starch as substrate [41]. The two-step process originally lacked high product yields because the equilibrium of the last reaction catalyzed by xylose isomerase was almost balanced between the substrate and product side. By designing a four-step linear enzyme cascade, in which the last step was irreversible and exergonic, high fructose yields were obtained [41]. Cassimjee et al. coupled the reductive amination of substituted benzaldehydes by amine transaminase with isopropylamine as amine donor with an alcohol dehydrogenase step to remove the by-product acetone, similar to an orthogonal cascade [42]. Thereby, they shifted the reaction equilibrium completely to the product side.

Parallel cascades can, for example, couple two reactions of synthetic interest with matching cofactor demands as an alternative to cofactor recycling. For example, the kinetic resolution of a chiral alcohol can be coupled to the reduction in a prochiral ketone both catalyzed by an alcohol dehydrogenase, resulting in a closed redox cycle [43]. A further example for an atom-efficient cascade architecture is a recycling cascade reported by Sehl et al., where a by-product serves as starting substrate of the overall cascade [39].

3.1.2. Enzyme-Selection and -Optimization

Once the cascade is designed and enzymes are found to catalyze these reaction steps, individual fine-tuning can be carried out in terms of activity, stability, selectivity, or possible inhibitions. This can be accomplished by choosing enzyme homologues or variants based on their properties, by protein engineering or *de novo* protein design followed by the adjustment of their concentrations. Selecting enzymes for a cascade that originates from the same organism increases chances that they prefer similar reaction conditions, which saves a great deal of optimization effort. However, this also limits the selection and exploitation of the benefits of various enzymes from the huge pool nature offers. Enzyme homologues originating from different organisms may differ in their catalytic properties [64,65], but their careful selection can substantially increase the performance of the reaction system. This was demonstrated during the development of an L-alanine producing cascade from glucose by five enzymes [21]. The previously selected enzymes [61] were sought to be replaced with mostly more active homologues and variants, which led to an improved economy in terms of enzyme loading and stability. Additional enzymes were substituted in order to circumvent bottlenecks caused by inhibition due to accumulating intermediates and to change cosubstrate selectivity (NAD- instead of NADP-acceptance) to ensure efficient cofactor regeneration. These alterations already resulted in an L-alanine yield of 90% after 12 h using the enzymes in a 1:1 activity ratio [21].

The operational stability of enzyme cascades can be increased by the use of thermophilic enzymes. According to literature, facilitated purification lowers the costs of the production process [66]. In addition to a possible higher reaction temperature and thus increased reaction rate, the enzymes' lifetime is enhanced, leading to higher TTNs. According to a study of Zhang et al., increased TTNs in turn reduce costs, i.e., if TTN_W (kg product per kg biocatalyst) $>10^6$, enzyme costs will be less than 1% of the production [63]. You et al. developed a four-enzyme cascade for the production of myo-inositol from starch with only thermophilic enzymes [67]. Selection of the catalysts was adjusted to the rate-limiting enzyme, which was highly active above 70 °C, but had low activity below 60 °C. The other three enzymes were chosen accordingly to catalyze the reactions optimally at 70 °C. High product yields of 99% were achieved even at large scale in a 20,000 L bioreactor [67].

Further customization of enzyme properties by protein engineering through rational design or directed evolution can make enzyme-catalyzed reactions even more efficient and open up new synthetic possibilities, especially with respect to properties relevant for application. Enzyme activity, stability, substrate specificity or selectivity can be tailored using protein engineering methods (as well as the acceptance of non-natural substrates) [68]. In addition, the expression levels of the enzymes are an important factor for optimization [69]. The nucleoside analog islatravir-producing cascade uses nine enzymes of which five were

altered through directed evolution [36]. The catalysts were selected by their acceptance for unnatural substrates and the final cascade synthesized islatravir from ethynyl glycerol with an overall yield of 51%. Compared to previous syntheses, the required reaction steps could be reduced to less than half and the atom economy was significantly improved.

It can be speculated that *de novo* design of proteins on demand might be the engineering method of the future with a more rapid and directed customization [27]. The computational approach AlphaFold shows the upcoming capability of predicting 3D proteins structure through machine learning and will be of great help when designing enzymes from scratch [9,70,71].

In addition to the catalyst selection, enzyme ratios and concentrations can be adjusted, which is a straightforward technique to avoid bottlenecks. Flux improvement and a reduction in the total amount of enzymes can be achieved if the activity of the biocatalysts is balanced. To perform rapid ratio screening of two enzymes by varying their amounts, a method using an inkjet printer was developed for portioning and identifying optimal concentrations of enzyme and substrate solutions [66]. Nevertheless, manual optimization is often performed as well, for example, by titrating each enzyme to find the optimal concentrations. For the L-alanine synthesis cascade, an adaptation of enzyme ratio led to improved performance and higher yields [21]. After further optimization of the buffer system and cofactor concentrations, nearly quantitative yields of >95% were reached. A rational approach for an optimized enzyme ratio was demonstrated for the synthesis of 2′3′-cyclic guanosine monophosphate–adenosine monophosphate (cGAMP). The concentrations of the four enzymes were adjusted according to their specific activities, which led to a twofold increase in product concentration [72]. Chen et al. determined an optimized ratio of enzyme amounts first by Taguchi orthogonal array design for the production of amorpha-4,11-diene [24]. Starting with mevalonate, six enzymes were used for the artemisinin precursor formation following the mevalonate pathway. Further investigation of enzyme activities revealed that enzyme concentrations of the two last steps were crucial for product formation, as they were rate limiting. Inhibitory effects and intermediate precipitation needed to be taken care of, which ultimately led to a conversion of 100%.

The selection of enzymes plays a crucial role for the performance of a cascade. Their characteristics influence most of the optimization goals such as the conversion, yield, rate, selectivity and stability. Balancing the activity ratios can optimize the flux of the system and can reduce the amount of enzyme needed. However, to fully exploit their catalytic properties, the reaction conditions have to be adjusted to the biocatalysts optima.

3.1.3. Reaction Conditions

An advantage of an *in vitro* enzymatic system is that it can be individually adapted to the needs of the selected enzymes, independent of the requirements of the living organism [73]. Although enzymes were developed under physiological conditions and thus in a similar, environmentally friendly environment, the requirements for the optimal reaction conditions for each step in a synthetic assembly may nevertheless differ.

The overlapping parameters, which are suitable for all reaction steps, define a window of opportunity of a cascade (Figure 5). This window changes by the number of reactions and parameters. Sometimes, it can be challenging to determine the window of opportunity of the cascade, especially if substrates and intermediates are not available, or if the reaction steps interact with each other in simultaneous mode. Typical parameters that can be interesting for optimizations are the solvent system [44,74,75], the concentration of reaction components (substrate [38], salts [24], co-solvents [76,77], cofactors [49], other additives), the pH of buffered reactions [49], reaction temperature [78], or also the necessity of cofactor regeneration. Choosing the reaction conditions for the whole cascade is always a compromise between the optimal reaction conditions for each enzyme of the cascade, especially if reactions take place in a simultaneous mode [79]. Fortunately, many enzymes require mild reaction conditions with aqueous solvents, temperatures between 20 and 37 °C and a pH around 7, which already reduces the degree of freedom. There are, however, enzymes

that tolerate more extreme environments regarding the pH, temperature, pressure, solvent, and salinity [80]. These higher stabilities can be of great benefit for synthetic purposes (e.g., when higher activity can be achieved due to higher process temperatures), but only when this is beneficial for the overall cascade. Working outside a tolerated milieu can decrease the activity and stability of each enzyme and, therefore, the overall performance of the multi-enzymatic reaction.

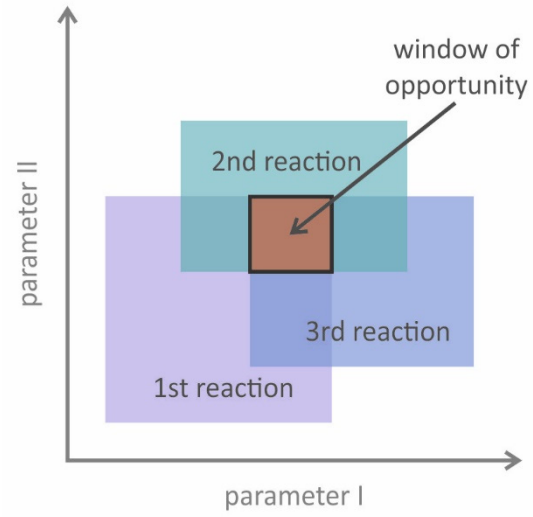

Figure 5. Each reaction of an enzymatic cascade is optimally catalyzed under certain conditions and the overlapping ranges define the window of opportunity of the whole system. This window of opportunity predefines the parameter ranges of the cascade and it can become narrower with more reactions and parameters to be changed. In this figure, only two parameter dimensions are displayed, although many more can define the tolerated window.

It is important to interconnect the optimization parameters with the optimization goals, especially for the reaction conditions: the use of, for example, unconventional media frequently decreases activity but enhances substrate/product solubility and downstream processing [74]. In general, it is important to keep in mind to weigh up stability optima against activity optima in order to achieve the best performance with regard to the optimization goals. In the end, a stable, robust overall process with high performance is most likely to be transferred to industrial application.

Solvent System

For choosing the solvent system, a recent tutorial review by van Schie et al. provides a guideline under which circumstances unconventional media are superior to buffered systems and when it is better to stick to buffered or biphasic systems [74]. One example showing that the switch from a buffered system to unconventional media can substantially increase substrate and product concentration is a cascade to aromatic vicinal diols originally published by Kihumbu et al. [81]. By transferring the reaction to a micro-aqueous reaction system (MARS) with methyl-*tert*-buthyl-ether (MTBE) as solvent, the substrate concentration was increased ten-fold compared to the solubility limit in water [44,82], resulting in an over 1600-fold increase in product concentration [44].

Optimization of the pH can be started with a rational investigation of the stability and activity optima of each enzyme, two parameters that are dependent on the pH. Either the limiting enzyme specifies the pH range or an experimental observation within the resulting window of opportunity provides a more detailed insight to the pH optimum of the cascade. The buffer system predetermines the range of pH and changing the buffer type can expand it but can also alter the cascades performance. The reaction conditions for the production of (*R*)-acetoin and ethylene glycol of D-xylose with seven enzymes was optimized regarding the buffer system, reaction temperature and pH [38]. In particular, a sensitivity of some

enzymes towards the pH and the buffer salts Tris and citrate phosphate could be observed. The maximal yields were detected for pH 8.5 and 7.5, respectively, with higher yields for the citrate phosphate buffer. Therefore, Jia et al. chose this buffer composition for further experiments [38]. After the optimization of substrate and cofactor concentrations, conversion of D-xylose was >99% and products were obtained with concentrations and productivities of 3.2 mM and 1 $mM \cdot h^{-1}$ (*R*)-acetoin (64% theoretical yield) and 5.5 mM and 1.7 $mM \cdot h^{-1}$ ethylene glycol (79% theoretical yield), respectively [38]. Additionally, for some types of reactions, a suitable pH value is of particular importance. For example, in oxidoreductions catalyzed by alcohol dehydrogenase, the pH selection can shift the reaction equilibrium to the keto- or alcohol side [83].

Reaction Temperature

The optimal temperature can be determined the same way, for example, as demonstrated for the conversion of UMP to UDP-GlcNAc with a total of five enzymes [78]. At higher temperatures up to 40 °C, an increased reaction rate and increased conversion were observed, which was confirmed by an Arrhenius model. However, there was a threshold at 50 °C, at which enzymes were inactivated within the investigated time [78]. In contrast, it was observed for a carbohydrate precursor synthesis using a four-enzyme cascade that a reduction of reaction temperature from 30 °C to 20 °C led to an increase in yield from 87% to above 95% [37]. The production of sesquiterpenes by Dirkmann et al. is another example for the importance of the reaction temperature since they found 30 °C to be the optimal reaction temperature [47]. Temperatures below reduced the conversion and above led to protein precipitation. The optimal temperature for an enzymatic cascade is a compromise between enzyme activity and stability. Usually, higher temperatures increase reaction rates but simultaneously decrease the operational stability [84]. Enzymes in a multi-enzymatic system must maintain their activity until the process goals are achieved.

Substrate and Cofactor Concentrations

Cofactors such as ATP, NAD(P)H or ions are important for the activity and stability of enzymes. The transfer of electrons, protons or chemical groups are enabled with these chemical components. A cascade's outcome is influenced by ion and metal type, ionic strength as well as concentration through interactions with the enzyme's amino acids. Metal ions as cofactors can stabilize the structure and they are often essential for the enzymes catalytic activity. Ions with equivalent strength are sometimes interchangeable, but might alter the activity of the enzyme. For example, Chen et al. titrated various ion types and concentrations to a six-enzyme cascade [24]. Starting from mevalonate, the cascade produces amorpha-4,11-dien, which is a precursor for the antimalarial drug artemisinin. Different amorpha-4,11-dien yields were observed for various cations and their concentrations not only for a single enzyme, but for the product yield of the whole cascade. Up to three-fold improvement was achieved by the appropriate selection of salt and subsequent adjustment of its concentration [24]. In addition to the individual influence of reaction conditions, interactions between them are also observed. The optimal cofactor concentration directly depends on the reaction temperature as was shown for Mg^{2+}. The optimal concentration in a five-enzyme cascade for UDP-GlcNAc synthesis varied dependent on the chosen temperature between 20 and 30 °C. This might be due to an altered metal complex formation or structural stabilization against thermal inactivation [78].

The substrate and cosubstrate concentrations play an important role in enhancing the performance of a multi-enzymatic reaction. Changes in the concentrations can shift the equilibrium of a reaction towards the product side, but can also increase the likelihood of inhibition. Titrating one substance at a time and investigating the cascades performance under these conditions is the most common approach. Lit et al. investigated the effect of the starting materials glycerol, pyrophosphate and D-glyceraldehyde sequentially on the product titer or the relative activity of their multi-enzymatic system [85]. With the substrates and four enzymes, the synthesis of rare ketoses such as D-sorbose and D-allulose

is possible. An increase in product titer or relative activity for each of these starting materials was observed with increasing concentrations, but the effect slowed down at certain concentrations. With the optimized reaction conditions and enzyme ratios, a conversion of 90% after 24 h and full conversion after 36 h was reached in a 10 mL scale [85]. This titrating approach was also chosen for optimizing the production of (*R*)-acetoin from D-xylose [38]. It turned out that the cofactor concentration had a negative influence on the product yield above a certain threshold of 0.01 mM. The substrate D-xylose had an optimal concentration of 10 mM. 5 mM below or 10 mM above that concentration led to a decrease in relative (*R*)-acetoin yield of about 25–45% [38]. A similar correlation was observed for the reaction rates, which decreased for increasing substrate concentrations, of a five-enzyme cascade for the production of isoprene from mevalonate [34]. However, in this case, a saturation curve was monitored for the cofactor concentrations. The consequential ideal ratio for substrate and cofactor was higher than the theoretically needed ratio [86]. This indicates the importance of validating the theoretical stoichiometry of the reactions in wet lab experiments.

To circumvent the usage of high cofactor concentrations in order to fulfill the stoichiometric need, one strategy is to regenerate the cofactors. Mordhorst and Andexer give a broad overview of the current enzymatic cofactor regeneration possibilities [87]. Regenerating systems can even be small cascades on their own [88,89]. It can make a multi-enzymatic reaction more complex and the optimization more challenging. Nevertheless, such regenerating systems give the advantage of using only small amounts of cofactors and cosubstrates. The costs, but also the risks, of inhibitory effects can be reduced, although a sacrificial substrate is often required for regeneration and further enzymes need to be expressed and purified. In conclusion, whether the addition of cosubstrates and/or cofactor regenerating enzymes is advantageous for the overall cascade depends on the goals of the cascade and has to be carefully considered.

3.1.4. Process Design

Beside the influence of enzymes and reaction conditions, process design strongly contributes to the performance of an *in vitro* biocatalytic system. Spatial separation, for example, can contribute to the avoidance of inhibitory effects and cross-reactions, but simultaneously may be a hindrance, shifting the equilibrium on the product side [56,90–92]. In addition, enzymes can be immobilized for an easier compartmentalization or reusability and often to increase the thermal and operational stability. Various techniques and systems are discussed elsewhere [93–95]. We herein address only examples of reaction modes, scale-up, and downstream processing with respect to performance optimization of enzyme cascades.

Reaction modes such as batch, fed-batch, and continuous processes also influence a cascade's potential. Feeding strategies are particularly important for substrate-inhibited reactions to keep concentrations low to prevent reductions in rate, conversion, yield, and titer due to excessive substrate concentrations. The same applies for product inhibition, which can be avoided by *in situ* product removal (ISPR). At the same time, product removal can shift the equilibrium towards the desired compound and often reduces the effort for further downstream processing [96]. Scherkus et al., for example, fed cyclohexanol to a three-enzyme cascade for the production of 6-hydroxyhexanoic acid (6-HHA), which is later polymerized to poly-ε-caprolactone [97]. *In situ* removal for the intermediate ε-caprolactone (ECL) by enzymatic hydrolysis was included since both the substrate and ECL would otherwise inhibit the cyclohexanone monooxygenase, the enzyme that produces ECL. The concentrations of both compounds were kept low and a 6-HHA concentration of 283 mM could be obtained in 20 h [97]. Another example for ISPR was demonstrated for the production of metaraminol by a two-step enzymatic reaction [98]. By *in situ* liquid–liquid extraction, the equilibrium was shifted towards the product side. Optimization of the extraction solvent and determining an operational window for the reaction conditions, the yield could be increased to 29% from 14% conversion [98].

For the scale-up of multi-step biocatalytic processes some considerations have to be made, because the scale-up can alter the performance of a cascade in an unpredictable way [63]. Molecular and gas diffusion, heat transfer, pressure, mixing rates, and other parameters are not linearly scalable and must be handled differently in a larger reaction volume [99]. This difference can be seen in the construction of a three-enzyme cascade starting from cinnamyl aldehyde to produce ester cinnamyl cinnamate. The production in a 25 mL lab scale yielded in 54% of the aromatic ester. In a scale-up to an 800 mL miniplant, the last esterification step showed an improved yield. However, the overall yield reached only 37% and the substrate conversion rate was slower. This reduced performance was attributed to the immobilization of the enzymes in this setup [100]. Nevertheless, scaling of a multi-enzymatic system to an industrially relevant scale is possible as demonstrated for the production of myo-inositol [67]. The four enzymatic steps were operated in a 20,000 L reactor at 70 °C. The final product concentration reached 95 $g \cdot L^{-1}$ after 48 h.

Purification of the final product plays an important role when developing a process. The degree of purity as well as the product recovery are key parameters for this step. The removal of the biocatalysts is often the first step for the downstream process, and can either be performed by separating immobilized enzymes, or removing enzymes in solution by filtration or centrifugation [101]. Subsequent product removal is often performed by chromatographic methods because of its robustness, scalability, and costs [102]. The product vidarabine 5′-monophosphate (araA-MP) of a three-step *in vitro* enzyme cascade was purified by a semipreparative chromatographic method [79]. It was obtained with 82.9 mM (95.5% conversion) in a 10 mL scale-up reaction. Immobilized enzymes were removed by filtration prior to the loading on a preparative HPLC. The purification of antiviral drug araA-MP resulted in a 55% yield and 90% purity. The main impurity was the stereoisomer adenosine monophosphate (AMP), a by-product of the biotransformation and difficult to remove by any purification technique. In contrast, Mahour et al. developed two cascades for the production of guanosine diphosphate L-fucose (GDP-Fuc) and purified it by ion exchange chromatography [103]. The method was optimized in terms of the pH of the equilibration buffer and the chromatographic gradient. The optimized chromatography helped to increase GDP-Fuc purity from 25 to 90.5%, which is in line with commercial standards of 91.8%.

In principle, considering process design already when setting up a cascade can have an immensely positive effect on the efficiency of the overall process. It can also change the optimal reaction parameters and the cascade design, if product purification is taken into account at an early stage [74]. Oeggl et al. showed in a cascade to chiral vicinal diols, that an integrated approach directly considering product crystallisation possibilities had a positive effect on economic and ecological process parameters in a techno-economic evaluation [45].

The process design next to the experimental approaches on *in vitro* enzyme cascade optimization with the aspects of cascade design, enzyme and reaction condition optimization can already result in highly productive cascades regarding conversion, yield, titer, or rate. However, optimization in only the wet lab can be very time, cost and resource consuming. An alternative or complementary approach is data and algorithm driven optimization, which relies on computational methods. Models of the systems can be used for simulations and other predictions of various scenarios. For example, enzyme, substrate and cofactor concentrations can be varied in a short amount of time. An algorithm can also be used for predictions of further experiments to be conducted such as the genetic algorithm.

3.2. In Silico-Based Optimization of Enzyme Cascades

Modelling of biochemical processes can provide quantitative data about the performance of biological systems, can provide benefit or feasibility analysis and allows the identification of bottlenecks and evidence-based decision-making. In order to describe reaction kinetics, ordinary differential equations are usually used. However, modelling of enzymatic reaction cascades requires the simulation of a number of reactions leading

to highly complex models with a large number of kinetic parameters. Limited examples exist for the empirical or mechanistic modelling of *in vitro* cascades, which is probably attributed to the complexity of modelling with a, simultaneously, often restricted amount of experimental data. Nevertheless, the more enzymatic steps are involved, the more complex are their synergistic effects that makes simulation essential for optimization. Generating and providing excellent data helps us to make better predictions for future experiments. Most model-based optimizations are combined with experimental approaches to achieve the best possible cascade performance quickly and cost-effectively. The objectives for optimizations are, for example, to achieve high productivity, to increase the overall yield or to minimize enzyme concentrations (Table 2). It is noticeable that the approach to optimizing the amount of enzyme appears to work quite well, but there are fewer examples of estimating the optimal reaction parameters.

Table 2. Model based approaches for enzyme cascade optimizations.

Number of Involved Reaction Steps	Substrate	Product	Optimization Method	Variable Parameters	Optimization Result	Reference
27	Glucose	Terpene	Kinetic model	Enzyme concentrations	95% yield, 15 $g \cdot L^{-1}$ titer	[33]
2	L-Alanine, 4-hydroxy-2-oxobutanoate	L-Homoserine	Kinetic model	Enzyme concentrations	3.2 $g \cdot L^{-1} \cdot h^{-1}$ (18% improvement), 80.1 $g \cdot L^{-1}$ (100% improvement)	[104]
5	Xylose	α–Ketoglutarate	Kinetic model	Enzyme ratio	98% yield (two-fold improvement)	[25]
10	Glucose	Dihydroxyacetone phosphate	Kinetic model	Enzyme ratio	88% increase in product concentration, decrease in cofactor concentration to one-fourth	[20]
3	Sucrose	Cellobiose	Kinetic model	Enzyme ratio, enzyme concentrations	62% yield in 10 h (ten-fold reduction in reaction time), 2.4-fold reduction of enzyme concentrations	[105]
2	D-Fructose 1,6-bisphosphate	D-Glyceraldehyde 3-phosphate, l-glycerol 3-phosphate	Kinetic model	Reactor type	100% conversion, 10.56 $g \cdot L^{-1} \cdot day^{-1}$	[46]
5	1,2,3-Trichloropropane	Glycerol	Dynamic simulations based on Michaelis-Menten kinetics	Enzyme ratio, enzyme variants	56% decreased enzyme loading	[106]
2	β-Hydroxypyruvate, glycolaldehyde	Erythrulose-aminotriol	Combined kinetic model and empirical model for process characterization	Enzyme ratio	100% yield	[22]
7	Mevalonic acid	Amorpha-4,11-diene	Statistical experimental design using Taguchi orthogonal array design	Enzyme ratio	100% yield (five-fold improvement)	[24]
13	Cellobiose	Hydrogen, carbondioxide	Multi-objective genetic algorithm	Enzyme ratio, temperature and cross-over inhibition of phosphate	87% yield, 355 $mmol \cdot L^{-1} \cdot h^{-1}$ (eight-fold improvement)	[23]
10	Glucose-6-phosphate	Hydrogen	Artificial neural networks, non-linear kinetic model fitted with a genetic algorithm	Enzyme loading, temperature	54 mmol H_2 $L^{-1} \cdot h^{-1}$ (67-fold improvement)	[107]
7	Glucose	Dihydroxyacetone phosphate	Artificial neural network	Enzyme ratio	63% flux improvement	[108]
7	Methyl-4-toluate	4-Tolyl alcohol	Genetic algorithm	Enzyme concentrations	90% yield (two-fold improvement)	[109]

3.2.1. Mechanistic Based Modelling of Enzyme Cascades

As the number of enzymes and compounds in cascade reactions increases compared to one-step biotransformations, the interdependencies between different variables (e.g., enzymes, intermediates) also increases. Stoichiometric optimization enables to maximize the efficiency of multi-enzyme processes while minimize the biocatalyst loadings [106]. A five-step reaction cascade from 1,2,3-trichloropropane to glycerol was optimized with regard to productivity and enzyme loading. Using dynamic simulations based on Michaelis-Menten kinetics expressed as differential equations, different enzyme variants and enzyme ratios were calculated. Finally, the enzyme loading was decreased by 56% while maintaining 95% productivity. Such models can have very good predictive power, as shown for a two-step cascade for the synthesis of glycerol-3-phosphate from D-fructose 1,6-bisphosphate [46]. The conversion was maximized to 100% with the aid of kinetic models. The model predictions correlated with the experimental values by nearly 99%.

In theory, those model-based optimizations are transferable to other cascades as well, however, often experimental and kinetic data are missing. In such a case, data from enzyme homologues might be useful, if available. Korman et al. successfully established a kinetic model for the *in vitro* terpene synthesis from glucose using kinetic parameters known from homologous enzymes, when the actual values were not obtainable [33]. This rough model was sufficient in order to explore the overall system behavior and to identify bottlenecks as well as the most sensitive parameters of the reaction cascade consisting of 27 enzymes. Another kinetic model was developed for an L-homoserine synthesis cascade in order to improve the volumetric productivity and final titer with regard to an industrial application [104]. The development of a kinetic model based on insights of homologous enzymes led to a more systematic reaction optimization and reduced the amount of experimental effort. Although the model parameters had to be adjusted several times to describe the experimental evaluation properly, the model fitted the experimental data well. The correction of kinetic parameters was required because the protein–protein interactions in the one-pot cascade reaction have an influence on the maximum reaction rates, the Michaelis and inhibition constants. The same observation has also occurred for several other model cascade systems such as the Weimberg pathway as *in vitro* cascade consisting of five enzyme-catalyzed reaction steps from xylose to α–ketoglutarate [25]. The enzyme kinetics were described for isolated enzymes and subsequently used for modelling a one-pot reaction cascade. Synergistic effects of several metabolites required an iterative adaptation of the model parameters.

To counter this issue and to enable a better description of such effects, Panke and coworker developed an experimental set-up for quantitative metabolic real-time analysis of *in vitro* multi-enzyme network dynamics [110]. This strategy enables to generate standard input functions applied to an enzyme reactor with simultaneous detailed data collection from the system's response via real-time mass spectrometry. Using this set-up, a mathematical model based on enzyme kinetics was developed and used to optimize a ten-enzyme cascade with regard to optimal enzyme and cofactor utilization [20]. This sophisticated approach could certainly be extended to the optimization of further reaction parameters in addition to those already investigated.

Nevertheless, a general issue of kinetic modeling is the extensive experimental characterization of enzymes to gain access to kinetic data. Additionally, it has been shown that the obtained data from individual reactions with pure enzymes are not equal to reactions in more complex reactions mixtures containing various enzymes, intermediates and cofactors [111]. Furthermore, cascade reactions are often carried out at different pH values, temperatures, buffer compositions or with co-solvents compared to the reaction conditions used during kinetic parameter investigation, so that the determined kinetic values are not transferable. In the course of the cascade, the reaction conditions, the concentrations of the reaction components (substrates, products, intermediates, by-products) and the stability of the enzymes change, which further increases the complexity. Combined approaches consisting of kinetic models for predicting the reaction progress with an integrated empir-

ical model for process characterization (e.g., reactant stability, reactant toxicity towards enzymes) can give guidance for process and biocatalyst development [22]. Therefore, mathematical models that are independent of kinetic data are also promising approaches for describing and optimizing enzyme cascades.

3.2.2. Empirical Models for Enzyme Cascade Optimization

The current state-of-the-art approach for empirically model enzyme cascades is to use genetic algorithms for the prediction of reaction optima, especially with regard to productivity and yield. Genetic algorithms are inspired by the principles of natural selection and reveal evolutionary relationships by an iterative solution approach [112]. Several studies report *in silico* simulations as a helpful approach to evaluate various scenarios as well as reaction and process conditions to increase the process performance. For example, the synthesis of hydrogen with a 13 enzyme synthetic *in vitro* metabolic pathway starting from cellobiose was optimized with regard to productivity by mathematical simulation of the one-pot batch process and modular processes [23]. It was shown that the adoption of enzyme concentrations and hence enzyme ratios can significantly increase productivity. The cascade was mainly controlled by the hydrogenase that is responsible for hydrogen formation. By increasing the concentrations of the hydrogenase, a 30-fold increase in yield was obtained corresponding to 87% of the maximum theoretical yield. The simulation was extended to study the influence of temperature and cross-over inhibition of phosphate to finally identify the maximal productivity with a multi-objective optimization using the multi-objective genetic algorithm in Matlab. The kinetic parameters and constants as well as enzyme degradation constants at different temperatures were obtained from literature. An *in silico* productivity of up to 355 $mmol \cdot L^{-1} \cdot h^{-1}$ was achieved, which is significantly closer to an industrially feasible process compared to the initial experimental values with a productivity of 0.6 $mmol \cdot L^{-1} \cdot h^{-1}$. This cascade for hydrogen production was further optimized by establishing artificial neural networks using empirical data available in the literature [113]. It turned out that several enzymes in the cascade were responsible for the optimal hydrogen production instead of only one enzyme as stated in previous studies. Optimization by a non-linear model fitted with a genetic algorithm using experimental data resulted in a productivity of 54 $mmol \cdot L^{-1} \cdot h^{-1}$ hydrogen starting from hexose as substrate after adjusting enzyme loading and temperature of the overall cascade [107].

The usage of artificial neural networks is a rather complex approach, but it is especially applicable for enzymatic reaction cascades where not all kinetic parameters are known [112]. Using a machine learning approach, an *in vitro* cascade of the upper part of the glycolysis consisting of four enzymes has been optimized with a flux improvement of up to 63% [108]. This achievement is based on the application of a novel artificial neural network approach that enables to extrapolate predictions. Typically, artificial neural networks are not useful for extrapolation and, therefore, miss a huge range of values. The new methodology enabled to expand the space for prediction and, in this case, the extrapolation of high fluxes. Interestingly, the flux improvement simultaneously reduced the assay cost of up to 25% by enzyme balancing.

Those simulation-based predictions are very useful for preliminary simulations to identify bottlenecks or as starting points for experiments as shown by a seven-step enzyme biotransformation for the synthesis of 4-tolyl alcohol from methyl-4-toluate [109]. The synthesis of alcohol from an ester was realized by hydrolysis and subsequent reduction using a multi-step reaction with an esterase, a carboxylic acid reductase with cofactor regenerating enzymes and an alcohol dehydrogenase. The overall yield of the cascade was increased of up to 90% by suggestions of a genetic algorithm that proposed random solutions for the concentration of each enzyme. The highest scoring solutions, defined by the highest yield with the lowest total enzyme concentration, was used to generate the next population of solutions. This procedure was repeated for 25 cycles. Although the predicted yields were not achieved in control experiments, the optimized cascade performed significantly better compared to the initial experiments. Other statistical approaches, such as the use of

Taguchi orthogonal array design, which is a type of a fractional factorial design, resulted in the optimization of a previously inefficient seven-step-cascade of mevalonic acid to amorpha-4,11-diene to yields as high as nearly 100% [24].

In general, optimization towards an industrially feasible production with a multi-enzyme cascade reaction requires extensive development effort. The application of model-based tools accelerates the progress even at an early stage of development. Nevertheless, validation of *in silico* data is, from the author's point of view, crucial, since no model represents the reality in its entirety and not all effects can be predicted, especially multi-variable reaction parameters and changes over time. In addition, *in silico* data is highly depended on experimental results of previous lab work. Only with reliable and robust data can precise predictions be made. Still, modelling can be of great help to detect bottlenecks and give a sense of limitations of an enzyme cascade, especially with respect to rate-limiting enzyme concentrations. By combining experimental-based optimizations with modelling, time, money, resources and effort can be saved compared to applying experimental-only approaches. Additional data and new (mechanistic) insights give valuable information for enhanced modelling for future cascades. Furthermore, especially for enzyme cascades consisting of multiple reaction steps, experimental-only approaches are not feasible with reasonable effort.

At the same time, careful selection and development of predictions must also be made. The more complex the cascade becomes, the more interactions take place between the reaction partners, which are increasingly difficult to describe by mechanistic models. Thus, it may be that an empirical approach might make more sense above a certain number of enzymes involved. To validate this hypothesis, the data basis is currently lacking; however, it might become available in the future due to the increasing interest in enzyme cascades.

4. Remaining Challenges and Future Perspectives

Over the past three decades, biocatalysis has evolved from one-step biotransformations to more complex and sophisticated cascade-like reactions. The establishment of enzyme cascades into the fundamental and application-oriented research is highly relevant, as the potential application fields are extremely broad, ranging from the synthesis of fine chemicals and active pharmaceutical ingredients to bulk chemicals. Due to their high selectivity, enzymes have clear advantages in the asymmetric synthesis of chiral (pharmaceutical) compounds. However, also in the bulk segment, although more economically challenging, enzymes can have an advantage, especially when it comes to using renewable substrates for more sustainable manufacturing in a bio-based economy. Advances in research on enzymatic cascade reactions have now led to systems that can be specifically designed, modeled and optimized for high economic and ecologic efficiency and selectivity. The use of enzymes in reaction cascades for organic synthesis has, therefore, become an emerging field for the development of new synthesis routes for already existing or completely novel products.

However, one has to keep in mind that as the number of involved enzymes and compounds increases, the number of molecular interactions and interdependencies between the components also increases. In order to manipulate such complex systems, optimize them and ultimately make them usable on a larger scale, they should be as fully understood as possible. Furthermore, this is where the challenge begins, as interactions are numerous. Phenomena that certainly cannot be mapped with kinetic information of individual enzymes are protein-protein interactions and macromolecular crowding effects as well as inhibiting or activating effects of small molecules in other parts of the cascade. The influence of all reaction parameters, which can even change in the course of the multi-step reaction, is also manifold and so far difficult to monitor or predict. Consequently, this leads to simulations in which there is little agreement between the experimental data and the originally estimated kinetic parameters.

To counteract this, more and more engineering approaches are being applied to complex multistep reaction systems, such as the application of genetic algorithms or

machine learning approaches. In the opinion of the authors, to improve the understanding, the enzyme cascades have to be described through combined experimental and model-based efforts. Furthermore, most of the considered systems are currently studied at the mL-scale and low substrate concentrations, which has little relevance for industrial application. Therefore, more effort is required for optimizing reaction conditions relevant to industrial scale in the future. In this context, scaling of multi-enzymatic reactions into demonstrator plants are highly welcome as proof-of-concepts and as a source of information and data for enhanced models/predictions.

Development and optimization of enzyme cascade reactions have already benefited and will continue to benefit greatly from advances in digitalization, miniaturization and automation for process intensification. Novel strategies for optimizing ecological and economic factors, for data acquisition, and for processing large data sets will certainly accelerate the development time of complex chemical reactions, making the use of enzyme cascades competitive in the chemical industry. Finally, the techno-economic assessment of multi-stage cascades—ideally even using life cycle analyses—enables the identification of economically and ecologically potent cascades and shows, whose challenges need to be addressed in order to make use of the full potential of enzyme cascades in future sustainable production.

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