Selin Kara
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Enzyme Cascade Design and Modelling

Springer

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Preface

In the last few decades, the use of natural catalysts—enzymes—in a cascading fashion has become of great interest to synthesize organic compounds. The mimic of nature, to prepare complex structures in a one-pot, is one of the main driving forces why the design of artificial enzyme cascade reactions is so popular. The recent developments in the field prompted us to highlight the advantages, disadvantages, and challenges of designed enzyme cascade reactions.

Although the proof-of-concept for multi-catalytic cascade reactions at laboratory scale looks promising, the number of examples that are applied at technical scales are still limited. From this perspective, we intend to join our forces in this textbook, which shall provide a fundamental background and comprehensive overview of recent developments achieved in the field of catalytic cascades (i.e., multi-enzymatic or chemo-enzymatic) from design (i.e., in vitro and in vivo applications) to kinetic and process modelling, reaction engineering as well as process control.

This textbook is written by experts from different backgrounds who develop and apply enzymatic cascade reactions in their research groups. They address various methodologies from design (i.e., retrosynthesis), kinetic modelling, and process modelling to analytical means for monitoring of enzymatic cascade reactions.

We hope that this book on Enzyme Cascade Design and Modelling will serve as a reference guide for academic and industrial researchers and provide a unique perspective on the design and modelling of enzymatic cascade reactions, both multi-enzymatic and chemo-enzymatic ones. With this, we anticipate to see more and more enzymatic cascades that arose from academic curiosity and were proven at small scales in the laboratories, to be implemented at industrial scales. This book shall be of assistance in the academic as well as in the industrial field for those who want to get an insight into the challenges of developing enzymatic cascade reactions. It is obvious that these challenges become more severe when more enzymes are involved in a synthetic route since more reaction parameters need to be optimized, more kinetic parameters need to be estimated, more degrees of freedom are available, and the complexity is increased. We hope to experience a trigger effect that makes it worthwhile for the readership, the authors, and the editors to have a second edition succeeding the first.

We sincerely hope that here presented knowledge and recent examples will inspire students and early-stage researchers to develop new enzymatic cascade applications providing our society with more sustainable solutions for the synthesis of compounds ranging from bulk chemicals to pharmaceutical intermediates.

We gratefully thank the authors who contributed to our joint "book project," who responded to our feedbacks promptly, who made this textbook ever possible. Finally, yet importantly, we thank our families for their support and tolerance during the time that we invested in the preparation of here presented textbook, which was partly completed during the COVID-19 pandemic that will certainly not be easily forgotten.

Aarhus, Denmark — Selin Kara
Vienna, Austria — Florian Rudroff
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Introduction

1

Selin Kara and Florian Rudroff

Abstract

Biocatalysis has become a key emerging field for the development of new synthesis routes for already existing or new-to-nature products to meet the dynamic needs and agendas of our globalizing society. Both fundamental and application-oriented insights obtained in different disciplines from the natural and engineering sciences have opened up new avenues for the use of enzymes in reaction cascades for organic synthesis. The great potential of enzymes becomes significant when they are coupled with other enzymes or with chemocatalysts to synthesize value-added products at industrially relevant product titers. Joining the forces from different disciplines for the use of enzymatic cascade reactions will pave the way for the use of nature's catalysts to produce products of our need.

The application of enzymes for the synthesis of chemicals is a key emerging field to meet the current and future needs of our society [1–3]. Nowadays, the challenges of using enzymes related to the establishment of single-step biotransformations can be overcome by means of molecular biotechnology techniques for designing biocatalysts with custom characteristics such as (1) high activity towards a non-natural substrate, (2) high stability at elevated substrate/product concentrations, and (3) high selectivity.

The design of multi-step cascade reactions has received great attention in the biocatalysis community. One of the main advantages of synthetic cascade reactions is the possibility to produce complex molecules that cannot be easily obtained from a single biotransformation. Additionally, cascade reactions offer solutions for a range of challenges faced in biocatalysis; the most important ones are:

1. reduced inhibition issues caused by intermediates (since they are formed in situ at low amounts and are immediately consumed),
2. no intermediate purification steps leading to low waste generation,
3. low consumption of resources (e.g., space, time, energy, and materials),
4. displacement of unfavorable thermodynamic equilibria, and
5. possibility to synthesize complex molecules from simple and readily available compounds.

Indeed, nature already uses an elegant and efficient synthetic strategy: Coupling enzymes in multi-step pathways without intermediate isolation and purification steps and with precise spatial

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control of catalysis. Hence, by mimicking nature the ultimate goal in multi-step biocatalysis is to design cascade reactions (multi-enzymatic and chemo-enzymatic) that run perfectly in balance with excellent yields and selectivities towards the final target product.

To transfer nature's synthetic strategy into the laboratory and industry has become a major focus of the biocatalysis community in recent years [4–12]. In fact, the potential application fields of multi-enzymatic reaction cascades are extremely diverse; they range from the synthesis of fine chemicals and active pharmaceutical intermediates (APIs) [13]—which is an established field of biocatalysis—to the conversion of renewable raw materials into platform chemicals.

Nature's wealth in biologically active and complex molecules has inspired synthetic chemists for centuries. They strived to imitate natural chemical processes and synthesize biomaterials through biomimetic synthesis, asymmetric catalysis, and natural product synthesis. The way in which chemists have designed chemical routes towards complex target molecules has been changed in the mid of the last century by the introduction of the concept of retrosynthetic analysis [14–16]. This theoretical approach is based on the systematic disconnection of strategic chemical bonds that link major components of a specific target molecule until simple or readily available starting materials are obtained. Crucial for each retrosynthetic step is the existence of a feasible chemical transformation in the synthetic forward direction. Consequently, this approach heavily depends on the knowledge of all organic reactions. Synthetic chemists are well grounded in retrosynthesis, as it is part of their education to design novel synthetic strategies for the preparation of complex molecules. This process has been aided by the formalization of how to generate "synthons" by homolytic or heterolytic cleavage of C–C or C–X bonds in the reverse direction. In addition, functional group interconversions (FGIs) have been introduced to prepare the molecule for the best retrosynthetic disconnection. This concept served as the basis for the development of a vast number of novel transformations in organic chemistry and constantly increases the toolbox of synthetic methodologies (e.g., metal- and organo-catalysis). On top of that, nature can serve as an additional pool for novel chemical transformations, especially with respect to exceptional chemo-, regio-, diastereo-, and enantioselectivity. The catalytic ability, natural diversity, and evolvability of enzymes extend the chemical space and complement the synthetic chemists' transformation portfolio [16–18]. Therefore, the continuously expanding biocatalytic toolbox has become an indispensable part of the chemical wealth.

Thanks to the pioneering work in molecular biology, microbiology, genetics, and biotechnology, tremendous progress in DNA technologies (e.g., directed evolution), high-throughput screening methods, and bioinformatics has been achieved, which led to the development of novel and tailor-made biocatalysts and enabled their production on industrial scale. During the past 15 years, more than 20 different types of enzymes (e.g., hydrolytic-, reverse hydrolytic-, oxidative-, reductive-, C–C and C–X bond-forming enzymes) have become commercially available and have been applied for the synthesis of chiral building blocks, pharmaceuticals, agrochemicals, polymers, and biofuels. These biocatalysts have been exploited in the targeted synthesis of complex molecules since they are exceptionally effective at catalyzing FGIs and often offer an appealing alternative to chemocatalysts. With this catalytic portfolio in hands, guidelines for the so-called biocatalytic retrosynthesis have been established (Chap. 2). Thereby biocatalysts as well as chemocatalysts and reagents are considered for key bond-forming steps in the targeted synthesis of complex molecules. Applying the principles of biocatalytic retrosynthesis, novel disconnections can be proposed that are not accessible by classical chemical catalysis, which facilitates the design of alternative synthetic strategies.

Nature's synthetic strategy is applied in a (bio)chemical laboratory by using either microorganisms (i.e., whole-cells) (in vivo) or isolated enzymes (in vitro). The main advantages of the in vivo approach are: (1) it is inexpensive as no enzyme isolation or purification is required,

(2) it offers high enzyme stability as enzymes are in their natural environment, and (3) there is no need for the addition of external cofactors as they are directly provided from the cell metabolism. However, there may be some major disadvantages, such as: (1) the molecular design of microorganisms (i.e., "designer bugs") [19–25] can be material and time-intensive, (2) the competitive reactions catalyzed by other intracellular enzymes in the host can lead to low selectivity and productivity, (3) high substrate and product concentrations—which are prerequisites at industrial scales—can be toxic to the cells, (4) adjusting the enzymes' expression levels is not straightforward, and (5) controlling and scaling up the bioprocess can be difficult.

Unlike the in vivo approach, in vitro reaction systems can be easily controlled and optimized, and high product yields and purities can be achieved due to the absence of aforementioned competing side-reactions. In principle, the advantages of the in vivo approach can be regarded as the disadvantages of the in vitro strategy. However, with the current technology and knowledge, it is possible to optimize in vitro systems by means of methods such as (1) automated protein purification, (2) reuse of expensive cofactors with a broad spectrum of regeneration techniques, (3) alteration of enzyme pH and temperature profiles, (4) enzyme stabilization (*via* immobilization, protein engineering, or use of additives or cosolvents), and (5) compartmentalization of incompatible enzymes and/or their reaction conditions, as nature does in cellular organelles or compartments.

Biocatalytic cascades are defined as two- or multi-step transformations carried out in the same reaction vessel (the so-called one-pot) using at least one enzyme; hence, they cover multi-enzymatic [5, 10, 26, 27], chemo-enzymatic [28–30], photo-enzymatic [31, 32], electro-enzymatic [33, 34], and enzyme-initiated spontaneous [35, 36] reactions. Among those, multi-enzymatic and chemo-enzymatic cascade reactions have been leading to higher productivities and hence will be of high interest for organic synthesis. However, it does not necessarily mean that the cascade reactions run concurrently; they can run in a sequential fashion as well. As long as a series of reactions takes place in a one-pot system, the term "cascade" can be used as a definition in the field of biocatalysis [37]. It is important to note that the term "reactor cascades" is commonly used in the field of engineering, whereby a reaction occurs in a series of linked reactors with each processing the output of the previous one. The biocatalytic cascade reactions reported so far have been categorized by their different designs, for which we hereby refer to excellent reviews summarizing them [4–12].

Whereas major attention has been put on the development of enzymatic cascade reactions in vitro as well as in vivo, the combination of two disciplines, chemocatalysis and biocatalysis, is surprisingly underrepresented in the literature, although both research fields cover a significantly different chemical space in terms of reactivity, selectivity, and productivity. In the last decade, intensive investigations for the introduction of bioorthogonal functionalities were made to gain deeper insights into cellular mechanisms. Different types of reactions, so far unknown in nature, such as metal assisted C–C couplings (e.g., Suzuki, Negishi, Sonogashira), cross-metathesis or copper-catalyzed [2+3] dipolar cycloaddition (Huisgen reaction), were explored. Conversely, biocatalysis offers the possibility of chemical transformations either unknown to or poorly understood by chemists—like the C–H activation of unactivated C–H bonds—or enables increased yields by improved regio-, stereo-, and chemoselectivity in already known reactions.

The combination of the two worlds of bio- and chemocatalysis would open a completely new way to synthesize complex molecules by taking advantage of their individual assets. Nevertheless, cascade type reactions involving both disciplines suffer from incompatibilities of their totally different windows of operation. Whereas many metal- or organocatalytic systems demand water-free conditions, the absence of oxygen or high substrate concentrations, enzymes mainly work in aqueous conditions at ambient temperature with a significant lower substrate load. Most critical is the inactivation of the catalytic system

by either the chemocatalyst or the enzyme, which has a severe effect on the overall reaction performance. Recent advances in protein engineering provided the community with tailor-made enzymes with improved stability, substrate scope, and selectivity. Also chemists improved the stability of chemocatalysts towards water and oxygen significantly. However, combination of both catalytic systems in a cascade fashion is still a challenging task (Chap. 5) [28–30, 37].

Spatial control plays a particularly important role for chemo-enzymatic cascades since biocatalytic and chemocatalytic reactions often run under significantly different reaction conditions, e.g., temperature, pH, and medium. It is also worth mentioning that spatial arrangements may also be required in enzymatic cascades when different enzymes need different optimal reaction conditions to work. In this context, the cell is perhaps the most prominent spatially separated organization, but a myriad of other systems like organelles or micro-compartments, anchoring or assembling mega-enzyme complexes, facilitate the control of complex multi-step reaction cascades. Spatial separation (the so-called modularization or compartmentalization) [38–42] of enzyme pathways allows suppressing side-reactions, alleviating the effect of reactive species and avoiding inhibition. Conversely, if the biocatalysts' optimal conditions are not significantly divergent, bringing the (bio)catalysts spatially together (the so-called co-localization) [9, 43] would provide efficient channeling of substrates between the catalytic steps.

Having highlighted the attractiveness of designing multi-step cascade reactions for organic synthesis, it has to be pointed out that complexity increases with the number of enzymes, chemocatalysts, and compounds in a reacting system since the number of dependencies between different variables multiplies. For such complex and interconnected multi-catalytic systems to work and to become fully applicable on a technical scale, it is important to view cascade reactions from a reaction engineering perspective. To understand and optimize them, kinetic modelling of all the numerous interdependencies will guide us in facing the challenges towards implementation of cascade reactions on a larger scale [44, 45]. By elucidating the kinetics bottlenecks and developing models to describe cascade reactions, it is possible to implement multi-step cascade reactions in their best suitable reactor and operation mode. For this purpose, enzyme kinetics modelling based proper bioreactor selection is necessary to operate a cascade reaction with high productivity and selectivity towards the target product. Challenges of cascade reactions related to cross-reactivity, cross inhibition, optimization as well as reaction control highly suggest that compartmentalization (or modularization), rather than one-pot synthesis, is a particularly attractive route for technical implementation [46, 47]. After the enzyme kinetics modelling guided reactor engineering, the next step is the cascade process design for the optimization of individual cascade modules and the complete multi-step multi-catalytic process.

For monitoring and controlling of multi-step enzymatic cascade reactions, process analytical technologies (PAT) are a powerful tool allowing immediate *online* or *inline* data collection, data processing, and fast response to (re)optimize the process. During the last decades, the developments in analytical techniques and (portable) devices has led to new levels of precision in detection of analytes, even at high dilutions. Multi-variate *inline* or *online* data are processed *via* chemometric models for the calculation of concentration data, which is sometimes provided with the analytical technique as an imbedded software tool. In order to get industrially relevant product titers in multi-step reaction cascades (multi-enzymatic or chemo-enzymatic) the use of non-aqueous media has attracted a great deal of interest in the biocatalysis community. Organic media and neoteric solvents are explored to enhance the productivity as well as sustainability of biocatalytic cascades as the perception of water as a green- and mild reaction medium has changed. This is simply because wastewater generated in enzymatic reactions is/should be considered as a bottleneck for the E-factor (=mass of waste generated per mass of product synthesized) [48, 49].

Alongside insights into the scientific revolution in the field of enzymatic cascades, this book provides a fundamental background and

comprehensive overview of recent developments achieved in the field of enzymatic cascades (e.g., multi-enzymatic or chemo-enzymatic). This ranges from design (i.e., in vitro and in vivo applications) to kinetic and process modelling, reaction engineering as well as process control. In the first part of the book (Chaps. 2–5), our main goal is to discuss the opportunities and challenges of building multi-step cascade reactions, whereby the latter are critically assessed in each chapter. The second part of this book (Chaps. 6–9) provides the problem-solving methods to alleviate these challenges on the way to implementation. By doing so, the ultimate goal of this book is to deliver a road map from challenge identification to overcoming them. As aforementioned, not only multi-enzymatic cascades but also chemo-enzymatic cascades are presented with the motivation of combining the strengths of these two worlds that cover selectivity, activity, and robustness and to assess the associated challenges. Chapter 10 is dedicated to the application of enzymatic cascade reactions in non-conventional media from an industrial perspective focusing on industrially relevant product titers and recent achievements in a technical environment.

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Enzyme Cascade Design: Retrosynthesis Approach

2

William Finnigan, Sabine L. Flitsch, Lorna J. Hepworth, and Nicholas J. Turner

Abstract

Retrosynthetic analysis for the design of synthetic routes towards target molecules is well-established in organic chemistry, and has been extended to include biocatalysis in recent years. The increasing number of transformations known to be catalysed by enzymes, whilst ultimately rendering biocatalytic retrosynthesis more powerful, necessitates the use of computational tools if biocatalysis is to reach its full potential. In the following chapter, we outline the pipeline required to go from pathway generation towards a target molecule, to construction of selected optimal pathways in the laboratory and the techniques currently used to analyse them. We compare manual vs. computer-assisted approaches for each step of the workflow. Current computational tools used for automated identification of suitable enzymes, such as molecular fingerprinting and structure-based substrate docking, and the evaluation of metrics that can be used to rank order the generated pathways, will also be discussed. Finally, we discuss a number of recent high-throughput analytical techniques for the experimental validation of potential pathways, leveraging the design-build-test-analyse cycle for pathway improvement.

Keywords

Biocatalysis · Retrosynthesis · Enzyme cascade · CASP

2.1 Introduction

The application of retrosynthesis during the planning and execution of preparative routes to target molecules is now an established and indispensable tool in organic synthesis. The concept was first introduced in the 1960s by E.J. Corey and emerged from a recognition that the careful and logical analysis of possible modes of construction of bonds could reveal a path from the target molecule back to potential starting materials in a step-wise fashion [1–3]. Having identified potential 'synthons' via the process, the next step was to correlate these synthons with real chemical building blocks that could be employed in the synthetic direction. In some cases it was necessary to carry out Functional Group Interconversions (FGIs) in order to reveal potential disconnections that might otherwise not be apparent. Retrosynthesis is a powerful way of teaching organic chemistry to undergraduate students [4]. After the initial shock of being asked to think backwards from target molecule to starting material, rather than vice versa,

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retrosynthetic analysis reinforces basic concepts such as mechanism and the nature of bond forming processes.

Implicit in the successful application of retrosynthetic analysis is a comprehensive understanding of exactly what reactions are available in the 'forward' direction once the required building blocks have been identified. In the early days of retrosynthesis, a highly skilled synthetic organic chemist may have been able to recall from memory, or from a quick read of the literature or a textbook, most of the important synthetic transformations that were required. However, the landscape of organic synthesis has changed dramatically during the past 50 years, with the invention of genuinely new reactions as well as reagents and, increasingly, catalysts for highly selective organic synthesis. Target molecules can also be structurally and stereochemically complex resulting in the generation of many possible routes for synthesis. As a consequence, in recent years there has been an attempt to apply computational methods to assist with both retrosynthetic analysis and also matching potential synthons with known building blocks and reactions/reagents/catalysts found in databases. Computational methods can of course handle very large data sets and can also incorporate machine learning and path finding algorithms in order to meet the challenge of 'computer-aided organic synthesis'.

2.1.1 Biocatalytic Retrosynthesis

In 2013 we proposed the concept of 'biocatalytic retrosynthesis' and suggested that the application of retrosynthetic analysis be extended to include biocatalysts [5]. By 2013 substantial developments had taken place globally in the discovery and development of new classes of biocatalysts for more general application in organic synthesis. In addition to the more established enzymes such as lipases, esterases, and dehydrogenases, many more (engineered) biocatalysts were becoming available including transaminases, oxidases, aldolases, oxygenases, P450 monooxygenases, and nitrilases [6]. This expansion of the biocatalytic toolbox has accelerated during the past few years with the arrival of new platforms such as imine reductases/reductive aminases, halogenases, P450 variants for C-H activation, peroxygenases, and carboxylic acid reductases amongst others [7]. Today there are *ca*. 100 different biocatalytic transformations that can be routinely carried out using commercially available biocatalysts [8] and hence the application of retrosynthetic tools in biocatalysis is very timely [9].

The power of biocatalytic retrosynthesis is that it can often result in the identification of completely new routes to target molecules when compared with the existing and more classical chemical routes. Some enzymes catalyse reactions that have no known (currently) non-enzymatic counterpart, e.g. ammonia lyases which catalyse the (enantioselective) addition of ammonia to the double bond of a cinnamic acid derivative to yield the corresponding L-aryl alanine product. P450 monooxygenases are able to insert oxygen into C–H bonds in ways which are unknown in organic synthesis. Recently these enzymes have been engineered to functionalise C–H bonds in a much broader sense enabling C–C and C–N bond forming processes, a development which will impact the way that biocatalysis can be employed in the future [10].

Interestingly, since biocatalysis is a relatively under-developed field, there are parallels with the early days of organic synthesis in the ways in which biocatalytic retrosynthesis is currently applied [11]. During the preparation of our recent book *Biocatalysis in Organic Synthesis—The Retrosynthesis Approach* we identified *ca.* 250 different 'retrosynthetic disconnections' which were possible using biocatalysis. This number is increasing and is probably closer to 300 at the time of writing (July 2020). With a good knowledge and understanding of organic chemistry and an awareness of what biocatalysts are available, it is currently possible to 'manually' apply biocatalytic retrosynthesis to analyse possible routes to target molecules. However, for reasons set out below, it is clear that biocatalytic retrosynthesis needs to embrace computational tools if it is to fully realise its potential and

widespread application. The application of biocatalysts in organic synthesis presents some special challenges where computational algorithms will become essential in the future. An obvious example is the increasing need to search large enzyme databases which are emerging from metagenomic and protein engineering programmes. Retrosynthetic analysis may identify the need for an imine reductase (IRED) in a key step. However, inspection of the database will reveal that there are >5000 different sequences known that potentially might catalyse the desired reaction! Another challenge, and indeed real opportunity, for biocatalytic synthesis/retrosynthesis is in the rapidly expanding area of enzymatic and chemo-enzymatic cascades where a multi-enzyme pathway needs to be designed, engineered, and optimised to convert the starting material to product.

2.1.2 Enzyme Cascade Design (Biosynthetic)

Nature exploits multi-enzyme cascade processes as a universal platform for creating the diversity of natural products that underpin life [12]. Compounds such as alkaloids, terpenes, and polyketides are all made from simple precursors by a sequence of enzyme-catalysed processes involving cofactors, coenzymes, and co-substrates. This process of natural product biosynthesis is remarkable from a synthetic chemistry perspective. Every step involves a (bio)-catalyst, no protecting groups are required, waste and by-products are minimised, and the final products often possess the molecular and stereochemical complexity necessary for biological activity (e.g. reserpine, penicillin, vitamin B12). The synthesis is also encoded (by genes) and can therefore be optimised through either engineering individual genes/biocatalysts or the whole pathway (or both). Reactions are carried out under mild conditions in aqueous environments, and are generally sustainable due to the fact that most reaction components are both biodegradable and renewable. Such an approach sounds like an excellent blueprint for organic synthesis!

The field of synthetic biology has sought to exploit this biosynthetic platform in order to improve the production levels of important and commercially valuable natural products (e.g. vanillin, resveratrol, D-methionine). Having established the pathway required for the biosynthesis of the target molecules in vivo, a range of different methods can then be applied to enhance production levels, e.g. gene duplication, gene deletion, altering circuitry, metabolic flux analysis, altering cofactor levels. Computational methods are widely applied in this field in order to search through the number of possible pathways and rank order the different options followed by experimental cycles of design-build-test-analyse (DBTA). Some spectacular successes have been achieved, notably the production of propane-1,3-diol (DuPont, United States).

2.1.3 Enzyme Cascade Design (Synthetic)

When moving to other sectors of the chemical industry, for example in the manufacture of intermediates for pharmaceuticals and agrochemicals, monomers for polymers, flavour components, fragrances, and fine chemicals, a major challenge emerges in that the target molecule is now ‘synthetic’, i.e. it is not ‘natural’, and hence no biosynthetic pathway will currently exist for its production. If a multi-enzyme cascade is to be used for its preparation, then a new pathway will have to be identified, constructed, and optimised. It may also be the case that the starting material required for its synthesis is ‘non-natural’ and hence the whole approach starts to resemble more closely that encountered in classical organic synthesis in which petrochemical-derived building blocks are converted via a series of reagents and catalysts to the target synthetic molecule.

Figure 2.1 sets out the workflow required to construct a multi-enzyme (chemo)biocatalytic cascade for the synthesis of any desired target

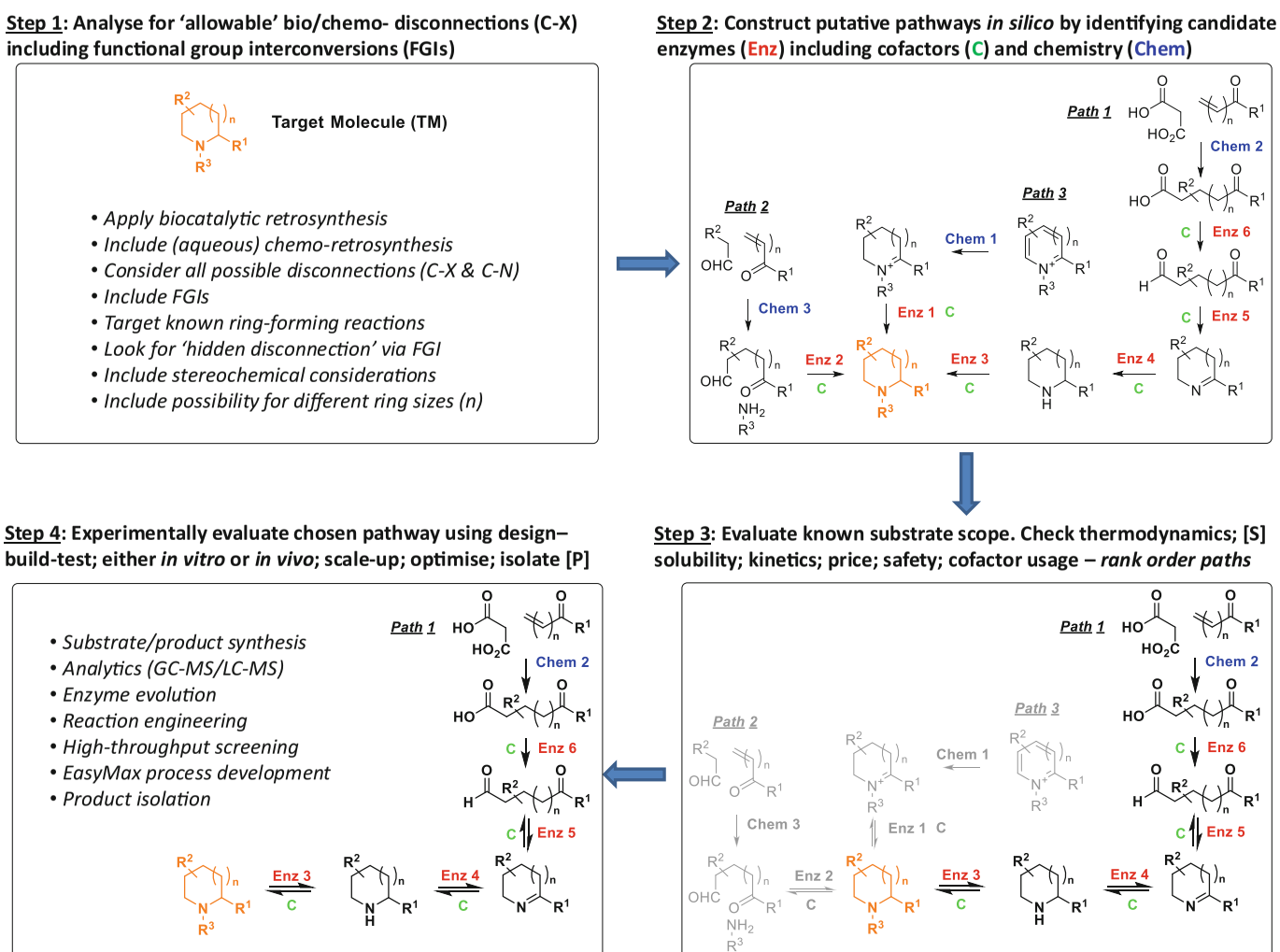

Fig. 2.1 Overview of workflow required for the design and validation of a biocatalytic pathway to a target molecule

molecule (TM). Step 1 involves the initial biocatalytic retrosynthesis in which the target molecule is subjected to a systematic analysis of how it might be disconnected into simpler precursors. At this stage both biocatalytic and aqueous compatible chemistries are included in order to potentially identify chemo-enzymatic as well as purely biocatalytic cascades. Note that it may also be necessary to introduce FGIs (e.g. alcohol to ketone, C–C to C=C) in order to generate additional possible retrosynthetic pathways by identifying 'hidden' disconnections. The data gathered from Step 1 is then fed into Step 2 where potential synthetic pathways are now constructed in silico for synthesis of the TM. For each step of the pathway either a biocatalyst (Enz) or chemical reagent (Chem) will need to be identified by interrogation of available databases. Cofactor requirements (C) can also be added at this stage together with any co-substrates required. Since several biocatalytic steps may be cofactor dependent it will be possible to generate cofactor networks to minimise overall net consumption of NADH, ATP, etc. The output from Step 2 is a complete set of putative pathways for further evaluation. In Step 3 additional parameters are added including, importantly, any known information concerning the substrate specificity and kinetics of the required enzymes. Other data such as substrate solubility, safety, commercial availability, and price can also be added. Each pathway will also be assessed for overall thermodynamics by adding known information concerning reversibility of each step together with the free energy of the reaction. At the end of Step 3 an overall assessment of each potential pathway will then be carried out enabling a rank order score to be assigned which will guide further work. In Step 4 the experimental begins in which pathways are now physically assembled in the laboratory, either in vitro or in vivo, to allow evaluation and screening for product and intermediate production. At this stage further enzyme evolution may be required in order to improve the kinetics of individual biocatalysts which are identified as rate limiting. Once a pathway has reached a proof-of-concept stage, in terms of meeting basic criteria for synthesis of the TM, it can be taken forward for further optimisation including reaction engineering and product isolation.

The purpose of this introductory chapter is (1) firstly, to map out the overall process and steps that need to be undertaken to design and construct a chemo-bio-catalytic cascade (Fig. 2.1) and (2) secondly, to highlight the various challenges and problems that need to be addressed in each of the individual Steps 1–4 in order to make the selection process as rapid and effective as possible. The subsequent sections in the book provide a comprehensive survey of the increasing number of chemo-bio-catalytic cascades (see Chaps. 3–5) that have been successfully designed and implemented and, in some cases, optimised for production on scale.

2.2 Retrosynthesis to Produce Pathways to the Target Molecule (Step 1)

2.2.1 Manual Retrosynthesis

We recently reported a comparison of six different biocatalytic routes for the preparation of the amino acid D-1 which is an intermediate in the synthesis of the antidiabetic drug sitagliptin [13]. In this example, 'manual retrosynthesis' was applied to generate the six pathways, all of which in this example used the same starting material, namely the aldehyde 2 (Fig. 2.2). Having conceptualised the different pathways, each was then constructed and executed experimentally in order to generate data to allow comparison between the routes in terms of overall yield and also green metrics. Interestingly, in this example, a pool of six different enzymes was used to create the six different pathways by appropriate choice of substrate and enzyme.

However, the example shown in Fig. 2.2 brings into focus the need to now move from 'manual retrosynthesis' to computer-assisted automated retrosynthesis for the following reasons: (1) the retrosynthetic analysis of D-1, and its subsequent synthesis from 2 using the six different routes shown, was greatly facilitated by

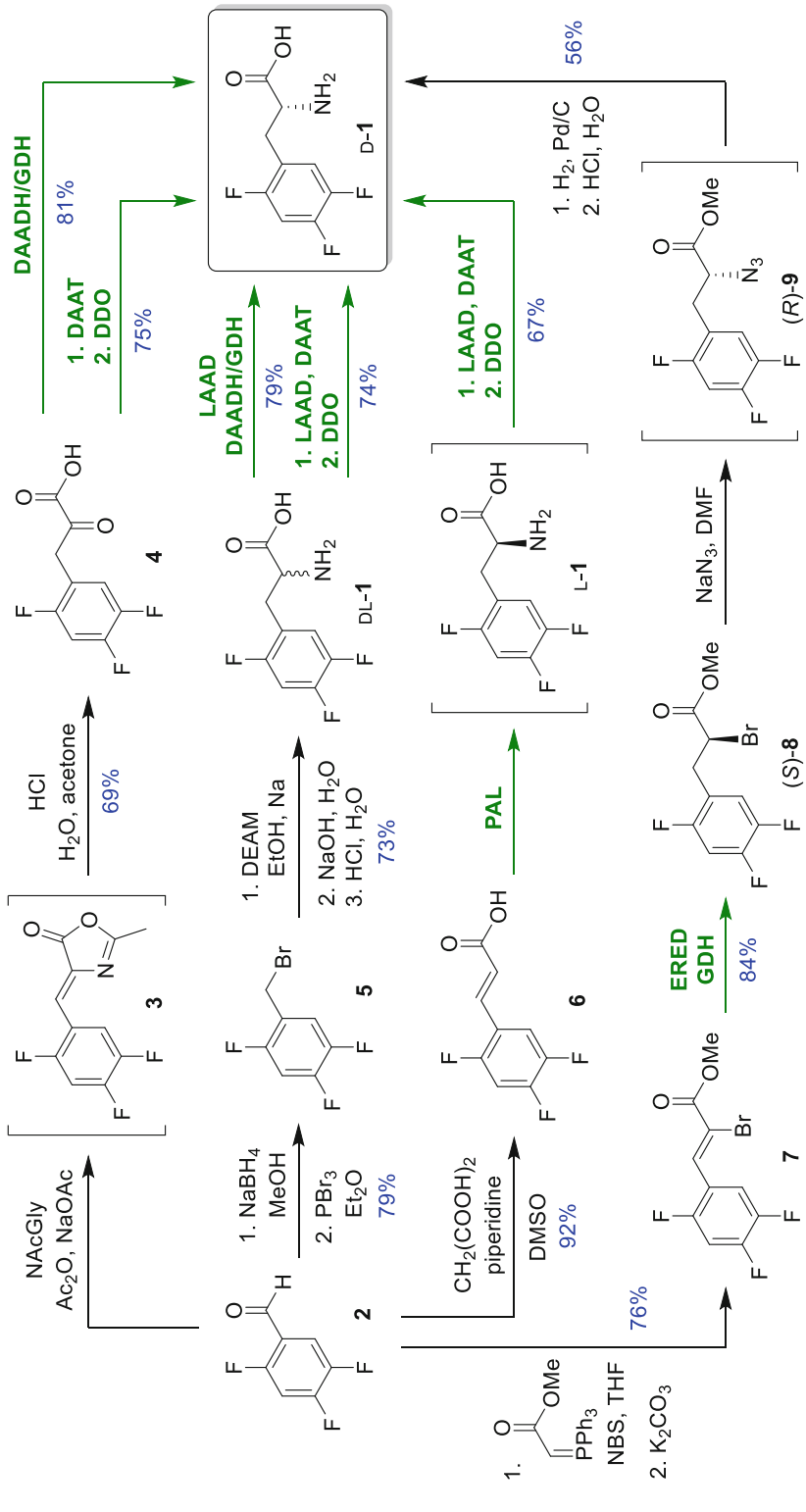

Fig. 2.2 Six complementary biocatalytic routes for the synthesis of α-amino acid D-1 from the aldehyde 2. (*DAADH* D-amino acid dehydrogenase, *GDH* glucose dehydrogenase, *DAAT* D-amino acid transaminase, *DDO* D-aspartate oxidase, *LAAD* L-amino acid deaminase, *ERED* ene reductase) [13]

in-house experience of working with the different enzymes that were ultimately chosen—for someone not so experienced in the application of these enzymes it would not be as easy to quickly assemble the different possible pathways; (2) for each of the six enzymes identified there will potentially be many candidate sequences that need to be considered with complementary substrate specificity and selectivity and hence, at this stage, it is highly desirable to be able to search databases of enzymes that might be suitable for application; (3) in the example shown there was no attempt to carry out a preliminary rank ordering of the six putative pathways prior to undertaking experimental work. Increasingly, and certainly in cases where retrosynthetic analysis generates a larger (>10) number of possible pathways, it will be desirable to apply filters and scoring functions in silico to initially gauge which pathways might be more suitable in terms of kinetics, thermodynamics, substrate availability, and many other parameters.

2.2.2 Moving Towards Automated Biocatalytic Retrosynthesis

In chemistry and metabolic engineering, increasingly researchers are turning to computer-aided synthesis planning (CASP) tools for the automated design of pathways to target molecules. Examples include Synthia (formally Chematica) and ASKCOS for retrosynthesis in organic chemistry [14], or RetroPath and BNICE for metabolic engineering [15, 16].

Most commonly, CASP approaches utilise a set of reaction rules or templates (encoding known functional group interconversions or bond (dis)connections) to guide synthesis planning (Fig. 2.3a), applying the reaction rules recursively until some stopping criteria are reached (Fig. 2.3b) [17]. The application of reaction rules in this way generates a network, or graph, which must be searched to identify promising pathways to the target molecule. The generation of the reaction rules can be carried out by automatically extracting them from databases such as Reaxys [18, 19], or simply by manually encoding them [20].

Reaction rules linking together many different chemical species have been described by some as forming the 'network of chemistry' [21]. A similar concept might be envisaged for the increasing number of possible biotransformations, which can be linked together into various cascades, as forming the 'network of biocatalysis' (Fig. 2.4). Traversing this network manually when performing biocatalytic retrosynthesis requires expert knowledge of increasing numbers of potential biotransformations. Automating this process through the use of a biocatalytic CASP tool would make the design of enzyme cascades more accessible to chemists who might be unfamiliar with biocatalysis, or could suggest pathways which might be missed by manual biocatalytic retrosynthesis. One such tool for automated biocatalytic retrosynthesis, RetroBioCat, is currently in development by us [22].

Biocatalytic CASP tools require reaction rules for the enzymes employed for biocatalysis, which describe the possible retrosynthetic steps in a computer readable fashion. One popular method for encoding reaction rules is the use of reaction SMARTS (Fig. 2.3a) [23], which can be applied to a target molecule to generate the product or products. For biocatalysis, the use of fairly general rules is likely necessary, as while feasibility may be initially limited by substrate scope, this is a challenge which in many cases can be overcome using enzyme engineering [24]. Furthermore, the context dictating whether a reaction is feasible or not is largely driven by the specific enzyme homologue chosen to perform the step, with different enzymes of the same type often showing different substrate specificities. Substrate specificity can be particularly broad where engineered enzymes are available [25].

It is often necessary or desirable to create chemo-enzymatic cascades, which feature both chemical and enzymatic steps [26]. A successful biocatalytic CASP tool will therefore need to include potential chemical steps as well. Interestingly, Reaxys includes a number of biotransformations, which may be included in tools able

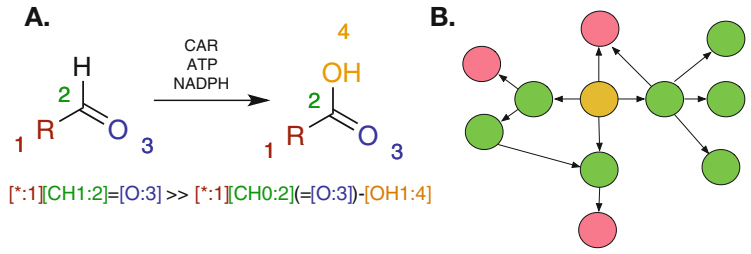

Fig. 2.3 Computer-aided synthesis planning (CASP). (**a**) A typical CASP tool requires a set of reaction rules or templates. Commonly these can be encoded as reaction SMARTS with atom-atom mappings (AAM). An example reaction SMARTS for the general reaction catalysed by CAR enzymes is shown. Reaction is shown in reverse for retrosynthesis applications. (**b**) A CASP program will iteratively apply the reaction rules to generate precursors (green and pink), resulting in a network of potential routes to the target molecule (yellow). Such a program can keep expanding the network until either a suitable starting material is found (pink), or a maximum number of steps is reached

to automatically extract reaction rules although they are likely lost amongst the more numerous chemical options [17, 18, 27].

Starting from the target molecule, a CASP tool applies the reaction rules recursively, generating potential precursors in iterative rounds until some stopping criteria are reached (Fig. 2.1b). For metabolic retrosynthesis the pathway is complete once it has reached the native metabolism of the host organism [28]. For other applications, the pathway may be considered complete upon reaching an inexpensive starting material [17], for which a database of possible starting materials and their prices is necessary. Alternatively, the CASP might be limited by simply a maximum number of steps.

One challenge often encountered by CASP tools iteratively applying reaction rules is that of combinatorial explosion. The application of focused rules, applying a filter to which reaction rules are applied, offers a route to limiting combinatorial explosion [17]. For example, RetroPath 2.0 scores reactions according to how well enzyme sequences can be retrieved to carry out the proposed transformation. Rules below a predefined score are then not applied [15]. Alternatively, GEM-path only accepts substrates if they are similar enough to the reference substrates for a reaction [29], as do other tools [30]. Another approach employed in chemical retrosynthesis is the use of metrics for molecular complexity, to focus searches towards less complex starting materials [31].

2.3 Identifying Enzymes to Fulfil Steps in the Proposed Pathways (Step 2)

2.3.1 Selection of Enzymes Manually by Literature Searches or Enzyme Screening

With a number of potential pathways in hand, specific enzymes must be selected to carry out each enzymatic step. For each step there is often a great many potential sequences which could theoretically complete it. Identifying the best candidate enzyme or enzymes from the vast number available can therefore be challenging. In the simplest case, enzymes might be selected based on what is easily available within the lab, what has been worked on before, or by manually searching the literature for examples of similar reactions (Fig. 2.5a).

Another approach adopted by some is the construction of enzyme screening panels using microtitre plates to quickly screen hundreds of enzymes against a new substrate (Fig. 2.3b) [32, 33]. Such panels are typically designed to

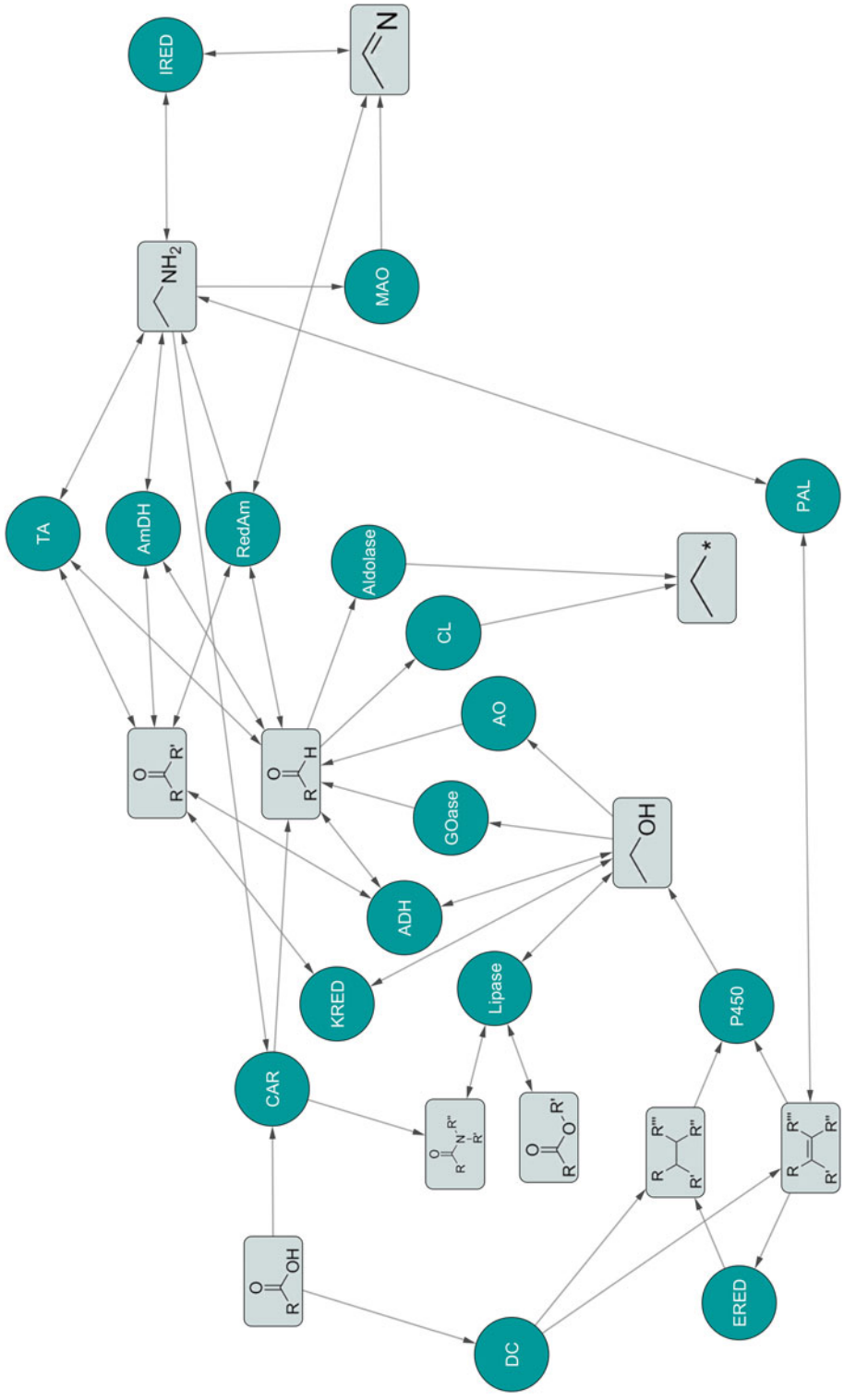

Fig. 2.4 Network of biocatalysis. A selection of some of the key enzymes available for biocatalysis, with a particular focus on functional group interconversion. Other biotransformations are also possible but are omitted for clarity. *ADH* alcohol dehydrogenase, *AmDH* amine dehydrogenase, *AO* alcohol oxidase, *CAR* carboxylic acid reductase, *CL* carboligase, *DC* decarboxylase, *ERED* ene reductase, *GOase* galactose oxidase, *IRED* imine reductase, *KRED* ketoreductase, *MAO* monoamine oxidase, *PAL* phenylalanine ammonia lyase, *RedAm* reductive aminase, *TA* transaminase

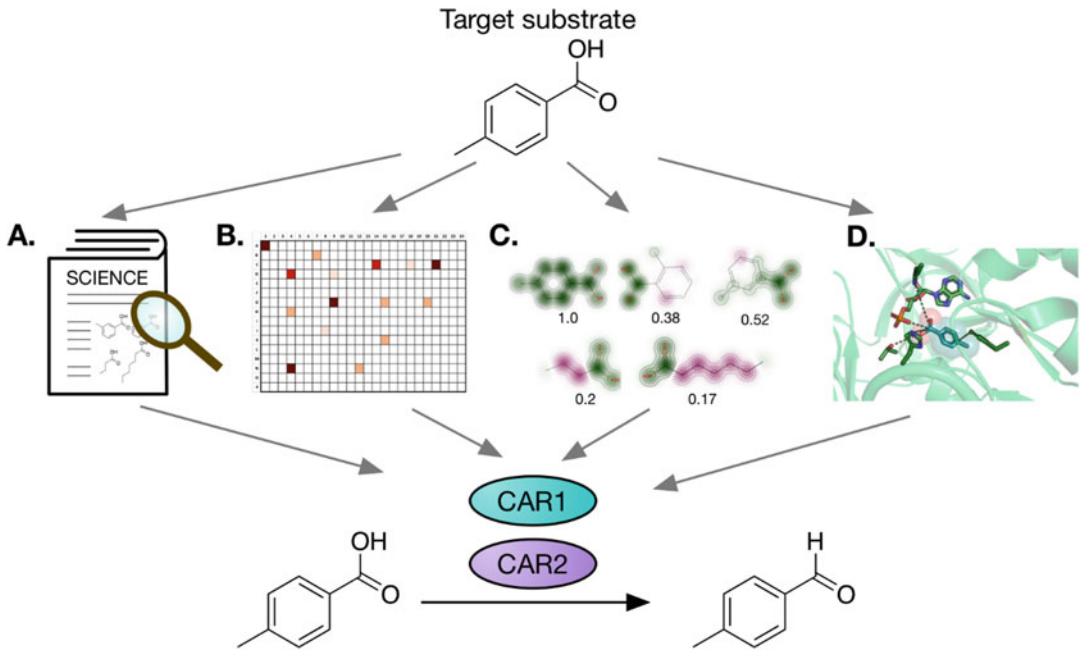

Fig. 2.5 Identifying enzymes for each step. Four routes for the identification of specific enzyme sequences to complete a biotransformation are illustrated, with carboxylic acid reductase (CAR) used as an example. In the example, two CAR enzymes are selected from many potential options for further testing in Steps 3 and 4. Options for selection include: (**a**) Selection using literature searches or enzymes already available 'in-house', (**b**) The use of enzyme screening panels using microtitre plates. Hits for activity are illustrated as dark red to light orange for strong to weak activity, respectively, (**c**) Molecular similarity can be used to find enzymes active on similar substrates. Visualisation of the atomic contributions to the Morgan fingerprint similarity between the target and known CAR substrates is shown, with the Tanimoto coefficient of similarity below. Analysis carried out using RDKit with the default options, and (**d**) (Inverse) virtual screening via substrate docking

encompass the phylogenetic diversity available for a specific enzyme [34], with the aim of capturing a wide range of substrate specificities [35]. Enzyme screening panels can be particularly powerful for assessing the substrate specificity of diverse metagenomic libraries [36]. In some cases, enzyme panels are available commercially. The enzyme with the best activity is then chosen from the panel and may be subjected to enzyme engineering to improve activity further [37]. However, screening can become challenging for pathways where an intermediate may not be readily available. In these cases, enzymes can be combined in a step-wise fashion, moving along the pathway as a suitable enzyme for each step is identified [38].

2.3.2 Selection from Databases Based on Molecular Similarity

Enzyme selection for the construction of metabolic pathways in synthetic biology has also been identified as a challenge, for which in silico tools have been developed [39]. This is primarily made possible due to a number of well-developed enzyme databases, which contain information predominantly on metabolic pathways and natural substrate specificity, for example, BRENDA or KEGG [40, 41]. With a target reaction encoded as machine-readable entries known as reaction SMARTS, tools such as Selenzyme can search for enzymes known to carry out similar reactions by evaluating molecular similarity, in addition to

Table 2.1 Summary table for pathway design highlighting both manual and automated approaches. A combination of manual and automatic approaches is possible

	Manual	Automated
Step 1: pathway generation	Retrosynthesis using disconnections and FGIs the researcher knows about. Based on expert knowledge	Pre-programmed reaction rules for general disconnections and FGIs (Fig. 2.1). Automatic retrosynthesis by iterative application of reaction rules
Step 2: enzyme identification	Selection based on what is available 'in-house', analysis of literature, or enzyme screening panels (Fig. 2.3a, b)	Use of molecular similarity, machine learning or structure-based substrate docking for enzyme identification from a database of known biotransformations (Fig. 2.3c, d)
Step 3: pathway selection	Manual evaluation of possible pathways using a small number of metrics or based on expert analysis	Automated evaluation of many pathway metrics (Table 2.2)
Step 4: experimental validation	Manual construction of one or a few pathways	Construction of many pathways simultaneously using liquid handling robots

Table 2.2 Example databases containing information specifically on enzymes for biocatalysis

Database	Description	Substrate specificity
RedoxiBase [44]	Contains sequence and structural information for peroxidase and other oxidoreductase enzymes	No
Lipase database [45]	Contains sequence and structural information for lipase enzymes	No
BioCatNet databases [46]	Collection of databases containing sequence and structural information for transaminase, P450, and IRED enzymes, amongst others	No
CAZy [47]	Contains sequence and structural information for carbohydrate-active enzymes	No
PrenDB [40]	Contains substrate, product and reaction information for prenyltransferase enzymes	Yes

scoring how closely related the host and source organisms are [42]. Such an approach for identifying enzymes for synthetic enzyme reactions in biocatalysis is limited only by the data available in the various enzyme databases.

A database dedicated to biocatalytic transformations, capturing successful and unsuccessful transformations on a sequence by sequence basis, would allow approaches utilising in silico methods such as molecular similarity to be adopted for identifying enzymes for synthetic transformations. Whilst some databases aimed at the biocatalysis community have been developed, none are comprehensive, and most do not provide the necessary information on the substrate specificity of individual enzymes (Table 2.2). However, the Kolb group has recently developed a substrate prediction database to facilitate the use of prenyltransferase enzymes in biocatalytic transformations (PrenDB) [43]. PrenDB reportedly automatically extracts information from newly published literature related to prenyltransferase reactions, and compiles the substrates, products, and reactions of the enzyme as SMILES and SMARTS strings. The information is subsequently used in a variety of algorithms to aid the wider application of this underrepresented family of synthetically useful enzymes. The compilation of databases similar in scope to PrenDB for the whole biocatalytic toolbox would surely enhance the development of new enzymatic cascade reactions.

As increasing amounts of data become available on rising numbers of enzymes, it becomes prohibitive for researchers to analyse all of it prior to selecting an enzyme for a transformation.

Automated enzyme selection, as is available for choosing enzymes for metabolic pathway construction, will therefore become increasingly necessary.

Critical to querying databases using chemical structures or reactions is the extrapolation of known substrate specificities to predict activity with new substrates. To an extent, researchers do this intuitively when tasked with a new reaction. For example, it is likely that a carboxylic acid reductase known to accept fatty acids with chain lengths of four or eight carbons will also accept a fatty acid with a six-carbon chain length [48]. One approach to allow this type of analysis to be carried out in silico is the use of metrics for molecular similarity, commonly utilised for virtual screening in drug discovery and more recently in both chemical and metabolic retrosynthesis (Fig. 2.5c) [15, 49].

To compare the similarity of one molecule to another in silico, molecules must first be encoded into vectors or fingerprints, which can then be compared using a similarity coefficient [50]. A number of methods can be used to encode molecules into vectors, which represent the molecules in 'chemical space'. Most simply, the use of descriptors such as LogP or pKa can be used, with the advantage of also being human-readable [51]. More commonly descriptors such as molecular fingerprints are used which offer further structural information (Fig. 2.5c) [52]. Other methods can also make use of 3D information [53]. Recently the use of autoencoders which use neural networks to encode and decode molecules into a continuous latent space has also been demonstrated [54]. With molecules encoded as a vector, a similarity coefficient can be calculated to score the similarity. A popular example is the Tanimoto coefficient, although many options exist, often with unique biases which should be taken into account [52].

Where multiple enzymes are to be tested in a pathway, phylogenetic diversity should be considered so as to deliver maximum sampling of the different substrate specificities within an enzyme family [55]. Sampling a diverse range of sequences is particularly important where no similar substrates have been previously tested, or no positive predictions can be made. Importantly, limited activity for a substrate should not rule that pathway or step out completely, as enzyme engineering can often be employed to improve activity. Indeed, the identification of an enzyme with even a low level of activity from a selection of many inactive homologues is an important first step towards engineering an enzyme [56].

Machine learning approaches could offer a more informative alternative to simply calculating a similarity coefficient. For example, recent work on glycosyltransferases demonstrated the creation of a chemo-bioinformatic machine learning model which coupled physicochemical features with isozyme-recognition patterns. The model was able to predict novel substrate specificities, with the use of decision trees in combination with 1D descriptors revealing the modulators of substrate specificity in a human understandable way [51].

2.3.3 Selection Based on Enzyme Structure

A complementary approach for the in silico determination and analysis of enzyme substrate specificity is to make use of the vast amounts of 3D structural information contained within publicly accessible databases such as the Protein Data Bank (PDB) [57] and similar repositories. The presence of bound ligands within the catalytic site of protein structures offers the opportunity to compare substrates of interest for novel biocatalytic transformations with a 'known' substrate of the enzyme, and intermolecular interactions between the protein and the ligand can be measured and compared. The 3D shape of a ligand to be docked into a protein is of special importance due to the inherent chirality of active site pockets and the molecules that interact and bind within them, and the need for shape complementarity to allow a receptor and ligand to form the critical interactions necessary for catalysis to occur [49]. In the event of a protein structure being available without any co-crystallised

molecules present, (inverse) virtual screening of 3D structures through molecular docking can be performed (Fig. 2.5d).

High-throughput (inverse) virtual screening (HTVS), analogous to high-throughput assays in the laboratory, enables the automated assessment of thousands of protein-ligand dockings and rank orders the output poses based on a scoring algorithm. Many tools are available to the researcher to perform these tasks [58–60], with some examples even allowing for online homology model generation for cases where a 3D structure of a protein of interest has not yet been elucidated [61, 62]. To make this process even simpler there exist several subdatabases containing context-specific protein binding site information [63], the most prominent of which being Screening-PDB (sc-PDB) [64], that are composed of high resolution protein–ligand complexes taken from PDB. These protein–ligand complexes are available for use in virtual screening pipelines, and the compilation of a subdatabase containing all known protein–ligand complexes elucidated in a biocatalyst context could streamline IVS for biocatalyst research.

Docking poses generated from (inverse) virtual screening are scored and ranked based on a number of factors [65]. If the protein structure contains a co-crystallised substrate, the docked substrate pose and the co-crystallised substrate are superimposed and the similarity in volume of the two compounds is measured. Any mismatch in the volume of the two compounds is considered a measure of dissimilarity [66], whilst any matched interactions between ligand and protein are a measure of similarity. In the absence of a crystallised ligand, active site elucidation can be achieved through pocket identification algorithms [67] or through prior knowledge regarding catalytic residues of the protein. Differing docking poses for a given substrate are most often ranked based on the free energy of binding (ΔG) for the protein–ligand complex, where van der Waals' forces, hydrogen bonding, electrostatic, and hydrophobic interactions are all considered [58].

Whilst great strides have been made in the past decade in the application of (inverse) virtual screening for drug discovery, the demands of using (I)VS in the search for biocatalysts capable of accepting target substrates are quite different. Molecular docking approaches can successfully calculate whether or not a ligand of interest is likely to bind within the active site of a protein, but it is much more difficult to predict whether this binding will lead to the required catalysis. However, with further advancements in the understanding of the forces that govern substrate–protein interactions and the increased accumulation of data relating to protein structures, it may become more commonplace for structure-based screening tools to be used for enzyme selection in the near future.

2.4 Selection of the Most Promising Pathways (Step 3)

With one or a few specific enzyme sequences selected for each step in the proposed pathways, further evaluation of the economic, ecological, and technical considerations is necessary to select only the most promising pathways for experimental validation in Step 4.

Manual pathway selection may be based only on a couple of factors, such as substrate scope, starting material cost, or metrics of green chemistry [13]. In other cases, manual selection may not even include a formal analysis of pathway metrics, but instead based on expert opinion of which pathway looks the most promising. Alternatively, a systematic and automated analysis of pathway metrics could allow a more informed decision to be made. Automated analysis becomes particularly necessary where there are many pathway options to choose from, such as where pathways have been generated using automated methods. Additional data pertaining to each step is therefore required for selection, which can be compiled into pathway-wide metrics for comparison with other pathways (Table 2.3).

A number of tools and resources are available that enable calculation or collection of a wealth of useful metrics to rank order the pathways generated earlier in Steps 1 and 2 of the workflow (Table 2.3). Where such sources are not yet

Table 2.3 Economic, ecological, and technical considerations for pathway evaluation

Metric for evaluation	Data source
Feasibility due to substrate scope	Specific activity (estimates if unavailable) or categorise each step as good, medium, poor, or chemically impossible
Substrate solubility	LogP prediction [68], COSMO-RS [69], hydrophilic-lipophilic balance (HLB) calculation [70], ESOL [71], general solubility equation (GSE) [72]
Enzyme stability	Melting temperature (T_M) (estimate if unavailable)
Waste generation	E-factor [73], atom efficiency [74]
Pathway length	Number of steps
Thermodynamics	eQuilibrator tool [75], group contribution estimates [76]
Starting material cost	Database of chemical prices
Cofactor usage	Stoichiometry of cofactor usage across the entire pathway
Sustainability of starting material	Bioderived or petrochemical
Possible side reactions	Automated or manual analysis of side reactions
Toxicity	(Estimated) IC50 values for cytotoxicity of a compound [77], structure–activity relationship models [78]

available, such as in the determination of the solvent-tolerance of an enzyme, the relevant literature can be consulted and used to inform the researcher. In cases where two or more given pathways rank very similarly to one another, additional practical properties of using the constituent enzymes may be taken into consideration, such as the presence of post-translational modifications or other factors that may restrict the soluble expression of the protein.

Cofactor usage of selected enzymes, and so the entire pathway, is often enabled by the incorporation of exogenous cofactor regeneration systems making use of additional enzymes and sacrificial substrates, for example a glucose dehydrogenase (GDH)/glucose system for the regeneration of NADPH [79]. However, since it is common for biocatalysts to be cofactor-dependent (e.g. carboxylic acid reductases are ATP- and NADPH-dependent enzymes) and/or reversible redox catalysts (e.g. alcohol dehydrogenases), it is increasingly possible to generate cofactor networks to enable low or net-zero usage of expensive cofactors.

The scoring of generated pathways can be achieved by rating each individual metric numerically, and then using the sum of the individual scores to reach a global assessment score for each pathway (Fig. 2.6). Pathways can then either be ranked solely on this global scoring, or each metric can be assigned a weighting by the researcher based on its importance within the scope of the current project. It is also possible to filter pathways using a primary criterion that the researcher has deemed the most important consideration for the work, such as substrate availability or sustainability, followed by rank ordering the 'best' candidates from this initial filter by using a secondary criterion, such as substrate cost. Ultimately, the final decision of which pathways to proceed with should be made by a human with insight into the aims, the resources available, and the capabilities of the project at hand.

Once a set of promising enzymatic pathways has been selected based on the scorings calculated for each metric and input from the researcher, the next step is to build the biocatalytic cascades in the laboratory and assess their suitability for the intended transformations.

2.5 Experimental Evaluation of Selected Pathways (Step 4)

The experimental tools for building biocatalytic cascades in the laboratory have dramatically grown in number over the past twenty years, in particular because of the emerging field of synthetic biology. Specifically, the genes encoding many biosynthetic cascades of natural products

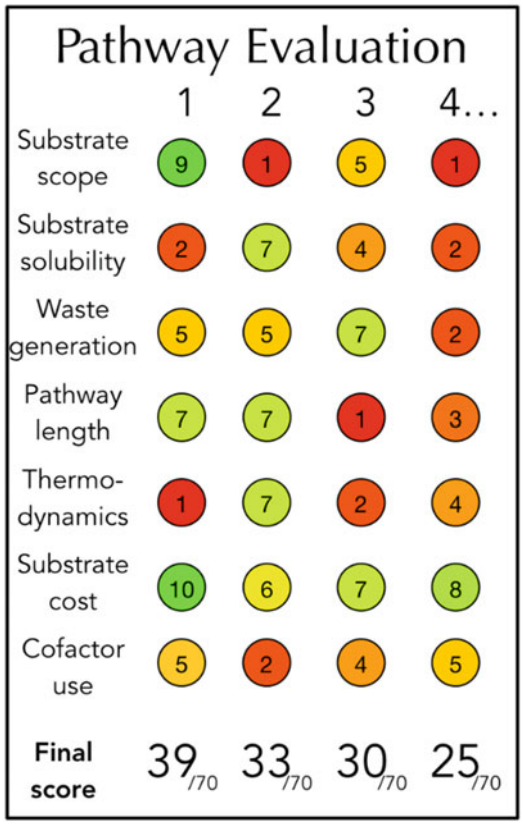

Fig. 2.6 Example score sheet for automated pathway evolution. Pathways could be ranked from best to worst using the sum of individual scores; however, the final decision of which pathways to move forward with should be made by a human with more insight into the potential for process and enzyme engineering. For example, pathway 1 might score badly on both substrate solubility and thermodynamics, which might be overcome by process engineering. In contrast, pathway 2 might become more attractive with enzyme engineering to improve substrate acceptance

and their analogues have been successfully heterologously expressed in engineered strains, using high-throughput cloning techniques that are now robust and easily accessible off-the-shelf. Many of these techniques can be adopted to de novo design of biocatalytic cascades, starting with the overall concept of design-build-test-analyse (DBTA) cycles (Fig. 2.7), which combines in silico design of pathways as described in previous sections, with fast cloning and analysis methods. The concept of the cycle acknowledges the increasing input of design and rationalisation of experimental findings, but also takes into account that pure design is still highly challenging. For the development of a successful cascade, the ability to quickly test hypotheses from modelling studies, with the ability to then explore multiple parameters which can feedback to refine the modelling, is very important.

2.5.1 Build and Test

A particularly important variable is the choice of biocatalyst for each step in the cascade. The study of natural biosynthetic cascades has shown us that all enzymes have co-evolved for their specific role and position as catalysts in the cascade in terms of expression, activity, stability, and substrate specificity. The complexity of the system makes it very challenging to predict these parameters in a de novo cascade context, and commonly several variants of enzymes obtained from mutagenesis and evolution campaigns [80], or homologues found in genomic databases, are tested. In a multistep cascade, the number of pathways to be tested by using biocatalysts in combinatorial fashion quickly leads to large numbers and experimental methodology needs to match the demand for high throughput. The implementation of de novo cascade reactions requires careful consideration, particularly with respect to whether the pathway is constructed in vitro or in vivo [81].

De novo enzyme cascades can be classified as (1) in vitro (see also Chap. 3), (2) in vivo (see also Chap. 4), or (3) hybrid, as shown in Fig. 2.8. The choice for any of these three options for a particular application relies on experimental constraints, including availability of gene sequences and good heterologous enzyme expression, cofactor availability, substrate and product transport into and within the cell, and the metabolic stability of individual components.

Often there are a number of options, but in vivo cascades generally have the advantage that less components are required. An example of a cascade that was published as a hybrid and in vivo cascade is shown in Fig. 2.9 [82, 83].

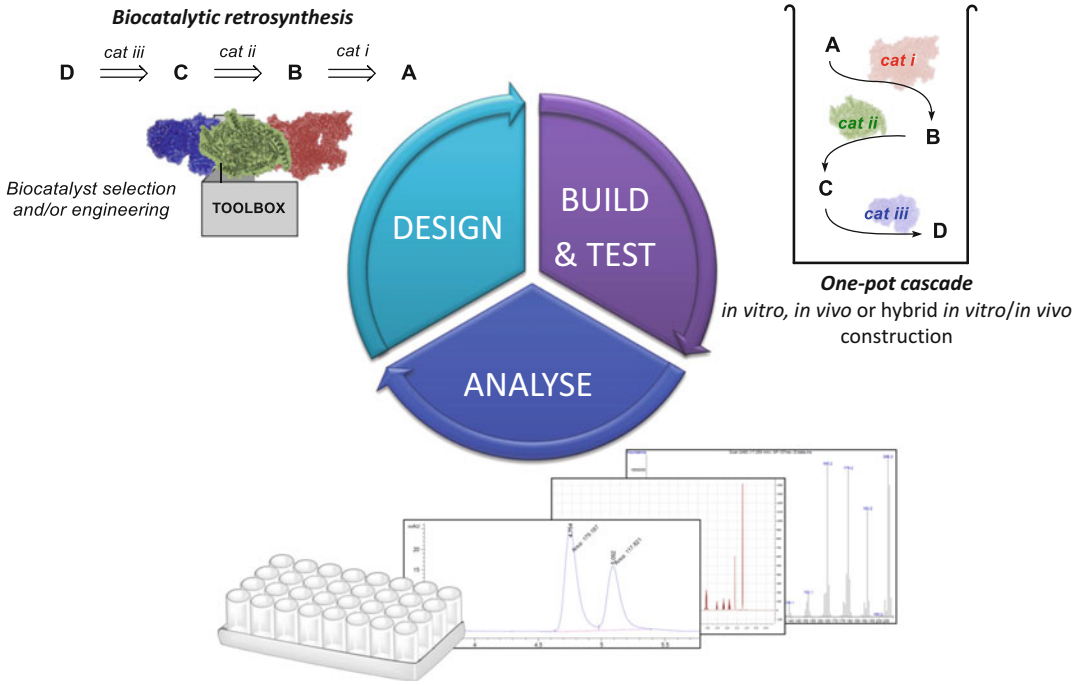

Fig. 2.7 DBTA cycles applied to de novo enzyme cascades [81]

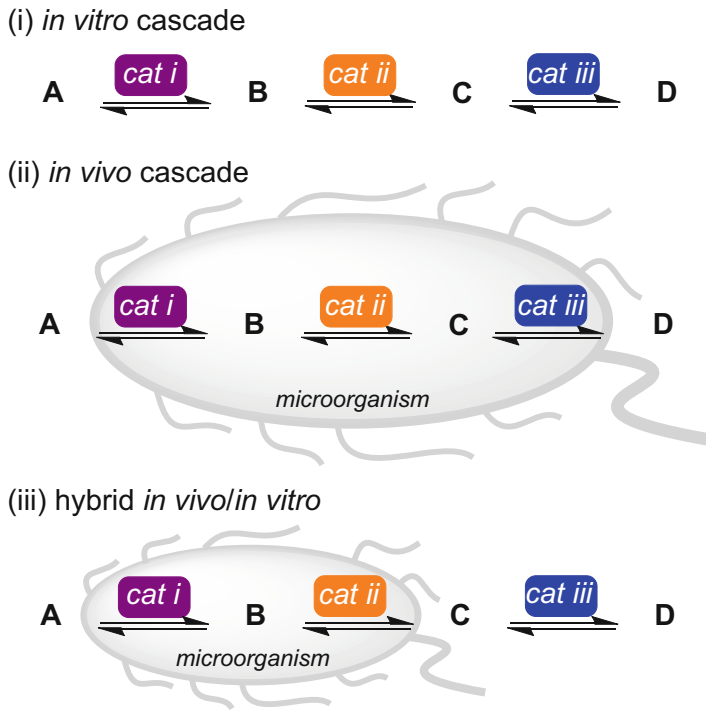

Fig. 2.8 Cascades can be classified as (1)–(3) [81]

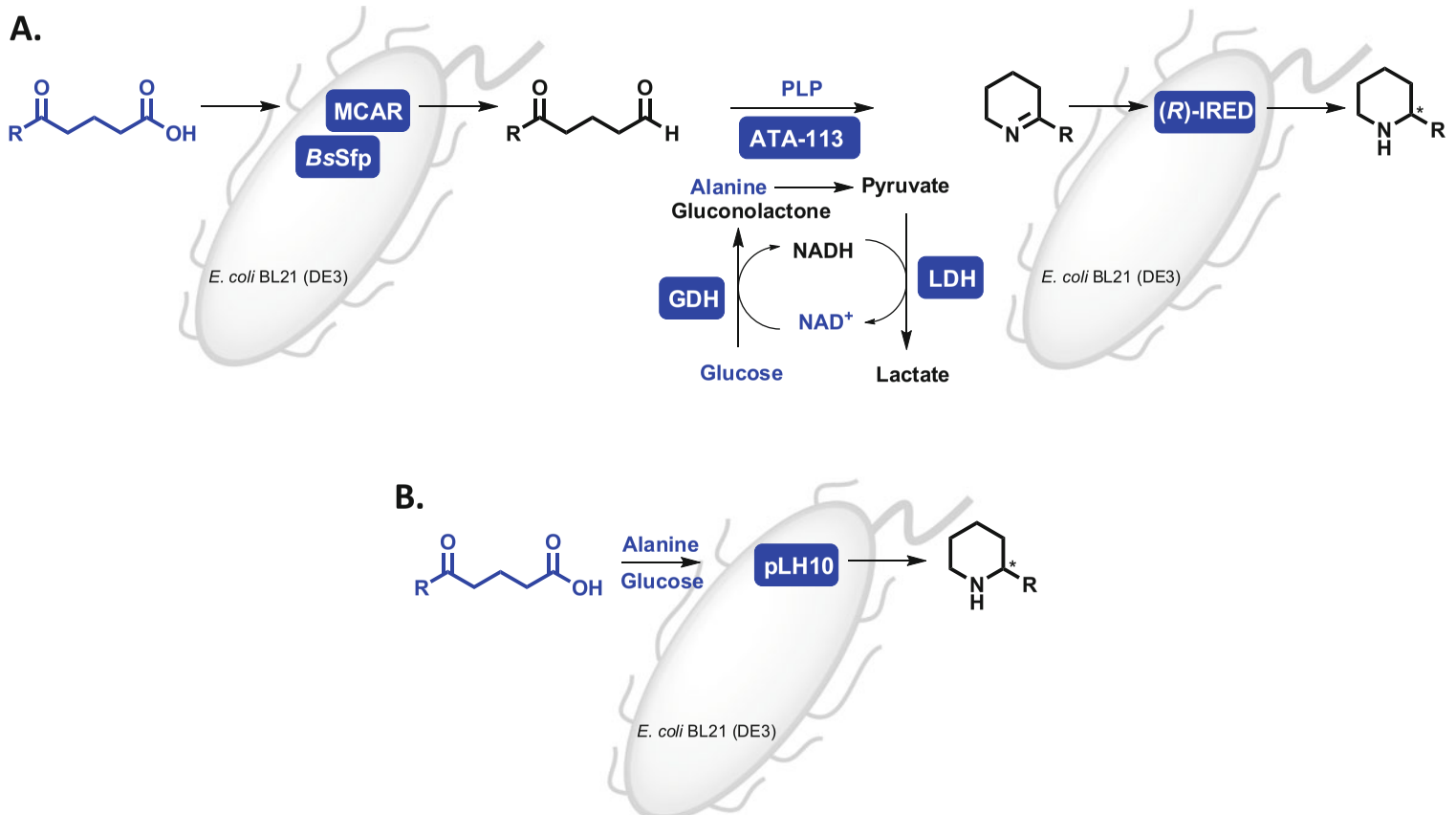

Fig. 2.9 Examples of an enzyme cascade designed as (**a**) hybrid and (**b**) in vivo. All components that need to be added to the reaction are shown in blue. pLH10 refers to a plasmid that contains the genes encoding for all enzymes necessary in the cascade [83]

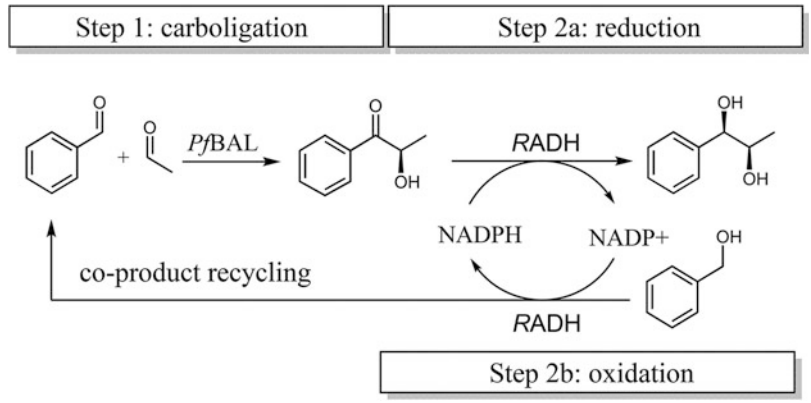

Fig. 2.10 One-pot two-step co-product recycling cascade for the synthesis of (1*R*, 2*R*)-1-phenylpropane-1,2-diol. Benzyl alcohol is used as a substrate for cofactor recycling generating starting material benzaldehyde. *PfBAL* benzaldehyde lyase from *Pseudomonas fluorescens*, *RADH* alcohol dehydrogenase from *Ralstonia* sp. [94]

The advantage of the in vivo cascade as shown in Fig. 2.9b is the low number of components that need to be added to the reaction mixture: apart from substrates, alanine (as a nitrogen source) and glucose, the catalyst is fully contained in one whole cell carrying the plasmid pLH10. In the hybrid cascade all enzymes need to be produced individually (in whole cell or isolated) and added to the reaction. The cascades in Fig. 2.9 also illustrate the issue of cofactors. The first and last reduction steps require ATP and NADPH, both of which are provided by the whole cell system. For the transamination, the hybrid cascade allows for cofactor recycling, whereas in the whole cell the equilibrium of the reaction is driven by excess amine donor. A hybrid system can also be easier to 'titrate', i.e. promoting reaction flow by increasing the amount of any slower enzyme. A whole cell system such as in Fig. 2.3b is more difficult to manipulate in terms of enzyme efficiency, but there are a number of options to increase activity, including changing promoters, terminators, or expression systems, and by gene duplication.

For the construction of plasmids in the 'build phase', the low cost of DNA synthesis provides access to tailor-made genes that can be designed with optimal codon usage for the desired expression system. Specific synthetic gene terminal sequences can offer fast integration into vectors using high-throughput cloning techniques such as Gibson cloning [84], LICRED [85] or restriction fragment-based methods such as 'BioBrick' assembly [86, 87]. *E. coli* remains a very popular host for initial experiments because of easily accessible vectors, tools and host systems that are well characterised [88]. There are a number of examples of successful enzyme cascades (for recent reviews see: [26, 81, 89–93]) generating product at preparative scale, demonstrating that the general concept is broad and feasible. A lot of the initial discovery studies are conducted at low mM concentrations, which allows for product isolation and characterisation. At this scale, the host cells are remarkably tolerant to synthetic intermediates, although metabolism can be observed, for example, reduction of active carbonyl intermediates [87]. Such issues can be overcome by strain engineering or increasing flow through the cascade avoiding accumulation of reactive intermediates.

Common cofactors such as NAD(P)H/ NAD $(P)^+$ and ATP have not been limiting at low mM concentrations in *E. coli*, and the addition of glucose to stimulate metabolism is often sufficient. An elegant concept to overcome cofactor limitations, in particular when developing processes for scale-up, is cofactor recycling, as shown recently in a one-pot two-step cascade (Fig. 2.10).

Molecular oxygen is also a common cofactor in biocatalysis [95] and gas–liquid mass transfer can be a major limitation for high space-time yields and can interfere with volatile substrates. An ingenious use of in situ O_2 generation via photosynthetic water oxidation, which allows a

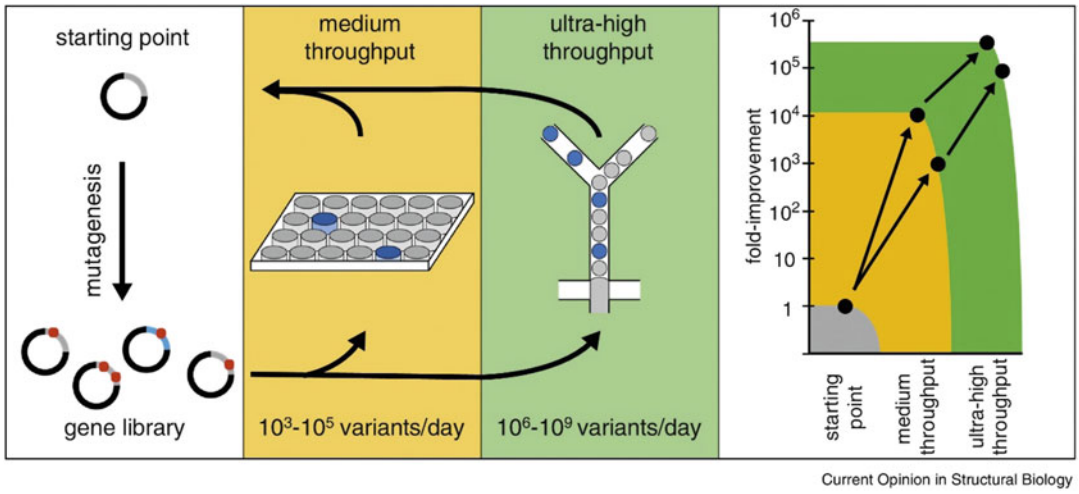

Fig. 2.11 Medium and ultra-high-throughput screening assays for enzyme activity [97]

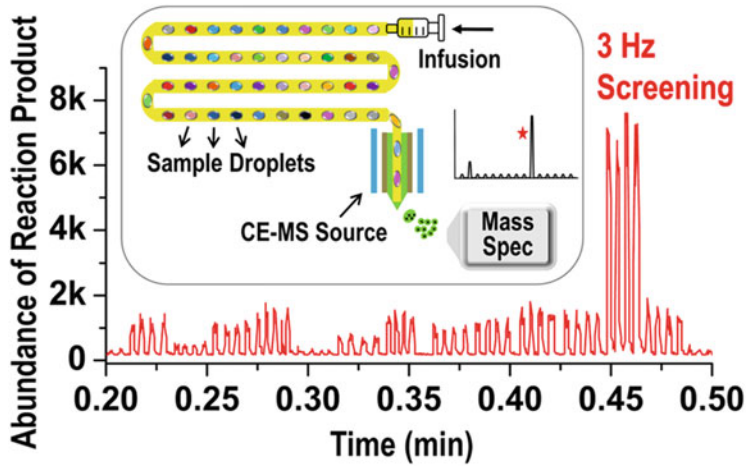

Fig. 2.12 Label-free high-throughput screening platform employing droplet microfluidics interfaced with electrospray ionisation (ESI)-MS [98]

non-aerated process operation, was recently reported and applied to alkane monooxygenase AlkBGT from *Pseudomonas putida* GPo1 [96].

2.5.2 Analyse

With the high data output from the design stage and fast cloning techniques that can sample enzyme cascades in combinatorial formats, analysis can become the bottleneck in the design-test-build-analyse cycle. Figure 2.11 shows schematically the timescale for screening enzyme activity from a recent review [97] and similar considerations can be applied to enzyme cascades. At the higher end of throughput, enzyme activity screens rely very heavily on fluorescent labelling, which is not always generic. Most of the published cascades have so far relied on label-free LC/GC-(MS) techniques, but this limits the opportunities for process optimisation and full exploitation of available biocatalysts dramatically.

New techniques are continuously being developed in enzyme evolution studies, and it is expected that these techniques will also be applicable to cascades. A recent example (Fig. 2.12)

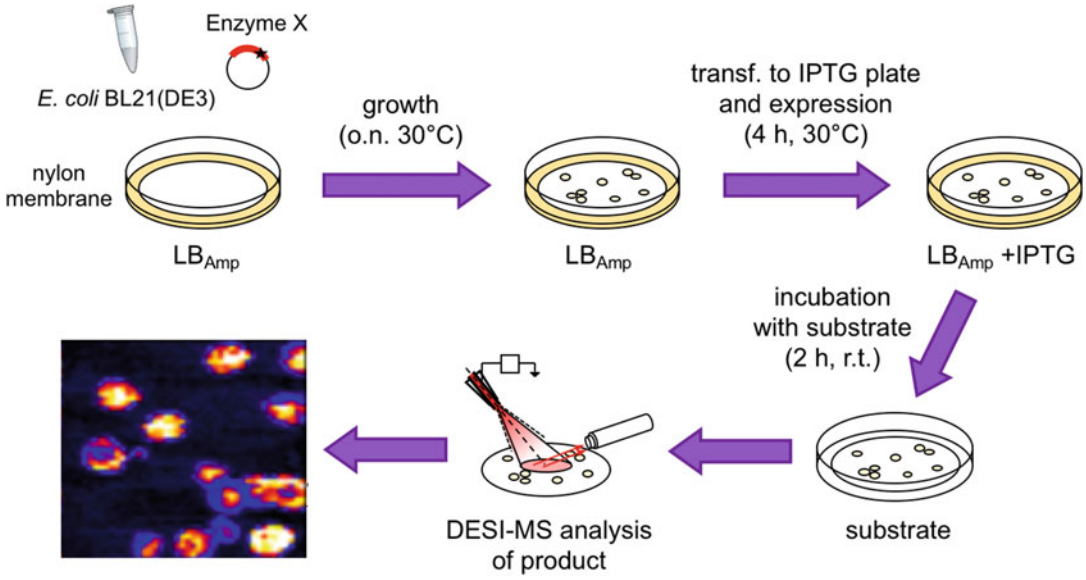

Fig. 2.13 Label-free high-throughput screening platform employing DESI-MS imaging at colony level [98, 99]

describes a droplet microfluidics interfaced with electrospray ionisation (ESI)-MS, which provides a label-free high-throughput screening platform that has been used for evolving new transaminases [98].

An alternative is direct screening of bacterial colonies from agar plates using label-free mass spectrometry techniques such as DESI-MS [98] in combination with Ion Mobility Spectrometry [99] (Fig. 2.13). Mass spectrometry as a read-out technique is particularly attractive for cascades because it can be multiplexed thus following all components of the cascade at the same time. Although mass spectrometry is not a quantitative technique in itself, isotope labelling can help provide reproducible quantitative measurements of analytes [99].

Another exciting technology is interfacing single-cell microfluidics and mass spectrometry, resulting in the possibility to quantify cellular productivity from living microbial cells in precisely controlled environments [100].

2.6 Conclusions and Outlook

There is no doubt that multi-enzyme cascade reactions represent a significant opportunity for biocatalysis. Enzyme-catalysed reactions operate under inherently similar reaction conditions in terms of media (aqueous system), temperature and pressure, and hence the notion of performing a telescoped process in a single vessel via this method seems a natural course for biocatalysis to follow in the future. This biocatalytic approach will then have significant advantages over conventional synthetic chemistry where changes in solvent regime, together with temperature, are often needed during a multistep synthesis. The real challenge for biocatalysis, as highlighted in this chapter, will be to increasingly automate the whole process of retrosynthesis followed by experimental evaluation of potential pathways using a design-build-test algorithm in order to select the best pathway(s) and also rapidly optimise them for eventual scale and application.

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Multi-Enzymatic Cascades In Vitro

3

Sandy Schmidt, Anett Schallmey, and Robert Kourist

Abstract

The combination of enzymatic reactions in a simultaneous or sequential fashion by designing artificial synthetic cascades allows for the synthesis of complex compounds from simple precursors. Such multi-catalytic cascade reactions not only bear a great potential to minimize downstream processing steps but can also lead to a drastic reduction of the produced waste. With the growing toolbox of biocatalysts, alternative routes employing enzymatic transformations towards manifold and diverse target molecules become accessible. In vitro cascade reactions open up new possibilities for efficient regeneration of the required cofactors such as nicotinamide cofactors or nucleoside triphosphates. They are represented by a vast array of two-enzyme cascades that have been designed by coupling the activity of a cofactor regenerating enzyme to the product generating enzyme. However, the implementation of cascade reactions requires careful consideration, particularly with respect to whether the pathway is constructed concurrently or sequentially. In this regard, this chapter describes how biocatalytic cascades are classified, and how such cascade reactions can be employed in order to solve synthetic problems. Recent developments in the area of dynamic kinetic resolution or cofactor regeneration and showcases are presented. We also highlight the factors that influence the design and implementation of purely enzymatic cascades in one-pot or multi-step pathways in an industrial setting.

Keywords

Biocatalysis · Enzymatic cascades · In vitro biotransformations · Enzymes · Cofactor regeneration

3.1 Introduction

Reaction systems combining two or more chemical steps in one pot without isolation of reaction intermediates are commonly referred to as cascades [1]. In such systems, individual chemical steps can be enzyme catalyzed or involve chemical (metal or organo-) catalysts. Accordingly, multi-enzymatic cascades include several

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biocatalytic steps, which can be either performed simultaneously or sequentially. In the first case, also called concurrent cascade or tandem reaction, all enzymes and reagents are present from the beginning of the reaction, meaning that all reaction steps take place at the same time. In contrast, in a sequential multi-enzymatic cascade, certain enzymes and/or reagents are added at a later point in time after a certain sequence is completed. Hence, all reaction steps of a sequential cascade are still performed in one pot but must be separated in time. The latter might be necessary if, e.g., two or more enzymes of the same cascade require different reaction conditions, one enzyme is inhibited by a compound appearing prior or later in the sequence, or to prevent undesired side reactions if cross-reactivities of the involved enzymes occur. Moreover, in vitro and in vivo multi-enzymatic cascades are distinguished depending on the biocatalyst preparation (compare also Chap. 4). Whereas in in vivo cascades all enzymes of the cascade are included in whole living cells, in vitro cascades make use of isolated enzymes in purified form, as cell-free extracts, freeze-dried preparations, immobilized versions, etc. [2].

In addition, cascade reactions in general can exhibit different topologies (Fig. 3.1) [1, 3]. In a linear cascade (Fig. 3.1a), the product of one chemical step serves as substrate of the subsequent chemical step. This is probably the most straightforward type of cascade reaction as it avoids the isolation of (unstable) reaction intermediates with the final goal to increase the overall product yield while saving time and resources.

Additionally, a linear cascade can be used to shift an unfavorable reaction equilibrium of one step by combination with a subsequent irreversible reaction step that pulls the product out of the reaction. A recent example is the combination of the hydroxynitrile lyase from *Manihot esculenta* for the synthesis of optically pure (*S*)-4-methoxymandelonitrile with the *Candida antarctica* lipase A-catalyzed acylation of the formed α-cyanohydrin yielding a stable ester product (Scheme 3.1) [4]. This way, the equilibrium of the hydrocyanation reaction could be shifted towards product formation and isolation of the unstable cyanohydrin intermediate was avoided.

Next to linear cascades, also orthogonal (Fig. 3.1b), cyclic (Fig. 3.1c), and parallel, interconnected (Fig. 3.1d) cascades have been described. In an orthogonal enzyme cascade, the conversion of a substrate into the desired product is coupled with a second reaction to remove one or more by-products. An example is the combination of a transaminase with lactate dehydrogenase, where the by-product pyruvate (when using alanine as amine donor) of the transaminase-catalyzed reaction is further converted to lactic acid in order to shift the equilibrium of the transaminase reaction (Scheme 3.2) [5]. In a cyclic cascade, one enantiomer out of a racemic substrate mixture is converted to an intermediate product, which is then transformed back to the racemic starting material yielding the unreacted substrate enantiomer as final product. The same applies if the unreacted substrate enantiomer is racemized to yield enantiomerically pure product (dynamic kinetic resolution). Hence, cyclic cascades are commonly applied in deracemization processes, e.g. of amino acids, hydroxy acids, or amines [6, 7]. Finally, in a parallel, interconnected cascade, two separate biocatalytic reactions are connected by complementary cofactor requirements of the two enzymes. Therefore, parallel, interconnected cascades are commonly associated with cofactor recycling systems. While in one biocatalytic step a substrate is transformed into the desired product, a cheap co-substrate is converted to a co-product in the parallel enzyme-catalyzed step to recycle the required cofactor for the first enzyme.

As briefly mentioned, cascade reactions offer several advantages compared to conventional reaction schemes [1]. This includes the avoidance of operational work up steps, which saves time, resources, and reagents, can reduce waste formation and, at the same time, allows for higher final product yields. Additionally, different reaction steps can be smartly combined in a cascade to solve synthetic problems of a reaction sequence such as cofactor regeneration, shift of reaction

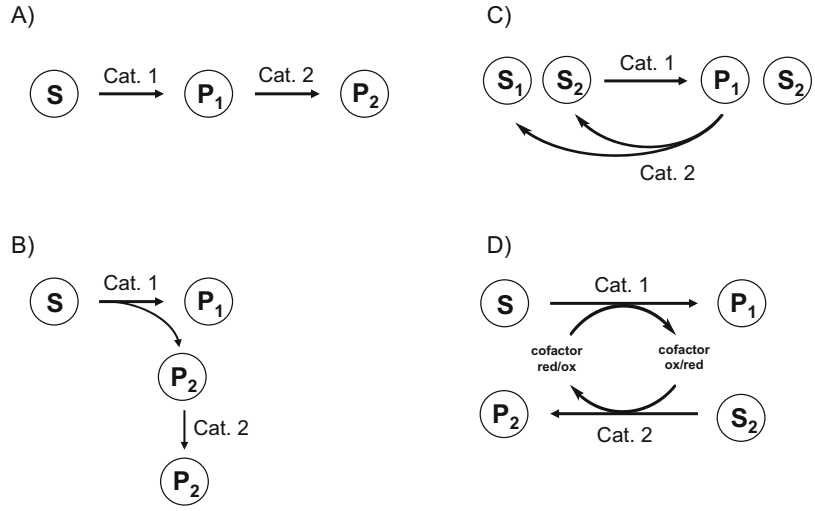
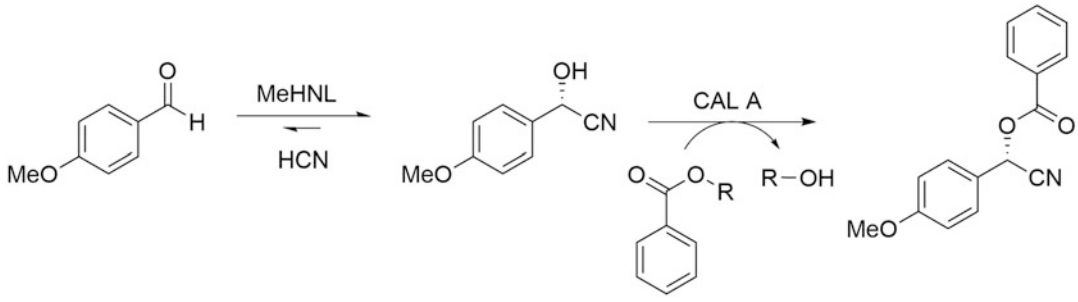


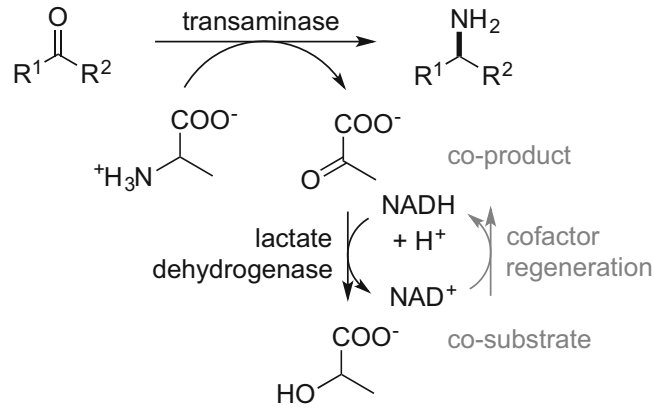
Fig. 3.1 Possible topologies of cascade reactions. (**a**) Linear. (**b**) Orthogonal. (**c**) Cyclic. (**d**) Parallel, interconnected. The scheme was adapted from [1]

equilibria, in situ generation of toxic or instable reagents, etc. On the other hand, the setup of efficient cascade reactions is usually a complex and challenging task [1]. Compatible reaction conditions have to be identified and, in case of a simultaneous cascade, the reaction rates of individual steps have to be balanced. Moreover, possible problems, such as the formation of undesired side products due to cross-reactivities of catalysts or the inhibition of an enzyme by a compound appearing earlier or later in the reaction sequence, can occur that have to be addressed. Multi-enzymatic cascades are usually easier to establish than chemoenzymatic or chemo-catalytic cascade reactions as enzymes commonly work in aqueous reaction media and often display similar temperature and pH requirements for optimal performance. Nevertheless, also several examples for the successful combination of enzymatic and chemical reaction steps in a cascade have been described in literature (compare also Chap. 5) [8].

In an impressive way, nature successfully evolved a multitude of highly complex concurrent cascade reactions. Living organisms built every minute thousands of highly complex molecules from simple precursors in an astonishing variety and efficiency. To achieve such high efficiencies, individual enzymatic transformations are arranged in cascading sequences (biosynthetic



Scheme 3.1 Combination of *Manihot esculenta* hydroxynitrile lyase (MeHNL) and *Candida antarctica* lipase A (CAL A) in a linear cascade to shift the reaction equilibrium of the first hydrocyanation reaction [4]



Scheme 3.2 Combination of transaminase and lactate dehydrogenase in an orthogonal cascade to shift the reaction equilibrium of the transaminase-catalyzed reaction

pathways) in living cells [9]. This strategy can be mimicked in vitro by the design of artificial metabolic pathways [10] through combination of multiple isolated enzymes in a homogeneous phase or by combination of two or more catalytic activities in a single protein, e.g., by fusion of genes encoding different enzymes or by crosslinking several enzymes [11–14]. Recently, a novel approach has been mentioned in literature named systems biocatalysis, which aims for the in vitro setup of synthetic metabolic cycles for the production of valuable compounds [15].

To illustrate the synthetic potential of multienzyme cascades, but also potential challenges in the development of cascade reactions, different cascade examples have been selected and are described in more detail in Sect. 3.2. Perhaps the most frequent motivation for currently used enzyme cascades is the concurrent regeneration of (redox-) cofactors or expensive reagents. A second application is the combination of isomerizing enzymes or catalysts with highly enantioselective enzymes for dynamic kinetic resolutions, which allow to overcome the yield limitation of 50% of kinetic resolutions. Cofactor regeneration and dynamic kinetic resolutions require concurrent cascades, which often makes it very challenging to provide optimal reaction conditions for both catalysts used. To overcome compatibility issues, compartmentalization is a possibility to enable different operating conditions for all reaction steps of a concurrent or step-wise cascade [8].

3.2 Cascades to Solve Synthetic Problems

3.2.1 Combination of Selective Enzymatic Steps with Isomerizing Reactions

Due to their availability and ease to use, hydrolases mainly constituted the first wave of biocatalysts in the first biocatalytic processes [16]. Kinetic resolution of racemic mixtures was often the preferred reaction form (Fig. 3.2) [17]. While this reaction is simple and robust, it suffers from an intrinsic limitation of 50% yield, which in turn requires the physical separation of substrate and product. The addition of a second catalyst, which racemizes the unreacted substrate, but not the product (Fig. 3.2) in a cyclic cascade allows for the complete conversion of starting material to the desired product enantiomer. The depletion of the faster-reacting substrate enantiomer leads to an increased conversion of the slower-reacting substrate enantiomer as its relative concentration increases, which reduces the optical purity of the product. To prevent this, the racemization should be one order of magnitude faster than the enantioselective reaction. This makes the racemization steps often the bottleneck of dynamic kinetic resolution reactions.

In 1997, Bäckvall et al. combined Ruthenium-catalyzed hydrogen transfer reactions for the racemization of aryl aliphatic secondary alcohols with their lipase-catalyzed kinetic resolution in a

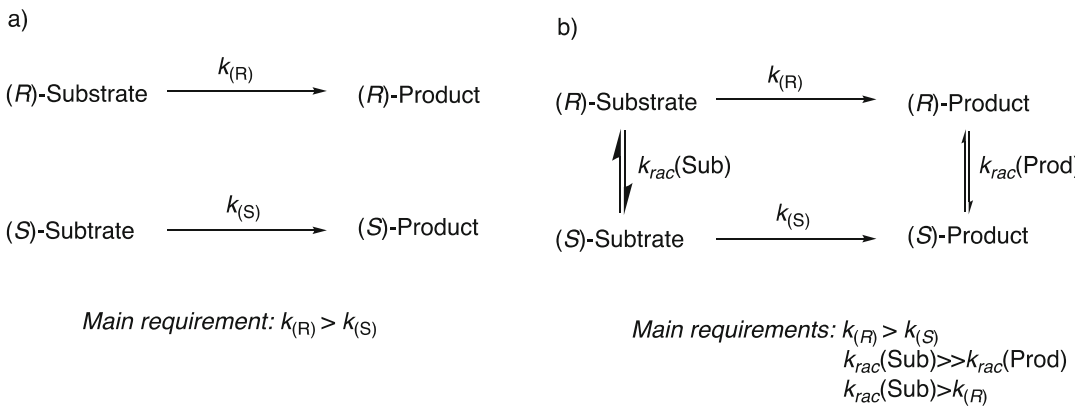
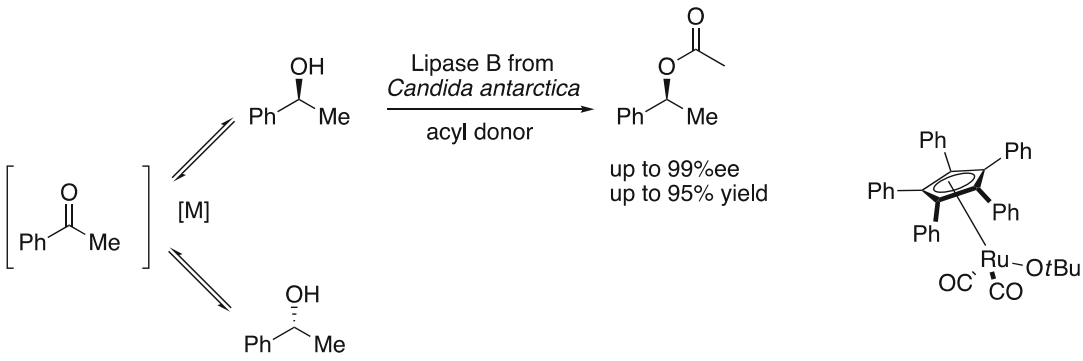


Fig. 3.2 Schematic representation of kinetic resolution (**a**) and dynamic kinetic resolution (**b**)

concurrent fashion to obtain the corresponding esters in high optical purity and yield (Scheme 3.3) [18]. The racemization of the secondary alcohol proceeds via intermediary oxidation to the corresponding ketone. Important parameters are the activity of the Ruthenium-catalyst and its stability. A high stability is desirable particularly against highly reactive acyl donors such as vinyl acetate that drive the transesterification towards ester formation [19]. By choosing the appropriate metal catalyst, excellent reaction rates, yields, and optical purities were achieved. The preference of most lipases for the formation of the (*R*)-enantiomers of secondary alcohols limited this approach to the formation of the respective (*R*)-esters. Instead, the use of proteases allowed to access the opposite (*S*)-enantiomers. The reaction concept was later extended to the transesterification of primary alcohols and aliphatic amines [20]. Lipases are frequently used for the kinetic resolution of esters either from chiral alcohols or chiral carboxylic acids. As shown, the racemization of secondary alcohols via redox reactions allowed the establishment of highly efficient dynamic kinetic resolution (DKR) reactions. The analogous DKR of the esters of α-chiral carboxylic acids, however, is much more challenging. Here, racemization of the stereocenter by a redox reaction is difficult to achieve. Racemization by acid-base catalysis is possible, but needs very special catalysts and conditions to avoid the base-catalyzed hydrolysis of the carboxylic ester. It is also not trivial to find conditions for an efficient base-catalyzed racemization that are compatible with the stability of the lipase [21]. This example underlines that concept for reaction sequences is often highly substrate-dependent. While the combination of metal-catalyzed racemization and lipases could be demonstrated with high yields and optical purities, the applicability to other enzyme classes beyond lipases and proteases is somewhat limited due to the requirement of the metal catalyst for organic solvents.

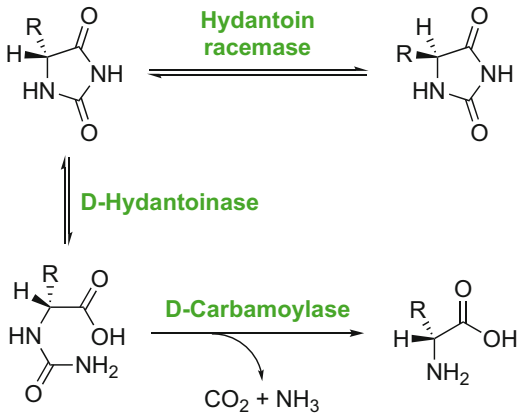
A prominent example for a fully enzymatic dynamic resolution is the so-called Hydantoinase process for the production of optically pure D- or L-amino acids, for which in vitro as well as in vivo approaches have been developed [22]. In general, D- or L-amino acids can be synthesized by chemical or enzymatic procedures. However, the chemical synthesis gives racemic mixtures of amino acids with low yield. The production of specific amino acids by fermentation of microorganisms is only useful for a few natural amino acids and depends on the microorganism. Thus, many chemical companies have embraced biocatalysis for manufacturing enantiomerically pure amino acids. This strategy involves aminoacylases, amidases, and hydantoinases. In 1980, the company Ajinomoto was first in purifying and immobilizing the enzyme that hydrolyzes hydantoins to optically pure amino acids. The mechanism was not understood at that time and they noted that more than one enzyme might be involved. Thereafter, several patent applications reported the production of



Scheme 3.3 Linear chemoenzymatic cascade for the dynamic kinetic resolution of secondary alcohols combining hydrolases with metal-catalyzed racemization

D- or L-*N*-carbamoyl-α-amino acids indicating that the reaction takes place in two steps [23–25]. In 1982, Olivieri et al. described in detail how D-amino acids are produced from the corresponding hydantoins catalyzed by three enzymes (Scheme 3.4) [26]. The enzymatic racemization of the inexpensive racemic hydantoin is crucial to achieve a complete conversion of the starting material to the product.

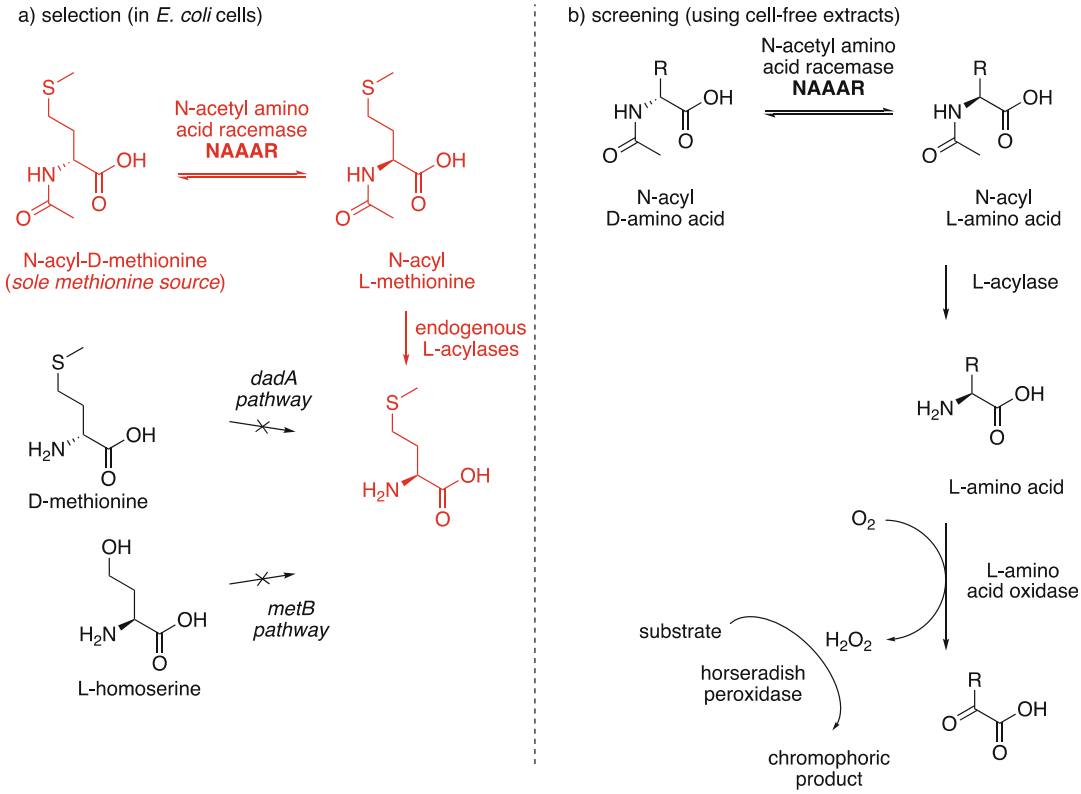
An alternative strategy for the synthesis of optically pure amino acids lies in the combination of N-acyl amino acid racemases (NAAAR) with stereoselective N-acetyl amino acid acylases (Scheme 3.5). Similar to the hydantoinase process, enzymes for the production of both enantiomers are available, which also allows the synthesis of the D-enantiomers. The bottleneck is often the low activity of the NAAAR. By performing a selection assay for racemizing activity, Campopiano et al. increased the specific activity of a microbial racemase [27, 28]. They used an *E. coli* strain with deleted ability to produce endogenous L-methionine. As the strain had several strictly L-selective acylases, feeding of N-acetyl-D-methionine allowed to couple racemizing activity to L-Met availability and hence growth. A variant with sixfold activity identified in the selection assay allowed the complete conversion of a 50 g/L solution of N-acetyl-DL-allylglycine into D-allylglycine within 18 h. For a more accurate characterization of the reaction in high-throughput format the same group also developed an in vitro assay that coupled formation of an L-amino acid to formation of hydrogen peroxide by use of an L-amino acid oxidase. The hydrogen peroxide could be easily detected by horseradish peroxide. This example underlines the importance of protein engineering to provide catalysts with optimal performance for their function in a cascade.



Scheme 3.4 Linear cascade for the production of optically pure amino acids by the hydrolysis of racemic D,L-5-mono substituted hydantoins. In the initial hydantoin molecule, R could be an aliphatic or aromatic residue, either substituted or unsubstituted. The scheme was adapted from Olivieri et al. [26]

3.2.2 Cascade Reactions for Cofactor Regeneration

Redox enzymes usually require expensive cofactors like NAD(P)H for the electron transfer



Scheme 3.5 Enzyme cascade reactions for selection (left) and screening (right) assays for amino acid racemases

from one molecule (reductant) to another molecule (oxidant). In organic synthesis, such biocatalysts can be employed either as isolated enzymes in combination with an appropriate cofactor recycling system or in whole cells, expressed in their original microorganism or recombinantly [29–32]. Generally, for such reactions various concepts have been developed that rely on electron supply via the metabolism of living heterotrophic cells. In synthetic applications, the nicotinamide cofactors are recycled by using energy-rich organic molecules as electron donors. In most cases, only a small fraction of the electrons provided by these sacrificial cosubstrates is utilized, resulting in a poor atom efficiency. Moreover, when glucose is supplied as sacrificial substrate for the recycling of NADPH, the often-used glucose dehydrogenase utilizes only a part of the electron pairs supplied by each glucose molecule. In contrast, many enzymatic in vitro cascade reactions have been developed in order to achieve an efficient regeneration of the required cosubstrates such as nicotinamide cofactors or nucleoside triphosphates. A vast array of two-enzyme cascades have been designed by coupling the activity of a cofactor regenerating enzyme to the product generating enzyme [1, 33]. By using this approach, the unfavorable reaction equilibrium is shifted by the second enzyme and thus drives the reaction to the desired product [34]. However, the necessity for sacrificial cosubstrates as well as an additional enzyme makes these cascade strategies less favorable. On the other hand, in vitro cascades can be designed in a way that they are redox neutral, i.e. the cofactor consumed in the first enzymatic step is regenerated by the second enzyme reaction. That offers the advantage that such redox-neutral cascades are simplified since no additional recycling system has to be supplemented.

One example for such redox-neutral cascade reaction has been recently reported by Turner and

coworkers [35]. In this example, an alcohol dehydrogenase (ADH) was employed to oxidize the alcohol starting material to the corresponding ketone. The cofactor NADH regenerated during the first step was used in the subsequent step, in which an amine dehydrogenase (AmDH) catalyzed a reductive amination of the intermediate ketone. Overall, the cascade is redox neutral and the hydrogen abstracted in the first step was reinstalled in the second in order to regenerate the NADH [36, 37]. However, it was necessary to run the cascade reaction in vitro with purified enzymes instead of cell-free extract since side reactions catalyzed by endogenous proteins in the crude cell preparation occurred. These side reactions sequestered and oxidized the NADH cofactor required for the second step and thus disrupted the hydrogen-borrowing nature of the cascade. Overall, moderate to excellent yields (30–91%) and high *ee* values (82 to >99%) could be achieved when running the reactions on preparative scale (100–126 mg of the ketone).

An example where the concept of a "mini" metabolic pathway was successfully applied is the synthesis of 6-aminohexanoic acid, which is the open-chain form of ε-caprolactam (the precursor for Nylon-6) reported by the Kroutil and coworkers [38]. In their work, a two-step system consisting of cascading enzyme sequences was build. In the first module an alcohol dehydrogenase (ADH) and a Baeyer–Villiger monooxygenase (BVMO) were combined to synthesize ε-caprolactone (ε-CL). This first reaction module can be considered as redox neutral, since the NADPH resulted from the ADH reaction was directly used by the BVMO. In the second module the produced ε-CL was further converted to 6-aminohexanoic acid via an in situ capping and uncapping step. The biocatalysts were either used as purified enzymes (in lyophilized form) or as freeze-dried cells containing overexpressed enzyme.

The same group reported a cascade for the amination of primary alcohols [39]. In this work, a thermostable ADH (ADH-hT) from *Bacillus stearothermophilus* was combined with an ω-transaminase (ω-TA) from *Chromobacterium violaceum* and an L-alanine dehydrogenase from *Bacillus subtilis* in order to recycle the amine donor in a strictly "non" buffered system. It could be shown that this cascade is redox neutral by applying a proper cofactor recycling system. Thus, the equilibrium of the cascade was shifted toward the product side by adding ammonia as cheap amine donor in excess. Under optimized conditions, they were able to fully convert 50 mM 1-hexanol to 1-hexylamine and 3-phenyl-1-propanol to 3-phenyl-1-propylamine. After further optimization of the reaction conditions, the authors were able to convert up to 50 mM octanediol or 1,10-decanediol to the corresponding diamines.

The direct oxidation of cycloalkanes to cycloalkanones employing a P450 monooxygenase and an ADH represents another successful enzyme cascade that is redox neutral or redox self-sufficient [40]. Key to success of this enzymatic cascade was the protein engineering of the P450 monooxygenase (BM3 from *Bacillus megaterium*). Two variants (19A12 and F87V) were identified as best candidates for cycloalkane hydroxylation. Although the initial proof-of-concept was successful, and the combination of the P450 variants with the ADH from *Lactobacillus kefir* resulted in the production of 6.3 mM of cyclooctanone, the low activity of the P450 monooxygenase turned out to be the bottleneck of this cascade reaction. Pennec et al. reported an extension of this cascade consisting of a cytochrome P450 monooxygenase for the initial oxyfunctionalization of a cycloalkane coupled with an alcohol dehydrogenase for ketone production and a Baeyer–Villiger monooxygenase for the subsequent conversion to the corresponding lactone [41]. By varying the cofactor dependence of the biocatalysts and the cofactor regeneration system, final product concentrations of around 3 g/L enantholactone from cycloheptane could be obtained within 12 h of reaction.

The combination of an ADH with a BVMO for the conversion of cyclohexanol to ε-CL has been strongly investigated since ε-CL is a valuable precursor for polymer synthesis [42–48]. The resulting biodegradable polymers, such as polycaprolactone, are of interest for applications

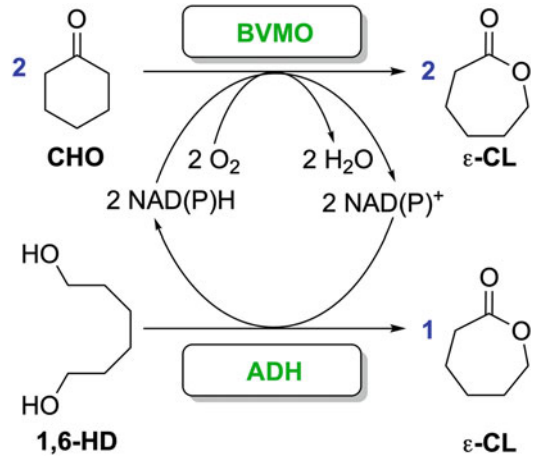
such as tissue engineering and drug delivery [49]. In all these examples, the ADH and the BVMO have been coupled in a redox-neutral fashion with respect to the nicotinamide cofactor. Advantageously, molecular oxygen is the only stoichiometric cosubstrate in the reaction. Two reports in 2013 showed the general feasibility of the cascade (94% conversion of 60 mM substrate in one case, 80% conversion of 10 mM in the other) [42, 43]. However, the cascade itself is limited since at high substrate concentrations, substrate as well as product inhibition has been observed. This limitation was addressed in a follow-up study by combining the ADH/BVMO reaction with lipase A from *Candida antarctica*, which converted the produced ε-CL in situ to oligomers of ε-CL and thereby removing it from the reaction mixture [45]. However, this cascade has been performed as hybrid in vivo/in vitro cascade.

Kara and coworkers developed a new class of redox-neutral reactions designated as convergent cascade involving a bi-substrate and a single product without intermediate formation was described [50]. This system involves a Baeyer–Villiger reaction catalyzed by a BVMO for the oxidation of cyclohexanone to ε-CL and an ADH for the simultaneous regeneration of NAD(P)H by oxidation of 1,6-hexanediol which serves as "double-smart cosubstrate" (Scheme 3.6).

In a follow-up study, the reaction parameters have been optimized by using design of experiments and an aqueous/organic biphasic reaction system [51]. These improvements led to an increase of the NADPH turnover number and the ADH by a factor of 50 and 10, respectively.

In order to gain as much redox equivalents as possible out of a single molecule for the regeneration of the cofactors, Kara et al. also reported a three-step oxidation of methanol to carbon dioxide (Scheme 3.7) [52].

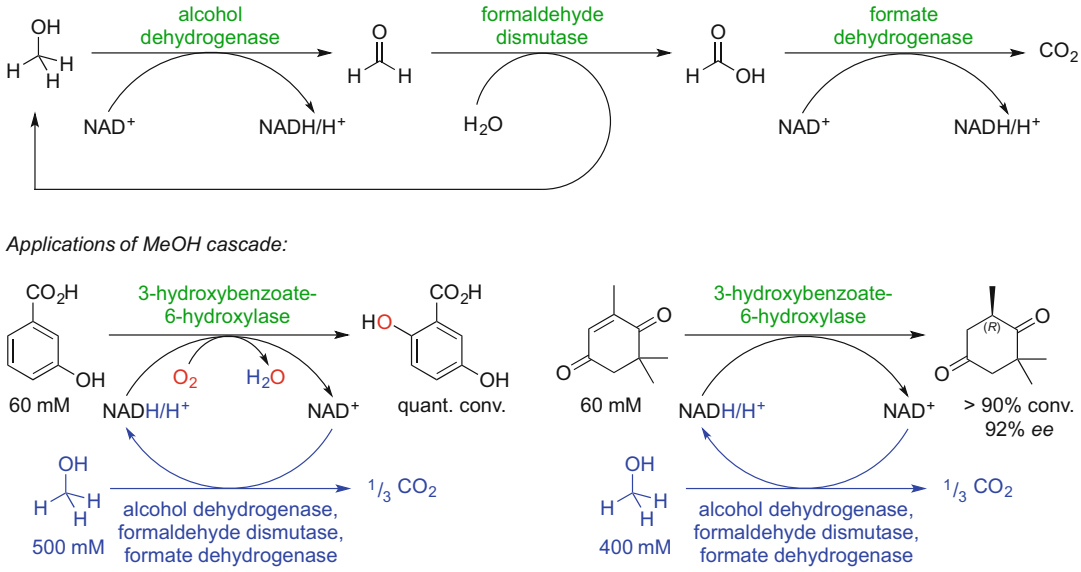
In this study, an ADH from yeast, a formate dehydrogenase (FDH) from *C. boidinii,* and a formaldehyde dismutase from *P. putida* were combined. The formaldehyde dismutase catalyzes the redox-neutral dismutation of formaldehyde into methanol and formic acid thereby providing a link between the ADH and FDH reactions. The



Scheme 3.6 "Double-smart cosubstrate" approach for the synthesis of ε-CL through a convergent cascade system. A BVMO-catalyzed oxidation of cyclohexanone (CHO) is coupled with an ADH-catalyzed oxidation of 1,6-hexanediol (1,6-HD) to ε-CL for the regeneration of two equivalents NAD(P)H. The scheme was adapted from Bornadel et al. [50]

overall cascade produces three molecules of NADH from NAD^+ for each molecule of methanol that is oxidized. This cofactor recycling system has been coupled on the one hand to an oxyfunctionalization reaction catalyzed by a monooxygenase and on the other hand to a C=C-bond reduction catalyzed by an ene-reductase. However, in both cases 500 mM MeOH must be added to convert up to 60 mM substrate. It has been assumed that this limitation can be attributed to the exceedingly high K_m value of the yeast ADH for methanol (>300 mM).

An intrinsic challenge for an efficient regeneration of the cofactors (recycling between 100 and 10^6 times) is given by the usually low long-term stability of the cofactors, even if a complete cascade is cofactor neutral and regenerates the cofactor during the course of its reaction [33, 53]. The stability of the cofactors can be influenced by the temperature, pH values, buffers, and salts of the reaction. Especially in terms of industrial applications, the pressure to perform enzymatic reactions at higher temperatures (50 °C and above) rises in the last years [54]. Possible solutions to solve this problem could be either the use of nicotinamide cofactor analogues

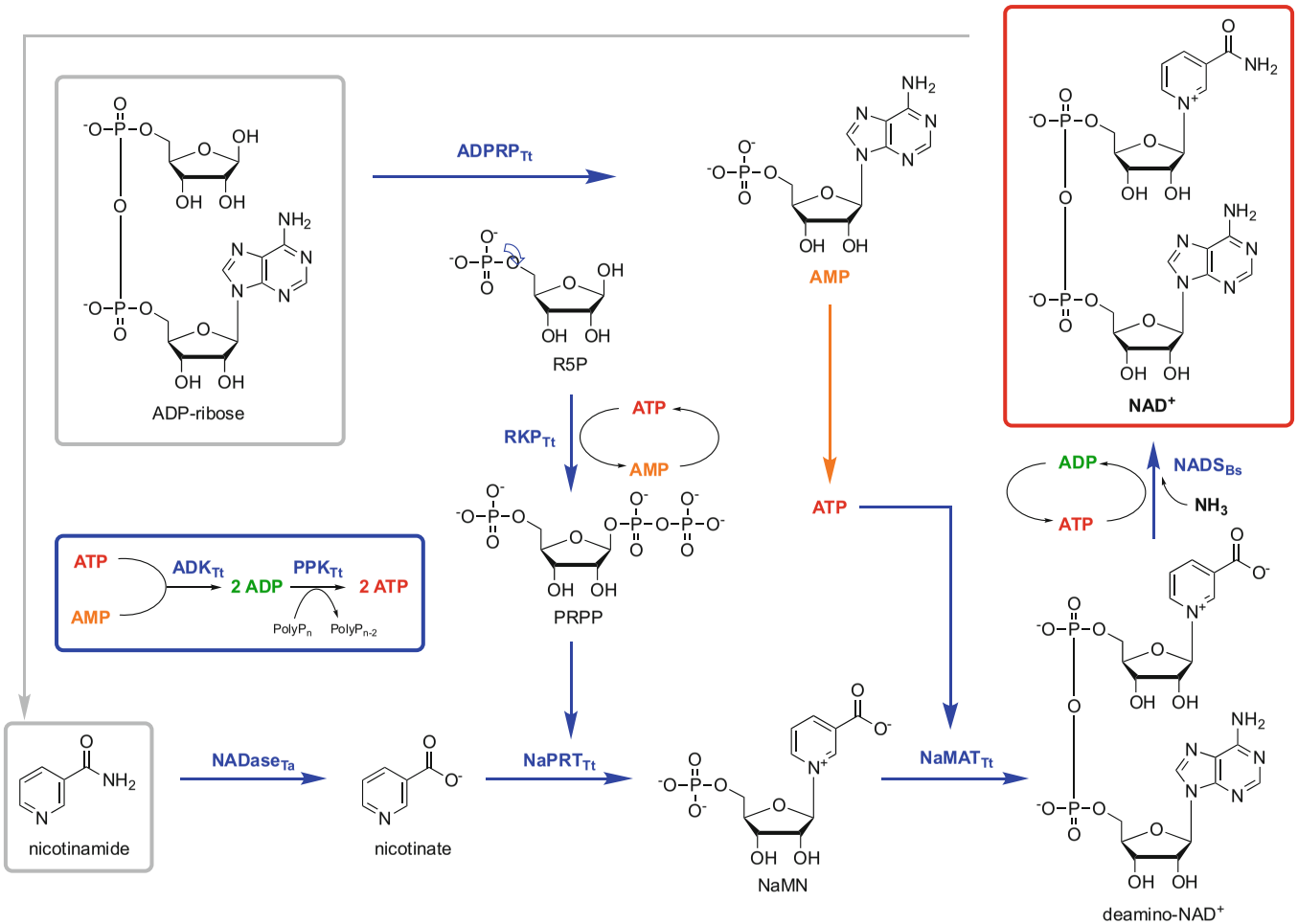


Scheme 3.7 Biocatalytic three-step cascade for the transformation of methanol to carbon dioxide for the regeneration of three equivalents NADH. The scheme was adapted from Kara et al. [52] and Schrittwieser et al. [1]

(so-called mimics), but their general applicability is limited since only a small fraction of enzymes is able to bind and convert them [55]. A promising cascade strategy to tackle this problem has been designed by Honda and coworkers, that combined eight different enzymes from thermophilic origins in order to construct an artificial metabolic pathway for the synthesis of NAD^+ from its degradation products [56]. With that approach, it was possible to keep the NAD^+ concentration constant for almost 15 h at a temperature of 60 °C.

Although this enzyme cascade replenishes the NAD^+ pool that is diminished by unwanted side reactions, there is still the problem that even perfectly balanced pathways loose reducing equivalents (e.g. NADH) over time by spontaneous oxidation [57]. Opgenorth et al. recently developed a molecular purge valve module as a strategy to overcome this problem (Scheme 3.8) [57]. This purge valve module was created in order to keep a balanced production and consumption of NADPH and NADH by applying two different pyruvate dehydrogenases that selectively accept either $NADP^+$ or NAD^+ in combination with an NADH oxidase that does not oxidize NADPH. With that system, high NADPH concentrations were maintained for reduction purposes while simultaneously allowing an independent carbon flux from pyruvate to polyhydroxybutyrate (PHB) or isoprene by purging of excess NADH.

Not only regeneration systems for nicotinamide cofactors have been studied intensively over the past couple of years, but also recycling approaches for adenosine-5′-triphosphate (ATP)-dependent reactions have gained increasing attention recently. ATP-dependent enzyme-catalyzed reactions can be widespread found in nature. Thus, ATP-dependent enzymes have an intrinsic potential for use in synthetic applications. Although regeneration systems for ATP starting from adenosine-5′-diphosphate (ADP) are in general available, certain limitations exist for in vitro applications [58]. Most available ATP regeneration systems start from ADP although methods for the regeneration of ATP from adenosine-5-′-monophosphate (AMP) have a high potential for cascade reactions. However, such systems are not well established yet. Those would enable ATP-dependent catalytic processes such as S-adenosylmethionine-dependent reactions or reactions where pyrophosphate (PP_i) is transferred to acceptor molecules. Most ATP



Scheme 3.8 In vitro enzyme cascade reaction for the salvage synthesis of NAD^+ from nicotinamide and ADP-ribose. Blue arrows: ATP regeneration reactions, which are shown in detail in the inner panel. *NaMATTt* Nicotinate mononucleotide adenylyltransferase, *NaPRTTt* nicotinate phosphoribosyltransferase, *ADPRPTt* ADP-ribose pyrophosphatase, *RPKTt* ribose-phosphate pyrophosphokinase, *ADKTt* adenylatkinase. The scheme was adapted from Honda et al. [56]

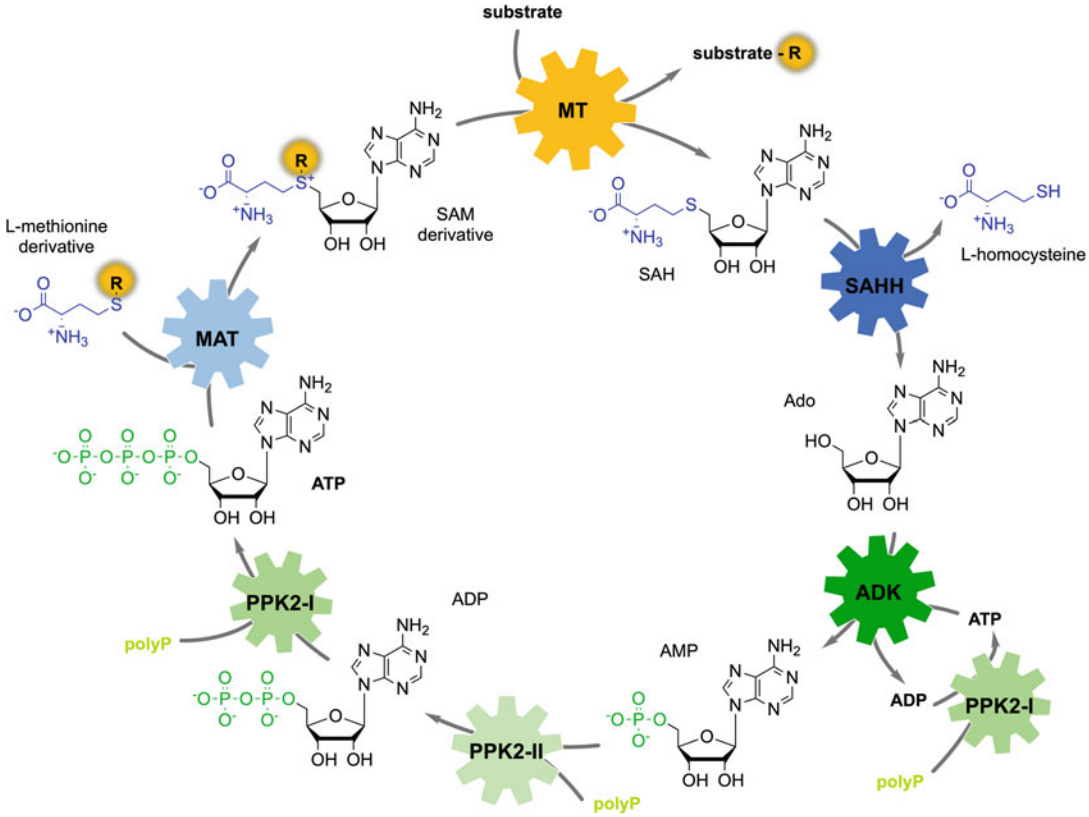
regeneration approaches rely on kinases starting from cheap raw materials. These enzymes usually transfer the γ-phosphate group of ATP or any other nucleotide to an acceptor molecule. The reverse reaction is commonly applied to phosphorylate ADP to yield ATP. Most important for an efficient regeneration approach based on these enzymes is the availability of a cheap and stable phosphate donor as well as the kinetic properties of the kinase. In order to create a recycling system starting from AMP, usually two or more enzymes are combined in a cascade. A very early example has been reported by Whitesides and coworkers that used a combination of adenylate kinase, adenosine kinase, and acetate kinase with acetyl phosphate as donor to achieve the triple phosphorylation of adenosine to ATP [59]. The system using an acetate kinase and a hexokinase or a glycerol kinase with acetyl phosphate as donor is one of the most frequently used examples for ATP regeneration in biocatalytic in vitro reactions [60–62]. Furthermore, pyruvate kinase and phosphoenolpyruvate (PEP) as phosphate donor can be employed for ATP regeneration [63, 64].

Andexer and coworkers reported an enzymatic reaction that combines the SAM-dependent methylation of several catechol derivatives with a coupled cyclic cascade that fuels the SAM-dependent O-methyltransferases (O-MTs) with the required cofactor ATP starting from the more stable SAM precursor adenosine (Scheme 3.9) [65]. The developed SAM regeneration cycle starts with the SAM-dependent alkylation of the substrate catalyzed by the O-MT resulting in the alkylated product as well as S-adenosylhomocysteine (SAH). The SAH is converted by a SAH hydroxylase, resulting in the formation of adenosine and the release of L-homocysteine. In subsequent steps, ATP was generated by a cascade reaction comprising adenosine kinase (ADK) from *Saccharomyces cerevisiae*, polyphosphate kinase (PPK2-II) from *Acinetobacter johnsonii* and polyphosphate kinase (PPK2-I) from *Sinorhizobium meliloti*. In order to drive the ATP formation and to shift the reaction equilibrium, an excess of polyphosphate and catalytic amounts of AMP (1:50 relative to the O-MT substrate) were added. Thus, to close the SAM regeneration cycle, a methionine adenosyltransferase (MAT) from *Escherichia coli* was used to catalyze the conversion of ATP to SAM using methionine as substrate. Overall, up to 25% conversion were achieved. This corresponds to a more than 10-fold regeneration of SAM and was observed for all tested methylation and ethylation reactions.

3.3 Examples of In Vitro Cascades from Industry

Biocatalysts are nowadays employed in a wide variety of industrial processes ranging from bulk chemical manufacture to fine chemical synthesis [66–71]. In particular, enzymes provide a powerful tool in order to produce enantiomerical pure compounds mainly through their high chemoselectivity, regioselectivity, and stereoselectivity [72]. The majority of these processes involve a single-step transformation catalyzed by one enzyme, followed by product isolation and purification. Industrial examples of de novo multi-step and multi-enzyme reactions being truly concurrent are still rare, but recent progress in cascade processes is paving the way for a greater industrial scope of the processes in the future [2]. However, a small number of cascade reactions have already been successfully implemented and reported in industrial laboratories [73]. One example involves a ketoreductase (KRED) for the synthesis of hydroxynitrile that is a key intermediate for atorvastatin using a multi-enzyme process.[69] This drug is a member of the statin family and lowers the cholesterol level by inhibiting the cholesterol synthesis in the liver. Atorvastatin is currently marketed by Pfizer under the trade name Lipitor® [69]. Codexis developed a two-step process consisting of three pre-evolved enzymatic steps, namely halohydrin dehalogenase (HHDH), glucose dehydrogenase (GDH), and KRED (Scheme 3.10).

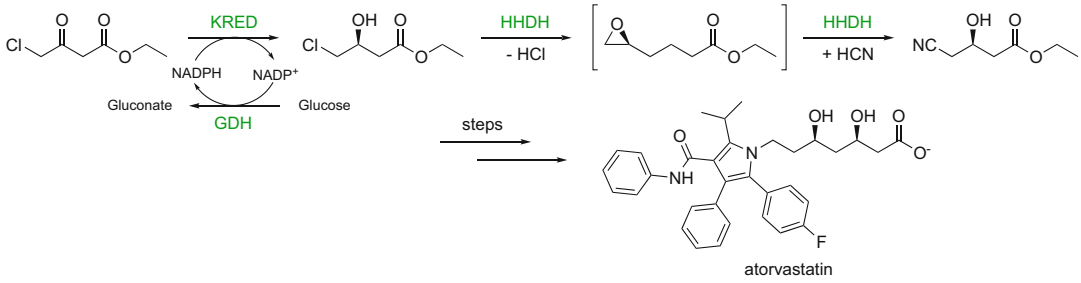
In the first step, the KRED catalyzes the reduction of ethyl-4-chloroacetoacetate that is coupled with the regeneration of the cofactor NADPH by the GDH. In a subsequent reaction catalyzed by



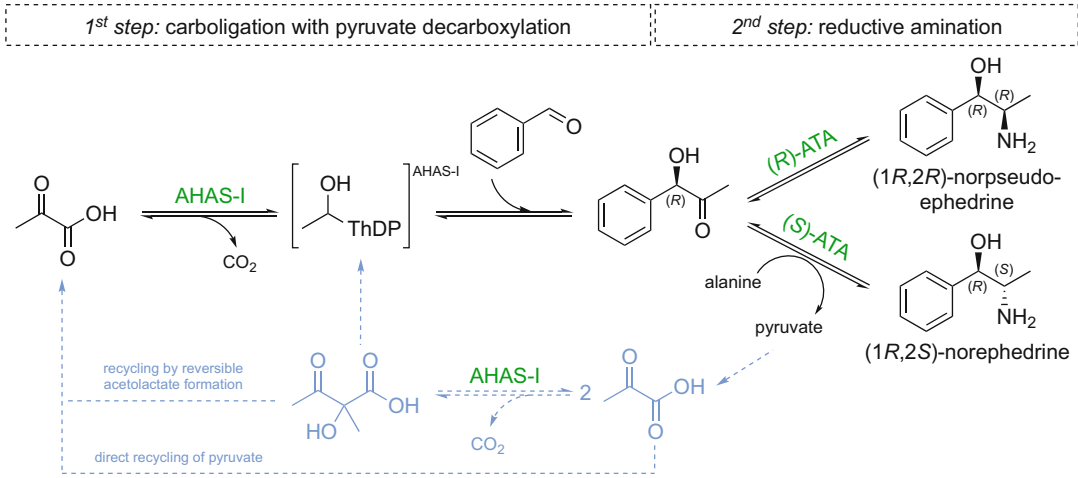
Scheme 3.9 The biocatalytic regeneration cycle of SAM. The enzyme-catalyzed alkylation combined with cofactor regeneration is powered by polyP and uses L-methionine (or a derivative) as alkyl donor. Methionine (or ethionine) and the corresponding substrate for the methyl transferase (MT) are added in stoichiometric amount, along with an excess of polyP and a catalytic amount of AMP as a precursor for the respective cofactor. The scheme was adapted from Mordhost et al. [65]

the HHDH, the product of the KRED is further converted to ethyl (*S*)-4-chloro-3-hydroxybutanoate that is used as a precursor for atorvastatin.

Due to the growing number of enzymes that can be utilized for versatile reactions to produce pharmaceuticals or fine chemicals, attempts at developing one-pot processes based on multi-enzyme reactions are increasing [1–3, 70, 74]. One-pot processes in general offer the advantage of high enantioselectivities while circumventing the need for multiple steps, thus being highly efficient. Especially amine transaminases (ATAs) have been used in a multitude of cascade reactions [75], and are mostly coupled to redox enzymes for cofactor regeneration. A recent example is an one-pot cascade comprising an ATA and a monoamine oxidase (MAO) for the synthesis of chiral 2,5-disubstituted pyrrolidines [76]. Another advantage of one-pot cascade reactions is represented by the possibility to start from simple, inexpensive, and achiral starting materials. The synthesis of nor-pseudoephedrine (NPE) and norephedrine (NE) has been recently demonstrated starting from simple materials such as benzaldehyde and pyruvate through a combination of an ATA and acetohydroxyacid synthase I (AHAS-I) (Scheme 3.11) [77]. By using an (*R*)- or (*S*)-ATA, the stereoisomers of NPE and NE were synthesized with high enantioselectivity (>99%). The by-product from



Scheme 3.10 Multi-enzyme in vitro cascade for the production of the hydroxynitrile intermediate for atorvastatin synthesis using engineered KRED, GDH, and HHDH. The scheme was adapted from Ma et al. [69]



Scheme 3.11 Synthesis of (1*R*,2*R*)-norpseudoephedrine and (1*R*,2*S*)-norephedrine, respectively, by an one-pot two-step cascade reaction combining acetohydroxyacid synthase I (AHAS-I) and a (*R*)-or (*S*)-selective amine transaminase (ATA) with internal pyruvate recycling. The scheme was adapted from Sehl et al. [77]

the ATA reaction (pyruvate) was recycled by the AHAS-I reaction. Moreover, a multi-enzyme network comprising an ADH, an ATA, and an alanine dehydrogenase (AlaDH) connected through redox-recycling has been reported [78].

Another unique example of the power of multi-enzymatic reactions is the commercial production of trehalose from starch. Trehalose, which is a disaccharide consisting of (α-1,1)-linked two glucose units with a relative sweetness of 45% compared to sucrose, while being more thermostable and having a wide pH-stability range compared to other saccharides. Moreover, trehalose does not undergo the Maillard reaction, which makes it an attractive material in the food industry [79]. In 1995, Murata and coworkers paved the way for the industrial production of trehalose by an enzymatic cascade reaction. The necessary enzymes, namely maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase) have been identified and isolated from the trehalose producing bacterium *Arthrobacter* sp. strain Q36. On the basis of this, the Hayashibara Company (Okayama, Japan) started the commercial production of trehalose from starch by using two additional enzymes (isoamylase and cyclodextrin glucanotransferase). The current production scale is assumed to be more than 30,000 tons/year [73].

Thanks to the power of synthetic biology, many patents have focused on optimizing this process by building recombinant plasmids for the co-expression of these three enzymes in one microbial host. Moreover, protein engineering enables further optimization of the biocatalysts by improving their activity, stability, stereoselectivity, and yield. Despite the highly motivating achievements that have been made for multi-enzymatic in vitro reactions, the industrial application of such systems has yet been limited to three biocatalysts in the cascading sequence. This can be mainly attributed to the limitations caused by the complexity of the process including the enzyme-purification procedures. However, the recent advances in genetic and enzyme engineering offer the potential to provide more convenient and less expensive approaches for the purification and the assembly of such multi-enzymatic artificial pathways in vitro [73].

3.4 Conclusion and Outlook

Enzyme cascade reactions have emerged as a widely used synthetic tool. Saving unit operations for the isolation and purification of intermediate products allows for tremendous savings in terms in cost, energy, and waste formation. As most enzymatic reactions require rather similar reaction conditions—they proceed in water and at moderate temperatures—the combination of enzymes in cascades is a generally applicable principle. Yet, the complexity of cascades as well as frequently encountered cross-reactivities and the still limited compatibility of the biocatalysts and their optimal reaction conditions have somewhat delayed the implementation of the concept. Cascade reactions have been routinely applied for redox cofactor regeneration and dynamic kinetic resolutions since decades. More recently, the application of cascades has been expanded to a wide range of different reaction types, and the number of industrial processes utilizing cascades is increasing. An intensive interdisciplinary collaboration between molecular biotechnology, biocatalysis, protein engineering, and process engineering facilitates overcoming the difficulties associated with cascades and is expected to pave the way towards a general application of the concept.

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Multi-Enzymatic Cascades In Vivo

4

Lydia Suchy and Florian Rudroff

Abstract

An immense number of chemical reactions are carried out simultaneously in living cells. Nature tackles the complexity by cascading assemblies of reactions in finely tuned metabolic networks. Multistep cascades in living organisms commonly function without separation of intermediates; concentrations of all reactants are kept low, which allows high selectivity and avoids by-product formation. Taking Nature as a model, application of cascade reactions in organic synthesis offers several advantages over the classical step-by-step approach. In this chapter, pros and cons of enzymatic cascades in living cells are described and the current status of the design and the application of them are highlighted.

Keywords

In vivo biotransformations · Biocatalysis · Enzymatic cascade reactions · Whole-cell biocatalysis · Artificial metabolic pathways

4.1 Introduction

4.1.1 Biocatalysis

Efficiency and sustainability currently represent the most relevant factors for the development of new catalysts. Biocatalysis still represents an underdeveloped branch in catalysis (see Chap. 2). In recent years the demand for the sustainable production of complex compounds has led to the development of environmentally friendly and efficient catalytic systems and has opened up the research in the field of biocatalysis. A biocatalyst can be defined as a natural catalyst (most commonly an enzyme) which is produced from renewable sources. These natural catalysts have been applied since many years in industrial processes, e.g. for the production of drugs, flavor and fragrance compounds, and polymers [1]. Furthermore, enzymes are being used more frequently as catalysts in organic synthesis [2–4]. When compared to conventional metal- and organocatalysis in chemistry, biocatalysis offers a more attractive alternative, due to milder reaction conditions as organic solvents can be avoided and no pressure or high temperature have to be applied. Additionally, synthetic transformations yet unknown by chemical means are possible (Chap. 2). Thereby, it reduces pollution, costs and creates greater sustainability [5]. The main advantage of a biocatalyst is that it is chemo-, regio-, and stereoselective; however, chemoselectivity may be also considered a

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negative aspect as enzymes tend to have a limited substrate scope. Biocatalysis often allows for the shortening of synthetic routes by avoiding protecting group manipulations, chiral resolutions, by-product formation, etc. [6, 7]. Another positive aspect is that there is a big diversity in possible enzymes. Advances in DNA manipulation technologies and in bioinformatics (e.g. DNA synthesis and high-throughput screenings) have led to the discovery of new enzymes and redesign of biocatalysts [8]. Therefore, several limitations of using enzymes as catalysts can be overcome by the development of engineering methods [9].

4.1.2 Biocatalytic Cascade Reactions

Shortening of synthetic routes by application of multistep reactions and thereby improving the efficiency of a process is considered the "holy grail" in chemocatalysis. Unfortunately, this is often difficult to realize due to the incompatibility of reaction conditions [10]. However, developments in biocatalysis have led to the advancement from single step reactions of simple molecules to multi-enzyme cascades producing a variety of compounds [11]. These synthetic enzyme cascade pathways are inspired by Nature and designed by combining biocatalytic reactions that are metabolically unrelated in Nature in a one-pot fashion. Advantages are the reduction of time because reaction intermediates do not have to be isolated and purified (as already mentioned in Chap. 3) and consequently also the reduction of waste as the use of organic solvents can be minimized. This ultimately results in decreased production costs [12].

Another positive aspect by applying cascade-type reactions is the shift of reaction equilibria to the product side by implementation of an irreversible intermediate or last reaction step [7]. Thereby, the overall reaction is energetically favored and overall yield is increased. In this way, (enzymatic) cascades offer the possibility to directly transform low-value chemicals into highly valuable products (e.g. chiral compounds) due to their excellent chemo-, regio-, and stereoselectivies [13].

In practice, either isolated enzymes can be used for the realization of a biocatalytic cascade reaction (in vitro, see Chap. 3) or a microorganism which overproduces the desired enzymes can be used as host cell (in vivo). A third option would be a combination of these two approaches in a hybrid system (Fig. 4.1). Additionally, the combination of enzymatic and chemical reaction steps in a cascade-type reaction represents another interesting opportunity for selected reactions (see Chap. 5).

4.1.3 In Vivo Cascades

Living organisms use enzymatic cascade-type transformations for the building up of highly complex molecules. Metabolic networks in an organism are assembled by such enzyme cascades in order to assure growth and survival [10]. Glycolysis represents a typical example of such a multistep transformation in which glucose is converted to pyruvate. In *E. coli* and many other organisms, pyruvate is a major metabolic intermediate linking carbohydrate catabolism to various biosynthetic routes and is being produced in an ancient, highly efficient and highly regulated 10-step route from glucose (Scheme 4.1). The flux through the pathway is adjusted in response to conditions both inside and outside the cell.

For the conception of artificial cascades, Nature was taken as a model and enzymatic cascades were implemented as an alternative to step-by-step approaches in chemical synthesis [10]. For the generation of valuable chemicals from simple precursors by an enzymatic cascade, either the metabolic network of an organism can be engineered, or heterologous enzymes can be incorporated and expressed in a host organism. Thus, it can be distinguished between cascade biotransformations in vivo and a fermentation/metabolic engineering approach. The difference between the two approaches is that biotransformations in vivo are unlinked to the cellular metabolism. The introduced substrate is only used for the desired product synthesis and has

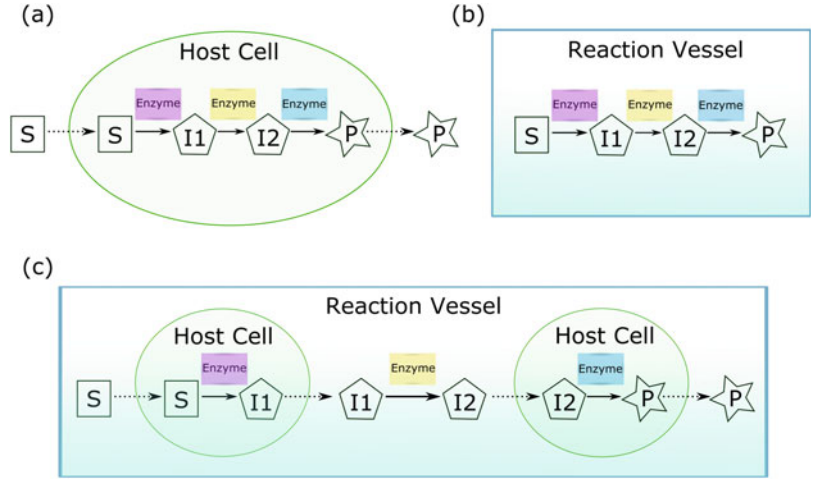


Fig. 4.1 Schematic representation of (**a**) in vivo, (**b**) in vitro, and (**c**) hybrid enzyme cascades. Substrate *S* is converted by different enzyme biocatalysts to the product *P* via the intermediates *I*1 and *I*2

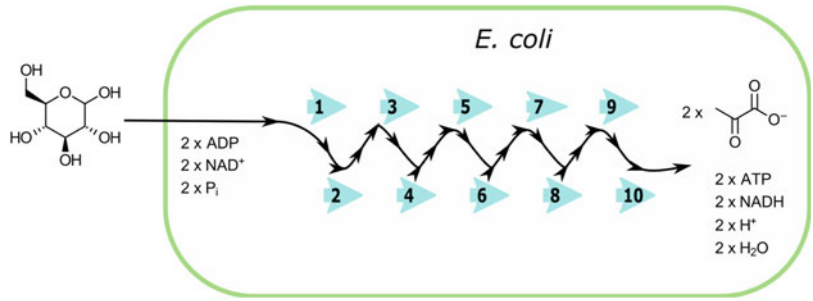
no other role in the cell [14]. Thereby, in vivo cascade biotransformations circumvent eventual defense mechanisms and a downregulation of the process by the host metabolic network. They are realized by expression of multiple recombinant proteins. For this purpose, the respective recombinant DNA construct has to be genetically engineered in a single strain of the host organism. Thus, the cascade biotransformation is performed as whole-cell biocatalytic system [13]. However, the borders which define in vivo enzymatic cascades cannot be drawn precisely. Therefore, whole-cell enzymatic cascades can be regarded as a combination of biocatalysis with synthetic biology, metabolic-, and protein engineering [10].

4.2 Enzymatic Cascades In Vivo: Advantages and Challenges

4.2.1 Comparison to In Vitro Systems

As already mentioned, enzymatic cascades can be put into action either by an in vitro or an in vivo approach. Both alternatives come with their advantages and disadvantages.

Employing in vitro/cell-free systems, reaction conditions can be manipulated and changed faster. It is easier to balance enzyme activities, simply by adjusting the amount of added enzyme. As a result, they often yield higher productivity through higher concentration of biocatalyst. Product purification is simpler and the application of purified enzymes avoids any complications that arise from the complex metabolic pathways operating in living whole-cells [5, 15]. On the other hand, in vitro systems require the laborious



Scheme 4.1 Simplified glycolysis pathway in *E. coli*. The cascade consists of ten catalytic steps (only three of them being irreversible)

preparation of the enzymes, which involves the (heterologous) expression, isolation, and purification from host cells. Moreover, many biocatalysts require cofactors, which have to be added in stoichiometric amounts or complemented with a suitable recycling system. As the enzyme is not in its natural environment, it is strongly affected by solvent, pH, and temperature and thereby its stability or activity can be diminished. Consequently, the most important downside of in vitro enzymatic cascades is the costs involved.

Whole-cell/in vivo biocatalysis bypasses these preparation steps and cells can be cultivated at low cost. Moreover, the microbial host metabolism supplies and recycles expensive coenzymes/cofactors (e.g. $NADP^+$/NADPH) and the biocatalyst proves to be more stable as the host cellular environment protects the enzymes from harsh reaction conditions. But, the realization of enzymatic cascades in vivo is far less explored than applications in vitro. In the whole-cell the balancing of enzyme activities is more difficult than adjusting protein amounts in a cell-free system. Promoters or expression systems have to be altered on the genetic level. Another problematic topic arises due to toxicity of the cascade substrate, intermediate, or product. This can lead to side reactions and thereby lower productivity of the cascade. Besides, unwanted interactions with the host background can occur due to competing reactions in the native metabolism. Even though the cellular environment and the cell membrane stabilize and protect the enzyme from potentially harmful reaction conditions, it can also act as a mass transport barrier making product isolation cumbersome. As a result of the mentioned complications, the scale-up in vivo can be difficult when comparing to in vitro systems.

All in all, in vivo biocatalysis is more cost-effective but requires much longer lead times. Therefore, it seems to have no advantage when considering one-step biotransformations. While in vitro systems seem to be easier to manipulate and offer highest flexibility and immediate control, whole-cell systems offer some benefits when multiple enzymes are combined in a cascade-type reaction [10, 16]. The whole-cell puts the reactants into closer proximity and immobilization techniques can further enhance the stability or the performance of the reaction pathway [1, 17]. As DNA synthesis, genetic engineering and development of high-throughput cloning techniques get easier and cheaper, new possibilities for pathway tuning become apparent.

4.2.2 What to Consider When Designing a Biocatalytic Cascade in the Whole-Cell

There are many factors which have to be considered during the implementation of a biocatalytic cascade in the whole-cell [13]. Apart from the design of the cascade pathway itself, either by a retrosynthetic approach (see Chap. 2) or by a "forward design" starting from an initial substrate, the thermodynamic feasibility of the overall cascade and especially the last reaction step has to be considered. The next aspect to consider is of course the host for the realization of the in vivo cascade reaction. Prominent organisms of choice which are "easy-to-use" are the bacterium *Escherichia coli* (*E. coli*) because of its rapid growth on inexpensive nutrients and its well-researched genetics [18]. Moreover, *Saccharomyces cerevisiae* (*S. cerevisiae*), *Pichia pastoris*, *Corynebacterium glutamicum*, *Bacillus subtilis,* and *Pseudomonas* sp. are examples of microorganisms that have been used successfully as host for in vivo biotransformations [19–22]. Cyanobacteria represent a promising new host and the "greenest" of all approaches for whole-cell biocatalysis, because they metabolize CO_2 as sole carbon-source and produce energy by photosynthesis [23, 24]. Moreover, they provide a high supply of cofactors (e.g. NADPH) and molecular oxygen (enabling, e.g. oxyfunctionalization reactions) [25, 26]. After selection of the host organism, the strain which co-expresses the desired enzymes has to be designed by genetic engineering. This is usually done by constructing expression vectors using advanced cloning techniques as, e.g. Gibson assembly or sequence-and ligation-independent cloning (SLIC) [27]. The respective genes can be constructed as

polycistronic operon (with a single promoter and terminator for all co-expressed genes), as multiple monocistronic operons (with individual promoters and terminators) or in a pseudo-operon configuration (with individual promoters for each gene but just one overall terminator). Moreover, genes can be knocked out or integrated into the genome of the host, e.g. by using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology [28]. Usually, resting cells which are metabolically active but non-growing are used for the realization of biotransformations in vivo. This offers several advantages: Since the cells are washed, undesired growth metabolites and nutrients are removed. Moreover, carbon and energy sources are not used for growth anymore, enabling higher productivity of the artificial reaction pathway [14]. On the other hand, fermentation processes use growing cells which combine enzyme expression and biotransformation in one step, but often suffer from inhibitory effects, e.g. by substrate or product toxicity [13, 29].

4.2.3 Challenges: What Can Go Wrong

A challenge for both, in vitro and in vivo enzymatic cascades arises by potential *cross-reactivity* of the implemented enzymes and problems in the compatibility of the biocatalysts and their preferred *reaction conditions* (see Chap. 3). Differences in activity and stability of enzymes (depending on solvent, pH, temperature, etc.) can complicate the search for suitable reaction conditions.

Probably the most prominent problem arising during the implementation of an enzymatic cascade in vivo is the expression of the pathway enzymes (Fig. 4.1). The active expression as well as *balanced enzyme stoichiometry* is equally important for a functioning synthetic pathway. Balancing the protein expression can even be of greater importance than overexpression itself. The target enzymes for a de novo pathway must be individually produced or co-expressed in the host in sufficient amounts balanced for individual activities. If the enzyme stoichiometry is unbalanced, (toxic) cascade intermediates can accumulate and the overall flux through the cascade is reduced [30].

One main challenge of recombinant protein expression is that the introduction of metabolically nonrelated enzymes might interfere with the metabolic host network (resulting in dead-end metabolites) which on the other hand might affect the production of the recombinant proteins themselves [16, 31, 32]. The expression of multiple recombinant proteins from individual plasmids can lead to a high *metabolic load/burden*, which induces *stress responses* in the host leading to a reduction of productivity in the synthetic pathway [30]. Additionally, enzymes in the host can react to the artificially introduced ones which can lead to *cross-inhibition*, cross-linking of protein domains, mutagenesis, damaging of other enzymes, etc. [7].

Another issue is the *reactivity/toxicity* of reaction substrates, intermediates, or products (e.g. activated alkenes with a conjugated carbonyl group or aldehydes). Unbalanced heterologous enzyme production or different enzyme kinetics can lead to the leaking or accumulation of potentially reactive/toxic pathway intermediates. The metabolic background of the host responds to accumulated toxic intermediates that can lead to *side reactions* and the formation of byproducts [33, 34]. Therefore, an optimal carbon flux through the cascade is of high importance.

Another relevant aspect is the *availability* of essential pathway components as *cofactors/coenzymes*. The addition of glucose to the reaction medium to stimulate the metabolism of the host can be sufficient for cofactor generation (as already mentioned in Chap. 3). However, metabolic enzymes compete with pathway enzymes for these cofactors. Many whole-cell enzymatic cascades employing redox enzymes are performed under resting-cell conditions. On the one hand, coenzymes are not produced in a sufficient amount any more, leading to a loss of productivity [7]. On the other hand, the cell does not have to invest the energy (e.g. ATP) and metabolic resources (e.g. NAD(P)H) for the production of biomass. In order to overcome resulting limitations in cofactor availability,

recycling in vivo will be important for the scale-up of a process [35].

Moreover, as already mentioned in the previous subchapter, the transport of the substrate into the cell and of the product out of the cell can be difficult as the *cell membrane often serves as a barrier* (Fig. 4.2).

4.2.4 Improvements: How to Tackle these Challenges

Reaction engineering offers a general possibility to improve a biocatalytic process. This can be achieved by modification of temperature, pH, the type of buffer, reagents and co-solvents or the substrate loading.

Balancing of *enzyme stoichiometry* is of great importance for the success of a synthetic enzyme cascade in vivo. Either the transcription or translation of the enzymes can be modulated by implementation of different regulatory elements, e.g. by altering the sequences of promoter or ribosome binding sites (as an example, tools can be based on the BioBricks principles [36, 37]). Apart from promoter and ribosome binding sites, nearby sequences also can influence target gene expression, which is referred to as context dependency [38]. A second option to balance expression levels is the adjustment of the copy number of the genes, either by using plasmids with different copy numbers or by gene duplication [13]. A third option would be the containment of the components of the enzymatic cascade within distinct whole-cells in a mixed-culture approach [39]. This can facilitate control over the expression of the individual enzymes and reduce the metabolic burden on the respective cells, but on the other hand, mass transfer limitations are enhanced due to the additional cell membrane barriers [15].

As already mentioned, the introduction of metabolically nonrelated enzymes can lead to interference with the host background and thereby to *unwanted side reactions*. When considering cellular stress in general, there are repair mechanisms which play a role in the survival of stressed cells and therefore could be useful for the optimization of cascades in living cells [40, 41]. Moreover, the metabolic background can be reduced by creating so-called minimal genomes which include only the genes essential for life [42]. In order to have a maximum flow through the cascade, side reactions of reactive intermediates need to be avoided [43]. Low productivity represents a big issue for the realization of industrially convenient processes. Flux balance analysis in combination with metabolic flux analysis helps to create "whole-cell biocatalysts by design" by predicting the carbon flow and identification of putative bottlenecks [29]. Redirecting of the carbon flux through the synthetic cascade and thereby eliminating unwanted background reactions can be done by strain engineering, incorporation of additional enzymes, or reaction engineering [30, 34, 44]. With increasing knowledge about metabolic networks in host organisms, strategies have been developed to *remove target genes* from the genome in order to inhibit unwanted side reactions (e.g. by rational knock-out of genes) [30]. As an example served an engineered *E. coli* strain which tolerated the accumulation of aromatic aldehydes through the knock-out of six ketoreductases, engineered by Kunjapur et al. [33]. Alternatively, carbon fluxes can be rerouted by the *introduction of a reversing enzymatic activity*. An example is given by Bayer et al. by the equilibration of the formation of toxic aldehyde species using enzymes with opposing functional group transformation activity. The combination of the enzymes alcohol dehydrogenase and carboxylic acid reductase antagonizes the respective reduction and oxidation of the reactive aldehyde species by the host background [30, 34]. *Spatial organization* represents another possibility to decrease side reactions of unstable/toxic intermediates, as proximity of the pathway enzymes can enhance the flux through the cascade by reducing the diffusion of intermediates [45]. This can offer a possibility to increase productivity. Enzymes can be co-localized inside a cell by linkers, protein scaffolds or by separation from the cellular environment into microcompartments such as cellular organelles (e.g. peroxisomes, carboxysomes, mitochondria, etc.) or artificial compartments [13, 46–48].

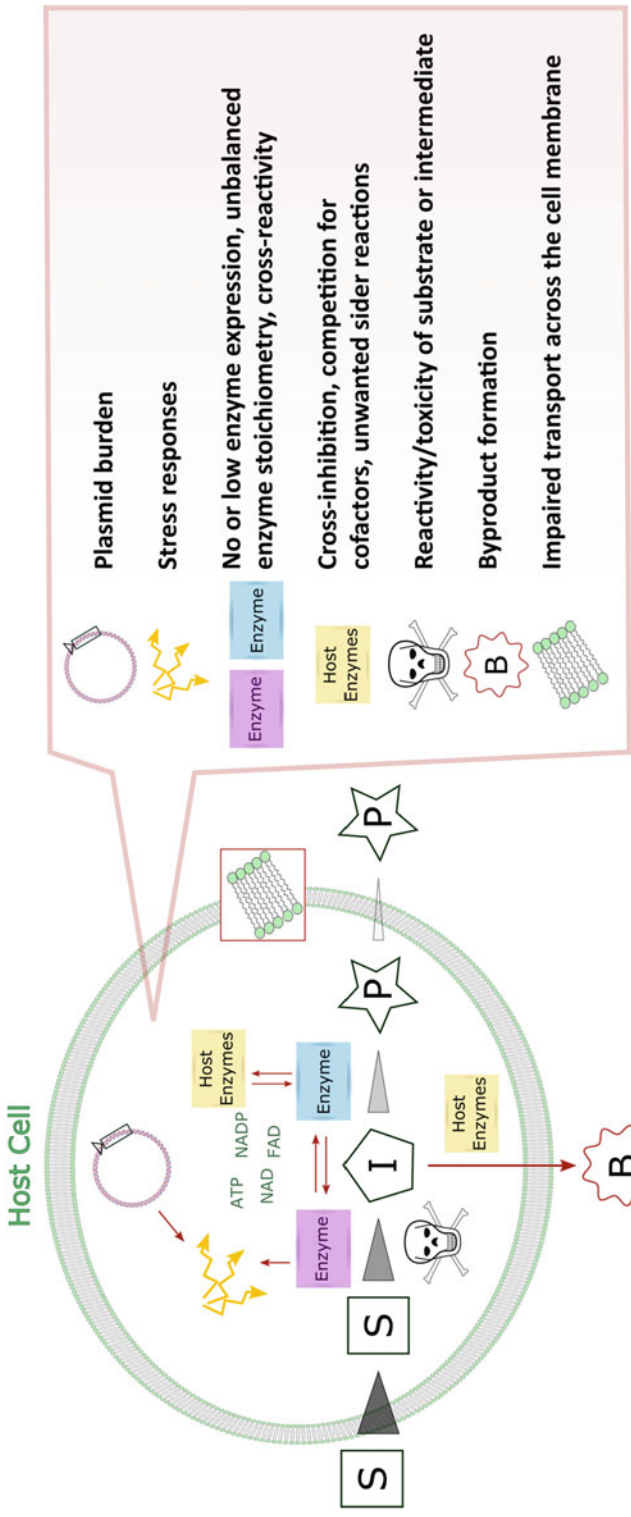


Fig. 4.2 Challenges in a whole-cell biocatalytic cascades. Substrate S is converted via the intermediate I to the product P or the byproduct B

Cofactors/coenzymes which are not self-regenerating *need to be regenerated* in order to keep up high productivity. For oxidoreductase-catalyzed enzymatic cascades, in situ regeneration of the cofactor by incorporation of additional cofactor recycling enzymes is often necessary. Moreover, knock-out of reactions which compete for the same cofactors can be applied. The development of self-sufficient redox systems, in which oxidizing and reducing reactions are coupled, offers an elegant alternative [29]. An example of a self-sufficient redox conversion of alcohols to the corresponding amines was given by Klatte et al. [49, 50].

To improve *substrate uptake/product export*, surfactants can be added or membrane transporters/porins can be introduced [30, 51, 52].

The efficiency of synthetic enzymatic cascades can be altered by engineering of the enzymes (directed evolution), the recombinant cells (genome engineering), or the reaction conditions (process engineering) [13]. Progress in technologies such as genomics, metagenomics, quantitative proteomics, metabolic engineering, and synthetic biology provides tools to manipulate microbial systems in order to allow the engineering of complex cascades [5].

4.3 Examples for In Vivo Cascades on the Laboratory Scale

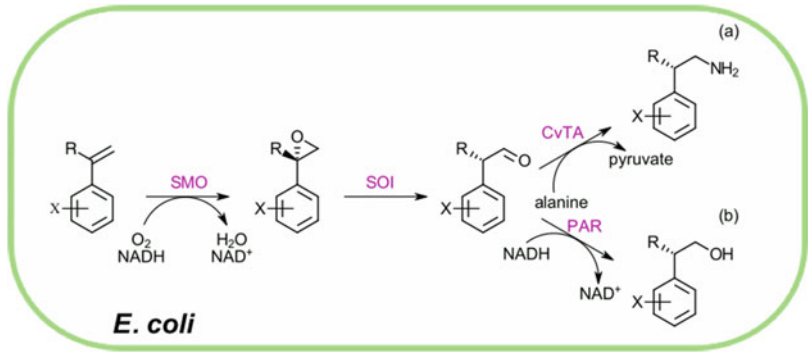
The recent development of whole-cell cascade biotransformations enabled the synthesis of a wide range of bulk as well as fine chemicals. Especially the opportunity to synthesize enantiopure chiral chemicals has encouraged researches to develop new multi-enzymatic routes. A variety of examples was recently summarized by Wu et al. [13]

An example for a 3-step cascade was developed by Wu et al. [53] For a formal anti-Markovnikov hydroamination, styrene monooxygenase SMO and styrene oxide isomerase SOI (from *Pseudomonas* sp.) were combined with an ω-transaminase CvTA (from *Chromobacterium violaceum* and *B. subtilis*) in *E. coli* to yield phenethylamines. The cascade enzymes were co-expressed with alanine dehydrogenase AlaDH (from *B. subtilis*) for pyruvate recycling. For the respective anti-Markovnikov hydration, the enzymes SMO and SOI were combined with a phenylacetaldehyde reductase PAR (from *Solanum lycopersicum*) obtaining phenethyl alcohols (Scheme 4.2). Yields for most substrates were higher than 80% (up to 99%) with anti-Markovnikov selectivity of >99:1.

Alcohols serve as a perfect starting material for the synthesis of many value-added chemicals. In this regard, Bayer et al. recently developed a 2-step cascade combining the oxidation of an alcohol by alcohol dehydrogenase AlkJ (from *Pseudomonas putida, P. putida*) and the subsequent carboligation by aldolase Fsa1 (from *E. coli*) to obtain chiral α-hydroxylketones [34]. To counteract the oxidation of the toxic aldehyde intermediate to the corresponding acid by host enzymes, a third enzyme NiCAR, a carboxylic acid reductase (from *Nocardia iowensis*) was incorporated into the pathway (Scheme 4.3). *E. coli* resting cells co-expressing these enzymes (and additionally phosphopantetheinyl transferase for the posttranslational modification of NiCAR) performed this enzymatic cascade with a yield of 70%.

The synthesis of enantiomerically pure chiral amines is of great interest for the synthesis of various bioactive compounds. In this regard, Both et al. reported a stereoselective C-H amination via a 3-step enzymatic cascade (Scheme 4.4) [43]. An engineered P450 monooxygenase (Y96F), *R*- and *S*-selective alcohol dehydrogenases *Lb*RADH (from *Lactobacillus brevis*) and *Re*SADH (from *Rhodococcus erythropolis*), and an ω-transaminase ATA117 (from *Arthrobacter* sp.) were heterologously expressed in *E. coli* in order to convert 4-substituted ethylbenzene into amines via the alcohol and ketone intermediates (with overall yields up to 26%). Apart from the substrate and the amine donor isopropyl amine, no additional cofactors had to be supplied.

An enzymatic 3-step cascade has been developed as a hybrid in vivo/in vitro system by France et al. (already mentioned in Chap. 3) [54]. First,

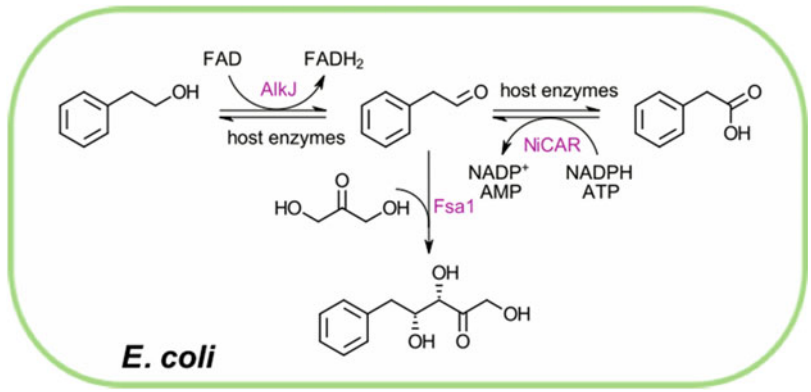


Scheme 4.2 Formal anti-Markovnikov hydroamination (**a**) and hydration (**b**) of styrenes [53]

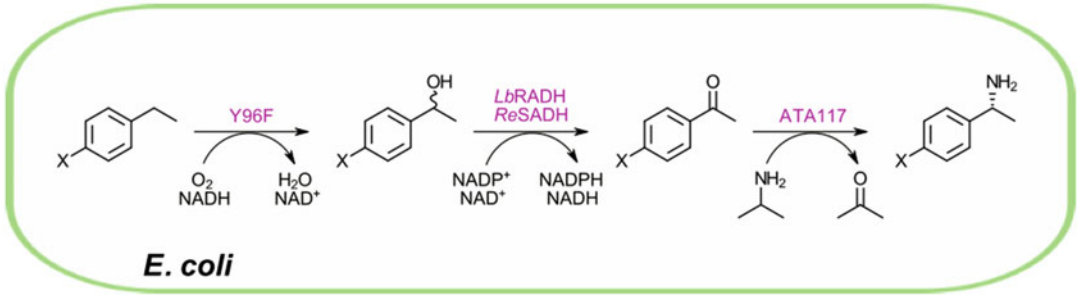
keto acids were reduced to keto-aldehydes by a carboxylic acid reductase CAR (from *Mycobacterium marinum*). Second, the amination of aldehydes and subsequent reduction of cyclic imines were performed by an ω-Transaminase ATA (from Codexis) and imine reductase IRED (from *Streptomyces* sp.). Thereafter, Heptworth et al. transferred the cascade into a single whole-cell version using *E. coli* as host organism (Scheme 4.5) [55]. In this way, the preparation of the catalyst could be simplified and the addition of supplementary cofactors (NAD^+, pyridoxal phosphate and enzymes lactate dehydrogenase and glucose dehydrogenase) could be avoided. Whereas cofactor recycling promotes the transamination step in the hybrid cascade, an excess of amine donor was applied to shift the equilibrium in the whole-cell approach.

Oxyfunctionalization (hydroxylation and consequent oxidation) of terpenes represents another important cascade step for the synthesis of oxygenated derivatives useful in the production of, e.g. pharmaceuticals or flavors. Oberleitner et al. developed an example for the utilization of limonene as waste product from orange peel as starting material in a mixed-culture approach (Scheme 4.6). Limonene was converted to a chiral carvolactone, a precursor for thermoplastic polyesters using *P. putida* cells expressing a cumene dioxygenase CumDO (from *P. putida*) and *E. coli* cells co-expressing an alcohol dehydrogenase (RR-ADH), an enoate reductase (XenB), and a Baeyer–Villiger monooxygenase CHMO (from *Rhodococcus ruber*, *P. putida,* and *Acinetobacter calcoaceticus,* respectively) [39]. Up to 6.3 mg carvolactone per g orange peel (29% yield over all 4 steps) could be produced.

Recently, a D-carbamoylase was identified by Liu et al. for an efficient way to produce bulky amino acids as, e.g. D-tryptophan via the previously developed hydantoinase process (Scheme 4.7, already mentioned in Chap. 3) [56]. Nozaki



Scheme 4.3 Redox equilibrium and subsequent carboligation yielding a chiral α-hydroxylketone [34]



Scheme 4.4 4-Step enzymatic cascade yielding enantiomerically pure amines [43]

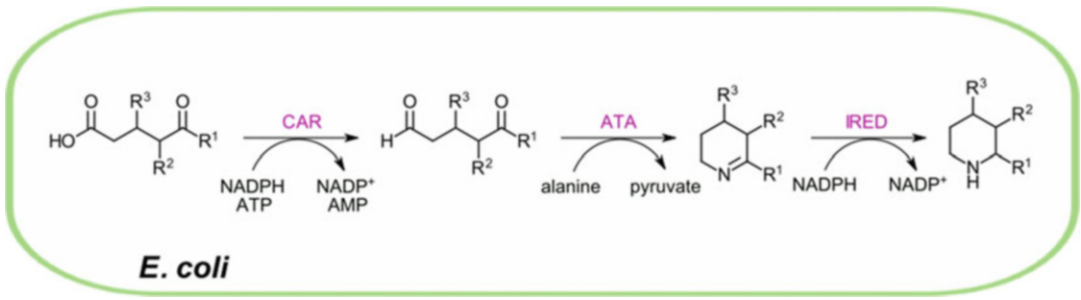
et al. first reported the application of this process in a single whole-cell by co-expressing the enzymes hydantoin racemase (from *Microbacterium liquefaciens*), D-hydantoinase, and D-carbamoylase (both from *Flavobacterium*) in *E. coli* [57]. The process produces enantiopure D-amino acids obtaining up to 98% yield.

4.4 Examples from Metabolic Engineering: Industrially Applicable Cell Factories

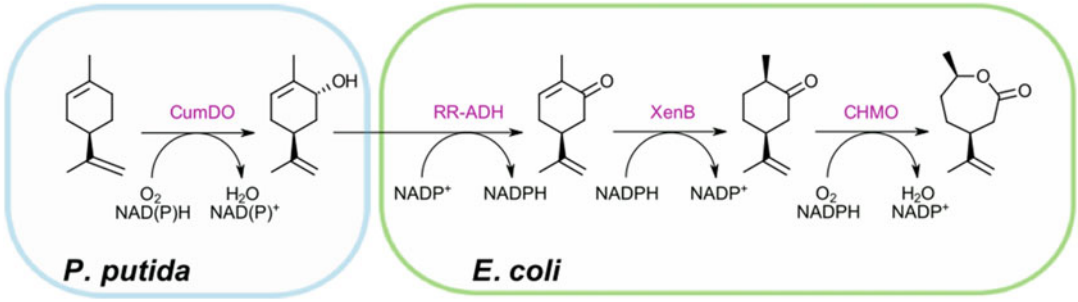
Biocatalysts are being used more and more in industrial processes ranging from bulk chemical manufacture to fine chemical synthesis. High chemoselectivity, regioselectivity, and stereoselectivity are some of their beneficial attributes. However, industrial multi-enzyme reactions are still rare. A prominent example for a multi-enzyme cascade in vitro is the synthesis of a precursor of the drug Atorvastatin (see Chap. 3) [58]. Biotransformation processes need to be efficient to comply with expected standards in industry. Concerning cascade transformations in vivo, low productivity is still an obstacle which has to be overcome in order to reach the potential for industrial processes [30]. The biggest challenge for the applicability in industry is to maintain cell viability while providing efficiently the desired synthesis product. Through the challenges during the development of enzymatic cascades in vivo mentioned above, it becomes clear that it is very difficult to fulfill industrial demands.

As mentioned in the introduction, metabolic engineering can be seen as related field to in vivo synthetic enzyme biotransformations. In this regard, microbes are promising hosts for the industrial production of, e.g. bulk chemical building blocks and biofuels (Scheme 4.8). However, here again limitations mentioned above prevent their success.

One example for the renewable production of chemicals and fuels was given by Zhou et al. who reconstructed efficient pathways for conversion of fatty acids to alkanes (titer of 0.8 mg/L) and



Scheme 4.5 3-Step conversion of keto acids to substituted cyclic imines [54, 55]



Scheme 4.6 Conversion of limonene to a chiral carvolactone utilizing orange peel as starting material [39]

fatty alcohols (titer of 1.5 g/L) in *S. cerevisiae* [59].

Moreover, 2,3-Butanediol (2,3-BDO) is a valuable chemical precursor in industry which can be upgraded to gasoline, diesel, and jet fuel. Its microbial production via the 2,3-butanediol biosynthesis pathway utilizes three enzymes to convert pyruvate into 2,3-butanediol. Yang et al. successfully expressed the enzymes in *E. coli* and *Zymomonas mobilis* while the latter resulted in higher efficiency of the pathway reaching a titer of more than 10 g/L [5, 60].

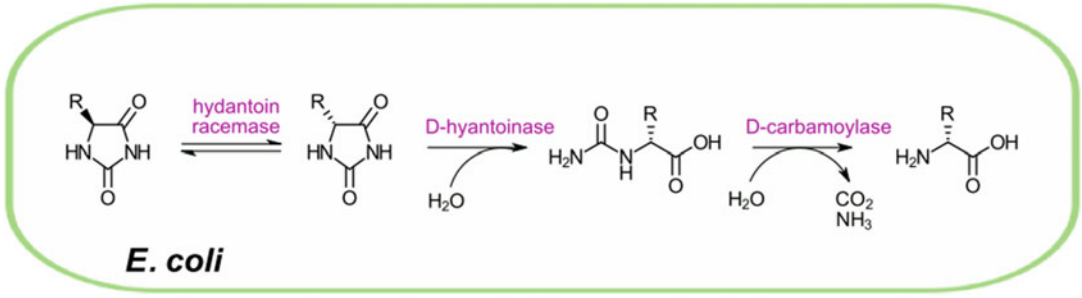
Apart from biofuels and other bulk chemical building blocks, metabolic engineering has paved the way for efficient production of precursors for drugs and food supplements among others. A famous example is the production of artemisinic acid, a precursor for the antimalarial drug artemisinin by engineering of *S. cerevisiae* for high-yielding biological production (titer of 25 g/L) [61, 62].

Another example is the production of eicosapentaenoic acid, an omega-3 fatty acid. Xue et al. engineered the oleaginous yeast *Yarrowia lipolytica* providing a sustainable non-animal source of the precious acid. The strain produced lipids with eicosapentaenoic acid at 56.6% of the total fatty acids [63].

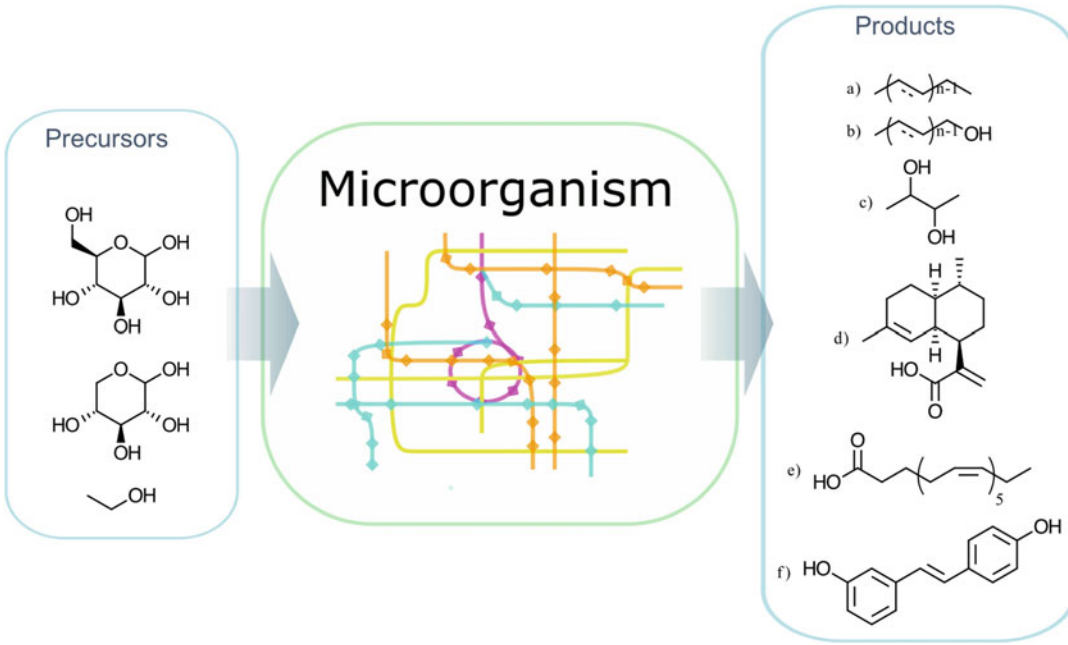
The production of resveratrol, an antioxidant compound used as food supplement and cosmetic ingredient represents a final example. Li et al. engineered the yeast *S. cerevisiae* to produce resveratrol directly from glucose or ethanol via a tyrosine intermediate (achieving maximum titers of approximately 530 mg/L) [64].

4.5 Conclusion and Prospects

Ultimately, the decision between in vitro, in vivo, or hybrid systems for the implementation of a synthetic enzyme cascade depends on several factors. As discussed above, an in vivo approach would offer several advantages (easier workability by avoiding the addition of cofactors, easier catalyst preparation by avoiding enzyme isolation and purification, protection from harsh reaction conditions by the cell wall, etc.). But



Scheme 4.7 Production of enantiopure D-amino acids from hydantoins [56, 57]



Scheme 4.8 Metabolic engineering enables the production of value-added chemicals from simple precursors (glucose, ethanol, or xylose). Chemical structures of (**a**) alkanes; (**b**) fatty alcohols [59]; (**c**) 2,3-butanediol [60]; (**d**) artemisinic acid [61, 62]; (**e**) eicosapentaenoic acid [63]; (**f**) resveratrol [64]

then again, limitations could prevent the success of the cascade (balanced enzyme expression, toxicity of substrate, intermediate or product, permeability of the cell membrane, etc.). However, all of these limitations can be addressed and improved.

For the development of whole-cell cascade catalysis, it is important to increase the knowledge of the microbial host and the respective pathway enzymes. A big problem is that enzyme cascades in vivo do not measure up to industrial expectations as, e.g. high productivity. A deeper insight into the metabolic network of the host will be crucial for the development of efficient synthetic whole-cell cascades for industrial applications. Whereas metabolic engineering approaches show some industrial applicability, purely synthetic enzyme cascades in vivo still fail to comply industrial demands. Bottlenecks (as the lack of coenzymes or unwanted side reactions) have to be identified and tackled [65]. Interdisciplinary approaches as systems metabolic engineering, which combines metabolic and genetic engineering with systems biology and synthetic biology, offer great potential for the optimization of enzyme cascades in vivo. In this regard, a lot of advancements are to be expected in the next years [66].

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Design and Development of Chemoenzymatic Cascades

5

Harald Gröger

Abstract

A broad range of tools have been developed enabling chemoenzymatic one-pot processes, in particular under combination of chemo- and bio-catalysis. A key issue for developing such processes is to gain initially insight into the compatibility of the reaction steps being considered to be combined. It is noteworthy that for many chemoenzymatic reactions with an initial chemocatalytic step and a subsequent biotransformation, a high compatibility of the biocatalyst with the chemocatalyst or components from the chemocatalytic step has been found. Such a compatibility then enables to conduct both reactions in a one-pot fashion, either in a sequential one-pot or tandem-type one-pot mode. However, also when it turned out that the two transformations planned to be combined are not compatible with each other, a range of solutions have been developed to realize one-pot or one-pot like processes. A favored method is based on compartmentalization, and a range of concepts have been developed in this field, which enable the combination of chemo- and bio-catalytic reactions without the need to isolate and purify intermediates. This review focuses on an overview of such tools, which now exists for the design and optimization of chemoenzymatic one-pot processes.

Keywords

Biocatalysis · Cascade reactions · Chemocatalysis · Compatibility · Compartmentalization · Enzyme catalysis · One-pot processes

5.1 Introduction

Among the most impressive features of biosynthesis and fermentation processes are the concurrently running reactions which enable the synthesis of the desired target molecules in a cell. A key prerequisite to achieve this goal within a cell is either compatibility of the reaction steps with each other or compartmentalization of the reaction steps. In the latter case, then a sufficient mass transfer between the compartments has to be ensured. Inspired by such natural cascades as well as the success of man-made catalysts to conduct efficiently non-natural types of reactions, in recent years increasing attempts have been made towards the combination of these two different "catalysis worlds" of enzyme catalysis and chemocatalysis (Scheme 5.1).

It is noteworthy that, in particular in the last 20 years, numerous examples underlining the synthetic power of this approach have been

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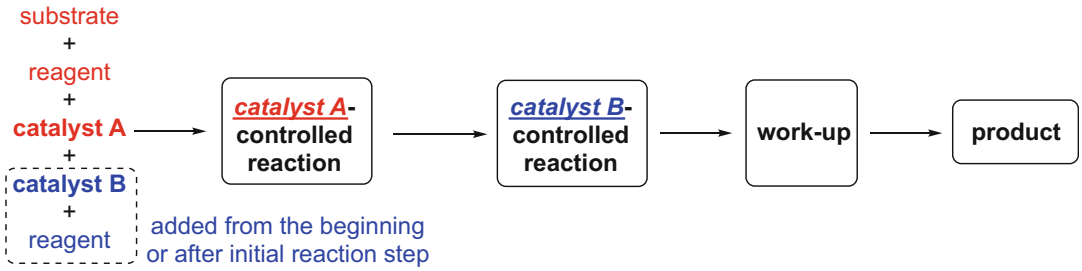
demonstrated by numerous research groups. Addressing this success, this research field has been comprehensively reviewed [1–9]. Taking into account these existing reviews on chemoenzymatic one-pot synthesis [1–9], the focus of this book chapter will be more on the fundamental strategies on how to design chemoenzymatic one-pot processes and how to overcome the hurdles when focusing on the development of such syntheses.

5.2 "Flow Scheme" for Developing Chemoenzymatic Cascades

As the topic of this book is centering on design and modeling of cascades, at the beginning of this chapter a "flow scheme" for developing chemoenzymatic cascades will be given (Scheme 5.2). The type of design of the chemoenzymatic one-pot process strongly depends on the issue of compatibility of the reactions, which should be combined. Thus, typically in the initial stage of the development of a chemoenzymatic cascade consisting of an initial chemocatalytic step and a subsequent biotransformation, it will be investigated if the components of the chemocatalytic step will have an impact on the enzyme, and, thus, biotransformation. The same work-flow has to be conducted for the chemocatalyst in an analogous fashion (in particular if the cascade is planned to be done in a concurrent mode or if the reverse sequence is considered). The latter step is less trivial than it appears. Whereas it is widely expected that enzymes can be affected by chemocatalysts, e.g., metals, in turn also the activity of chemocatalysts can be influenced by proteins or the components being involved in the enzymatic reaction. It also should be added that this phenomenon is not so rare and was described as a challenge in the development of chemoenzymatic one-pot processes by several groups (and representative examples will be given below).

After gaining an insight into these individual reactions and potential "cross inhibitions" and deactivation of a (bio- or chemo-)catalyst by components of the other, complementary step, one can conclude if a "process window" for the desired combination of chemo- and bio-catalyst exists. If compatibility and, thus, such an option for a one-pot process with a direct contact of chemo- and bio-catalyst exists, then two strategies are conceivable. First, the one-pot process can be carried out in a sequential mode. In this set-up, at first one of the two (or more) reactions is conducted in an individual fashion, and after completion the catalyst for the second reaction is added. Second, both catalysts are added already at the beginning, and, thus, both reactions are running concurrently. In general, there is no best choice among those two concepts, and both of them show advantages and drawbacks. For example, tandem-type one-pot processes are favored in case of labile intermediates, whereas sequential-type one-pot processes are favored when undesired pathways occur (e.g., consumption of the substrate for the first step also by means of the catalyst of the second step).

When the initial compatibility study revealed no compatibility of the two reactions steps, which are planned to be combined, a subsequent task could be the evaluation if the critical component causing this incompatibility (in the subsequent reaction step) can be removed after or during the first step. The removal of such a critical component for the catalyst used in the second step then enables the addition of the catalyst for the second step after removal of the critical component, leading to a one-pot process running in a sequential mode. For the removal of such a critical compound, various strategies are conceivable, such as e.g., evaporation (in case of volatile components) or extraction. If such a removal step is not possible, an option for still combining these two reactions within a one-pot process consists of a compartmentalization of the two reaction steps. For this option also a range of



Scheme 5.1 Basic principle of the combination of bio- and chemo-catalysis towards one-pot processes

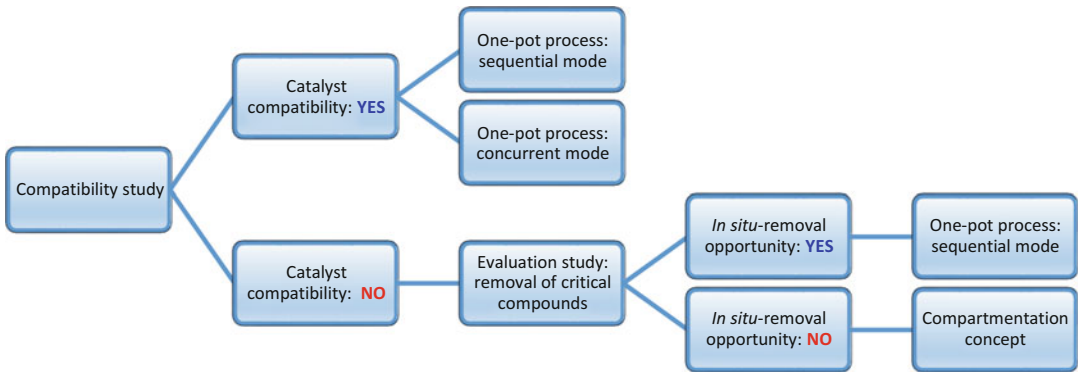
methods exist, and an overview will be given below.

5.3 Studies on Catalyst Compatibilities and Conclusions for the Set-up of One-Pot Processes

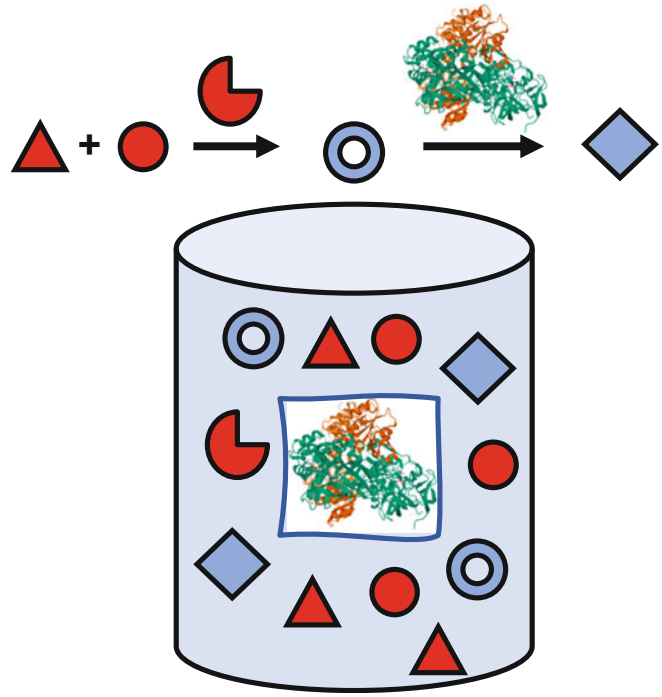
The knowledge of the influence of the various components of the envisaged one-pot process based on chemo- and bio-catalytic transformations is a key prerequisite for designing one-pot processes with such reaction types. The complexity of combining reactions, e.g., chemo- and bio-catalytic reactions, towards a process running in a one-pot fashion is illustrated in Scheme 5.3. Whereas in a "classic" reaction sequence with intermediate isolation, the reaction parameters can be adjusted just to the need of the desired transformation of the intermediate as second reaction step, in a one-pot process also all components for the synthesis of the intermediate are present. Thus, the requirements on catalyst stability are much higher in such a case, as the catalyst might be also deactivated by any of the components used in the first reaction step under synthesis of the intermediate.

Besides a potential negative impact caused by reaction components of the first step, also other reaction parameters relevant to the first step (e.g., solvent, pH, temperature) might play a role for the second (biotransformation) step. At the same time, at least in part, these reaction parameters might be re-adjusted to the need of the second reaction step when conducting the one-potprocess in a sequential form with addition of the (bio-) catalyst for the second step after completion of the first step.

A chemoenzymatic one-pot process, which has been studied very intensively in terms of compatibility of the individual reaction components of a certain reaction step with the catalyst of the other step is the combination of



Scheme 5.2 "Flow scheme" and basic research questions when developing chemoenzymatic one-pot processes under combination of bio- and chemo-catalysis



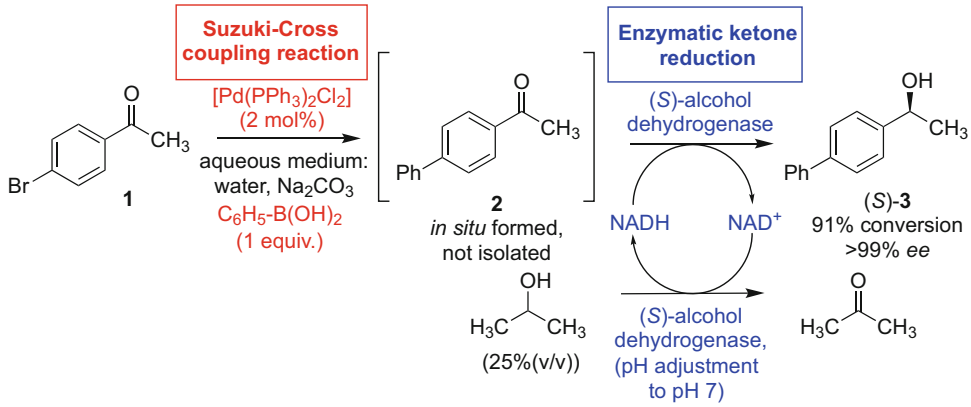
Scheme 5.3 Illustration of the increased complexity of potential catalyst deactivation effects when combining bio- and chemo-catalysis towards one-pot processes

an initial Suzuki coupling reaction as an important palladium-catalyzed reaction [10, 11] with a subsequent biocatalytic reduction in the presence of an alcohol dehydrogenase, while conducting both steps in an aqueous medium (Scheme 5.4) [12]. In the first metal-catalyzed step biaryl ketones are formed, which are then converted in situ without isolation into the desired chiral biaryl alcohols with excellent enantioselectivity.

Since the Suzuki cross-coupling reaction represents the initial step, the compatibility of the individual components of this palladium-catalyzed transformation with the alcohol dehydrogenase used as a biocatalyst has been studied. Since this class of enzyme is cofactor-dependent, this study has been conducted by means of measuring the enzymatic activity through spectrophotometry. Towards this end, the activity data of the enzyme for the substrate in the absence of any additives have been compared to the ones when adding the individual components of the Suzuki reaction (Scheme 5.5). Surprisingly it was found that the palladium did not severely hamper enzyme activity, whereas boronic acid as a reagent used in the Suzuki reaction step revealed to have a strong negative impact on the enzyme.

As boronic acid is used as a reagent in the first step, this hurdle could be overcome by developing a Suzuki reaction which operates at an amount of boronic acid of exactly one equivalent, thus being fully consumed at the end of the reaction, which proceeds with >95% conversion. The addition of the enzyme was then done just after completion of the Suzuki reaction and adjustment of the pH, leading to a high overall conversion of 91%. This case study also shows the advantages of the concept of a sequential one-pot process in such cases of inhibition or deactivation of the catalyst used in the second step by components (e.g., substrates, reagents) needed for the first step. Furthermore, this study illustrates the advantages of individually studying the impact of the various components being involved in a one-pot process on the catalysts in order to design a suitable one-pot process.

In the following years, several groups contributed to the development of a range of complementary variants of this type of one-pot process, which addressed in particular the issue of

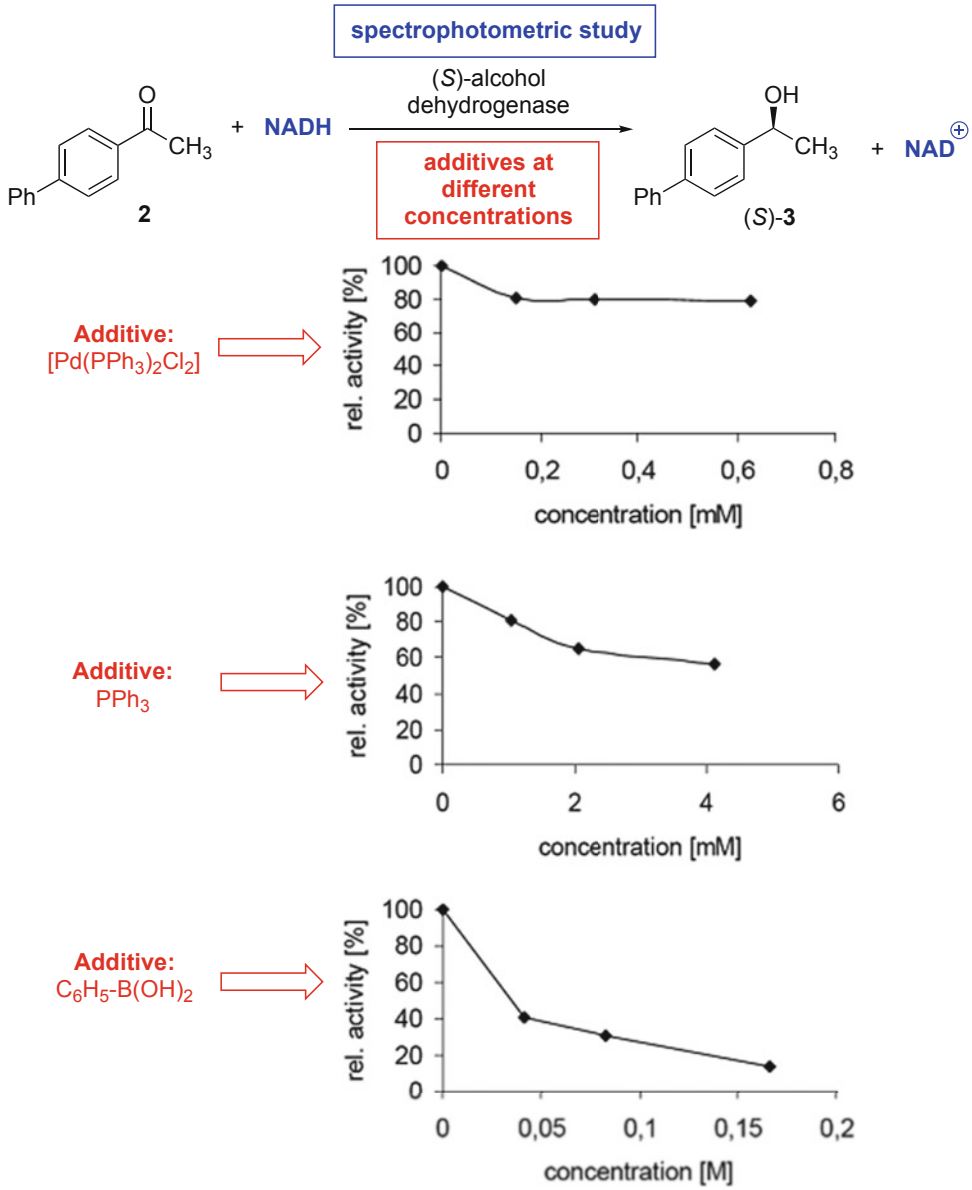


Scheme 5.4 Design of a chemoenzymatic one-pot cascade consisting of an initial palladium-catalyzed Suzuki cross-coupling reaction and a subsequent enzymatic ketone reduction

further improvement of its overall efficiency. Focusing on the use of modified palladium catalysts, the Cacchi group succeeded in efficiently using palladium nanoparticles, which are stabilized by embedding them into a protein cavity, thus being soluble in water. This catalyst, which represents an alternative to palladium catalysts with phosphine ligands, turned out to be highly compatible with the biocatalytic ketone reduction and led to high yields and enantioselectivities of >99% ee [13]. This elegant work exemplifies that in general the option to use modified ligands sphere is not only attractive for fine-tuning the catalyst properties (thus, increasing activity or selectivity) but also represents a valuable tool for improving the compatibility of catalysts in case of one-pot processes with combined chemo- and bio-catalytic transformations. It should be added that besides expanding this chemoenzymatic synthesis to the preparation of analogous diols bearing a biaryl unit [14], also attempts to conduct the Suzuki cross-coupling reaction at lower temperature were done. Addressing this issue, a Suzuki reaction running at room temperature when utilizing the water-soluble palladium-TSPP complex as a catalyst turned out to be suitable. This palladium catalyst catalyzes the reaction efficiently at low temperature and does not show a negative impact on the enzyme used in the subsequent ketone reduction step [15].

A further strategy for improving the process efficiency is by means of engineering the reaction medium, which also turned out to be valuable for the increase of the process efficiency of this combination of a palladium-catalyzed Suzuki reaction and an enzymatic ketone reduction. These achievements will be discussed more in detail below in the subsequent sub-chapter about designing one-pot processes by solvent engineering.

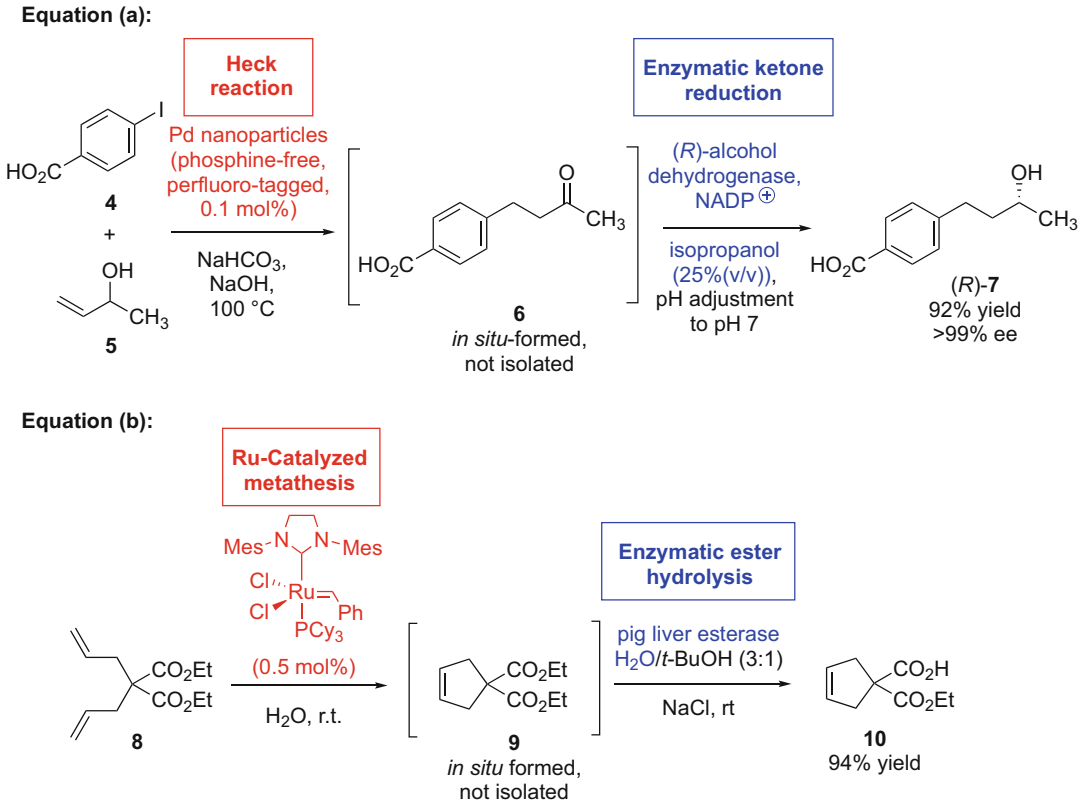
It should be added that a range of other related one-pot processes based on the combination of metal-catalyzed C-C bond forming reactions comprising, for example, other palladium-catalyzed reactions such as Heck reactions and ruthenium-catalyzed transformations such as metathesis reactions with various types of biotransformations have been successfully developed. Representative examples of such combinations of Pd- and Ru-catalyzed reactions with biotransformations are shown in Scheme 5.6. For example, the Heck reaction [10, 11] as a further important Pd-catalyzed transformation was successfully combined with a biotransformation by the Cacchi group, enabling an efficient access to styrenes from aryl halides [16, 17]. Different process options have been applied, and, for example, the one-pot process can be carried out in aqueous reaction medium when utilizing a phosphine-free, perfluoro-tagged Pd nanoparticle as catalyst for the Heck reaction in combination



Scheme 5.5 Study of the compatibility of an alcohol dehydrogenase with the components of the Suzuki reaction used for the preparation of the substrate for the biotransformation (Figures are taken from ref. [12])

with an alcohol dehydrogenase. This Pd catalyst turned out to be compatible with the biocatalyst, and the chemoenzymatic synthesis furnished, e.g., the styrene (*R*)-**7** in 92% yield and with excellent >99% ee (Scheme 5.6, equation (a)).

The first example of an olefin metathesis reaction [18] with a biotransformation was reported jointly by the groups of Schatz and Gröger, and the proof-of-concept for such a process was exemplified for the combination of a ruthenium-catalyzed metathesis with a subsequent selective esterase-catalyzed hydrolysis to the monoester (Scheme 5.6, equation (b)) [19].

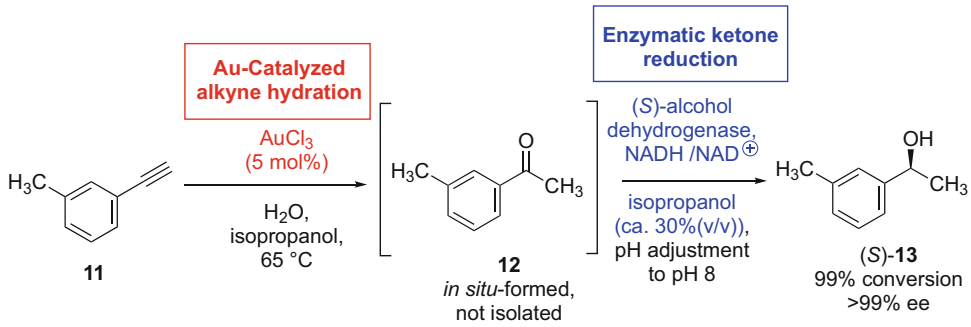


Scheme 5.6 Further examples for the combination of palladium- and ruthenium-catalyzed reactions with enzyme-catalyzed reactions

It also should be added that in the meantime a range of further metals used in catalysis turned out to be compatible with enzymatic transformations. An elegant work has been reported by the Mihovilovic group, demonstrating that also gold catalysis can be effectively combined with biocatalysis (Scheme 5.7) [20]. The designed one-pot process consists of an initial hydration of an alkyne in the presence of a gold catalyst, followed by a subsequent enzymatic reduction of the in situ-formed ketone moiety. Over these two steps, the resulting chiral secondary alcohols, e.g. (*S*)-**13**, were formed with conversions of up to >93% and excellent enantioselectivities of >99% ee in all cases. It is noteworthy that suitable enzymes have been found for the (*R*)- as well as (*S*)-enantioselective formation of the alcohols, and that no negative impact of the gold catalyst on the enzyme has been observed.

Besides these examples, in the meantime numerous further one-pot-type processes based on the combination of metal and enzyme catalysis in aqueous reaction media have been developed and this research area also has been already reviewed [3–9].

A related study focusing on the impact of an organocatalyst on the applied biocatalyst was also done when investigating the combination of an asymmetric organocatalytic aldol reaction under formation of a β-hydroxy ketone, and a subsequent enzymatic reduction of this intermediate, thus forming 1,3-diols in highly diastereo- and enantio-selective fashion [21–24]. The combination of the enantioselective aldol reaction in the presence of the Singh-catalyst with the subsequent diastereoselective



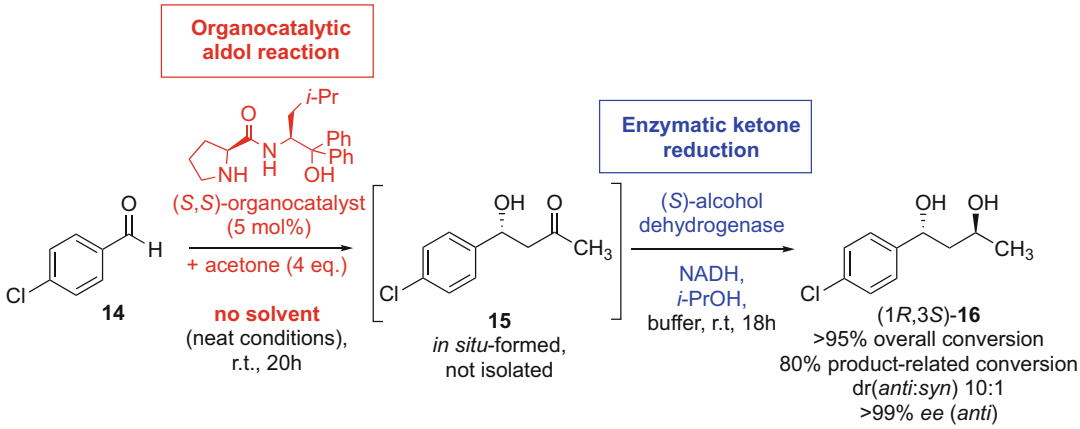
Scheme 5.7 Combination of gold-catalyzed alkyne hydration and enzymatic reduction for enantioselective synthesis of secondary alcohols from alkynes

biocatalytic reduction can be done in various ways. The first developed example consists of conducting the initial organocatalytic transformation under neat conditions, followed by addition of the aqueous phase and components for the biotransformation to the reaction mixture (Scheme 5.8) [21]. In such a one-pot set-up running in a sequential mode, the 1,3-diol product (1*R*,3*S*)-**16** was formed with a conversion of 80% related to the formation of this product, a diastereomeric ratio of dr(*anti*:*syn*) = 10:1 and with >99% ee.

Also for the field of combining organo- and bio-catalysis within one-pot processes, a range of further examples have been reported in the meantime and a review covering this research area has been reported very recently [1]. Representative examples comprise the combination of an enantioselective organocatalytic Mannich-type reaction with a diastereoselective enzymatic reduction for the synthesis of compounds bearing a γ-aminoalcohol subunit reported by the groups of Faber, Kroutil, and Pietruszka [25] and various combinations of organocatalytic oxidations of secondary alcohols with biocatalytic ketone reductions towards a deracemization of secondary alcohols reported by the groups of Lavandera, Gotor, Gotor-Fernández, and González-Sabín and Rebolledo, respectively [26, 27].

The insight into the compatibility of chemo- and bio-catalysts and the various reaction components being involved in the individual transformation steps also enabled the design of chemoenzymatic one-pot processes running in a tandem fashion, in which both chemo- and bio-catalytic transformations occur concurrently. The pioneer work in this field combining homogeneous chemocatalysts and enzymes in a one-pot process in aqueous medium has been developed by Entrechem researchers, who combined a Ru(IV)-catalyzed isomerization of allylic alcohols with an enzymatic reduction of the C=O double bond of the in situ-formed enone intermediate (Scheme 5.9) [28]. The resulting chiral alcohol products were obtained in enantiomerically pure form and the obtained yields were of 60–86%. Both catalysts turned out to be able to co-exist while maintaining their catalytic activity, but adjustment of the reaction rates turned out to be important and for a high overall conversion a fast rate of the initial isomerization step turned out to be beneficial. Thus, this example also underlines the importance of recording kinetics for the design of one-pot processes.

This one-pot concept has been extended by the Entrechem researchers to the analogous synthesis of related amines through combination of the Ru-catalyzed isomerization of allylic alcohols with a transaminase-catalyzed conversion of the in situ-formed enones to the corresponding amines, leading to high yields and excellent enantioselectivity [29]. In this case, however, the transaminase was added after completion of the first step and dilution of the reaction mixture,



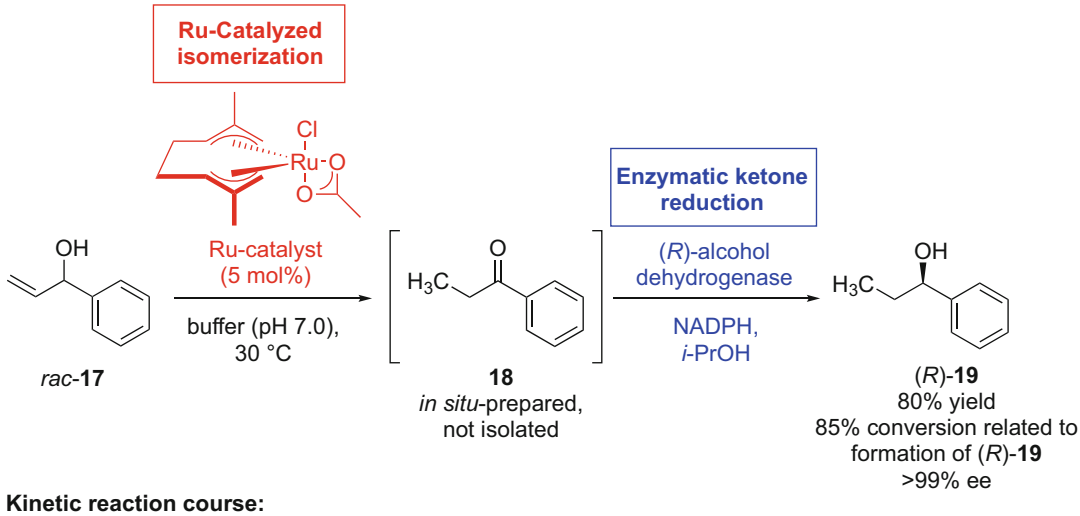
Scheme 5.8 Design of a chemoenzymatic one-pot cascade consisting of an initial asymmetric organocatalytic aldol reaction and a subsequent enzymatic ketone reduction

thus representing an example for a sequential-type one-pot process.

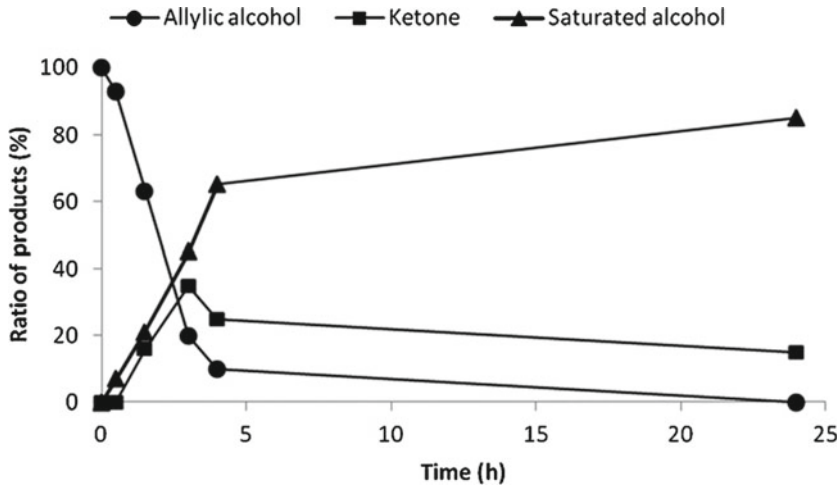
It also should be added that compatibility of enzymes and chemocatalysts is not limited to aqueous medium but has also been achieved in organic solvents as reaction medium. The most prominent example in this field is the chemoenzymatic dynamic kinetic resolution of secondary alcohols through combination of a redox-based racemization step catalyzed by a homogeneous ruthenium catalyst and a lipase-catalyzed enantioselective acylation of the secondary alcohol [30–33]. Compatibility of the chemocatalyst being homogeneously dissolved in the organic solvent with the biocatalyst plays a key role. The most widely used biocatalyst in this field of chemoenzymatic one-pot synthesis in organic media is the heterogenized lipase from *Candida antarctica* B, which is absorbed on a resin as a solid support and turned out to be highly stable in organic media. As a favored chemocatalyst for the racemization, the Shvo catalyst [34] has been used very successfully by the Bäckvall group [30–33]. This catalyst racemizes the secondary alcohol through a redox sequence, in which the alcohol is oxidized to the corresponding prochiral ketone species and reduced again to the racemic alcohol. It is noteworthy that this homogeneous ruthenium catalyst turned out to be highly compatible with the lipase, thus enabling the direct use of both of them in one-pot for this type of dynamic kinetic resolution of secondary alcohols. The concept of this technology, which has been demonstrated for numerous synthetic examples, is shown in Scheme 5.10 and a technical application of this process technology has also been reported [32]. Other chemocatalysts being suitable for the racemization step have been reported as well for this type of chemoenzymatic dynamic kinetic resolution, for example, a BINOL-based aluminum complex as demonstrated by the Berkessel group [35]. In addition, the Bäckvall group extended this technology towards the dynamic kinetic resolution of secondary amines [36, 37].

5.4 Studies on Undesired Side-Reactions As Well As Unfavored Thermodynamics and Conclusions for the Set-up of One-Pot Processes

When combining two reactions towards a one-pot process, one has to take into account also the option that the catalyst of a specific step also converts reagents and/or substrates from the other step(s). Accordingly, novel undesired side-reactions can occur under such conditions which would not be observed when conducting the

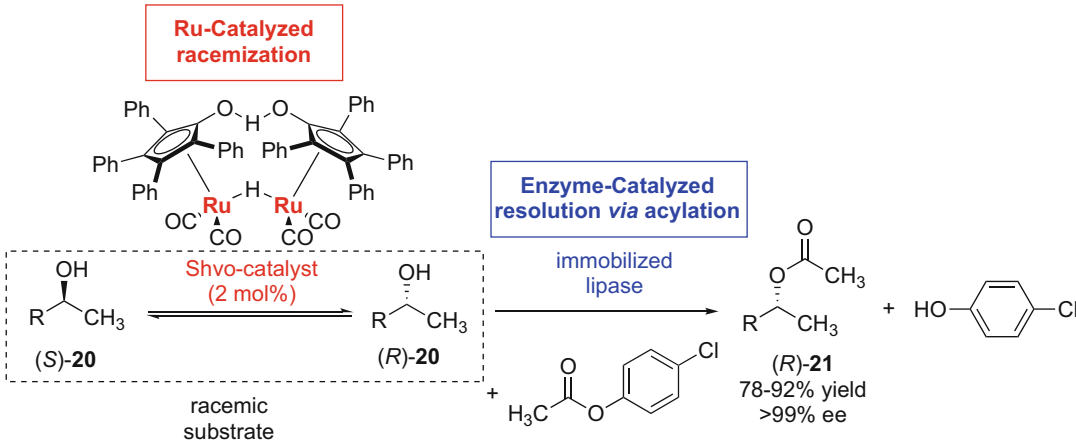


Kinetic reaction course:



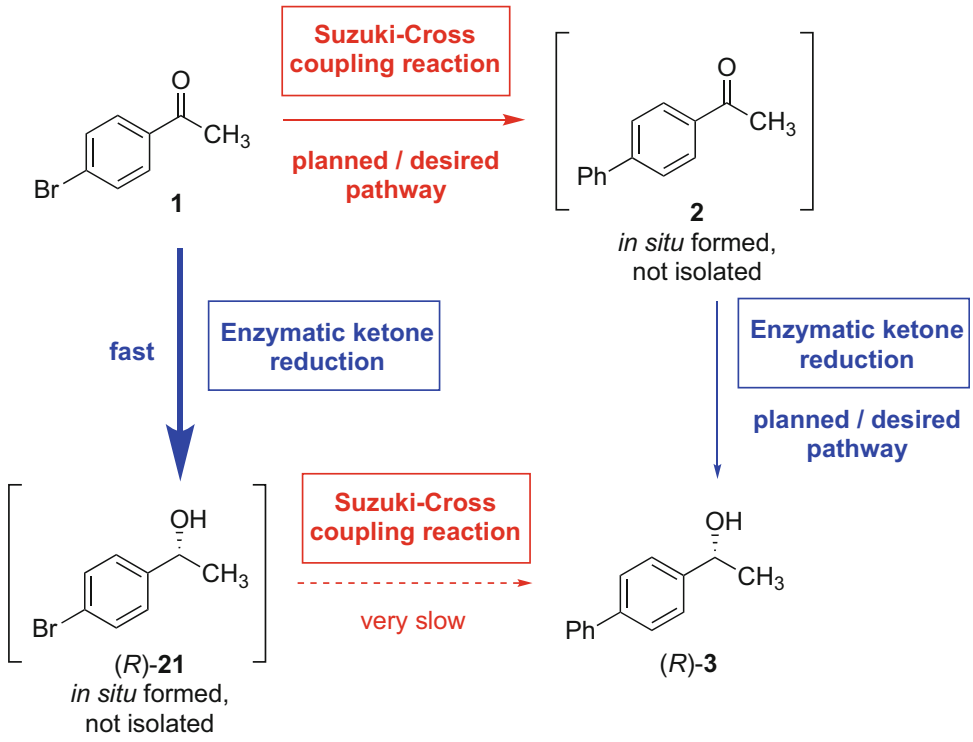
Scheme 5.9 Combination of chemo- and bio-catalysis in a tandem-type one-pot process with concurrently occurring reactions for the synthesis of enantiomerically pure alcohols (Figure with reaction course was taken from ref. [28])

reaction sequence in a subsequent manner with intermediate isolation. A representative example is again the chemoenzymatic cascade consisting of an initial palladium-catalyzed Suzuki cross-coupling reaction and a subsequent enzymatic reduction, which has been discussed above as a sequential one-pot process in the previous section and which is visualized in Scheme 5.4 [14, 15]. When conducting such a one-pot process, however, in a tandem-mode, it is noteworthy that besides the desired cascade sequence shown in Scheme 5.4, in turn as an alternative (but undesired) pathway at first the enzyme could reduce the 4-halogenated acetophenone under formation of the corresponding alcohol **21**, followed by a Suzuki reaction under formation of the desired product (*R*)-**3** when using an (*R*)-enantioselective alcohol dehydrogenase (Scheme 5.11) [38]. Although in both cases (desired and undesired pathways), the final product (*S*)-**3** is the same, the competing new sequence represents a problem as the enzymatic step converting **1** is favored over the one for the 4-phenylacetophenone in the desired sequence,



Scheme 5.10 Dynamic kinetic resolution of secondary alcohols in organic medium by combination of a Ru-catalyzed racemization and a lipase-catalyzed resolution via acylation

thus leading to an accumulation of the alcohol **21**. However, the Suzuki reaction for this alcohol **21** is rather slow, thus making completion of the cascade very difficult and leading to a significantly lower overall conversion in the tandem-type one-pot process (shown in Scheme 5.11)



Scheme 5.11 Example for novel side-reactions when combining chemo- and bio-catalytic reactions in a one-pot process running in tandem-mode instead of sequential mode

compared to the sequence-type one-pot process (shown in Scheme 5.4) [38]. Thus, this cascade represents an example for a more beneficial one-pot process when being conducted in a sequential one-pot fashion under addition of the catalyst for the second step not prior to the completion of the first step.

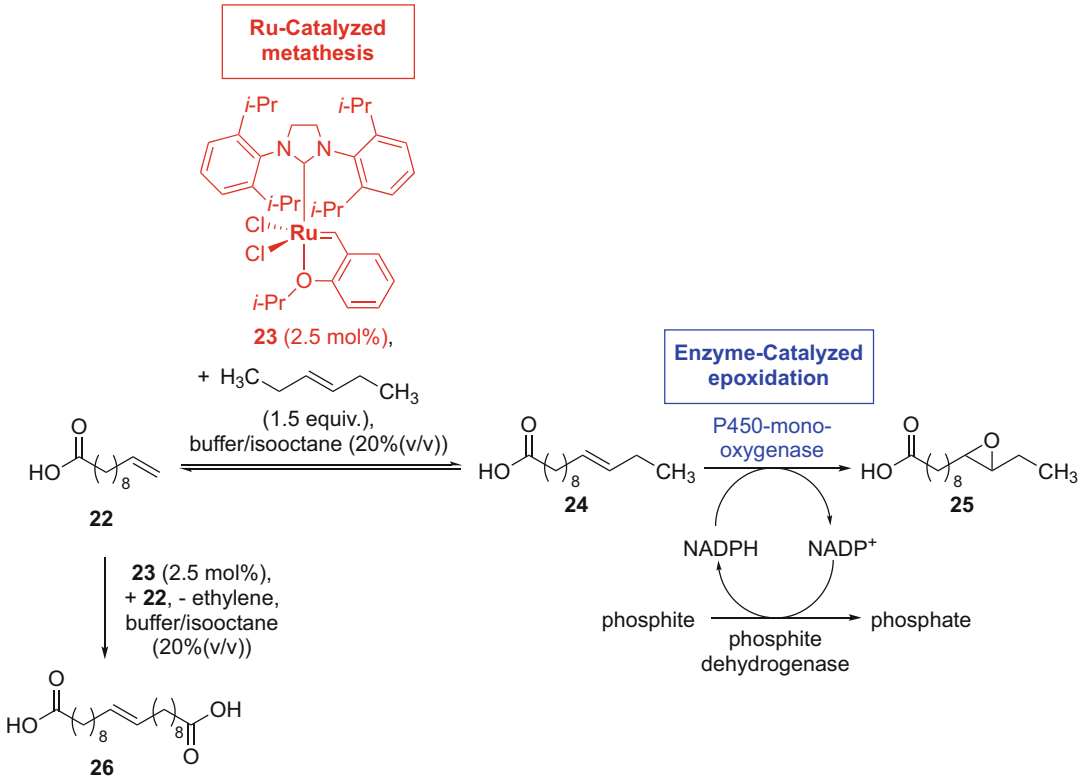
Another example illustrating the benefits of a tandem-mode one-pot process as well as in general the opportunities to shift the one-pot process into the desired reaction when choosing the right one-pot mode by understanding the individual reactions is the synthesis of fatty acids bearing an epoxy unit reported by the Zhao and Hartwig groups (Scheme 5.12) [39]. The first step of this one-pot cascade represents a metathesis of an unsaturated fatty acid, e.g., **22**, with an alkene, which is a reversible reaction and suffers from undesired irreversible self-metathesis of **22** under formation of ethylene. However, this hurdle can be overcome when coupling this step in situ with an irreversible second reaction. Such an irreversible reaction is the epoxidation catalyzed by a P450-monooxygenase, which has been chosen as a second step. Thus, by means of this enzyme, which turned out to be compatible with the water-stable ruthenium catalyst **23**, the metathesis reaction can be shifted towards the desired product side.

This chemoenzymatic synthesis represents an example underlining the advantages of one-pot processes running in a tandem-mode, since compared to the sequential mode here the conversion of the initial (reversible) step can be influenced by coupling with the second (irreversible) step, thus enabling higher overall conversions. Accordingly, in this work the epoxide product **25** was obtained in a 1.6-fold higher yield (which was 48%) compared to the theoretical yield, which has been calculated from the two separated reaction steps.

5.5 Studies on the Impact of Solvents and Conclusions for the Set-up of One-Pot Processes

For the design of biocatalytic processes in general and accordingly also chemoenzymatic processes with enzymatic key steps therein, also the search for the "right solvent system" plays an important role. Therein, two major issues regarding the impact of the solvent have to be considered. First, the solvent can have a strong impact on each of the two (or more) individual catalytic reactions of the one-pot process. Thus, the impact of the solvent on the individual catalytic reaction has to be studied as it can influence the catalyst, e.g., the enzyme directly or indirectly through, e.g., an increase of the substrate solubility, which then is affecting again the reaction rate (in a positive or negative way as, for example, the reaction rate might be increased when having high K_M-values and enabling the increase of the solubility of a hardly water-miscible substrate goes hand in hand with an increase of the enzymatic activity, or in a negative fashion if an elevated solubility of a component caused by a solvent leads to inhibition or deactivation effects at the biocatalyst). Second, the solvent choice can also have an impact on the separation of reactions in different phases, thus representing a tool for compartmentalization of different reactions steps. This topic will be discussed more in detail below.

Starting with the initial issue on fine-tuning specific reactions by means of solvents, such a strategy has been successfully applied by the Schmitzer and Kroutil groups for the process development of the one-pot process consisting of an initial Suzuki cross-coupling reaction and a subsequent biocatalytic reduction. Originally this process has been conducted in aqueous medium (see sub-chapter above and Scheme 5.4 therein). In spite of the compatibility of the two reactions, only water as reaction medium makes the separation and recycling of the chemo- and bio-catalysts after the reaction difficult. Addressing this issue, Schmitzer and Kroutil et al. utilized ionic liquids for the Suzuki reaction



Scheme 5.12 Tandem-type one-pot process combining a reversible metathesis reaction and an irreversible enzymatic epoxidation

to recycle the chemocatalyst in this one-pot process [40]. Towards this end, the Suzuki reaction is carried out in an ionic liquid as reaction medium. The subsequent enzymatic reduction of the in situ-formed ketone then runs in a two-phase system, which consists of the ionic liquid from the first step and added aqueous buffer solution. What makes this two-phase system in particular interesting is the fact that both phases can be recycled. The efficiency of the recycling has been shown for four reaction cycles, leading to yields of the biaryl alcohol being in the range 94–98% in combination with excellent enantioselectivities of >99% ee (whereas the fifth reaction cycle led to a decreased yield of 44%).

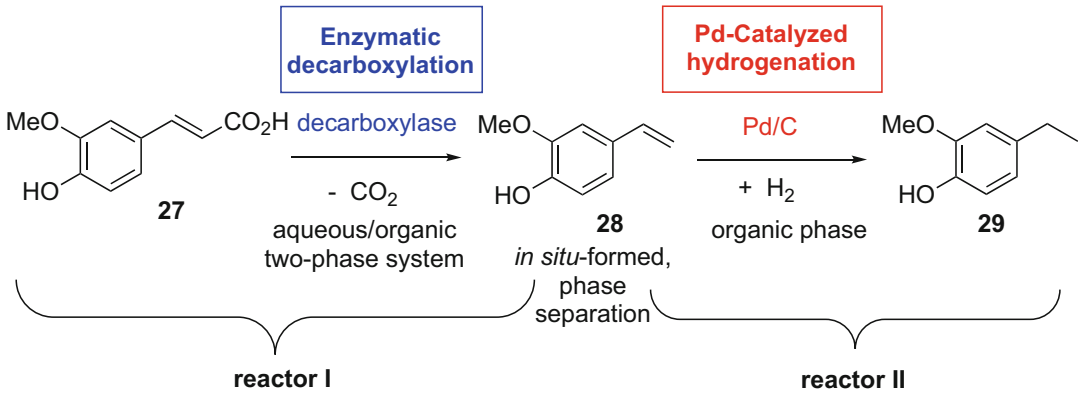
Besides ionic liquids, in recent years also deep eutectic solvents (DES) gained a lot of attention as an alternative co-solvent for improving biotransformations [41]. The class of DES consists of mixtures of, for example, quaternary ammonium salts such as choline chloride as a low-cost component and uncharged hydrogen bond donors such as urea, glycerol, or sorbitol, which then form a hydrogen bond network throughout the solvent. Advantages of DES solvents are the high thermal stability, low vapor pressure, and easy recovery and re-use as well as the high solubility of hydrophobic compounds being used as substrates in the biotransformation. The pioneer example of using DES in the field of chemoenzymatic one-pot syntheses has been reported by González-Sabín and co-workers for the combination of a ruthenium-catalyzed isomerization and an enzymatic reduction of the in situ-formed ketone component (which originally has been done in buffer, see also sub-chapter above and Scheme 5.9 therein) [42]. It is noteworthy that the use of a DES-buffer mixture contributes to improve the biotransformation, which proceeds efficiently even at a buffer content of only 20%

(w/w) and led to excellent conversions of >99% and enantioselectivities of >99% ee. As DES components, mixtures of choline chloride and glycerol (1:2) or choline chloride and sorbitol (1:1) were used [42]. Furthermore, the application range of DES in the field of chemoenzymatic one-pot processes has been studied and extended by the González-Sabín and Gröger groups to the combination of the Suzuki coupling reaction with an alcohol dehydrogenase-catalyzed alcohol synthesis and a transaminase-catalyzed amine synthesis, respectively [43, 44].

A further key area of application of organic solvents (and resulting biphasic solvent systems) is their utilization to improve those reactions, which suffer from inhibition or deactivation of the catalysts by a specific reaction component. In biotransformations, many of such inhibition and deactivation effects are known. Dependent on the partition of such a component in a two-phase system, however, its concentration in the aqueous phase can be dramatically reduced in the presence of an organic phase, thus ensuring an improved enzymatic reaction. The successful application of this concept for the design of improved chemoenzymatic one-pot cascades has been demonstrated by the Liese, Kara, and Faber groups for the synthesis of the hydrophobic product 4-ethylguaiacol starting from hydrophilic ferulic acid as a substrate (Scheme 5.13) [45]. In the initial biocatalytic oxidation reaction, the decarboxylase is strongly inhibited and deactivated by the formed product 4-vinylguaiacol, **28**. This challenge has been solved by means of a two-phase system and continuous in situ-product removal of the hydrophobic 4-vinylguaiacol (but not ferulic acid) into the organic phase, which is then directly transferred into another reactor. Therein, the organic solvent is distilled off and recycled for the next extraction step. The second reaction step consisting of a Pd/C-catalyzed hydrogenation of intermediate **28**, which accumulates as a crude product in the organic phase, can be carried out in the organic phase. The combination of these two reactions linked to each other by an in situ-product removal step without the need for an intermediate purification then furnished the desired 4-ethylguaiacol, **29**, in 70% overall yield.

5.6 Compartmentalization as a Process Concept to Overcome Hurdles of Incompatibility

In case that incompatibilities of a catalyst for a specific reaction with components from other reactions being present in the reaction medium cannot be solved, compartmentalization is a promising strategy for still realizing one-pot processes in spite of such problems. A range of compartmentalization strategies have been developed, which are complementary to each other and

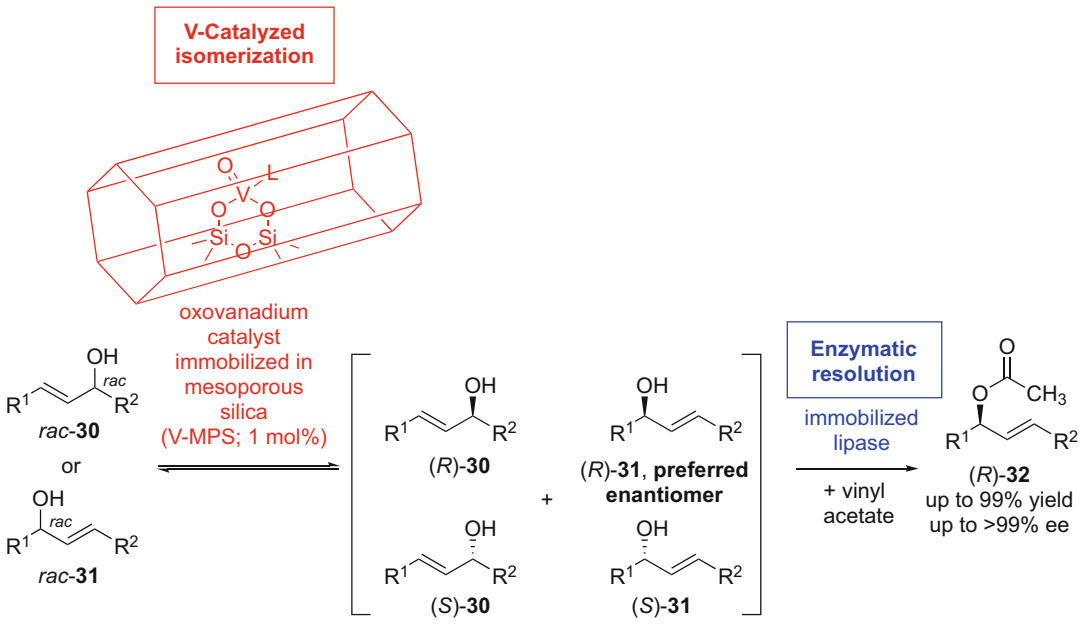


Scheme 5.13 Combination of bio- and chemo-catalysis for the synthesis of 4-ethylguaiacol utilizing a two-phase system for the biotransformation with in situ-product removal

which are based on compartments of very different sizes.

To start with the concept that the chemocatalyst is immobilized in a defined compartment on a nano-scale dimension, such a concept can provide various advantages. In terms of compatibility, a direct contact of such an entrapped chemocatalyst with the biocatalyst is avoided, which is of importance in case of a deactivation of one of such catalytic components by the other one. In addition, typically separation of such heterogenized chemocatalysts from the reaction mixture is much easier and often also the option for a re-use exists.

One efficient example for such a concept is the compartmentalization by embedding chemocatalysts into porous materials and their combination with enzyme catalysis, which has been successfully demonstrated by the Akai group for the dynamic kinetic resolution of secondary alcohols, in particular allylic alcohols (as shown for representative examples in Scheme 5.14) [46, 47]. In detail, the concept consists of the preparation of heterogeneous oxovanadium-type mesoporous catalysts and the combination of these heterogeneous systems with an immobilized lipase for the dynamic kinetic resolution of allylic alcohols [46, 47]. In earlier work, homogeneous oxovanadium-complexes were used as catalysts for the racemization of the alcohols in this DKR, which occurs by means of a reversible "formal removal" of the hydroxy moiety under formation of a prochiral carbenium ion as an intermediate [47–49]. However, compatibility with the enzyme remained a challenge due to the (at least in part) inactivation of the enzyme by the oxovanadium complex. Addressing this limitation, the Akai group could avoid a direct contact to the enzyme and, thus, a negative interaction leading to a deactivation of the biocatalyst, by embedding the oxovanadium catalyst in a mesoporous silica matrix [46, 47]. The size of the mesoporous silica scaffold can be fine-tuned according to the need of the process and the substrate used therein. The resulting chemoenzymatic dynamic kinetic resolution process with this mesoporous oxovanadium catalyst and the lipase from *Candida antarctica* B in also immobilized form turned out to proceed very efficiently and required a catalyst loading of

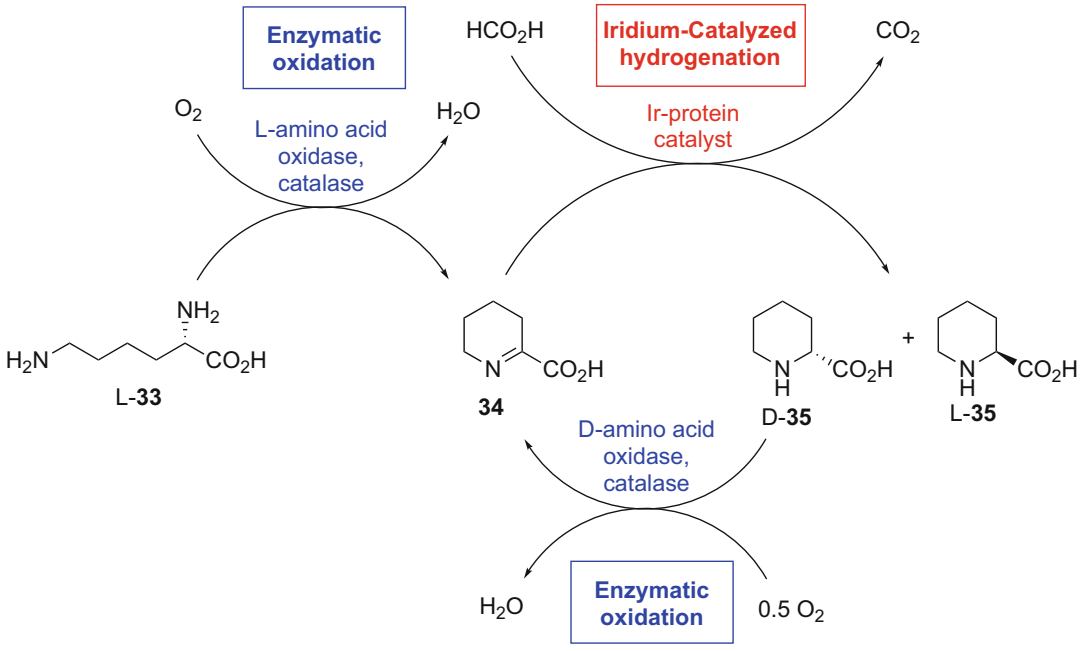


Scheme 5.14 Dynamic kinetic resolution of secondary alcohols by combination of oxovanadium-catalyzed racemization and enzymatic resolution via acylation

only 1 mol% [46, 47]. Furthermore, a very low leaching of <0.0003% of the vanadium was observed. The desired products of, e.g., type (*R*)-**32**, have been obtained in high yields of up to 99% and with excellent enantiomeric excess of up to >99% ee [46, 47]. In addition, this heterogeneous catalyst is very stable and, thus, can be recycled. The Akai group demonstrated a re-use of this catalyst leading to excellent yields of 99–100% and enantioselectivities of 99% ee over six reaction cycles. In the seventh reaction cycle, the yield, however, decreased to 85%.

It should be added that recently this concept has been expanded towards the DKR of tertiary alcohols, which represents the first example of a DKR of this type of substrates [50].

A complementary approach, which has been reported by the Ward, Turner, and Hollmann groups to avoid such negative interactions on the enzyme by the chemocatalyst consists of "surrounding" the chemocatalyst of choice with a protein scaffold. This concept has been exemplified by the preparation of a metalloprotein, which consists of an iridium metal complex for hydrogenations bound to streptavidin via a biotin-type linker, and the use of this artificial metalloprotein in combination with various biocatalysts for a one-pot synthesis of L-pipecolinic acid (L-**35**) starting from L-lysine (Scheme 5.15) [51]. By means of this methodology, compatibility of the catalysts has been reached successfully. The whole cascade then consists of an initial oxidation of L-lysine in the presence of an L-amino acid oxidase, thus forming imino acid **34**. The next steps consist of the hydrogenation with the artificial iridium-based metalloprotein, which proceeds in a non-enantioselective fashion and furnishes the racemic α-amino acid *rac*-**35**, and the subsequent re-oxidation of the D-enantiomer with a D-amino acid oxidase in combination with a catalase to decompose the formed H_2O_2. Thus, in the overall process, the L-amino acid is left untouched and remained as product in this multi-step cascade. In summary, this cascade consisting of three enzymes and one artificial metalloprotein is an elegant example for the design of chemoenzymatic cascade processes based on

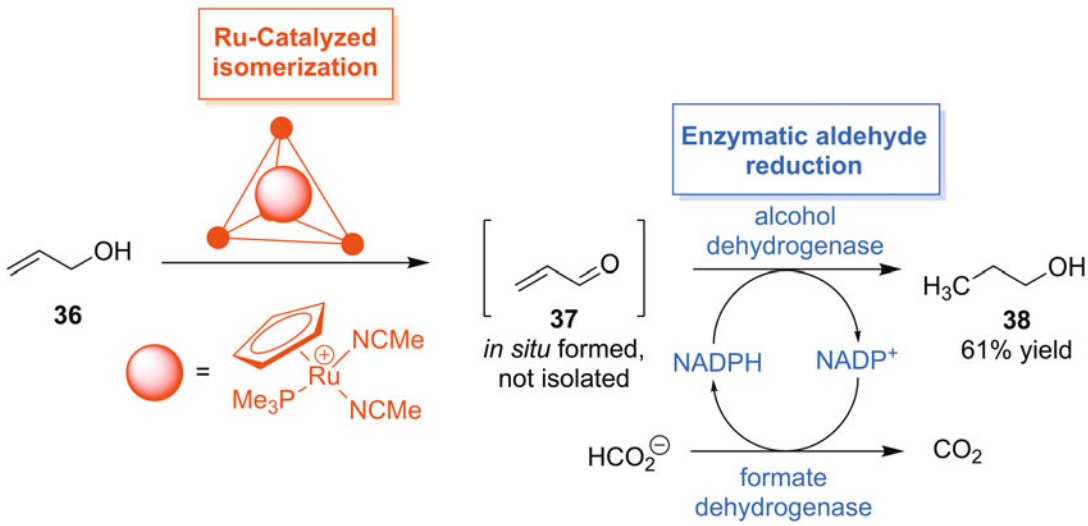


Scheme 5.15 Design of chemoenzymatic cascades through integration of a metal catalyst in a protein scaffold and its combination with biocatalysts exemplified for L-amino acid synthesis

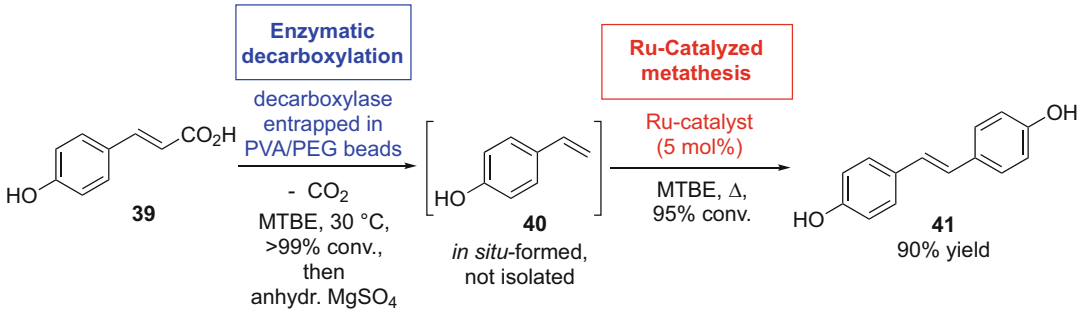
achieving compatibility of enzymes with a chemocatalyst through its integration in a protein structure.

A complementary strategy, which also follows the idea of shielding the enzyme from the chemocatalytic species as a potentially inhibiting or deactivating agent, has been reported by the Bergman, Raymond, and Toste groups [52]. In this case, however, instead of a protein environment the "protecting shield" is realized by means of a supramolecular host–guest complex. In detail, a Ga_4L_6-type tetrahedral complex bearing a Ru(II) moiety, which is capable to isomerize an allylic alcohol, was used as such a supramolecular cluster (Scheme 5.16). In this tandem-mode cascade, the supramolecular host–guest complex catalyzes the transformation from allyl alcohol to propanal as initial step. The subsequent enzymatic transformation by means of an alcohol dehydrogenase in combination with a formate dehydrogenase for the in situ-recycling of the cofactor NADH then led to the formation of propanol in 61% yield. It should be added that this methodology turned out to be suitable also for other combinations of chemo- and bio-catalysis as demonstrated by the authors for a range of other chemoenzymatic syntheses utilizing such Ga_4L_6-type supramolecular host–guest complexes [52].

A further concept for compartmentalization, which is in particular applicable for immobilization of the enzyme component, consists in the entrapment of the catalyst in polymer beads. As such beads can contain an aqueous phase inside (being separated from the organic medium outside by a membrane), this immobilization method is attractive for enzymes as they can still operate in their native aqueous reaction environment inside the beads whereas at the same time an organic medium can be used outside of the beads. The whole process set-up then allows to conduct the chemoenzymatic process in an organic reaction medium containing the homogeneously dissolved chemocatalysts (which are often more suitable for organic media) with such polymer beads as heterogenized biocatalysts. Thus, the polymer beads protect the enzymes from the organic phase and, vice versa, the chemocatalyst from the aqueous phase. Such a one-pot process has been developed by Kourist et al., exemplified for a one-pot synthesis of 4,4-′-dihydroxy-*trans*-stilbene, **41**, via biocatalytic decarboxylation and ruthenium-catalyzed metathesis starting from *para*-coumaric acid, **39**



Scheme 5.16 Combination of a chemocatalyst applied as a supramolecular host–guest complex with enzymes exemplified for a one-pot process for propanal from allyl alcohol



Scheme 5.17 Combination of polymer beads containing a decarboxylase and a metathesis catalyst in a one-pot process for a stilbene running in organic reaction medium

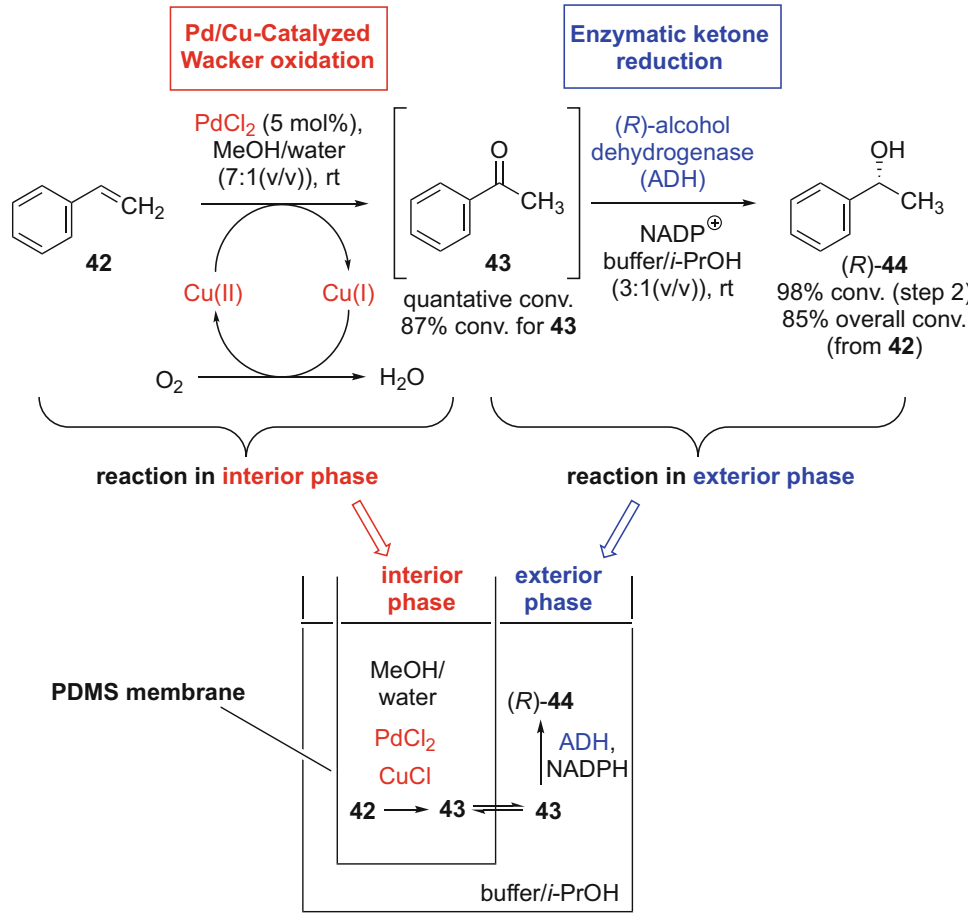
(Scheme 5.17) [53]. Toward this end, the decarboxylase was embedded into a polyvinylalcohol/polyethylene glycol (PVA/PEG) cryogel matrix and removed after the biotransformation. After removal of these enzyme-containing polymer beads the organic phase has been dried prior to the addition of the (water-sensitive) metathesis catalyst, thus furnishing product **41** in a high yield of 90%.

It should be added that in general further efficient encapsulation strategies for enzymes into beads exist. One of such efficient methods is based on UV-cured polyurethanes as matrix as recently reported by the von Langermann group for immobilization of alcohol dehydrogenases. This elegant immobilization method and the use of such polyurethane-based materials have been already applied for enzymatic cascades [54].

Instead of beads, also larger volumes can be separated by means of membranes. Such a type of more "macroscopic compartmentalization" being based on the use of polydimethylsiloxane (PDMS)-thimbles has been previously applied by the Bowden group for combining non-compatible "classic chemical" and/or chemocatalytic reactions [55–57], and was recently extended towards chemoenzymatic one-pot processes by the Gröger group [58]. By means of these hydrophobic PDMS-membranes, two different volumes in a reactor bearing aqueous phases can be separated from each other (Scheme 5.18). The hydrophobicity of the membrane then only allows the hydrophobic components of the reaction (e.g., the intermediate) to pass the membrane. In contrast, the water-soluble components remain inside or outside of the membrane, thus avoiding a direct contact of such components with each other. This separation of water-soluble components is beneficial in case of deactivation effects such as an enzyme deactivation by a water-soluble metal component.

Such a case occurred for the combination of a Wacker oxidation of styrene in the presence of palladium and copper components as catalytic system with a biocatalytic ketone reduction. It turned out that copper salts strongly deactivate the enzyme. However, by means of this compartmentalization both reaction steps could be done in a one-pot process enabling the synthesis of the enantiomerically pure alcohols with high overall conversions and excellent enantioselectivity. Separated by the PDMS-membrane, the Wacker oxidation proceeds inside of the thimbles, whereas the biotransformations is conducted outside of the thimble in the same reactor.

This PDMS-thimble-based compartmentalization method for conducting chemoenzymatic one-pot processes in one reactor but different reaction compartments has been further applied by various groups. The Micklefield and Greaney groups reported the application of this technique for the combination of an enzymatic aryl halogenation with a Pd-catalyzed Suzuki cross-coupling reaction in order to prepare aryl-substituted indole heterocycles [59]. Another application is the combination of the Wacker oxidation and an enzymatic transamination for the one-pot

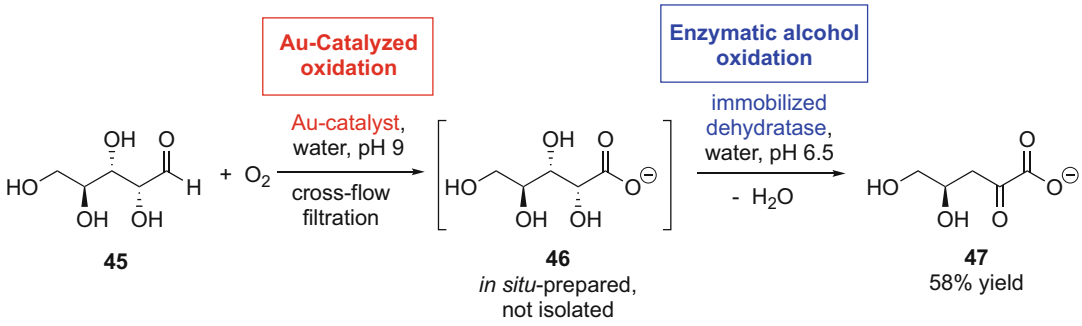


Scheme 5.18 Macroscopic compartmentalization by means of polydimethylsiloxane-thimbles applied for the combination of a Wacker oxidation of styrene with a biocatalytic reduction

transformation of styrene into 1-phenylethylamine with up to 93% conversion and 99% ee developed by the Gröger group [60]. Furthermore, Rudroff and Mihovilovic et al. combined a Pd-/Cu-based metal assisted Liebeskind–Srogl coupling reaction with enzymatic ketone reductions, thus leading to the desired alcohols in yields of up to 99% and with excellent enantioselectivities of 99% and >99%, respectively. For conducting the two reactions in a concurrent mode, a tailor-made membrane reactor with two chambers being separated by means of a PDMS-membrane has been designed for this one-pot process [61].

As an alternative related way of membrane-type compartmentalization, a "tea bag"-like system developed by the Deska and Bäckvall group turned out to be also suitable to separate chemo- and bio-catalytic transformations [62]. In this case, isooctane and water, thus representing a two-phase solvent system has been utilized as a reaction medium. This concept has been applied in a chemoenzymatic dynamic kinetic resolution of carbocyclic allylic alcohols [62].

Although most chemoenzymatic one-pot processes have been designed in a batch-mode, the utilization of flow processes represents a further option and turned out as a versatile tool for combining chemo- and bio-catalytic reaction steps, which are non-compatible with each other. Such an example has been recently reported by the Sieber group, exemplified for the synthesis of a

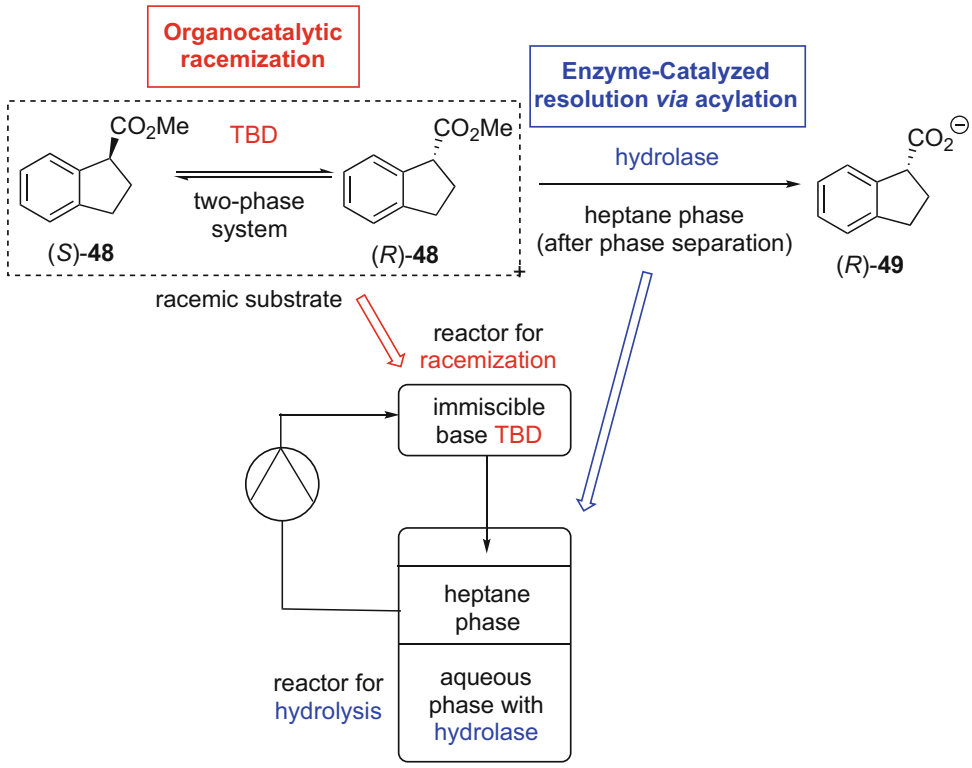


Scheme 5.19 Chemoenzymatic synthesis via compartmentalization of a gold-catalyzed oxidation and an enzymatic dehydration running in a flow-mode

variety of 2-keto-3-deoxy sugar acids starting from corresponding sugars [63]. A representative example of this type of process, which combines a gold-catalyzed oxidation of a carbohydrate to the corresponding acid and a subsequent enzymatic dehydration leading to an α-keto acid (e.g., product **47**), is shown in Scheme 5.19. For the first step, Au/Al_2O_3 (0.5%) as a heterogeneous gold catalyst is used. Since the protein buffer salts turned out to hamper the Au-catalyzed oxidation step and at the same time the by-product hydrogen peroxide, which is generated in the Au-catalyzed oxidation, is deactivating the enzyme, it has been a challenge to find conditions to combine the two reaction steps. Furthermore, the pH and temperature requirements of the chemo- and bio-catalytic step differ significantly. The reported solution consists in a flow process with compartmentalization of these two reaction steps. The filtrate resulting from the initial Au-catalyzed reactions step is then collected in a reaction vessel containing a catalase, which leads to the decomposition of the H_2O_2 being formed in this Au-catalyzed reaction step. Thus, both reactions can be combined without the need to isolate the intermediate and with the opportunity to conduct both reactions under favored conditions for each step. This type of chemoenzymatic flow process then leads to an efficient synthesis of the desired products and, for example, 2-keto-3-deoxy arabonate, **74**, was obtained in a yield of 58% (Scheme 5.19).

However, compartmentalization of aqueous and organic phase can also be done without a "physical separation" by means of a membrane. As an alternative concept, integrating a phase separation step in combination with a biotransformation running in a two-phase system can also enable the combination of two reactions running under different conditions within a one-pot(-like) process. Designing such a chemoenzymatic one-pot process with two reactions operating under strongly differing pH values has been reported by the Pietruszka group for a dynamic kinetic resolution of carboxylic esters (Scheme 5.20) [64]. The challenge was to combine a base-catalyzed racemization requiring a high pH with an enzymatic hydrolysis running at physiological conditions.

This task has been addressed successfully by conducting the enzymatic hydrolysis of the racemic substrate methyl 2,3-dihydro-1*H*-indene-1-carboxylate, *rac*-**48**, in an aqueous-organic two-phase system, leading to the formation of carboxylate (*R*)-**49**. The remaining, non-converted ester substrate **48** then remains in the organic phase and is continuously separated and pumped through a second packed-bed-type reactor containing the base 1,5,7-triazabicyclo [4.4.0]dec-5-ene (TBD), which is not soluble in hexane under the applied conditions. In this second reactor, then the desired racemization occurs. The combination of these two compartmentalized reactions leads to a chemoenzymatic process, which gave the product (*R*)-**49** in 95% yield and with >96% ee.



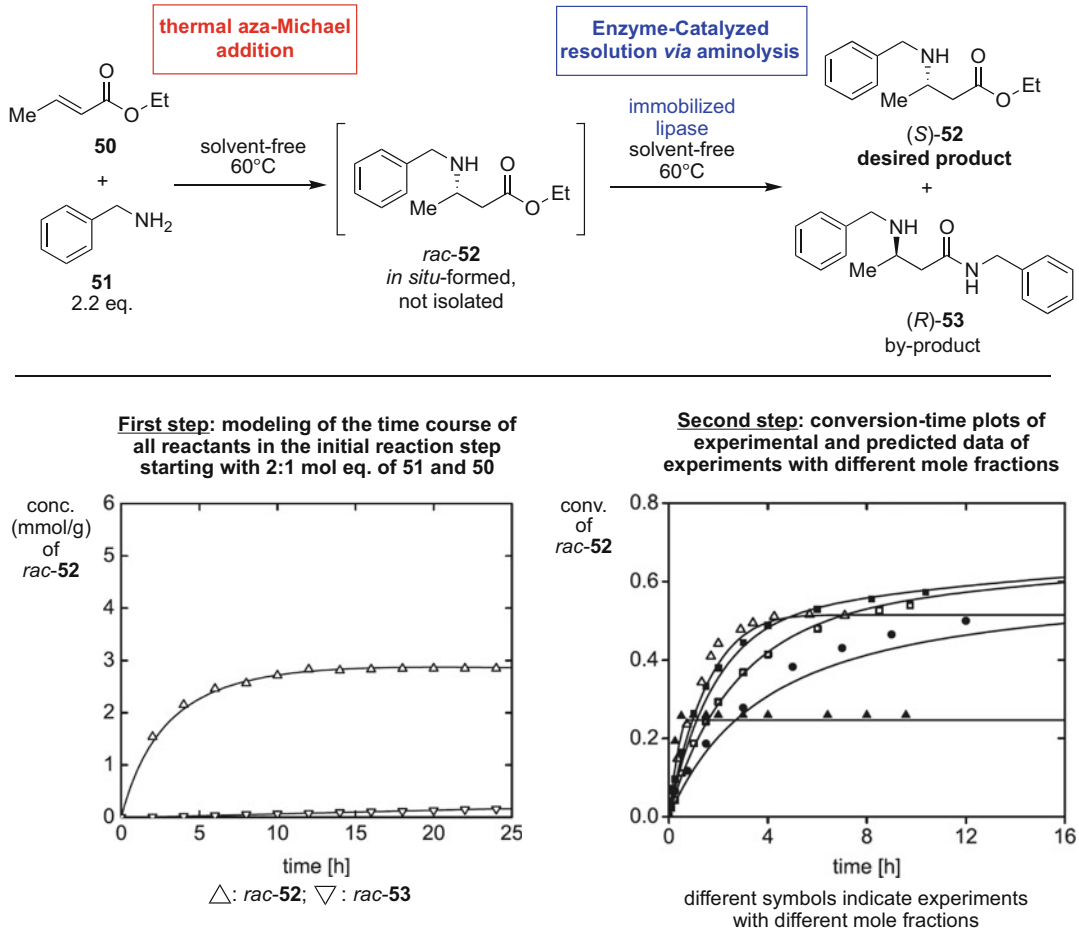
Scheme 5.20 Compartmentalization based on phase separation of a two-phase system for a biotransformation and chemocatalytic racemization when using the separated organic phase

5.7 Modeling of Processes

Up to now, modeling of chemoenzymatic processes based on kinetic data has been done only to a very rare extent. At the same time, as for many other reaction engineering work, such tools are extremely helpful for setting up also chemoenzymatic one-pot processes. A pioneer work in this field has been reported by the Liese group, which was done within a collaboration project with the Gröger group for the combination of a thermal (non-catalyzed) Michael addition of an amine to an α,β-unsaturated compound, followed by a lipase-catalyzed aminolysis reaction (Scheme 5.21) [65, 66]. Both transformations run in continuous fashion and under solvent-free conditions. Modeling of these reactions in combination with process development work then led to a highly efficient process, which operates for more than 80 h without significant loss of activity. In this process, the desired β-amino ester was formed with a high space–time yield of 1.8 kg ($L * d$). Taking into account the excellent fit of the modeling data with the experimental data, this example underlines the high potential of reaction modeling for optimizing chemoenzymatic one-pot processes.

5.8 Conclusions and Outlook

In conclusion, a broad range of tools have been developed enabling chemoenzymatic one-pot



Scheme 5.21 Modeling and experimental data for the chemoenzymatic sequence combining a aza-Michael addition and a lipase-catalyzed resolution for β-amino acid ester synthesis (Figures were taken from literature, see ref. [65])

processes, in particular under combination of chemo- and bio-catalysis. A key issue for developing such processes is to gain initially insight into the compatibility of the reaction steps being considered to be combined. It is noteworthy that for many chemoenzymatic reactions with an initial chemocatalytic reaction and a subsequent biotransformation, a high compatibility of the biocatalyst with the chemocatalyst or components from the chemocatalytic step has been found. Such a compatibility then directly enables to conduct both reactions in a one-pot fashion, either in a sequential one-pot or tandem-type one-pot mode. However, also when it turned out that the two transformations planned to be combined are not compatible with each other, a range of solutions have been developed to realize one-pot or one-pot like processes. A favored method is based on compartmentalization, and a range of concepts have been developed in this field, which enable the combination of chemo- and bio-catalytic reactions without the need to isolate and purify intermediates. Thus, numerous tools now exists, which also serve as a basis for the development of further chemoenzymatic one-pot processes in the future. At the same time, an intensified application of modeling tools for the development of chemoenzymatic one-pot processes represents a task for the future. Certainly, the integration of such tools in process development work will contribute to expand the scope of chemoenzymatic one-pot processes and to

enhance their synthetic efficiency, thus matching also data required for technical purpose such as high substrate loading and space–time yield.

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Enzyme Cascade Kinetic Modelling

6

Martina Sudar and Zvjezdana Findrik Blažević

Abstract

Cascade multi-step reactions have gained a lot of attention in the last decade due to their numerous advantages against traditional organic synthesis methods. Indeed, they are excellent from the viewpoint of sustainability. Nevertheless, one has to bear in mind that as the number of enzymes and compounds increases, the number of dependencies between different variables also increases. For such complex systems to work, and to become fully applicable on larger scale, it is important to understand them from within, i.e., from the viewpoint of reaction engineering. The path towards development of these complex processes can be challenging, with many open questions, but with the aid of modelling all the numerous interdependencies can be described and understood. Using models, reactions can be optimized faster and at low cost. This chapter presents the methodology for the multi-step process development via kinetic modelling, with challenges and problems addressed, and potential solutions offered.

Keywords

Multi-step reactions · Kinetic modelling · Reaction engineering · Enzymes · Process optimization

Abbreviations

BP	Biocatalyst productivity [$kg_{product}$ $kg_{biocatalyst}^{-1}$]
c	Molar concentration [mM]
v	Mass concentration [g L^{-1}]
K_i	Inhibition constant [mM]
K_M	Michaelis constant [mM]
Q_P	Volumetric productivity [g L^{-1} h^{-1}]
r	Reaction rate [mM min^{-1}]
V_{max}	Maximum reaction rate [U mg^{-1}]
$Y_{product}$	Product yield [%]

6.1 Enzyme Kinetic Models and Their Benefits

Nowadays, biocatalysis is a viable method to produce chemicals and is very often used in industry [1, 2]. The need for enantiopure compounds and shorter reaction routes has paved the way for biocatalysts possessing high enantio- and diastereoselectivity with remarkable catalytic efficiency under mild reaction conditions [3, 4]. A newer concept of biocatalytic reactions is evolving fast, i.e. cascade reactions,

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often referred to as **systems biocatalysis.** In systems biocatalysis enzymes are organized in vitro to construct complex reaction cascades for an efficient, sustainable synthesis of valuable chemical products [5]. Systems biocatalysis concept imitates Nature where these kinds of cascades take place as a part of cell's metabolic pathways [5, 6]. The advantage of in vitro approach is that substrates and enzymes do not necessarily have to exist in Nature. Furthermore, within these artificial cascades of enzymes new products, that are difficult to obtain through the conventional organic chemistry methods, can be synthesized [7, 8]. As already introduced at the beginning of this book, multi-enzymatic cascade reactions offer numerous advantages over traditional chemical procedures, such as simplified downstream processing without the intermediate product recovery steps, simpler and cheaper reactor set-up consisting of one instead of numerous vessels, mild and controlled reaction conditions, lower chemicals consumption, and overall improved environmental impact [9–11]. Due to all that, cascade reactions continue to provoke interest of both scientific and industrial community, which is evident from a comprehensive review articles that cover all the cascade reactions studied and published in the last decade [9, 12], another discussing their development possibilities via engineering approach [13], as well as book chapters [14].

A decade ago Findrik and Vasić-Rački [11] reviewed the existing literature on cascade reactions in general with the purpose to evaluate the state of the art. Modelling was not the focus of that work, even though it was the initial idea, since the literature was really scarce then. This is understandable because modelling techniques are still not commonly used for biocatalytic processes even though their benefits are widely recognized [15]. By reading the existing new literature (after 2000), one can see that a lot has changed, and that research topics employing modelling are broadening fast. Modelling and engineering approaches are gaining momentum. It has been known for a long time that reaction engineering is an efficient and effective methodology to design and size the reactors in the chemical industry [16]. It is therefore expected that the same can be applied for biocatalysis. Surely, wider industrial application of biocatalysis is hindered by the extensive effort required to develop a competitive process [17]. For an efficient cascade process, it is important to balance enzyme kinetics, enzyme stability, and system's thermodynamics [7]. This presents a challenging task as it requires the application of multidisciplinary methodologies such as substrate, medium, protein (enzyme), biocatalyst (formulation), biocatalytic cascade, and reactor engineering [18, 19]. These challenges can be resolved through interdisciplinary approach linking chemistry, biology, and engineering which will surely pave the way for faster commercialization of biotechnological processes [20]. To achieve a successful process implementation, protein and process engineering have to be employed simultaneously as they offer complementary solutions to the process design problem [7]. Namely, protein engineering can significantly improve enzyme kinetic parameters [21], which basically determine process outcome. Reaction engineering can be used to minimize reaction time, to reduce by-product formation, to affect enzyme stability, product concentration as well as other process metrics; e.g. with aspiration towards high product concentrations, substrate loadings (see Chap. 10) can be arranged through different feeding strategies. All this can be investigated through simulations, and without the need of extensive experimentation. Simulations of different reaction scenarios are helpful in gaining better understanding of the interactions between reaction compounds and can facilitate the analysis and assessment of suitable operating points in complex reactions at an early stage. In this manner, the experimental effort during process development is efficiently reduced [22–24]. Woodley and co-workers [25] have demonstrated how mathematical modelling in biocatalysis can be applied not only in process optimization, but also in evaluation of process economics and its environmental effect. This capacity of mathematical modelling should be used to establish efficient, robust, and self-sufficient cascade reactions. These are important features for new processes

desired to be competitive with chemical syntheses [26]. By employing mathematical models to estimate the potential costs and profits of a process at early stages of development, valuable information on process feasibility can be obtained [27]. This is especially important in pharmaceutical industry where more money is spent for product development that fails than on successful products. Since this clearly depicts the importance of reducing cost and time for the development of industrial processes [28], any aid in evaluation and development can have a tremendous effect. Thus, mathematical modelling should be more employed, and its advantages used more, especially for the establishment of complex multi-enzyme cascade reactions.

6.2 Setting-up Conditions of an Enzymatic Cascade System and Kinetic Model Development

Multi-enzyme cascades are complex reactions and to set them up efficiently a clear strategy is needed. In the literature [13] technology options and strategies related to enzyme format options, reactor and process options, as well as operational options can be found, but the focus of this chapter is on the development of multi-enzyme cascades via kinetic modelling. The strategy [29] towards setting-up a multi-enzymatic cascade reaction discussed in this chapter is presented in Fig. 6.1 in its simplified form [15].

6.2.1 Selection of Reaction Conditions

The first step when dealing with several catalysts/enzymes in one reactor is the choice of the appropriate reaction media and temperature. The influence of different buffers at different pH values and the influence of temperature on the activity and the values of apparent kinetic constants (Table 6.1, [30]) of all enzymes have to be taken into account [31–33]. This applies to multi- or chemo-enzymatic reactions that can and will be performed simultaneously in one pot. Usually this decision implies a compromise, i.e. selecting conditions at which all catalysts, either all biocatalysts or the combination of biocatalysts and chemical catalysts, are sufficiently active to catalyse the reaction [34–37]. The choice of reaction conditions is very important because kinetic parameters, e.g. maximum reaction rate (V_{max}), Michaelis constant (K_M), and enzyme operational stability can differ significantly at different reaction conditions [30, 38]. Thus, conditions at which the multi-enzyme cascade will be investigated (buffer, pH, temperature) must be chosen prior to the next step which is kinetic characterization of enzymes. Also, the addition of organic co-solvents to the reaction mixture must be considered during this decision. Co-solvents are sometimes necessary due to the insolubility of hydrophobic substrates in aqueous media, but they may have a negative effect on enzyme activity and stability [39–41].

The complexity of one-pot multi-enzyme cascades is often underestimated [42] and the choice of the proper reaction conditions for a multi-enzyme cascade can be a very complex decision, but a systematic approach will offer the right solution. In the case of multi-enzyme cascade reactions that do not take place simultaneously in one pot, the situation is less complicated; different pH values or temperatures can be selected for each step of the cascade since it can be adjusted after each step according to the preferences of the enzyme in the next step [43–46].

6.2.2 Enzyme Kinetics and Kinetic Model

The selection of reaction conditions enables the next step in the set-up of a multi-enzymatic cascade reaction. It includes a detailed evaluation of kinetics of each reaction in the cascade at the carefully chosen reaction conditions. Following the evaluation of kinetics is the estimation of kinetic parameters. Development of a useful kinetic model for a single enzymatic reaction can be troublesome, which makes it an even

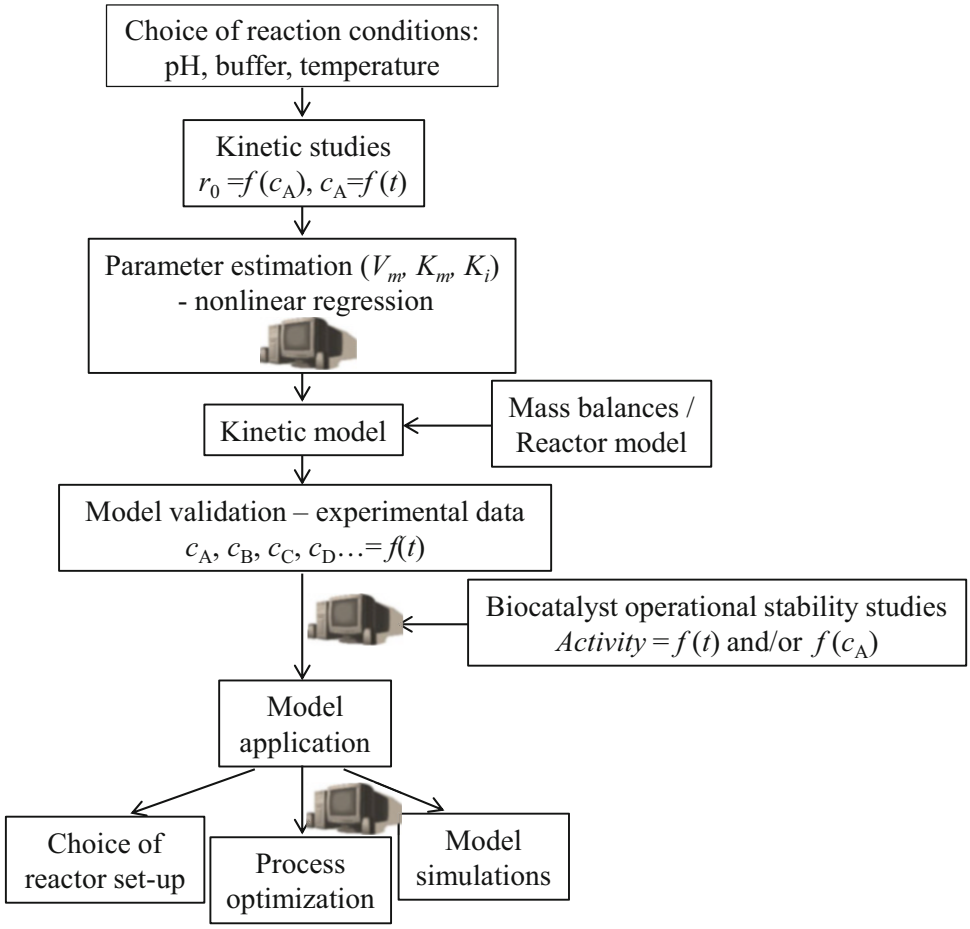

Fig. 6.1 Simplified methodology towards a mathematical model set-up

more complicated task for a multi-enzymatic system. Issues in simpler reactions, e.g. one enzyme and two substrates, sometimes can be resolved intuitively. But, for complex multi-step and multi-enzyme systems understanding of the pathway, as well as interdependencies of different variables, is necessary [47, 48]. Complex reactions imply complex models and the size of the model also has significance, while too many reactions make the process computationally inefficient. Therefore, to overcome this problem, simplifications should be used [48]. An important postulate from Octave Levenspiel says: '*Unless there are good positive reasons for using the*

Table 6.1 Estimated apparent kinetic parameters for different pH values and in different buffers [30]

	Phosphate (50 mM) buffer	TEA-HCl (50 mM) buffer	Tris-HCl (50 mM) buffer
pH	7.0	8.0	9.0
V_{max} [U mg^{-1}]	18.680 ± 0.673	16.409 ± 0.717	15.918 ± 0.769
$K_{M,\ NADH}$ [mM]	0.007 ± 0.001	0.005 ± 0.001	0.015 ± 0.003
$K_{M,\ Oxygen}$ [mM]	0.0042 ± 0.0012	0.0045 ± 0.0019	0.0003 ± 0.0001
$K_{i,\ NAD+}$ [mM]	0.251 ± 0.046	0.142 ± 0.045	0.495 ± 0.117

more complicated of two equations, we should always select the simpler of the two if both fit the data equally well [49]'.

From the viewpoint of biochemistry, it is important that kinetic parameters are obtained with purified enzymes and substrates so that all side-reactions and influences can be excluded. Usually they are obtained at limited substrate concentration ranges which correspond well to those in enzyme's natural environment. Additionally, they are estimated with enzyme's natural substrates. The reaction rate equations derived from the biochemistry measurements are called **intrinsic rate equations** and the acquired kinetic constants are related to the mechanism of the reaction [50–52]. Reaction mechanisms can be very complex as well as the corresponding equations [51, 52]. Since the intrinsic kinetic parameters are obtained at different conditions, as opposed to the conditions of the technical application of an enzyme, they are not always useful in obtaining the realistic picture of the studied reaction system [50]. Additionally, mechanistic models contain a significant number of parameters and substantial experimental and computational efforts are required for proper prediction of the reaction rate [15, 53]. Furthermore, enzymes are not always used in purified form in technical application, which may lead to side-reactions [54] that need to be evaluated as well. Purified substrates are also not always available, and kinetic parameters estimated from such experimental data are different from the intrinsic ones. Very often they are referred to as the **apparent kinetic parameters**, and the reaction rate as the overall or formal reaction rate [50]. Equations describing overall reaction rate do not necessarily relate to the reaction mechanism, but present a simplified version, such as **multi-substrate Michaelis–Menten equation** including substrate/product inhibitions, as well as some other unspecific influences.

6.2.2.1 Determination of Kinetic Parameters

Kinetic investigations are essential part of fundamental studies in the development of any (bio)-chemical process. They provide better understanding of the reactions and give dependencies of reaction rates on the concentration of reacting compounds [55]. Numerous enzymes have been characterized and data on enzyme kinetic constants can be found in the literature. According to a paper from 2010 [56], enzyme information system BRENDA had 103,706 entries for K_M values at that time. A paper from 2012 [57] indicates the increase of these entries in BRENDA of approx. 36% in the span of five years (2008 to 2012). Another database, SABIO-RK, also collects kinetic parameters, as well as kinetic laws used to describe reactions [58]. This clearly illustrates the increasing interest in biocatalysis and the recognition of the value of kinetic models in the academic community.

Experimental method to collect the kinetic data in a batch reactor is the slowest and most labour-intensive method; nevertheless the use of a batch reactor mode is the most robust technique in terms of wide applicability [16]. One of the methods often used to evaluate kinetic parameters is the initial reaction rate method. The method is represented by short measurements that give a lot of important data and enable estimation of kinetic constants such as $V_{\max}$ *(maximum reaction rate)*, K_M (Michaelis constant), and K_i (inhibition constant). It is excellent for investigation of complex reactions and its application can be very elegant, particularly if measurements can be performed on a spectrophotometer, which is timesaving. The selection of enzyme concentration is of great importance for evaluation of reaction's kinetics. For the **initial reaction rate experiments**, it is important to use the concentration of enzyme, which will enable slow linear increase of product concentration and substrate conversions $<10\%$. Only in this way can the concentration of product be regarded as irrelevant, and its potential inhibitory effect on the reaction rate disregarded. Furthermore, the choice of concentration range of substrates and/or inhibitors for kinetic measurements must be made very carefully. Concentration ranges of interest for further application on larger scale have to be considered so that the model can be applied in as broad range as possible [50, 59].

Once experimental kinetic data are obtained, kinetic parameters can be estimated by using **non-linear regression methods**. Even though linear regression methods can also be applied, these methods include transformation of the original data which can generate errors. This error depends on the quality of the experimental data and can vary. Approaches to estimate the values of kinetic parameters from the initial reaction rate experiments, as well as from **progress curves** by integral method are presented in detail in the literature [60]. An elegant newer alternative method shown in the literature consists of combining microscale kinetic experiments and parameter estimation using the genetic algorithm [61, 62].

One must be careful in the interpretation of results since alternative approaches are possible. An example is presented in Fig. 6.2 for a double-substrate reaction in which A and B are substrates and additionally, an inhibition with substrate A is present. The influence of concentration of both substrates on the specific enzyme activity was evaluated and the kinetic parameters were estimated by using non-linear regression analysis. Usually, kinetic parameters are estimated using single-substrate Michaelis–Menten equation (Eq. 6.2, Table 6.2), or single-substrate with included excess substrate inhibition (Eq. 6.1, Table 6.2), and separate data series for each substrate are used for that purpose. In the case of substrate inhibition, we propose using double-substrate Michaelis–Menten kinetics for the estimation of parameters (Eq. 6.3, Table 6.2). To illustrate this claim, kinetic parameters were estimated in both ways (single- and double-substrate equations) and are presented in Table 6.2 with two values for V_{max} when single-substrate equation is used, each corresponding to data series obtained for one substrate. Figure 6.2 shows that there is no significant difference in the simulations (Fig. 6.2, line and dotted line); however, parameters (Table 6.2) show a different picture. This is especially pronounced for the maximum reaction rate which is different for each approach. The value estimated by using the double-substrate equation (Eq. 6.3, Table 6.2) is unique and presents the right value. The explanation for this conclusion is very simple; when the double-substrate equation is used for estimation of parameters, it takes into account the influence of the substrate-inhibitor A. This is important because the influence of substrate B on the specific activity of the enzyme cannot be measured without the presence of substrate A, which cannot be in surplus due to inhibition, and that is why there is an error in the estimated values of V_{max} (Table 6.2). The value of the estimated V_{max} will be incorrect if single-substrate Michaelis–Menten kinetics is used for the estimation of parameters in cases like the presented one. Consequently, this will cause disagreement between the model and the experimental data during model validation. Moreover, estimation of many parameters from a small set of experimental data always leads to uncertainties. Hence, it is always advisable to have an abundance of experimental data to estimate kinetic parameters. This is especially important in the case of substrate inhibition where more parameters are estimated from a single set of experimental data.

For a complete and accurate model of a multi-step and/or enzyme reaction, it is necessary to evaluate the influence of every compound present in the system on each enzyme. Substrate, product, or any other compound present in one enzymatic reaction can have a negative influence on the enzyme catalysing another reaction in a cascade or vice versa. Also, any other compound present in the system, i.e. compounds used as stabilizers of commercial substrates [29], different impurities or by-products, can affect the reaction rate. Of course, in many cases these compounds do not affect the reaction rate. Therefore, an alternative approach in building a model may be in evaluating the effect of these 'extra' compounds only if the initial model proves to be inadequate during its validation.

To highlight the importance of investigating the influence of all compounds in the reaction system on the reaction rate, an example is presented in Fig. 6.3. It shows the effect of a **non-competitive inhibition** with a stabilizer of the substrate on the reaction outcome. Model simulations (Fig. 6.3) were obtained by using the kinetic model without (dotted line) and with (solid line) inhibition. Since the stabilizer is present in the commercial stock solution of the

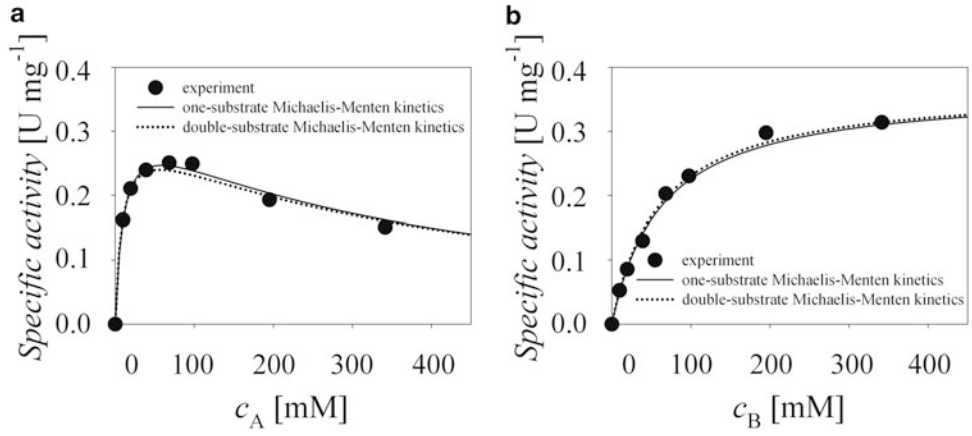

Fig. 6.2 Estimation of kinetic parameters in reaction with two substrates: single-substrate vs double-substrate Michaelis–Menten kinetics

substrate at a constant concentration, at lower substrate concentration the effect of inhibition is not that significant (Fig. 6.3a) and could easily be missed. High substrate concentrations (Fig. 6.3b and c) imply that the inhibitor is present at higher concentration as well; the inhibition is more pronounced, and it cannot be neglected. Thus, for the complete picture of the reaction system, inclusion of this inhibition was essential for the kinetic model to describe the data well. During investigation of this system, after recognition that the model without inhibition did not describe the data well, first trials to resolve the issue were done in silico, by adding the inhibitory effect. Model with the added inhibition by the stabilizer showed very good agreement with the experimental data. Furthermore, very similar value of kinetic constant of stabilizer inhibition was obtained from these in silico trials, as well as from the independent inhibition data estimated from the initial reaction rate experiments, which were carried out later to confirm the assumption [29].

Enzymes in the multi-enzymatic cascades can perform unwanted reactions with substrates and/or products of some other reaction step, which should also be experimentally evaluated. This is especially important for technical enzymes that are not pure. An appropriate type of inhibition model should be selected based on experimental investigation or literature. By evaluating all these influences, inhibition constants can be estimated, concentrations of the problematic compounds can be adjusted, and their influence on the reaction rate can be incorporated into kinetic models.

Another challenge one can face when determining enzyme kinetics of a multi-step enzymatic reaction is the unavailability of chemical

Table 6.2 Comparison of the kinetic parameters estimated from Fig. 6.2 by using single-substrate and double-substrate Michaelis–Menten kinetics

	Model	
Parameter	$r = \frac{V_{max} \cdot c_A}{K_{M,A} + c_A + \frac{c_A^2}{K_{i,A}^A}}$ (6.1); $r = \frac{V_{max} \cdot c_B}{K_{M,B} + c_B}$ (6.2)	$r = \frac{V_{max} \cdot c_A \cdot c_B}{\left(K_{M,A} + c_A + \frac{c_A^2}{K_{i,A}^A}\right) \cdot (K_{M,B} + c_B)}$ (6.3)
V_{max} [U mg^{-1}]	A: 0.367 ± 0.011 B: 0.335 ± 0.024	0.503 ± 0.032
$K_{M,A}$ [mM]	10.409 ± 2.232	8.769 ± 1.716
$K_{M,B}$ [mM]	62.836 ± 5.157	60.033 ± 6.143
$K_{i,A}$ [mM]	330.543 ± 69.871	364.405 ± 73.881

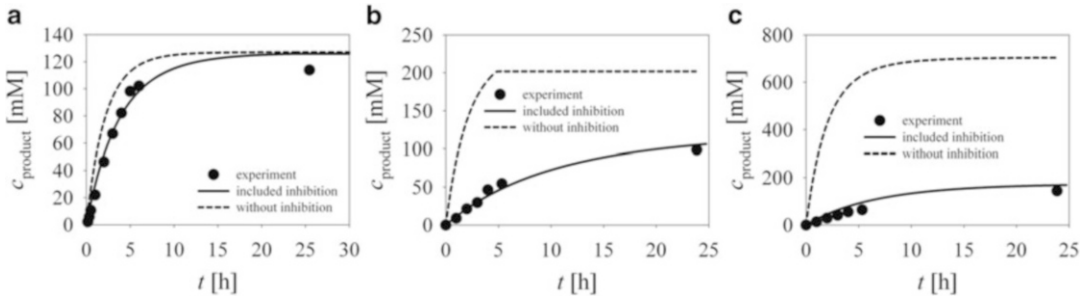

Fig. 6.3 The effect of non-competitive inhibition by the stabilizer found in the commercially available substrate on the product concentration. (**a**) 172.0 mM, (**b**) 233.3 mM, and (**c**) 749.7 mM of substrate (data adapted from [29])

(s) needed for experimental measurements; for example, an unstable intermediate difficult to isolate and purify. In these cases, it may be impossible to evaluate the right values of kinetic constants and modelling might be a difficult task, but some experience with similar systems might help, as similar models can be applied for similar reactions. So, the model parameters can be estimated from the entire concentration vs time curve. Something similar Sudar et al. [35] showed in their work where they successfully applied previous experience and theoretical model in alcohol dehydrogenase-catalysed oxido-reduction to select appropriate conditions for reaction optimization, even though they used this information later for statistical optimization of multi-enzyme cascade reaction.

Substrates are frequently not soluble enough to reach industrially relevant concentrations in the reactor and strategies on how to address this are discussed in Chap. 8. This also presents a challenge for evaluation of kinetic constants while saturation with substrate cannot be achieved. Additionally, for poorly soluble substrates analytics can present a problem [63]. In these cases, estimated parameters may be less reliable because kinetic data contain higher errors. Potential solutions for the choice of analytical method to follow the reaction are reviewed in Chap. 9.

Final things to keep in mind when developing a kinetic model of a reaction are the following: (i) if an equilibrium reaction is investigated, then both directions of the reaction need to be investigated; (ii) side-reactions occurring in the system have to be evaluated as well [64], while some are relevant and some are not; (iii) stability of reaction components is very important to consider, while substrates or products can undergo unwanted unspecific transformations that affect the final outcome of the process. Thus, it is important to know how fast these reactions are and to describe them with appropriate kinetic equations or neglect them if possible, keeping in mind the previously mentioned postulate from Octave Levenspiel [49]. One can conclude, from the previous discussion, that high amount of data should be available for a proper analysis of a complex reaction system.

6.2.3 Mathematical Model Development and Validation

Kinetic parameters, as well as the reaction scheme, serve as a base for the kinetic model development. When combined with mass balance equations for a specific reactor mode, they form a mathematical model of the reaction system in the reactor. Details on the mass balances for ideal reactors and their application are discussed in Chap. 7. The methodology of kinetic model development in a four-enzyme cascade reaction converting D-methionine to L-methionine was demonstrated in detail by Findrik and Vasić-Rački [65]. In this system every reaction step was investigated, and kinetic model was validated for each reaction step separately. Afterwards, the model of the cascade was devised and validated for the whole cascade reaction. In this example, purified substrates and enzymes were used, and

no side-reactions were detected; therefore, no difficulties were encountered. Model development and validation is not always a straightforward task due to complications that can occur during kinetic investigations which will be discussed below.

Experimental data from the literature or databases like BRENDA can also be used to develop mathematical models. This makes these models theoretical with limited applicability. However, they can still be very useful for preliminary simulations, for deciding on the starting points for future experiments, as well as for finding bottlenecks in the system. This is what Chen and co-authors [66] did in a two-step reaction for the synthesis of aminotriol catalysed by transketolase and transaminase. Their model included the equations calculating the toxic effect of substrate on the enzyme, substrate degradation, as well as kinetics of enzymatic reactions. Model simulations provided useful and descriptive information which led them to the decision on the best reactor mode and gave guidelines on enzyme activity expression levels in a cell host needed for future successful application of the investigated reaction system.

If used for optimization, mathematical model requires experimental validation [15, 67]. Performing multiple experiments at different (low and high) substrate and enzyme concentrations is the best way to ascertain broadness of its applicability. It is important to emphasize that the same data cannot be used for parameter estimation and model validation. A common problem in model validation is disagreement of the model of the entire multi-step reaction with the experimental data even if the model of the separate reaction steps shows broad applicability. Even so, in these cases the model still can be used for obtaining insights about the system and finding the right strategy towards improvement of the process metrics, but with a certain error. There are different reasons for non-compliance of the model and experimental data, and it is not always possible to find a reason. One of the most common reasons is the enzyme activity loss that occurs during time, i.e. operational stability decay. This is especially pronounced for enzymes used at industrial conditions which are much harsher than the conditions in the enzyme's natural environment—within the cell. Under these harsh conditions, enzyme decomposition can also occur. Thus, additional experiments, preferably independent from the other experimental data, should be done to evaluate **enzymes' operational stability**. Equations describing enzyme operational stability decay rate (k_d, deactivation rate constant) should be incorporated in the model to improve fitting between experimental and simulation results. In most cases, the rate of decay of enzyme operational stability can be described by the first-order kinetics [68]. Operational stability decay rate constants can be estimated directly from the concentration versus time data in the reactor but can also be evaluated from the independent measurements by following enzyme activity during the reaction. Just like with the initial reaction rate experiments, these experiments also must be well prepared and planned. Alternative substrates may be used if needed for these measurements, especially if they ease the data collection (for example, spectrophotometric method). Details on such approach are demonstrated in the literature [29]. An effect of operational stability decay on the reactor performance is presented in Fig. 6.4 for the batch (Fig. 6.4a) and continuous stirred tank reactor (Fig. 6.4b). The simulations in the batch reactor show that it is difficult to notice enzyme operational stability decay by merely observing the concentration vs time curve. One can make wrong conclusions that the reaction is too slow, or that there is an equilibrium, etc. However, this is not the case for the continuous stirred tank reactor, where the concentration vs time curve has a specific shape (Fig. 6.2b solid line) and it is very easy to observe enzyme operational stability decay (Fig. 6.2b long dashed and dotted line). Simulations of the reaction with included operational stability decay rate can show at what time during the reaction course will the enzyme become ineffective, i.e. the reaction can be planned to end before the enzymes lose their activity or additional enzyme could be added to the reactor to prevent the decline of the

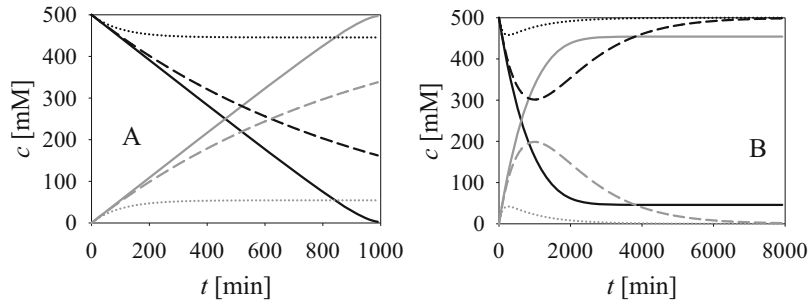

Fig. 6.4 The effect of enzyme operational stability decay rate on the experimental results in the (**a**) batch reactor and (**b**) continuous stirred tank reactor (solid line: $k_d = 0$ min^{-1}, long dashed line: $k_d = 0.001$ min^{-1}, dotted line: $k_d = 0.01$ min [1]

conversion and productivity [69]. The investigation of enzyme operational stability decay is an important part in the development and validation of mathematical models and should not be neglected, but often is.

Enzyme operational stability can also be affected by the substrate itself [29, 50, 70–76]. It is therefore important to investigate the effect of its concentration on the enzyme activity and use the data to mathematically formulate this in a model. This is of crucial importance for proper selection of the reaction conditions in later stage of model development, as well as for reactor selection.

As for other potential reasons for model non-compliance, scientists discuss factors such as protein–protein interactions, substrate channelling among cascade enzymes, and macromolecular crowding [77–79]. In practice this may lead to difference between the estimated kinetic parameters of individual enzymes and the kinetic parameters in the mixture of enzymes [80, 81]. This consequently leads to simulations that cannot describe the experimental data with the originally estimated kinetic parameters. Zhong and co-authors [42] developed a kinetic model for one-pot biotransformation of cellobiose from sucrose in a three-enzyme cascade and they found that kinetic parameters of individual enzymes are not the same as the apparent values of the parameters in the cascade reaction containing all enzymes. They found it necessary to adjust the values of two kinetic parameters, i.e. whereas catalytic constant of one enzyme was found to be approx. 25% higher, the catalytic constant of the other enzyme was found to be approx. 50% lower in the cascade than in separate reactions. Besides previously mentioned reasons, the authors explained that the differences in the kinetics parameter values might be due to the presence of a third enzyme, responsible for increased catalytic constant of the first enzyme (documented in the literature), and the presence of proteins in the latter case, which was experimentally determined. Baker et al. [82] showed that enzyme complexes in a multi-enzyme mixture can be formed which can then enable substrate transfer between enzymes' active sites without the release to the bulk solvent. This can surely explain some of the discrepancies in the values of the estimated kinetic parameters in the cascade reaction in comparison to single-enzyme systems. Nevertheless, as was stated before and shown with examples from the literature, even not completely accurate models or theoretical ones can be very useful for gaining insights of the investigated system, finding the starting points towards improvement of the process metrics, as well as for finding bottlenecks. Finally, when all the relationships are mathematically revealed, and the model experimentally validated, it is ready to be used in process optimization.

6.2.4 Model Application

Mathematical model can be used to predict reaction profiles in different reactors and at different conditions and thus provide insights for reactor selection, process control, and optimization. Different hypothetical reaction conditions can be explored in silico and time and money can be saved. For example, Fig. 6.5 (data not published) shows the effect of concentrations of two

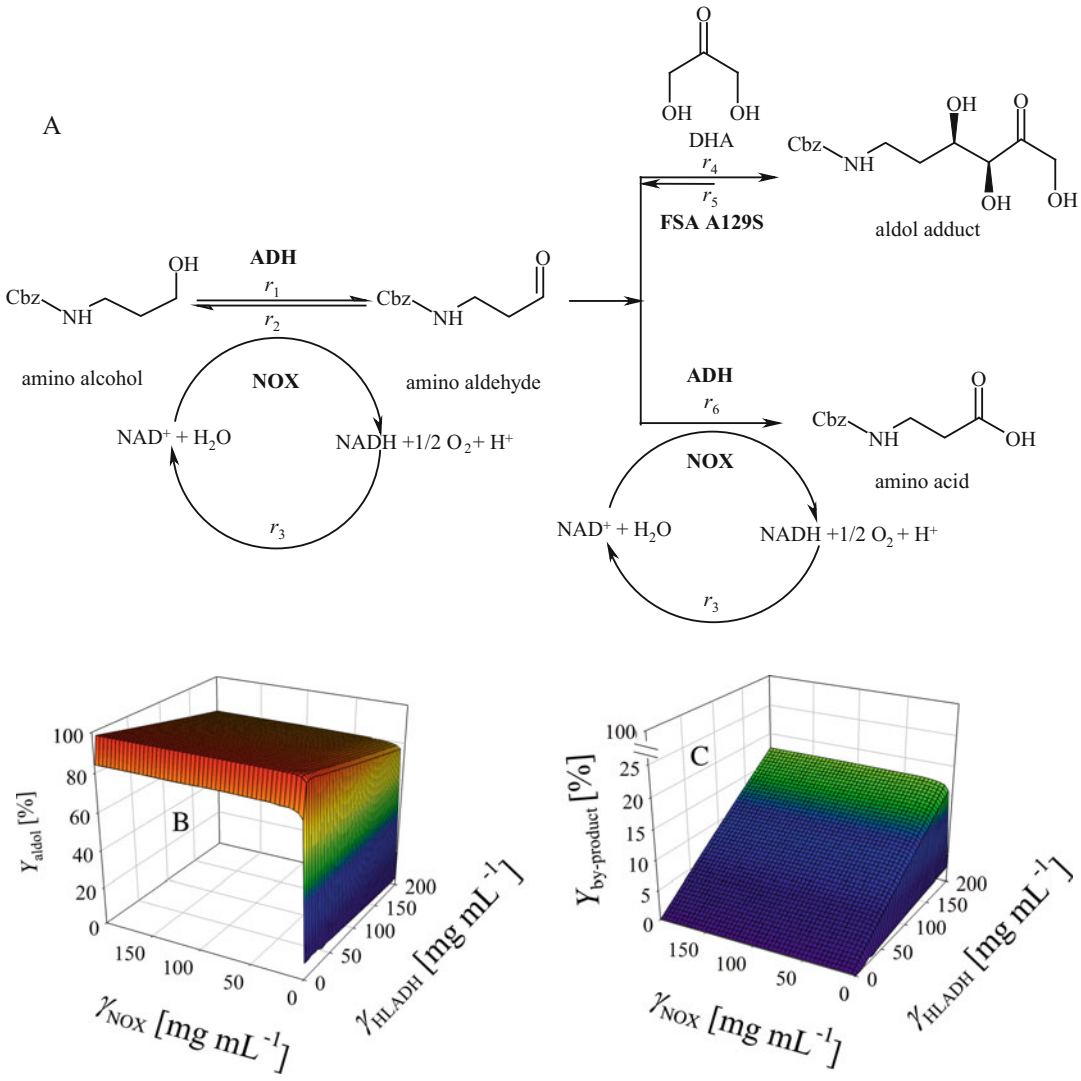

Fig. 6.5 (**a**) Reaction scheme. Influence of concentrations of horse liver alcohol dehydrogenase and NADH oxidase on the yield of target aldol product (**b**) and by-product, i.e. Cbz-*N*-3-aminopropanoate (**c**) in the three-enzymatic cascade reaction

enzymes (horse liver alcohol dehydrogenase, HLADH and NADH oxidase, NOX) used in the first oxidation step of the cascade towards an aldol product [35] on the reaction outcome, i.e. on the concentration of the main product and by-product. The simulation enables careful choice of reaction conditions to minimize the concentration of the unwanted by-product.

Parameter sensitivity analysis can help identify the parameters with the most pronounced influence on the reaction outcome [83]. Furthermore, the ones that are found to be irrelevant can be omitted from the model [15]. Accordingly, based on these data, specific kinetic parameters of enzymes can be improved by protein engineering [83].

Identification of kinetic models for separate reaction steps of a multi-step or multi-enzyme cascade reaction allows them to be used in modular form, i.e. one enzyme in the reaction can be easily replaced by another with the appropriate kinetic parameters. This also enables

identification of the best enzyme candidate by merely doing simulations. Model simulations can also be used to evaluate if a process could be improved with occasional enzyme addition, or even if this is economically justified. Different reaction scenarios in various reactor set-ups, feeding strategies of substrates and/or enzymes, etc. can be evaluated by using the modelling approach. Evaluation of a process from the point of process metrics, as well as from the economic point of view can be complicated even in a single-enzyme system, while in the multi-enzyme cascade reaction the number of constraints increases [13]. So, mathematical models, as an aid, can be used for process assessment. With an integral analysis, modelling can be also used for further scale-up.

Existing literature (some of which was already mentioned) that covers multi-enzymatic cascade reactions shows increasing recognition of the mathematical modelling value and the application of mathematical models. Even though it shows the advantages of a modelling approach, frequently the concentration of substrates used in these reactions is very low. For example, Dvorak et al. [84] described a five-step biotransformation of 1,2,3-trichloropropane to glycerol in a three-enzyme system with a model containing 16 kinetics parameters. They evaluated three variants of the enzyme for the first reaction step and applied the modular approach by exchanging kinetic parameters for the three variants. By doing so, they were able to choose the best enzyme to improve the process. Substrate concentrations used in this work were only up to 2 mM. Finnigan et al. [85] developed a model for seven-enzyme, three-step biotransformation. Some of the kinetic parameters were extracted from the literature and some were experimentally estimated. Genetic algorithm was used for model-based optimization to minimize the concentration of enzymes needed to obtain 90% yield. Concentrations of substrate of up to 4.5 mM were used. A one-pot synthesis of optically pure D-glyceraldehyde 3-phosphate and L-glycerol 3-phosphate was designed using three-enzyme system [64]. Performance of different reactor modes was evaluated by combining the developed model which included the kinetics of enzymatic reactions and non-enzymatic decomposition of the product. As in the previous example, very low substrate concentrations were used. An example of kinetic modelling application in systems biocatalysis is enzymatic pathway consisting of five enzymes for the production and regeneration of guanosine diphosphate (GDP)-mannose from mannose and polyphosphate with catalytic amounts of GDP and adenosine diphosphate (ADP) studied by Rexer et al. [86]. Their cascade optimization resulted in 71% yield of mannose to GDP-mannose.

More significant concentrations of product, up to 50 g L^{-1}, were obtained by Zheng et al. [81] who developed a semi-mechanistic model for the enzymatic hydrolysis of lignocellulosic biomass. Enzymatic hydrolysis of lignocellulosic substrate, as well as adsorption of cellulose onto chemically pre-treated cellulose material was included in the model. Scherkus et al. [87] investigated a linear three-step three-enzyme cascade system. The kinetic model was used to choose a proper reactor set-up for the reaction, i.e. fed-batch, due to the severe inhibition of one enzyme. This improved the productivity and shifted the equilibrium towards the product. Relatively high concentration of product (283 mM) and conversion (>99%) was obtained. Oxido-reductions consisting of a single step with coenzyme regeneration are surely the most studied and modelled two-enzyme systems. Kragl et al. [88] used the kinetic model of the two-enzyme system to optimize the reactor in continuous mode. 98.4% substrate conversion and 290 g L^{-1} d^{-1} of product were obtained in two enzyme membrane reactors connected in series. An excellent example of the application of multi-enzymatic cascade reaction modelling on process development and scale-up is the paper published by Van Hecke et al. [89]. Their model included the oxygen mass transfer, as well as enzyme kinetics and was used to find the optimal reaction conditions for the up-scaled reactor, i.e. mixing rate correlated with $k_L a'$, temperature enzyme activity ratio, mediator concentration, and substrate feed. The modelling results obtained at 0.2 L scale were successfully applied to study the process in 20 L-reactor and the final substrate conversion

obtained was >97% with volume productivity at 74.4 g L^{-1} d^{-1}.

With the progress of genetic engineering the design of de novo artificial metabolic pathways and conversions of unnatural substrates within in vivo cascades has gained momentum. For the successful implementation of these systems, mathematical modelling can provide great benefits while fine-tuning of the expression systems is required to obtain appropriate enzyme concentrations within the cell [62]. Rios-Solis et al. [62] studied the synthesis of two chiral amino-alcohols in a cascade reaction. By detailed kinetic characterization of enzymes within original cells, followed by the development and validation of the kinetic model in the batch reactor, they were able to predict the needed activity ratio of enzymes within newly designed genetically modified cells of *E. coli* containing both enzymes needed for the cascade, as well as scale up the syntheses to 50 mL-volume. As the kinetic analysis revealed substrate inhibition, fed-batch reactor was selected for the scaled-up synthesis and the reaction conditions were optimized. An excellent agreement between the model and experimental data was obtained with product concentration of 140 mM. This work highlights the importance of the application of kinetic modelling in synthetic biology, and it is expected that modelling will increase the speed of development and practical application of these systems. Similar approach was demonstrated by Milker et al. [90] who applied the kinetic model developed from in vitro experiments to investigate in vivo artificial pathway, as well as to identify problems and ways to resolve them merely by doing simulations. However, the concentrations they worked with were in a range of few mM. Opinions on the transferability of parameters measured in vitro to in vivo systems are divided between scientists [91], but there are examples in the literature that prove it can work.

Ardao and Zeng [33] investigated hydrogen production in silico in a process containing 13 enzymes. They compared one-pot and modular process, the effect of enzyme inhibitor consumption in a separate reactor which led to 8-fold increase in productivity in comparison to one-pot process. Simultaneous optimization of 16 variables in this reaction was performed by using the genetic algorithm and they were able to find the process conditions at which productivity of 355 mmol H_2 L^{-1} h^{-1} (0.71 g L^{-1} h [1] can be achieved with 94.2% yield. Similar system was investigated experimentally by Rollin et al. [92] who combined ten purified enzymes into an artificial cascade starting from sugars to produce H_2. They also used genetic algorithm for their research and based on model simulations they were able to improve productivity to 54 mmol H_2 L^{-1} h^{-1} (0.11 g L^{-1} h^{-1}) which is of industrial relevance [93]. The examples mentioned in this chapter clearly describe the possibilities and potential of modelling in the development of multi-enzymatic systems.

Even though the number of references covering multi-step reaction modelling increases every year, modelling related to process industry is not easy to find. To implement a biotransformation on industrial scale, the process has to comply with certain goals and reach certain process metrics (Table 6.3 [17]). Typical metrics for a biocatalytic process include product yield ($Y_{product}$), volumetric productivity (Q_P), specific productivity, product concentration ($c_{product}$), biocatalyst productivity (*BP*), etc., and it is clear that their optimization cannot be achieved without experimental and extensive modelling effort. Usually the objective of the optimization must be a compromise between several goal functions. Product

Table 6.3 Minimum goals of process optimization for different kind of chemicals produced by biocatalysis (adapted from [17])

Chemicals	Pharma/fine	Specialty	Bulk
$c_{product}$ [g L^{-1}]	50–100	100–200	200–400
$Y_{product}$ [%]	>80	>80	>99
BP [$kg_{product}$ $kg_{biocatalyst}^{-1}$]	10–100	100–1000	1000–10,000

concentration designated in Table 6.3 is relevant when crude or immobilized enzyme is used, whereas for whole cells, targets are lower [17]. These goals give a straightforward answer to question what dependent process variables need to be optimized and what is the purpose of modelling for industrial application. Volume productivity is usually expected to be above 2 g L^{-1} h^{-1} for small volume products [7].

6.3 Conclusions

In this work methodology of enzyme cascade modelling was described together with major issues and problems that occur. From the existing literature one can see that an increasing effort is being made to implement and apply engineering approach on complex multi-step reaction systems such as artificial metabolic pathways [86, 94], synthesis of building blocks or chemicals used in different industries [35, 37, 65, 87, 89, 90], synthetic pathways for the use of biomass, i.e. hydrogen production, or degradation of biomass to produce other value added products [92, 95, 96], degradation of toxic compounds [84], and sugar biotransformation [42, 94, 97, 98]. Application of modelling in multi-enzyme system is growing but is still in its early stages. Most of the systems are studied on mL-scale and by using relatively low and industrially insignificant substrate concentrations in the batch mode of operation which is acceptable for some fields of science (such as systems biology) but not for process engineering which should lead to industrial exploitation of these systems. Considering low concentrations of substrate applied in most systems presented in the literature, it is understandable that operational stability of enzymes is mostly neglected in these examples.

Construction of genetically modified microbial cells containing the desired enzyme activities to perform natural and non-natural multi-enzymatic biotransformations can be simplified and facilitated by kinetic modelling, while their concentrations in the cells can be finely tuned by this approach. Metabolic pathways can be evaluated and understood by kinetic modelling. The availability of kinetic behaviour of enzymes makes it possible to make preliminary matches between enzymes to form new multi-enzymatic systems without any additional experimental effort. Available scientific literature and databases offer a pool of substrates and enzymes for potential application. Even in the case of new substrates with unavailable data, preliminary evaluation can be performed by using known substrates, and conclusions can be drawn from there, based on the experience of the researcher. The potential of experimentation in silico is enormous, while it requires less time and money invested to reach results. Also, when combined with other engineering methods for optimization such as genetic algorithm or statistical optimization, the future of mathematical modelling seems even brighter.

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Enzyme Cascade Reaction Engineering

7

Philipp Petermeier and Selin Kara

Abstract

Biocatalytic transformations are of growing interest due to their high chemo-, regio-, and enantioselectivity, sustainability, and biocompatibility. In order to build on these advantages, to reinforce them, and not to weaken them, as well as to ensure the fulfillment of economic framework conditions, it is important to harmonize the reaction with its best suitable reactor and operation mode. In this chapter, three ideal reactor types and their biocatalytic conversion equations are presented for different inhibition scenarios, facilitating the proper bioreactor selection. Important aspects in the implementation of an enzymatic cascade are discussed and the introduced equations are exemplarily illustrated for a cascade reaction. In order to highlight different emerging approaches for cascade engineering, miniaturized flow bioreactors are discussed, and examples of recent publications are presented.

Keywords

Enzyme cascades · Reactor configurations · Kinetic modelling · Michaelis–Menten kinetics · Flow biocatalysis · Enzymatic process optimization

7.1 Introduction

Enzyme kinetics is the main decisive parameter for the design of biotransformations in different reactor types and different operation modes, for both single-step and multi-step catalysis implemented in cascade reactions. This chapter focuses on how to run enzymatic cascade reactions efficiently based on kinetic constraints. Designing cascade reactions is additionally useful for the displacement of unfavorable thermodynamic equilibria, which in this context will not be discussed in detail. For this, a recent review is advised for further reading [1]. In order to provide fundamental understanding of ideal reactors and their operation mode, the following parts focus on mass balances and rate equations for single-step catalysis, which represents an introduction to enzymatic cascade reactions. Ideal reactors display the following characteristics:

- Mixing is sufficiently intense and uniform,
- Controlled and constant process conditions (e.g. T, pH, pressure, etc.),
- No mass-transfer limitations with immobilized enzymes,
- No mass-transfer limitations in multi-phasic reaction systems.

From the biocatalyst point of view, ideality means:

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- No enzyme deactivation under process conditions,
- No enzyme leakage from immobilized preparations.

Deviations from ideality (from both the reaction and biocatalyst viewpoints) are observed when the following situations appear:

- Incomplete mixing (stirred-tank reactors) and back-mixing (plug-flow reactors) by, e.g.:
  - Channeling of fluid,
  - Recycling of fluid,
  - Creation of stagnant regions in the vessel.
- Non-isothermal operation due to inefficient mixing—poor heat transfer to the entire reaction medium,
- pH gradients due to poor mixing, H^+ generation or consumption during the biocatalytic reaction,
- Mass-transfer limitations in case of immobilized enzymes and in multi-phasic reaction systems,
- Enzyme denaturation under process conditions,
- Enzyme leakage from immobilized enzyme preparations.

Within this section, only ideal conditions will be handled, and the corresponding assumptions are given with the calculations.

7.2 Single-Step Enzymatic Catalysis: Reactor Configurations and Operation Mode

7.2.1 Batch-Wise Operated Stirred-Tank Reactor (BSTR)

In a BSTR, reactions start either with the addition of the enzyme E or (co)substrate S and usually 20–30% (v/v) of the reactor volume is left for the headspace. At the end of the reaction, the enzyme is inactivated (via T or pH change) and removed from the media. Recovery and reuse of active enzyme is possible via filtration when using immobilized enzymes. The characteristics of an ideal BSTR are:

(a) *Unsteady-state operation*: substrate and product concentrations change with time,
(b) *Perfect mixing*: uniform composition throughout the reactor at any instant time.

The mass balances and the rate equation used for a BSTR are derived as indicated below and the terms are listed in Box 7.1.

Mass Flux Analysis for BSTR (Fig. 7.1)

$$\begin{aligned}\text{input(S)} = {} & \text{output(S)} \\ & + \text{consumption(S) by reaction} \\ & + \text{accumulation(S) in reactor}\end{aligned}$$

$$F_{S,0} = F_S + (-r_S) \cdot V + \frac{dN_S}{dt} \tag{7.1}$$

(No input and output during batch operations: $F_{S,\,0} = F_S = 0$)

$$(-r_S) \cdot V = -\frac{dN_S}{dt} \tag{7.2}$$

(use: $dN_S = d(N_{S,\,0} \cdot (1 - X_S)) = -N_{S,\,0} \cdot dX_S$)

$$t = N_{S,0} \int_0^{X_S} \frac{dX_S}{(-r_S) \cdot V} \tag{7.3}$$

(*Assumption: constant fluid density)

$$t = C_{S,0} \int_0^{X_S} \frac{dX_S}{(-r_S)} = -\int_{C_{S,0}}^{C_S} \frac{dC_S}{(-r_S)} \; (*) \tag{7.4}$$

(Substituting $-r_S$ with $m_E \cdot v$ and using Michaelis–Menten equation for v. $-r_S$ varies since composition varies with time.)

Example on terms and units:

$$\left[-r_S\left(\frac{\text{mmol}}{\text{L} \cdot \text{min}}\right) = m_E\left(\frac{\text{mg}}{\text{mL}}\right) \cdot v\left(\frac{\mu\text{mol}}{\text{min} \cdot \text{mg}}\right)\right]$$

$$\begin{aligned} t &= -\int_{C_{S,0}}^{C_S} \frac{dC_S}{(m_E \cdot v)} \\ &= -\int_{C_{S,0}}^{C_S} \frac{dC_S}{m_E \cdot \left(\frac{v_{\max} \cdot C_S}{K_M + C_S}\right)} \end{aligned} \tag{7.5}$$

(**Assumption: m_E and $v_{\max}$ are constant)

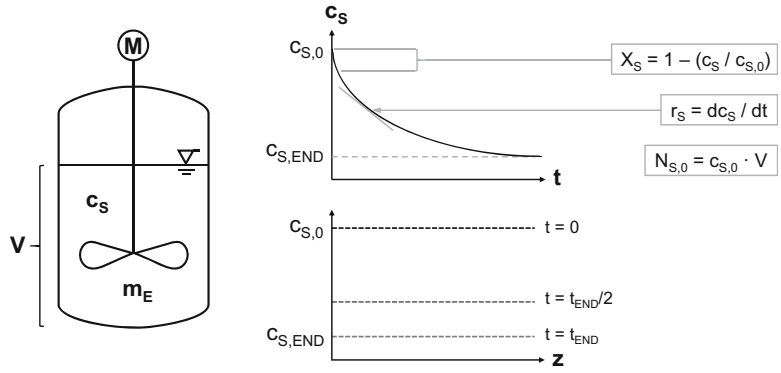

Fig. 7.1 Substrate S, enzyme E, and co-substrate are all homogeneously distributed in the reaction volume V of the ideal BSTR. The stirrer counteracts any local gradients and therefore maintains the homogeneous state. The substrate concentration c_S decreases over the course of the reaction and therefore with time. For an ideal reaction S → P, without any by-product formation, the accumulation of P follows the reverse trend of S. Due to perfect mixing, there are no differences along the arbitrary space coordinate z. Other abbreviations: X_S . . . conversion [–]; $c_{S,0}$ . . . initial substrate concentration [mmol/L]; r_S . . . reaction rate [mmol/L/min]; $N_{S,0}$ . . . initial amount of substrate [mol]

Assumption for Michaelis–Menten Equation: Irreversible, single-step, and single substrate enzymatic reaction without any inhibition

$$t = -\frac{1}{(m_E \cdot v_{\max})} \int_{C_{S,0}}^{C_S} \frac{dC_S}{\left(\frac{C_S}{K_M + C_S}\right)}$$

$$= -\frac{1}{(m_E \cdot v_{\max})} \int_{C_{S,0}}^{C_S} \left(K_M \frac{dC_S}{C_S} + dC_S\right) \quad (**) \tag{7.6}$$

(use: $C_S = C_{S,\,0} \cdot (1 - X_S)$)

$$t \cdot m_E \cdot v_{\max} = -K_M \cdot \ln(1 - X_S) + C_{S,0} \cdot X_S \tag{7.7}$$

Equation (7.7) can be applied to calculate the required enzyme amount (m_E) for a target substrate conversion (X_S) in a fixed operation time (t) (Table 7.1). The above given equations are valid with the assumptions that m_E, v_{max} (i.e. no change in biocatalyst amount and stable enzyme over process time), and fluid density are constant (i.e. no change in reaction volume). The terms used in the equations are:

Box 7.1

F_S Flux $\frac{N_S(\text{moles})}{t(\text{time})}$	**X_S Conversion** $\frac{N_{S,0} - N_S}{N_{S,0}}$ (–)
C_S Substrate concentration $\frac{N_S(\text{moles})}{V(\text{reation volume})}$	**r_S Specific reaction rate** $\frac{N_S(\text{moles})}{t(\text{time}) \cdot V(\text{reaction volume})}$

7.2.2 Continuously Operated Packed-Bed Reactor (CPBR)

This reactor type is also called an *ideal tubular reactor* or *plug-flow reactor* and exhibits the following characteristics:

(a) *Ideal plug-flow regime*: no mixing or diffusion along the flow path, residence time in the reactor to be the same for all elements of the fluid,

(b) *Concentration–location profile* through a CPBR is analogous to the concentration–time profile in a BSTR.

In a CPBR, the reactor volume can be envisioned as a set of volume elements that follow each other in plug-flow propagation direction

Table 7.1 Equations for reactors and operation mode to calculate the enzyme amount (m_E) to get a target substrate conversion (X_S) in a fixed operation or residence time (t *or* τ)

Reactor and Operation Mode *Case 1: No inhibition*	$t \cdot m_E \cdot v_{max}$ for BSTR $\tau \cdot m_E \cdot v_{max}$ for CPBR or CSTR	Eq. no.
BSTR	$C_{S,\,0} \cdot X_S - K_M \cdot \ln(1 - X_S)$	(7.7)
CPBR	$C_{S,\,0} \cdot X_S - K_M \cdot \ln(1 - X_S)$	(7.15)
CSTR	$C_{S,0} \cdot X_S + K_M \cdot \frac{X_S}{1-X_S}$	(7.24)

representing a series of sequential batch reactor with different initial substrate concentrations. Operation time (t) in a BSTR corresponds to fluid residence time (τ) in a CPBR. Immobilized enzymes [void fraction of 40–60% (v/v)] are densely packed in a CPBR and the reaction medium/fluid is pumped through the reactor continuously. The mass balance equations are derived for a CPBR as follows. Additional terms are given in Box 7.2.

Mass Flux Analysis for CPBR (Fig. 7.2)

$$\begin{aligned}\text{input(S)} = {} & \text{output(S)} \\ & + \text{consumption(S) by reaction} \\ & + \text{accumulation(S) in reactor}\end{aligned}$$

In a CPBR, the composition of the fluid varies from point to point along its flow path. Consequently, the material balance for a reaction component must be set up for a differential volume element dV:

$$F_{S,0} = (F_{S,0} + dF_S) + (-r_S) \cdot dV + \frac{dN_S}{dt} \quad (7.8)$$

(No accumulation at steady-state: $\frac{dN_S}{dt} = 0$)
(use: $dF_S = d(F_{S,\,0} \cdot (1 - X_S)) = -F_{S,\,0} \cdot dX_S$)

$$F_{S,0} \cdot dX_S = (-r_S) \cdot dV \quad (7.9)$$

$$\int_0^V \frac{dV}{F_{S,0}} = \int_0^{X_{S,f}} \frac{dX_S}{(-r_S)} \quad (7.10)$$

$$\frac{V}{F_{S,0}} = \int_0^{X_{S,f}} \frac{dX_S}{(-r_S)} \quad (7.11)$$

$$\frac{\tau}{C_{S,0}} = \int_0^{X_{S,f}} \frac{dX_S}{(-r_S)} \quad (7.12)$$

(*Assumption: constant fluid density)

(Substituting $-r_S$ with $m_E \cdot v$ and using Michaelis–Menten equation for v. $-r_S$ varies since composition varies with length)

$$\begin{aligned}\tau &= -\int_{C_{S,0}}^{C_S} \frac{dC_S}{(-r_S)} \; (*) = -\int_{C_{S,0}}^{C_S} \frac{dC_S}{(m_E \cdot v)} \\ &= -\int_{C_{S,0}}^{C_S} \frac{dC_S}{m_E \cdot \left(\frac{v_{max} \cdot C_S}{K_M + C_S}\right)}\end{aligned} \quad (7.13)$$

(**Assumption: m_E and v_{max} are constant)

$$\tau = -\frac{1}{(m_E \cdot v_{max})} \int_{C_{S,0}}^{C_S} \left(K_M \frac{dC_S}{C_S} + dC_S\right) \; (**) \quad (7.14)$$

(use: $C_S = C_{S,\,0} \cdot (1 - X_S)$)

$$\begin{aligned}\tau \cdot m_E \cdot v_{max} = {} & -K_M \cdot \ln(1 - X_S) \\ & + C_{S,0} \cdot X_S\end{aligned} \quad (7.15)$$

Box 7.2

$\dot{Q}$ **Flow rate** $\frac{V(\text{volume})}{t(\text{time})}$	$F_{S,\,0}$ **Mole flow rate of** S $F_{S,0}(\frac{\text{moles}}{\text{time}}) = \dot{Q} \cdot C_{S,0}$
τ **Residence time** $\frac{V(\text{volume})}{\dot{Q}(\text{flow rate})}$	**Consequential relation** $\frac{V}{F_{S,0}} = \frac{\tau}{C_{S,0}}$

The Equation (7.15) above is applied to calculate the enzyme amount (m_E) to get a target substrate conversion (X_S) in a fixed residence time (τ). The main difference between Eq. (7.15) and Eq. (7.7) is that "τ" is used in case of a CPBR, whereas "t" is applied in case of a BSTR (Table 7.1).

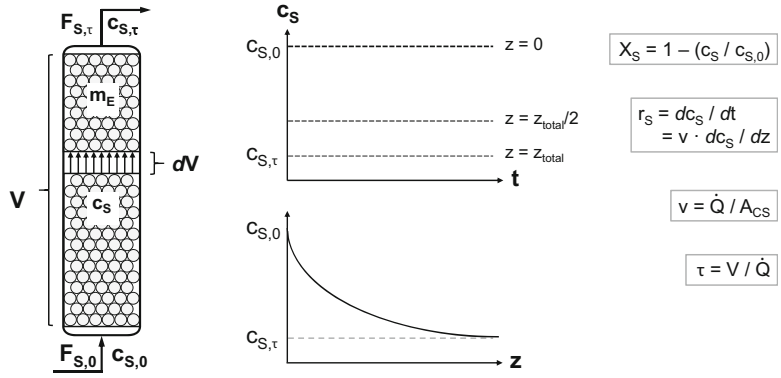

Fig. 7.2 A feed with substrate S and potentially necessary co-substrate is subjected to the CPBR. It passes through a densely packed bed of carrier with immobilized enzyme E and exits after residence time τ. The filled structure of the packed bed prevents a laminar, parabolic flow profile but ideally causes a constant flow velocity v across the whole cross-section of the tubular reactor. The constant flow vectors are illustrated in the segment dV. Due to ideal radial mixing by the turbulent flow, the interesting space coordinate z points in flow direction. In stationary operation, the local substrate concentration is constant with time but decreases along the reactor length. The time axis of the BSTR and the space axis of the CPBR are linked by the flow velocity v, which can be derived from the volume flow $\dot{Q}$ and the cross-sectional area A_{CS} of the packed bed. Other abbreviations: X_S . . . conversion [–]; $c_{S,0}$ . . . initial substrate concentration [mmol/L]; r_S . . . reaction rate [mmol/L/min]

7.2.3 Continuously Operated Stirred-Tank Reactor (CSTR)

The last reactor and operation mode to be analyzed is a continuously operated stirred-tank reactor (CSTR). Characteristically it has well-mixed conditions and different configurations are possible depending on enzyme and operational costs. Main characteristics of a CSTR are:

(a) *Runs at reactor outlet/exit conditions in steady-state*: constant high product and low substrate concentrations in the output,
(b) *Perfect mixing*: composition is uniform throughout the reactor (like BSTR).

Mass Flux Analysis for CSTR (Fig. 7.3)

$$\begin{aligned}\text{input(S)} = {} & \text{output(S)} \\ & + \text{consumption(S) by reaction} \\ & + \text{accumulation(S) in reactor}\end{aligned}$$

$$F_{S,0} = (F_{S,0} + dF_S) + (-r_S)\cdot V + \frac{dN_S}{dt} \quad (7.16)$$

(No accumulation at steady-state: $\frac{dN_S}{dt} = 0$)
(use: $dF_S = d(F_{S,\,0}\cdot(1 - X_S)) = -F_{S,\,0}\cdot dX_S$)

$$F_{S,0}\cdot X_S = (-r_S)\cdot V \quad (7.17)$$

$$\frac{V}{F_{S,0}} = \frac{X_S}{(-r_S)} = \frac{\tau}{C_{S,0}} \quad (7.18)$$

$$\tau = \frac{V}{\dot{Q}} = \frac{V\cdot C_{S,0}}{F_{S,0}} = \frac{C_{S,0}\cdot X_S}{(-r_S)} \quad (7.19)$$

(*Assumption: constant fluid density)

$$\tau = \frac{C_{S,0}\cdot X_S}{(-r_S)} = \frac{C_{S,0} - C_S}{(-r_S)}\ (*) \quad (7.20)$$

(**Assumption: m_E and $v_{\max}$ are constant)

(Substituting $-r_S$ with $m_E \cdot v$ and using Michaelis–Menten equation for v. $-r_S$ is constant since composition is constant during the reaction at steady-state)

$$\tau = \frac{C_{S,0} - C_S}{(m_E\cdot v)} = \frac{C_{S,0} - C_S}{m_E\cdot\left(\frac{v_{\max}\cdot C_S}{K_M + C_S}\right)}(**) \quad (7.21)$$

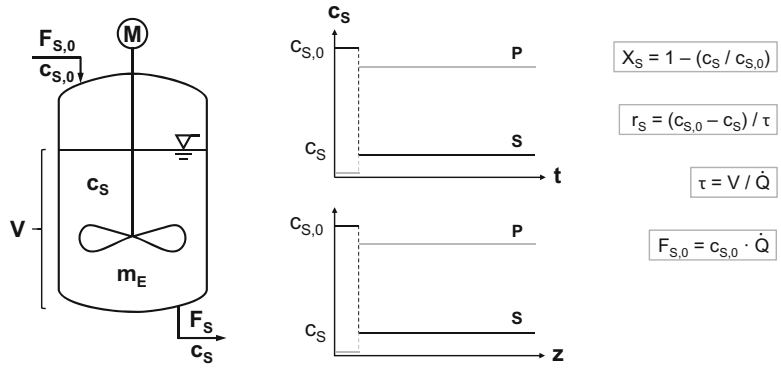

Fig. 7.3 The ideal CSTR runs with a constant supply of substrate S and possibly necessary co-substrate. Enzyme E may be fixed in the reactor or can be added constantly as well. This depends on its stability and productivity. As the graphs show, the substrate feed enters the reactor and is immediately mixed causing the sharp drop in concentration c_S down to a spatially and temporally constant concentration of substrate S within the reaction volume V. Again, the concentration profile of P is mirror-inverted when compared to S. This means that the reactor is operated under constant high product concentrations. The average residence time τ can be estimated from the reaction volume V and the total volume flow $\dot{Q}$. Other abbreviations: X_S . . . conversion [–]; $F_{S,0}$ . . . initial molar flow rate [mol/min]; r_S . . . reaction rate [mmol/L/min]

$$\tau \cdot m_E \cdot v_{\max} = \frac{C_{S,0} - C_S}{\left(\frac{C_S}{K_M + C_S}\right)} = \frac{(C_{S,0} - C_S) \cdot (K_M + C_S)}{C_S} \quad (7.22)$$

(use: $C_S = C_{S,\,0} \cdot (1 - X_S)$)

$$\tau \cdot m_E \cdot v_{\max} = \frac{(C_{S,0} \cdot X_S) \cdot (K_M + C_{S,0} \cdot (1 - X_S))}{C_{S,0} \cdot (1 - X_S)} \quad (7.23)$$

$$\tau \cdot m_E \cdot v_{\max} = K_M \cdot \frac{X_S}{1 - X_S} + C_{S,0} \cdot X_S \quad (7.24)$$

Table 7.1 summarizes the three equations derived for BSTR, CPBR, and CSTR.

Not only the kinetics but also other parameters such as operational costs, possibility for automatization, control of reaction conditions, etc., play an important role to choose which reactor to be used. Table 7.2 summarizes the main advantages and disadvantages of a BSTR, CPBR, and a CSTR.

So far, mass flux analysis for the combined determination of reaction times (τ or t) and enzyme loading (m_E) was done for a biocatalytic uninhibited system. However, often inhibition phenomena must be considered, what is done at the stage of the implementation of the Michaelis–Menten equation. The Michaelis–Menten equations to be applied for v in different inhibition scenarios are shown in Table 7.3.

A series of different equations that connect substrate conversion (X_S), reaction time (τ or t), and enzyme loading (m_E) with basic kinetic parameters like the Michaelis–Menten constant (K_M) and inhibition constants (K_i) can be derived for the different mentioned types of inhibition. An overview is presented in Table 7.4. The given equations are applied for single-step enzymatic reactions (based on the listed assumptions) in order to identify which reactor and operation mode to be applied.

Overall, C_S/K_M (i.e. first- or zero-order kinetics) and $K_{i,S}/K_M$ ratios (i.e. weak or strong inhibition) are crucial values to decide which reactor would be useful for a specific enzymatic reaction. Table 7.5 outlines in which scenarios a CSTR or a CPBR/BSTR should be used based on calculations with the equations from Tables 7.1 and 7.4. The determination of the most efficient

Table 7.2 Advantages and disadvantages of a BSTR, CPBR, and a CSTR

	Advantages	Disadvantages
BSTR	– High flexibility – High conversion – Less investment costs due to a general low demand on control expenditure – Easy pH and oxygen control	– Discontinuous operation – Low space-time-yields $g/(L \cdot d)$ due to long running and set-up times – High labor costs due to high personal costs
CPBR	– High space-time-yield $g/(L \cdot d)$ – Extensive automatization – Low labor costs – Small reactor volumes – Constant product quality	– Low flexibility – Difficult pH and oxygen control – High investment costs – High control expenditure
CSTR	– Control of reaction rate – Extensive automatization – Low labor costs – Small reactor volumes – Constant product quality – Easy pH and oxygen control	– Low flexibility – High investment costs – High control expenditure – Not suitable in case of product inhibition – Low conversion for $n \geq 1$ reactions

and economical reactor choice therefore allows for the solution with the highest output hence the least amount of enzyme (m_E) needed for a certain substrate conversion (X_S).

The efficient use of free, i.e. homogeneously dissolved enzyme in continuously operated reactors like the CSTR can be facilitated using ultrafiltration membranes of appropriate molecular weight cut-off (MWCO) specification. By this, the difference in molecular size of the biocatalyst and its substrate/product, respectively, are exploited, since the first is retained, whereas latter passes through the membrane. Thereby the residence times of catalyst and product are decoupled, and the catalyst can continue to be used, whereas the product is removed from the reaction [2].

As shown earlier, regarding enzyme kinetics the ideal BSTR and ideal CPBR behave the same, mathematically speaking. Their differences reside in their inverted space-time behavior and in their handling in non-stop operation. The BSTR requires set-up times for cleaning, feeding, and start-up between consecutive batches. For an ideal CPBR operation, i.e. without any enzyme deactivation, this is not needed. Therefore, the shorter the set-up times, the closer these reactor types become in terms of their productivity. Since these practical aspects may be taken into account at a later stage, these reactor types are treated mutually interchangeable in Table 7.5. Moreover, based on the same reasoning it can be concluded that the CPBR—and not the CSTR—is the continuous version of the BSTR.

Table 7.3 Michaelis–Menten equations used in different inhibition scenarios

Case 1: No inhibition	Case 2: Uncompetitive substrate inhibition	Case 3: Competitive product inhibition	Case 4: Non-competitive product inhibition
$\frac{v_{max} \cdot C_S}{K_M + C_S}$	$\frac{v_{max} \cdot C_S}{K_M + C_S \cdot \left(1 + \frac{C_S}{K_{i,S}}\right)}$	$\frac{v_{max} \cdot C_S}{K_M \cdot \left(1 + \frac{C_P}{K_{i,P}}\right) + C_S}$	$\frac{\frac{v_{max}}{\left(1 + \frac{C_P}{K_{i,P}}\right)} \cdot C_S}{K_M + C_S}$

Table 7.4 The equations needed for reactors and operation modes to calculate the enzyme amount (m_E) to get a target substrate conversion (X_S) in a fixed operation or reaction time (t *or* τ) for different inhibition scenarios

Reactor and Operation mode	$t \cdot m_E \cdot v_{\max}$ for BSTR $\tau \cdot m_E \cdot v_{\max}$ for CPBR or CSTR
Case 2: Uncompetitive substrate inhibition	
BSTR	$C_{S,0} \cdot X_S - K_M \cdot \ln(1 - X_S) + \frac{{C_{S,0}}^2}{K_{i,S}} \cdot X_S \cdot (1 - 0.5 \cdot X_S)$
CPBR	$C_{S,0} \cdot X_S - K_M \cdot \ln(1 - X_S) + \frac{{C_{S,0}}^2}{K_{i,S}} \cdot X_S \cdot (1 - 0.5 \cdot X_S)$
CSTR	$C_{S,0} \cdot X_S + K_M \cdot \frac{X_S}{1-X_S} + \frac{{C_{S,0}}^2}{K_{i,S}} \cdot X_S \cdot (1 - X_S)$
Case 3: Competitive product inhibition	
BSTR	$C_{S,0} \cdot X_S \cdot \left(1 - \frac{K_M}{K_{i,P}}\right) - K_M \cdot \left(1 + \frac{C_{S,0}}{K_{i,P}}\right) \cdot \ln(1 - X_S)$
CPBR	$C_{S,0} \cdot X_S \cdot \left(1 - \frac{K_M}{K_{i,P}}\right) - K_M \cdot \left(1 + \frac{C_{S,0}}{K_{i,P}}\right) \cdot \ln(1 - X_S)$
CSTR	$C_{S,0} \cdot X_S + K_M \cdot \frac{X_S}{1-X_S} + C_{S,0} \cdot \frac{K_M}{K_{i,P}} \cdot \frac{{X_S}^2}{(1-X_S)}$
Case 4: Non-competitive product inhibition	
BSTR	$C_{S,0} \cdot X_S \cdot \left(1 - \frac{K_M}{K_{i,P}}\right) - K_M \cdot \left(1 + \frac{C_{S,0}}{K_{i,P}}\right) \cdot \ln(1 - X_S) + \frac{{C_{S,0}}^2 \cdot {X_S}^2}{2 \cdot K_{i,P}}$
CPBR	$C_{S,0} \cdot X_S \cdot \left(1 - \frac{K_M}{K_{i,P}}\right) - K_M \cdot \left(1 + \frac{C_{S,0}}{K_{i,P}}\right) \cdot \ln(1 - X_S) + \frac{{C_{S,0}}^2 \cdot {X_S}^2}{2 \cdot K_{i,P}}$
CSTR	$C_{S,0} \cdot X_S + K_M \cdot \frac{X_S}{1-X_S} + C_{S,0} \cdot \frac{K_M}{K_{i,P}} \cdot \frac{{X_S}^2}{(1-X_S)} + \frac{{C_{S,0}}^2 \cdot {X_S}^2}{K_{i,P}}$

7.3 Enzymatic Cascade Reactions: Essential Characteristics and Examples of Recent Developments

The above explained derivations for single-step reactions are the basis to design enzymatic cascade reactions. This necessitates detailed consideration and analysis of a series of important factors:

- *Enzyme kinetics*
  (first-order or zero-order kinetics, substrate and/or product inhibition),
- *Enzyme stability*
  (enzyme deactivation or disintegration of the immobilization carrier due to high shear stress),
- *Enzyme form*
  (immobilization for stability, recovery and reuse, and for continuous processes, apparent kinetic parameters of immobilized enzyme, effective mass transfer while using immobilized enzymes),

Table 7.5 Scenarios of enzyme kinetics for the choice of bioreactors

	Case 1: No inhibition		Case 2: Uncompetitive substrate inhibition		Case 3 and 4: Competitive or non-competitive product inhibition	
	First-order kinetics $\left(\frac{C_S}{K_M} \ll 1\right)$ $\left(v \approx \frac{v_{max} \cdot C_S}{K_M}\right)$	Zero-order kinetics $\left(\frac{C_S}{K_M} \gg 1\right)$ $(v \approx v_{max})$	Weak inhibition[a] $\left(\frac{K_{i,S}}{K_M} \gg 1\right)$	Strong inhibition[a] $\left(\frac{K_{i,S}}{K_M} \ll 1\right)$	Weak inhibition[a,b] $\left(\frac{K_{i,P}}{K_M} \gg 1\right)$	Strong inhibition[a,b] $\left(\frac{K_{i,P}}{K_M} \ll 1\right)$
Recommended reactor type	CPBR/BSTR	CSTR or CPBR/BSTR	CPBR/ BSTR	CSTR	CPBR/ BSTR	CPBR/ BSTR
Enzyme form	Immobilized	Free or immobilized	Immobilized	Free or immobilized	Immobilized	Immobilized

[a]L.G. Lee and G.M. Whitesides, J. Org. Chem. (1986) 51, 25–36; A. Liese et al. Biotechnol. Bioeng. (1996) 51, 544–550
[b]At low conversions a CPBR/BSTR is slightly better than CSTR, at high conversions a CPBR/BSTR is significantly better than CSTR

- *Parameter control*
  (temperature and pH control as well as gas supply),
- *Investment and labor costs*
  (reactor volume, extensive automatization, complexity of process control, and personnel costs),
- *Process time*
  (fit to the industrial routine, running and set-up times of BSTRs),
- *Thermodynamics*
  (in situ product removal, e.g. extraction in multiple-phase reaction systems or reactive distillation; excess substrate supply; reaction coupling with a thermodynamically highly favored and hence spontaneous consecutive or simultaneous reaction providing an additional driving force).

Assuming that an enzymatic cascade reaction is planned to be established in a series of continuously operated reactors, the information from enzyme kinetics, i.e. v_{max} and K_M as well as the operational values m_E and τ have to be used to calculate X_S. In this case, the substrate concentration at the outlet of the first reactor will correspond to the substrate concentration at the inlet of the second. With this initial assessment, it can be evaluated which configuration would be better, e.g. a CSTR followed by a CPBR or vice versa. An exemplary calculation is provided below to illustrate this approach. For a more detailed analysis of different scenarios for cascade design Illanes et al. [3] is recommended.

Example: A biocatalytic transformation is envisioned to be run in two continuous reactors operated in series: a CSTR and a CPBR. The more favorable sequence shall be determined. Residence times, flow rates, and enzyme amount shall be equal for both reactors. The total amount of immobilized biocatalyst is 7 kg. The kinetic parameters are $v_{max} = 100\ \mu mol \cdot min^{-1} \cdot g_{cat}{}^{-1}$ and $K_M = 75.8$ mM. The initial substrate concentration is to be 250 $mmol \cdot L^{-1}$ and shall be fed at 120 $L \cdot h^{-1}$. The system does not suffer from any form of inhibition; hence these equations are used:

$$C_{S,0} \cdot X_S - K_M \cdot \ln(1 - X_S) = \tau \cdot m_E \cdot v_{max} \quad \text{(CPBR)}$$

$$C_{S,0} \cdot X_S + K_M \cdot \frac{X_S}{1 - X_S} = \tau \cdot m_E \cdot v_{max} \quad \text{(CSTR)}$$

Note: The right-hand term of the equation can be rewritten as shown below.

$$\tau \cdot m_E \cdot v_{max} = \left(V/\dot{Q}\right) \cdot m_E \cdot v_{max} = m_{E,abs.} \cdot v_{max}/\dot{Q} = 175.0$$

$$[s] \cdot [g/L] \cdot [mmol/s/g] = ([L]/[L/s]) \cdot [g/L] \cdot [mmol/s/g] = [g] \cdot [mmol/s/g]/[L/s]$$

In either scenario (CSTR/CPBR or CPBR/CSTR) the second reactor does not start at a conversion $X_{S,\ 0} = 0$. To account for this, the equations are adapted as follows:

$$C_{S,0} \cdot (X_S - X_{S,0}) - K_M \cdot \ln\left(\frac{1 - X_S}{1 - X_{S,0}}\right) = \tau \cdot m_E \cdot v_{max} \quad \text{(CPBR 2)}$$

$$C_{S,0} \cdot (X_S - X_{S,0}) + K_M \cdot \frac{X_S - X_{S,0}}{1 - X_S} = \tau \cdot m_E \cdot v_{max} \quad \text{(CSTR 2)}$$

Numerical solutions of these equations give the following intermediate and ultimate conversions of 49.4% and 78.4% for CPBR/CSTR and 45.1% and 81.7% for CSTR/CPBR, respectively. Therefore, the second is the better configuration.

Having introduced the theoretical background for designing enzymatic conversions in different reactor types and operational modes, the following part of this chapter focuses on recent developments in the implementation of enzymatic reactions in different operation modes and on different scales.

7.3.1 Enzymatic Cascade Reactions in Continuous Flow

Continuous flow technology has become a rapidly growing research area and recently attracted a deal of interest in biocatalysis for the synthesis of pharma and fine chemicals with high level of process intensification [4–11]. The following graph outlines the number of publications reported for the use of biocatalysis in continuous flow systems in the last 20 years (Fig. 7.4).

Miniaturized flow bioreactors (MFBRs) are applied for continuous processing, whereby reactions take place under rigorously controlled conditions in a confined space. The advantages of MFBRs over batch bioreactors are [12–14]:

- Small reactor dimensions result in enhanced heat and mass transfer by 1–2 orders of magnitude, that speed up reaction rates hence reduce reaction times and increase productivity,
- Small footprint from the viewpoint of equipment,
- Ease of increasing capacity by prolonging the reaction time (i.e. scaling out), or building series and/or parallel MFBRs (i.e. numbering up),
- Reduced risk associated with accumulation and storage of hazardous intermediates since their transient amounts are below the safety limits,
- Reduced product inhibition issues by continuous removal of product(s) with flow,
- High enzyme-to-substrate ratio achieved,
- Reduced attrition of enzyme activity compared to using immobilized enzymes under stirring conditions,
- Small reactor dimensions allowing precise monitoring and control of heat exchange,
- Easy reaction parameter (temperature, pressure, flow rate) set-up and monitoring resulting in more reliable and reproducible processes,
- Screening of reaction conditions in a time and material efficient way due to the small reactor volumes,
- Low manufacturing, maintenance, and operating costs with low power consumption.

MFBRs enable sustainable manufacturing since high-throughput optimization of reaction conditions can be done in a material-, space-, time-, and energy-efficient manner. MFBRs are *micro*- and *meso*-scale reactors known for their characteristic (dis)advantages [4]. The former mainly cover microfluidic devices displaying channels or tubes with inner diameters of 10–500 μm and reactor volumes of μL's, whereas latter have inner diameters between 500 μm and a few mm's corresponding to reaction volumes of a few mL's. Please note, that slightly different definitions for micro- and mesoreactors are also available in the literature [15]. In terms of their dimensions they resemble HPLC columns. Especially in case of cascade reactions, continuous flow technology has attracted great attention since a multi-catalytic and multi-step reaction poses a multi-parameter problem that has to be handled both in a holistic and efficient manner at the same time. However, the challenges related to continuous flow technology need to be addressed as well. Typical examples are:

- Efficient control of reaction conditions, e.g. pH changes,
- Efficient supply of gaseous substrates for enzymes, e.g. oxygen for oxidases, oxygenases, etc.
- Low tolerance towards the presence or formation of solid particulate matter in the reaction media,
- Presence of remaining non-converted reaction intermediates leading to cross-inhibition and difficult product isolation (a general challenge for cascades).

The above listed limitations can be overcome by bioprocess engineering. For example, tube-in-tube reactors allow to provide gaseous substrates via diffusion through a membrane into the aqueous media [16–18]. If an organic solvent as a substrate reservoir and/or product sink is needed in a so-called two-liquid-phase system (2LPS), continuous flow technology is significantly advantageous over batch operations. With the aid of continuous flow, fine dispersions of an organic phase can be achieved providing high

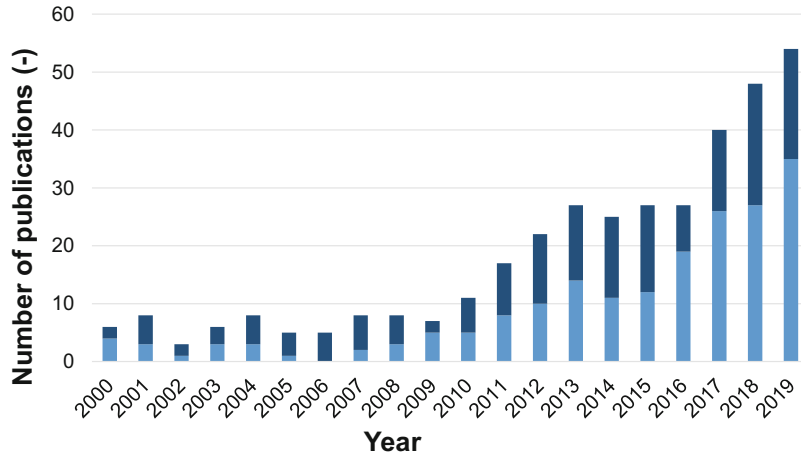

Fig. 7.4 Number of publications per year since 2000 in continuous flow biocatalysis. The overall bars represent the numbers found for publications with the key words "flow biocatalysis," whereas the lighter blue proportions represent these that used "continuous flow biocatalysis." (source: Web of Science, Data was retrieved in March 2020)

surface-to-volume ratios. Since mass transfer proceeds via the increased liquid interface, it allows for (1) high substrate loadings and (2) enhanced extraction of product(s) while simultaneously reducing the risk of enzyme denaturation caused by high-speed stirring in batch systems [19]. Overall, continuously operated MFBRs have great potential for process intensification in biocatalysis for "green" manufacturing.

A recent example of flow chemistry for a multi-enzymatic cascade by Zheng et al. [20] employed four enzymes in a continuous two-step two-pot system. Each "pot" contained two enzymes, which were co-immobilized (Fig. 7.5). Oxidation of chenodeoxycholic acid (CDCA) to the intermediate product 7-oxolithocholic acid (7-oxo-LCA) was catalyzed by co-immobilized 7α-hydroxysteroid dehydrogenase (7α-HSDH) and lactate dehydrogenase (LDH) in the first packed-bed reactor (PBR), whereas the subsequent selective reduction to ursodeoxycholic acid (UDCA) was catalyzed by co-immobilized 7β-hydroxysteroid dehydrogenase (7β-HSDH) and glucose dehydrogenase (GDH) in the second PBR. Epoxy-functionalized resins were used in this study.

For comparison, the productivity in a batch process was investigated using either free or immobilized enzymes. In the batch system, in order to avoid a reverse reaction, the two enzymes involved in the first step had to be inactivated by heat treatment, a cost- and time-intensive part of the cascade reaction. However, this could be eliminated by running the enzymatic cascade using immobilized enzymes in two serial packed-bed reactors. Parameters such as immobilization pH, time, and loading ratio of enzymes to epoxy resin were optimized, which gave 12- and 516-fold higher activity of LDH-7αHSDH and 7βHSDH-GDH, respectively, compared to the initial immobilization conditions. Under the optimized conditions, the yield of UDCA reached approx. 100% and lasted for at least 12 h in the packed-bed reactors, which was superior to that of the batch-wise operated system.

Another study by Brahma et al. [21] combined CALB-catalyzed hydrolysis of ethyl cyanoformate (NCCOOEt in Fig. 7.6) to HCN with hydroxynitrilase (HNL)-catalyzed synthesis of cyanohydrins in continuous flow. The first step involved the use of a commercially available immobilized *Candida antarctica* lipase B (CALB) available under the trade name Novozym 435, whereas the second step was catalyzed by wild-type *Arabidopsis thaliana* (*At*) HNL-expressing *E. coli* BL21 (DE3) cells.

By means of flow chemistry, HCN was provided for the HNL-catalyzed step in situ, eliminating the need for the use of highly toxic HCN gas. In addition to that, the flow system allowed to introduce a protecting group in an in-line fashion. The bi-enzymatic cascade and in-line chemical acetylation for stabilization of products run in continuous flow proved to be

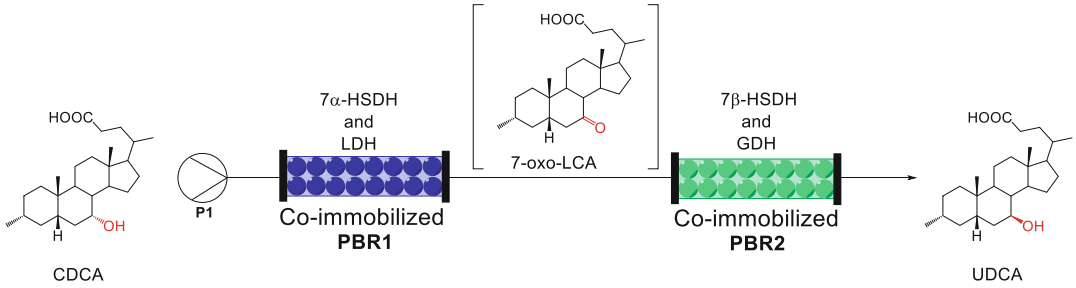

Fig. 7.5 Co-immobilized enzymes applied in two serial packed-bed reactors for the continuous synthesis of ursodeoxycholic acid (UDCA) from chenodeoxycholic acid (CDCA)

advantageous over the batch approach in terms of reaction time (40 min vs. 345 min) and ease of operation. *O*-acetylcyanohydrins were synthesized with very good conversions and *ee* values over three steps (75–99% conversion; 40–98% *ee*). In this respect, continuous flow has high potential for implementation of reactions, which have been avoided in batch operations due to safety reasons.

Spain and coworkers [22] developed a continuous chemo-enzymatic cascade running in three sequential packed-bed microreactors (μ-PBRs). The three-step reaction comprises an initial reduction of nitrobenzene by zinc to hydroxyaminobenzene (HAB), which is then re-arranged intramolecularly by immobilized HAB-mutase in the second reactor to form 2-aminophenol (Fig. 7.7). The final transformation involves the oxidation of 2-aminophenol to 2-aminophenoxazin-3-one (APO) catalyzed by immobilized soybean peroxidase using H_2O_2. The reaction set-up consisting of three cascading microfluidic chips was separately loaded with zinc powder (μ-PBR1) and enzymes immobilized on silica (μ-PBR2 and μ-PBR3). The observed low conversion of 19% and space-time yield (STY) of 4 g L^{-1} d^{-1} implied the need for optimization. However, it demonstrated the high potential of the reaction system and microfluidics for the screening of nitroarene conversions.

The continuously operated cascade-reactor approach was also shown to be powerful for multi-enzymatic reactions involving more than three reaction steps. The continuous conversion of 3-phospho-D-glycerate (3-PGA) into D-ribulose 1,5-bisphosphate (RuBP) was demonstrated in a series of 11 packed-bed reactors containing immobilized enzymes: phosphoglycerate kinase, glycerate phosphate dehydrogenase, triosephosphate isomerase, aldolase, transketolase, phosphatase, epimerase, and phosphoribulokinase by Bhattacharya et al.

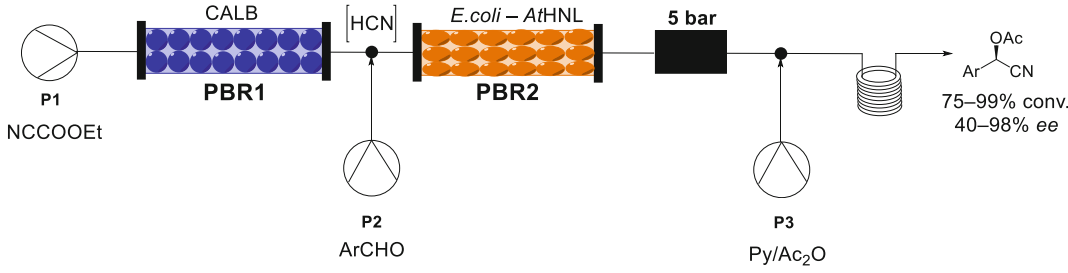

Fig. 7.6 In situ generation of HCN gas for asymmetric synthesis of cyanohydrins. Pump 1 (P1) supplies ethyl cyanoformate, P2 supplies aldehydes, and P3 provides the acetylating agent for stabilization of products. CALB: Novozym 435 and HNL: *E. coli* whole-cells containing *Arabidopsis thaliana* hydroxynitrilase (HNL)

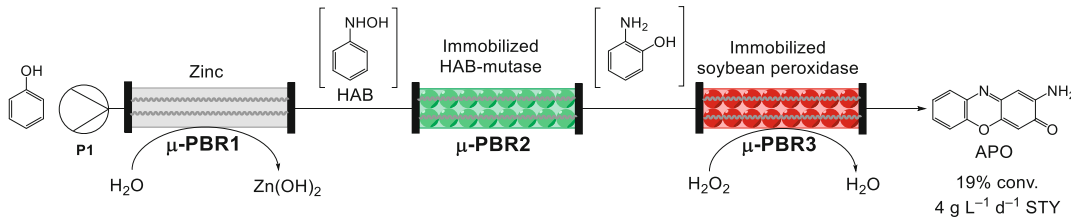

Fig. 7.7 Chemo-enzymatic synthesis of APO in a cascade of continuously operated packed-bed microreactors (μ-PBRs)

[23]. The ultimate motivation was to continuously regenerate RuBP, which acts as an acceptor for the biocatalytic fixation of CO_2 to synthesize 3-PGA, rendering the CO_2 fixation a continuous process. The use of 11 PBRs in series for the continuous synthesis of RuBP from 3-PGA gave 56 ± 3% overall conversion at 0.3 g L^{-1} d^{-1} STY, which demonstrated the applicability of such a sequence reaction in continuous flow.

Martínková and coworkers [24] demonstrated the synthesis of isonicotinamide as a by-product in the nitrilase-catalyzed conversion of 4-cyanopyridine to isonicotinic acid. In this study, two PBRs were coupled in series whereby the second reactor was loaded with an immobilized amidase from *Rhodococcus erythropolis*, which catalyzed the hydrolysis of the residual amide to isonicotinic acid (Fig. 7.8). Both reactions were carried out in an aqueous medium using a suitable buffer acceptable for both enzymes. Overall, a STY of 2.1 kg L^{-1} d^{-1} was obtained at full conversion and 99.8% purity of the desired product.

Strompen et al. [25] developed a sequential chemo-enzymatic process for a continuously operating production of the chiral β-amino acid ester ethyl (*S*)-3-(benzylamino)-butanoate. The reactor set-up combined a plug-flow reactor (PFR) for the thermal aza-Michael addition of benzylamine to *trans*-ethyl crotonate coupled to a subsequent packed-bed reactor for the immobilized CALB (Novozym 435)-catalyzed kinetic resolution of the racemic intermediate product (Fig. 7.9).

The coupled reactors were operated continuously for 80 h without significant loss of enzyme activity. The target β-amino acid ester was obtained with 92% conversion in the PFR and 59% conversion in the PBR at high *ee* of >98% and a catalyst productivity of 4.9 kg $kg^{-1}_{N435} \times h^{-1}$. A space-time yield of 0.4 kg × L^{-1} × d^{-1} was calculated for the total reactor system and 1.8 kg × L^{-1} × d^{-1} based solely on the volume of the packed-bed reactor. The continuously operating, solvent free process thus represents an efficient method for the enantioselective production of a value added (*S*)-β-amino acid ester starting from cheap substrates [25].

Moreover, high-throughput optimization enabled by MFBRs can be combined with means of computational approaches. In silico modelling of flow biocatalysis is challenging, especially for coupled biocatalytic reactions that pose complex systems, but it allows a more targeted and ultimately faster process optimization (Chap. 6). As Burgahn et al. [26] have shown, modelling goes even further than that and can provide detailed insights into reacting biocatalytic flow systems that can hardly be accessed otherwise. They studied a microfluidic reactor with a rectangular cross-section. The lower half of the channel was filled with a packed bed of ketoreductase (Gre2)-functionalized magnetic beads that were hold in place by magnets underneath the reactor. The packed bed was overflown by a feed of substrate, co-substrate, cofactor, and enzymatic cofactor regeneration system as shown in Fig. 7.10.

They developed a novel mathematical reactor model based on modular rate laws that allowed them to predict the influence of different parameters such as flow rate or catalytic bed dimensions on the final product composition.

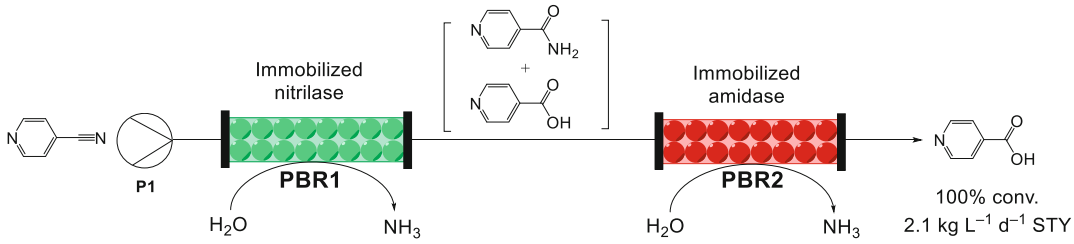

Fig. 7.8 Continuous hydrolysis of 4-cyanopyridine to isonicotinic acid in a cascade of two PBRs

Despite their complicated system, they were able to validate their model and even identify the bed thickness as bottleneck, limiting the local availability of NADPH. These findings from modelling allowed to reduce the required amount of biocatalyst and to increase the STY.

As shown, continuous flow processing in miniaturized reactors offers specific advantages in terms of material, energy and time efficiency. The use of enzymes in miniaturized flow reactors has enabled enormous developments in biocatalysis as highlighted by the number of publications reported during the last years (Fig. 7.4). Key enabling technologies have been joined with continuous flow technology resulting in the utilization of, e.g. 3D-printing, material sciences, microreactor technology, or online process analytical technology (PAT) tools (Chap. 9).

7.3.2 Enzymatic Cascade Reactions in Discontinuous Systems

A recent example on an enzymatic cascade was reported by Liese and coworkers for the synthesis of poly-ε-caprolactone running in a fed-batch operation [27, 28]. In the first part of the cascade, two oxidation steps starting with alcohol dehydrogenase (ADH) catalyzed oxidation from cyclohexanol to cyclohexanone and further oxidation to ε-caprolactone by means of a Baeyer–Villiger monooxygenase were coupled in a stirred-tank reactor (STR). As a third step, lipase-catalyzed hydrolysis of the lactone to 6-hydroxyhexanoic acid was performed. Two scenarios were realized; in one case, immobilized lipase was packed in a column, whereas in the second case lipase was added into the STR directly together with the other two enzymes, i.e. alcohol dehydrogenase and Baeyer–Villiger monooxygenase. With the fed-batch approach, severe substrate surplus and product inhibition could be circumvented by adding the cyclohexanol substrate and by in situ removal of lactone product by hydrolysis, respectively.

Another fed-batch synthesis approach has been demonstrated by Bornscheuer and coworkers [29] for the use of whole-cells harboring co-expressed ADH and a cyclohexanone monooxygenase CHMO. The bi-enzymatic

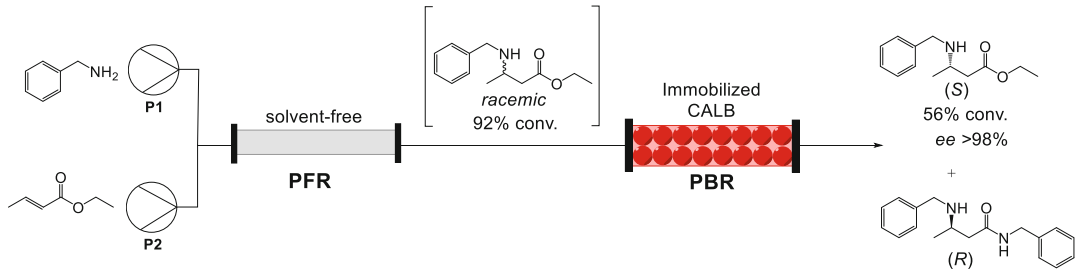

Fig. 7.9 Coupled reactor set-up for the continuously operating chemo-enzymatic production of ethyl (*S*)-3-(benzylamino) butanoate

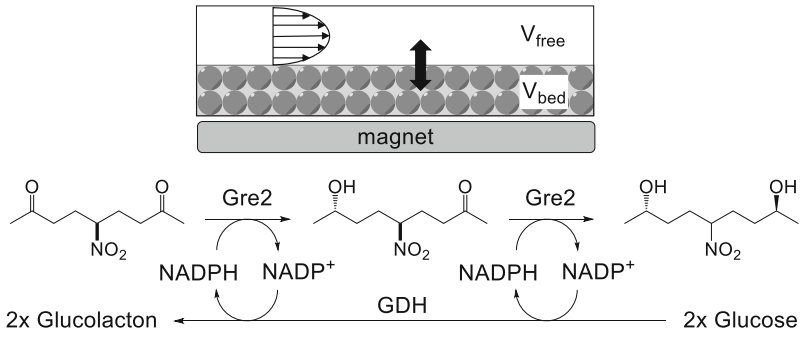

Fig. 7.10 This 2D illustration shows the axial cross-section of the set-up and modelled microreactor. The mass transfer between the packed bed and the free volume is indicated by the double-headed arrow. The reaction taking place at the active site of the immobilized Gre2 is shown below. The $NADP^+$ recycling was realized with GDH

cascade coupled ADH-catalyzed cyclohexanol oxidation to cyclohexanone with the CHMO-catalyzed oxygenation reaction resulting in ε-caprolactone. The optimized process gave 98% conversion of the starting substrate cyclohexanol and 20 g L^{-1} ε-caprolactone.

Ultimately, it can be concluded that not only the choice of reaction system but also that of the reactor and its operation have a significant influence on the outcome and success of a biocatalytic transformation. The advantages of different types of reactors, their combinations with each other or with other mentioned and constantly emerging tools of modern reaction engineering offer a multitude of synergistic possibilities, especially for enzymatic cascade reactions.

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Enzyme Cascade Process Design and Modelling

8

John M. Woodley

Abstract

In recent years, the use of multi-enzyme cascades to catalyze synthetic reactions has become increasingly commonplace in the laboratory as a means of enabling the synthesis of complex molecules of interest. When coupled with ex vivo cofactor regeneration, cascades provide the possibility for a potentially scalable synthesis. However, challenges of cross-reactivity and optimization as well as control suggest that modularization, rather than one-pot synthesis, is a particularly attractive route for scale-up. In this chapter, the concept of cascade process design using modularization will be further outlined and the specific role of mathematical modelling in both enzyme stoichiometric balancing, as well as optimization of individual cascade modules and the complete process will be discussed.

Keywords

Enzyme cascade · Process design · Process modelling · Kinetic modelling · Cascade modules

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8.1 Introduction

The past decade has seen enormous progress in the development of enzyme-catalyzed reactions to synthesize a whole range of chemical intermediates and products, for different industries from low-priced products to high-priced products. For lower-priced intermediates and products, the driver has been the need to improve process sustainability [41], for example, by using renewable and 'sustainable' feedstocks, as well as renewable catalysts. For higher-priced intermediates and products, such as pharmaceuticals [3, 14], the major driver has been the need to introduce more selective reactions, thereby simplifying reaction schemes and the subsequent product purification. Indeed today, the pharmaceutical industry routinely employs enzymes such as lipases for amide formation, transaminases for the synthesis of chiral amines, ketoreductases for chiral ester and alcohol formation, as well as nitrilases for resolution. Emerging enzyme classes include the use of imine reductases for reductive amination yielding chiral amines, oxidations of various types (using in particular oxidases and peroxygenases), as well as aldolases for carbon–carbon bond formation. Some of these emerging enzyme reaction classes are still in the laboratory, while others are already on the road to commercialization. Although the majority of industrial processes implemented so far have been in the pharmaceutical sector, new applications in other sectors are already

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forthcoming. The ability to tune enzyme properties using a variety of sophisticated protein engineering tools also provides enormous potential for the conversion of previously unknown molecules in nature, to valuable products [5], as well as improving specific traits to improve process compatibility and economics. Increasingly, such approaches can be enhanced using computational methods and most recently machine learning technologies [29].

8.2 Biocatalytic Cascades

A particularly attractive feature of biocatalysis is that enzymes usually operate under relatively similar operating conditions in terms of reaction media, pH, temperature, and pressure. This represents a marked difference from heterogeneous (and even homogeneous) catalysts, which frequently catalyze reactions under quite different conditions from each other. Likewise, the conditions under which biocatalysts usually work are far from most heterogeneous catalysts, in particular with respect to temperature [53]. This means that in a synthetic scheme (from feedstock, via intermediates to final product) it is frequently necessary to change reaction conditions when changing between different catalyst types carrying out the individual reactions. The consequence of this is that yield losses (often due to losses of intermediate(s) under unfavorable conditions) and other inefficiencies therefore result in an inefficient synthesis. In laboratory-based syntheses, such inefficiencies are usually acceptable, but this creates a major challenge when designing an industrial process, where sustainability and in particular economics are of key importance. One particularly interesting way to minimize the changes in operating conditions during a complete synthetic scheme is therefore to consider using enzymes for all the steps [19, 30]. Indeed, this has been one of the primary drivers for implementing in vitro multi-step enzymatic syntheses (perhaps better termed biocatalytic cascades). Nature has also inspired the design of in vitro cascades with features such as coupling which, by means of an auxiliary enzymatic reaction, can drive reactions, which would otherwise be thermodynamically unfavorable [2] as well as assist in the regeneration of expensive cofactors [4]. Hence, the aim is to make entire syntheses enzymatic, rather than just a few enzyme steps embedded in, an otherwise, chemical synthesis. An excellent review from 2015 describes some of the possibilities for the biosynthesis of terpenes, alkaloids, and polyethers, with numerous examples [42]. A more recent review on overcoming the current bottlenecks in the further application of biocatalysis also emphasizes the importance of telescoped multi-step processes (with improved step economy) due to high selectivity and the possibility to avoid molecular protection and deprotection steps [40].

Of course, nature also uses biocatalytic cascades inside cells (metabolic pathways) to its own end, but such systems require an understanding of the metabolic network and significant engineering in order to be synthetically useful. The importance of engineering pathways in cells to overcome the diversion of substrate to cell growth, rather than to product, is highlighted by efforts underway to enhance orthogonal pathways in order to allow higher yields towards the target compound [35]. Operating in vitro multi-step enzymatic cascades, free from the constraints of cellular metabolism, thereby afford higher yields.

For those working in vivo, the challenge is that in the microbial cell there are many possible pathways and therefore the need to ensure focus on the pathway of interest becomes the major target for metabolic engineering [28] from a synthetic perspective. However, from the perspective of the process some other challenges with in vivo operation include (1) the rate of entry of substrate into and exit of product from the cell itself (especially for new-to-nature compounds), (2) the limited amount of overexpressed protein that can be inside the cell, and (3) the need to balance redox and energy. While metabolic engineers continue to make significant progress, especially for products in the middle-price range, for many other chemicals, biocatalytic (in vitro) cascades represent an attractive alternative. A recent example concerns the two-step enzymatic asymmetric

synthesis of the unnatural amino acid, homoalanine, from cheap L-methionine used to produce both enantiomers of the product [43]. This illustrates very well the benefit of exploiting the superb selectivity of enzymes, which in this case was used to produce optically pure products (actually of both enantiomers) while simultaneously starting from a cheap substrate rather than the more expensive alpha-keto acid. The ability to start from cheap starting material is also a key feature of biocatalytic cascades. Likewise, other more complex cascades also illustrate the selectivity of enzymes to synthesize natural products and their derivatives such as bioactive naringen glucosides and quercetin rhamnoside from simple sugars [49].

In this way, biocatalytic cascades find a particular role in the synthesis of high-priced products where selectivity is of utmost importance and, interestingly also, lower-priced products where process yield is of importance. Figure 8.1 schematically illustrates the opportunities for multi-enzyme based processes and places biocatalytic (in vitro) cascades in context.

Many more biocatalytic cascades have been reported in the scientific literature. For example, a review of the opportunities for the asymmetric amination of secondary alcohols also highlights the extensive use of biocatalytic cascades, here involving between three and five redox enzymes, incorporating cofactor regeneration where required [47]. More recently, a comprehensive review of linear cascades was published [39], again emphasizing the many examples in the field. Still the vast majority of these cascades usually involve two steps, with just a few examples of higher numbers of up to six or more enzymes. Hence, although there are many examples of laboratory-scale enzyme cascades, many of which are also described in other chapters in this book, it is clear that there are still relatively few examples of industrial processes using enzyme cascades. One major reason for this is the complexity of designing such systems. It is self-evident that mathematical modelling of such systems can assist optimization (as described also in Chap. 6), but it is equally clear that the more enzymes in a cascade, the more parameters need to be estimated, the more degrees of freedom are available and the greater the complexity. Hence, in an attempt to address this, in this chapter a proposed systematic methodology will be described. The rationale behind this methodology is to simplify the design and optimization of biocatalytic cascades, to ensure they are fit for purpose in an industrial setting. The focus is on decomposing the cascade into manageable units (termed modules), each of which can be modelled and optimized, prior to analysis of the complete system. Identification of the modules forms part of a strategic design methodology, taking the cascade from the laboratory to an industrial process.

8.3 System Design

8.3.1 Strategic Considerations

The design of a biocatalytic cascade for the synthesis of a given target molecule of industrial interest is a complex endeavor. For the design of single-enzyme processes there are some rules to help the translation of a laboratory reaction into an industrial process [56], but most importantly there are precedents where today several hundred processes have been successfully implemented [41]. That is not the case for cascades, making design and scale-up much harder. Dividing the problem into manageable units (an approach based on decomposition, as used in many other engineering problems) allows significant simplification. The methodology includes (1) proof-of-concept cascade design, (2) scalable cascade design, and (3) process design. Figure 8.2 illustrates the strategy as well as information flow between the three steps.

8.3.2 Proof-of-Concept Cascade Design

It is proposed that the proof-of-concept cascade design stage involves identification of the reactions and the necessary enzymes to catalyze these reactions from feedstock to final product.

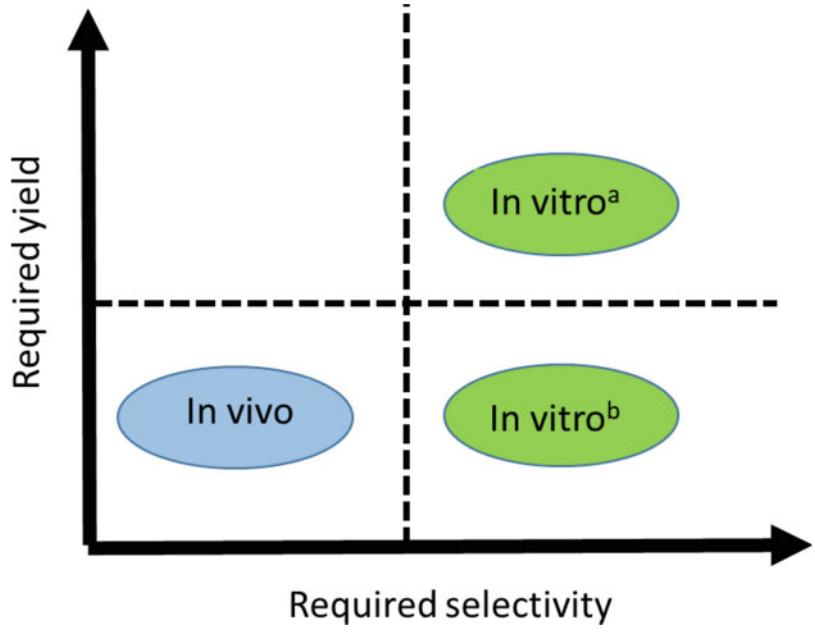

Fig. 8.1 Opportunities for multi-enzyme based processes. In vivo processes represent fermentation and whole-cell approaches to pathway cascades where selectivity and yield are often compromised by other cellular requirements. The suitable zone for such processes can be expanded by metabolic engineering, but still requires significant investment. In vitro processes represent enzyme cascades outside the cell. [a]High yield and selectivity required, especially for low-priced products. [b]High selectivity required, especially for high-priced products

Issues such as availability of the enzymes (at a small scale) and their ability to convert given substrate groups, as well as atom efficiency need to be considered at this stage. Retrosynthetic tools, incorporating biocatalysis should be of particular value. For the past few decades, synthetic organic chemists have constructed pathways by retrosynthesis, whereby a target product is decomposed into its constituent parts, which can be combined via known reactions to make the product. Repeated application of this procedure ultimately leads to the identification of suitable feedstocks for the product of interest, via a series of known reactions [12]. More recently, such an approach has been applied using known biocatalytic reactions to form new bonds and add functionality [52]. The selective nature of enzymatic reactions makes this particularly interesting, since the total number of steps (the so-called step economy) may be improved significantly. For now, the combination of catalytic and enzymatic approaches is also of great interest, since there

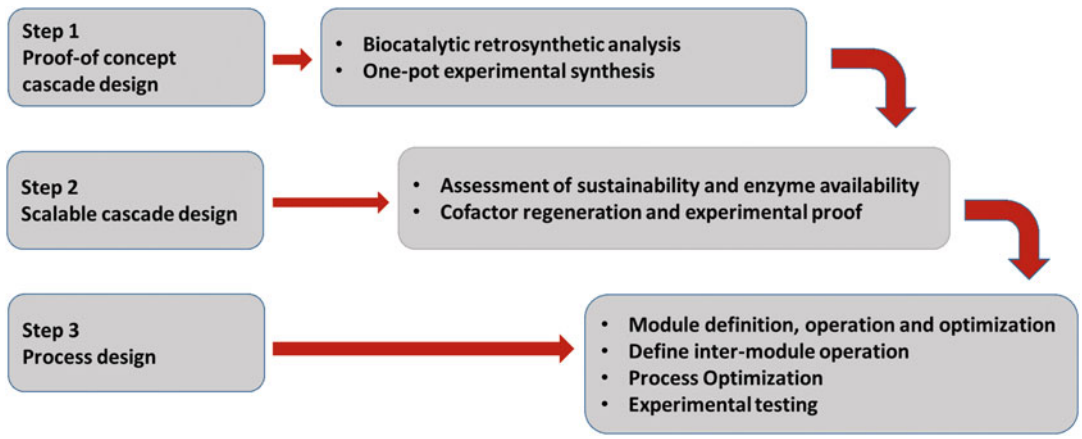

Fig. 8.2 A possible three-step strategy for systematic biocatalytic cascade design

are still too few established biocatalytic reactions in comparison to catalytic ones [21] (see also Chap. 5). In the longer-term the objective would be to have a wholly enzymatic retrosynthetic tool [13] (see also Chap. 2).

At this stage, the performance of the cascade in terms of economic metrics such as the process yield (mass of final product per mass of substrate used) and specific process yield (mass of final product per total mass of enzymes used) is not critical. In some cases, the rates (mass of final product per volume of reactor(s) and operating time) will also be low. However, what is important here is a proof-of-concept that it is possible to synthesize the product directly from the (readily available, renewable, and cheap) substrate via the cascade. The optical purity of the final product in the case of chiral molecules may also be important at this stage. Nevertheless, in the laboratory such a system can be (preferentially) operated in a single reactor (in a so-called one-pot reactor system). Some of these considerations are discussed elsewhere in this book and are an important first step in the strategy to design a cascade, see also Chap. 7.

8.3.3 Scalable Cascade Design

The second step is concerned with translating a laboratory reaction concept into a process and here it is important to ensure the scalability of the cascade. In this second step, considerations should include the following:

- **Sustainability:** At a larger scale, it is important not only that the substrate is renewable, but also sustainable. Here a sustainable substrate is defined as one which is renewable, readily available (not in competition with food or other important applications), and sufficiently cheap.
- **Enzyme availability:** Enzymes need to be available and also free from intellectual property (IP) restrictions (with freedom-to-operate (FTO)). Availability at this stage also means that the price needs to be low enough and likewise the scale of production large enough to meet demand. Some guidelines have been published concerning enzyme costs but at this stage only an estimate is required to ensure price and scale are adequate [25, 51, 57]. In some cases, this may prove a major bottleneck, because the enzyme(s) of interest are still only expressed at a small scale (and therefore high cost).
- **Cofactors:** In the laboratory cofactors may be added in stoichiometric amounts, but this is clearly too expensive at a larger scale. Hence at this stage cofactor regeneration methods need to be identified for the most expensive cofactors such as NAD(P)(H) [23] and ATP [4]. Many enzymatic methods are available for nicotinamide cofactor regeneration, such that the cascade can run redox neutral, but the key here is to select suitable co-substrates and co-products. In cases where they can be run as a redox neutral cycle there are obvious advantages. Some examples are given in Fig. 8.3.

Although cofactors will be used in such a way that either they can be regenerated or the cascade is redox neutral, it is still the case that some material will be required for start-up.

The final stage here involves the need to combine the suitable substrate, with available enzymes as well as cofactor regeneration methods in a small-scale cascade, as an experimental demonstration. Again, at this stage the economic metrics are not critical.

8.3.4 Process Design

Designing the process needs an understanding of how the cascade works. In reality, this is a decision about which parts of the cascade must be combined and which ones must not. There are two alternatives here, best described as the one-pot approach and the modular approach.

- **One-pot approach**

An interesting cascade reported in the scientific literature concerns a route from alcohols to

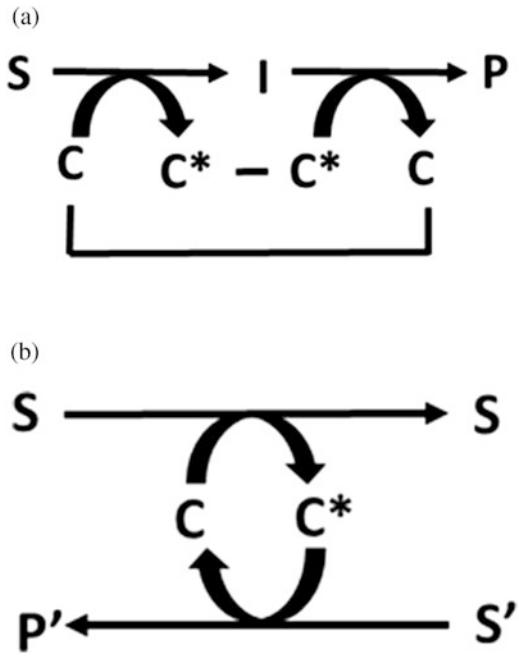

Fig. 8.3 Cofactor-neutral regeneration schemes. (**a**) Cofactor-neutral cyclic cascade from substrate, S, via intermediate, I, to product, P. Two enzyme reactions are required operating next to each other, one of which consumes cofactor form C and produces cofactor form C*. The subsequent reaction does the reverse. (**b**) Cofactor-neutral parallel cycle from substrate, S, to product, P. In so doing cofactor form C is consumed and C* produced. An auxiliary enzyme is required to convert auxiliary substrate, S′, to auxiliary product, P′. In so doing cofactor form C* is consumed and C produced

chiral amines by a combination of two enzymes, an alcohol dehydrogenase and an amine dehydrogenase, in a single-pot reactor. Operation in a one-pot reactor affords a redox neutral cascade. This was successfully demonstrated for the synthesis of a range of target molecules [33]. A similar approach was developed using a cascade composed of an alcohol dehydrogenase and a cyclohexanone monooxygenase, together with a further lipase-based hydrolysis in a two-liquid phase one-pot system to 6-hydroxyhexanoic acid, which was subsequently polymerized to give poly-ε-caprolactone [38]. The starting substrate is inhibitory and can be fed to the system, regardless of operation in a single-pot (see Chap. 7). However of greater importance is that this cascade also illustrates two of the real advantages of a multi-step one-pot synthesis. First, the cascade is redox neutral and then secondly the intermediate (ε-caprolatone), being inhibitory to the cyclohexanone monooxygenase, is coupled to the subsequent enzyme in the cascade (lipase) enabling the control of the intermediate concentration so that the system can run effectively. Still more recently the first two enzymes in the cascade, alcohol dehydrogenase and cyclohexanone monooxygenase, were fused to again demonstrate the effective synthesis of ε-caprolactone, without the need for stoichiometric cofactor addition [1]. Interestingly, intermediate inhibition was still a problem with the fused system. Overcoming this requires either a very careful balancing of kinetic parameters or else use of a subsequent enzyme. Nevertheless, a condition for such a cycle is that it must operate in a one-pot operation, which adds to the complexity.

- **Modularization**

Although the one-pot approach looks very attractive, cross-reactivity between one or more of the reaction components (substrate(s) and product(s)) and one or more of the enzymes may mean that reactions other than the desired ones can take place, solely by virtue of the fact that all components and cascade enzymes are together in one-pot. The more enzymes that are in the cascade, the more likely it is that cross-reactivity becomes a problem. Therefore, especially for longer cascades, cross-reactivity between molecules and enzymes in the cascade frequently represents a major limitation. For example, the substrate of one enzyme may also prove to be the substrate of another enzyme at a different point in the cascade. Such situations are relatively common and can lead to a lower process yield (mass of product per mass of initial starting substrate) and additionally more complex downstream product recovery. The obvious solution is therefore to divide the cascade into small sub-cascades, each with its own reactor. A sub-cascade is a part of a cascade, which can run independently, and in the context of this chapter will be termed a cascade "module." The modular approach was first introduced by Zhang and co-workers (see [58] and later [37])

and has been discussed in detail in the context of compartmentalization by Sieber and co-workers [45]. The principle of modularization is to split the cascade into smaller reactor stages or modules. Therefore, experimental assessment of cross-reactivity is essential at this stage to evaluate if some type of splitting of the cascade, or compartmentalization (as in microbial cells in nature), is required. At the smallest size, a module can be an individual enzymatic step. However, in some cases, especially where cofactor regeneration is required, it will prove essential to operate parts of the cascade together, and in the first instance, this should be the basis for identifying discrete modules (i.e. defining the enzyme composition of a given module). In other cases (especially where cross-reactivity is a risk) it will be essential to operate some steps separately and therefore as an initial design all reactions should be separated and only those where integration is essential to operation (i.e. for cofactor regeneration) will be combined. Many laboratory-based cascades today are operating at relatively low reaction rates. Addition of more enzyme to enhance the rate (productivity) may also prove complicated in a one-pot system where it is easy to imagine very high protein loadings, which might even limit the system. Likewise, the product concentrations attainable from cascades run in one-pot systems in the laboratory are relatively low. Indeed the use of higher concentration, especially of multiple substrates and reagents may lead to very high ionic strength, which may even lead to a major problem for some enzymes, resulting in salting out and precipitation.

An excellent review of enzymatic cascades, focused on the use of ω-transaminases, in combination with many other enzymes, ultimately to afford optically pure chiral amines outlined a very useful classification of cascades into linear and cyclic cascades of different sorts [44]. It is clear that parallel and sequential cofactor-neutral cycles as shown in Fig. 8.3 need to be run with the enzymes in close proximity to each other in a single module, due to requirements for cofactor coupling. In principle that is also required for convergent and divergent cofactor-neutral cycles as well. Likewise, reactions where an inhibitory intermediate is formed, or a reaction that is otherwise thermodynamically unfavorable, can also be advantageously linked to the subsequent enzyme in a single module. Other cascades can more easily be decomposed, into more than one module.

Another key principle behind modularization is to ensure that as many degrees of freedom as possible are available for optimization while also ensuring the cascade can be modelled in a simple way. Decomposing the problem into modules makes for a far easier optimization task and additionally gives a clearer understanding of the effects of parameter changes. Of course, there are also arguments for effective module economy (i.e. reducing the number of modules) but this can be developed at a later stage of process optimization.

Modules may be defined, as here, on the basis of spatial separation or in principle also on a temporal basis. However, alternative methods to control cascades in a one-pot approach increase complexity considerably. For example, stimulus-response techniques are being developed using changes in temperature, pH, and localized generation of free radicals, via light and magnetic field changes [11]. Such approaches might longer-term overcome the need for modularization, but require research on the technology to switch enzymes on and off, as well as dynamic modelling. While this exciting research is on-going, today for simplicity it is still best to operate modules on the basis of spatial separation, at least from the perspective of scalability.

Table 8.1 lists some of the potential advantages of the modular approach.

- **Inter-module operations**

Between modules, reagent addition and product (or by-product) removal are possible. Clearly, the aim should be to keep this to a minimum, but this can also allow extra operational possibilities.

One of the key requirements here is the separation or retention of individual enzymes in a given module. One of the most useful methods to achieve this is by enzyme immobilization.

Table 8.1 Potential advantages of the modular approach to process design of biocatalytic cascades

Process feature	Advantages of modular approach
Cross–reactivity	Can be avoided
Enzyme retention/recycle	Easier with fewer enzymes
Controlled feeding and product removal	Possible between modules
Operational flexibility	Increased
Operational mode	Continuous possible by linking modules
Modelling and optimization	Easier with decomposition

Attachment of the enzyme to a porous (or solid) support (covalently or otherwise) allows simple filtration to separate enzyme from the reaction mixture. In cases where all the enzymes work together in a single one-pot reactor it is hard to imagine immobilization on a single support since immobilization of multiple enzymes on a single support will require very high specific activity to avoid the problem of loading too little enzyme. Likewise, the optimal ratio of the enzymes, i.e. balancing the activities of the catalytic partners, must be known prior to immobilization. Another argument concerns the fact that different enzymes will have different stabilities and may even need to be replaced (and reimmobilized) at different times. All this points to the fact that immobilizing on a single support two enzymes might be reasonable, but more will prove difficult. Indeed several examples have illustrated well that two enzymes can be co-immobilized successfully and might be useful in some modules. In principle the use of soluble enzymes, using ultrafiltration membranes between each module as a means of separation and retention is also a possibility.

- **Module optimization**

For a single-enzyme-catalyzed reaction with one substrate and one product, a simple analysis to establish the basis of optimization is as follows: an amount of enzyme **E** can catalyze the conversion of an amount of substrate **A** to an amount of product **B** in volume **V** and time **t**. For effective process economics this can be optimized in terms of reaction yield (**B/A**), specific yield (**B/E**), product concentration (**B/V**) as well as productivity (**B/(V·t)**). This optimization will involve adjusting the variables of T and pH, as well as the ratios **A/V** and **E/V**. Additionally, the medium, in which the reaction is performed could also be a variable. Likewise, if the enzyme has significant residual activity at the end of the reaction, it can be recycled and this should be taken into account in measuring **B/E**. Targets for the performance metrics are dependent upon the value (cost/amount) of the substrate, product, and enzyme. Hence, different industry sectors will require very different performance metrics (see also Chap. 6). However, while this is a prerequisite for effective development (in particular of the enzyme) in many cases a deeper analysis is required. For example, where the substrate and/or product proves inhibitory or water-insoluble (or both) other operational modes will be required (see also Chap. 10). In principle, inhibition may be engineered out of a protein, but in many cases, this remains a difficult trait to engineer. Indeed, from a practical perspective, the relatively easy process solutions of continuous substrate feeding and in situ product removal may prove far simpler (see also Chap. 7). Several process (as well as cascade) solutions are available to address such limitations for a given enzyme (see Table 8.2).

In the case of a cascade, these solutions need to be considered for each individual module. An additional variable is the possibility of merging two modules to solve substrate supply or product removal problems. However, this is only possible where cross-reactivity allows the merging of modules, otherwise conventional solutions must be sought.

- **Example of cascade design**

A particularly interesting example of the development of a biocatalytic cascade is the

Table 8.2 Solutions for handling challenging enzyme and reaction features

Feature		Potential process solution	Potential cascade solution
Cofactor regeneration		–	Cyclic cofactor-neutral cascade
Substrate	Inhibition	Controlled substrate feeding	Feed from previous enzyme step
	PWS solid	Slurry reactor[a]	–
	PWS liquid	Two-liquid phase operation[b]	–
Product	Inhibition	In situ product removal[c]	Link to subsequent enzyme step
	PWS solid	In situ crystallization[a]	
	PWS liquid	Two-liquid phase operation[b]	

PWS, poorly water-soluble
[a]Potential problems of blockage in continuous flow reactor
[b]Potential problems for phase distribution in flow reactor
[c]Potential techniques, including in situ crystallization [24]

synthesis of Islatravir, developed by Merck and Co (Rahway, NJ, USA). The key motivation for using biocatalysis here was to avoid multiple protection and deprotection steps while ensuring complete stereoselectivity. The cascade involves 5 steps using a total of 9 enzymes, 4 of which are auxiliary enzymes. The details of the selected pathway and associated protein engineering improvements are given in a recent publication from the team, together with their collaborators at Codexis (Redwood City, CA, USA) [22]. The development illustrates very well the use of protein engineering to allow the effective conversion of non-natural substrates for these enzymes, as well as the ability to convert effectively at higher concentrations of substrate, and allow higher concentrations of product, avoiding inhibition. Aside from the importance of protein engineering the entire synthesis also demonstrates the power of retrosynthesis as a tool to create the initial, proof-of-concept cascade. In the context of this chapter though, the most interesting aspect of this work is the decomposition of the cascade into three modules (schematically illustrated in Fig. 8.4).

The rationale behind the module selection is essentially that the enzymes in modules 1 and 2 (galactose oxidase and pantothenate kinase, respectively), are essentially irreversible and hence can be operated independently. However the three enzymes in module 3 (deoxyribose 5-phosphate aldolase, phosphopentomutase, and purine nucleoside phosphorylase) are reversible and thereby benefit from operating together, as well as being combined with an auxiliary enzyme (sucrose phosphorylase) to pull the equilibrium. The enzymes in modules 1 and 2 each operate with additional (auxiliary) enzymes to allow their effective operation. The rationale for the auxiliary enzymes is given in Table 8.3, along with module composition.

Between the modules, the aqueous solution containing the intermediate is sent directly to the next module, without any isolation or purification. This enables the overall product yield (51%) to be far higher than other reported syntheses of this product, which require between 12 and 18 steps. Interestingly the authors also report that the large number of enzymes means that the concentration of protein in solution would become problematic if not retained along the way. Of course, improved specific activity through protein engineering can reduce the amount of protein; nevertheless, with nine enzymes there are considerable challenges. With this in mind, the enzymes in the first two modules were immobilized to allow their easy retention and thus removal from the aqueous stream moving forward to the subsequent module. In this way, this example illustrates very well, not only the power of cascade biocatalysis, but also the benefits of modularization.

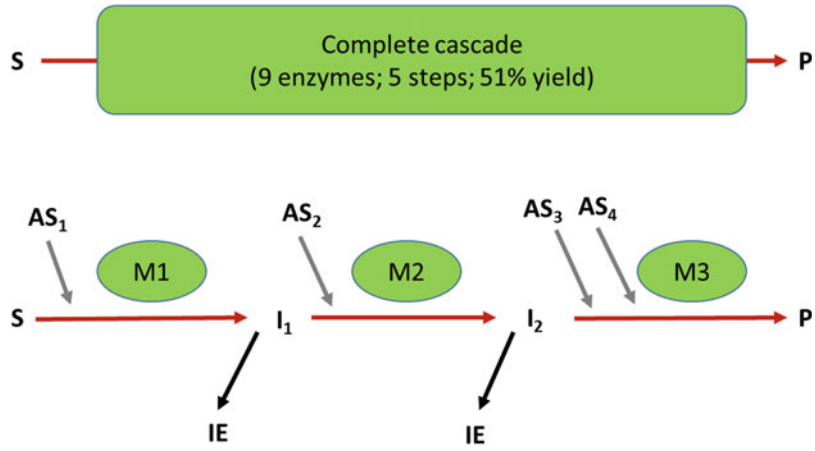

Fig. 8.4 Schematic representation of the modularization of the five-step enzymatic synthesis of Islatravir. S, substrate; I_n, intermediate n; P, product; AS_n, additional substrate n; M1, module 1; M2, module 2; M3, module 3; IE, immobilized enzyme for ease of retention/removal. Details of the module composition are given in Table 8.3. Data extracted from Huffman et al. [22]

8.4 The Role of Kinetic Modelling

8.4.1 Introduction

A necessary stage in the optimization and scale-up of all enzymatic reactions, including biocatalytic cascades, is modelling of the reaction kinetics. Its significance and more details are introduced in Chap. 6 of this book. Indeed, reaction progress kinetic analysis is an important methodology for establishing complex catalytic reaction behavior and is especially valuable for multi-step reactions [7]. One of the challenges with multiple enzymes is that the number of model parameters increases, making the mathematical treatment of such models, let alone finding optimal solutions, particularly difficult.

A recent paper describes a new approach to modelling enzyme-catalyzed reactions, with the aim of simplification. Even for reactions catalyzed by a single enzyme, many are known to be much more complex than the simple one-substrate, one-product reactions depicted in the well-established Michaelis–Menten expression. In the recent paper [16] a two-substrate, single-product reaction (the symmetric carboligation of 3,5-dimethoxybenzaldehyde to *R*-3,3′,5,5′-tetramethoxybenzoin catalyzed by benzaldehyde lyase) was modelled. Bayesian learning and uncertainty quantification were used for model calibration, selection, and reduction. The reduction of models into simpler forms is of particular importance in the context of cascading multiple enzymes, in order to reduce the number of parameters to be estimated and fitted.

The proposed methodology presented here, focused around modularization, also enables kinetic modelling to be simplified and used to great effect in designing the optimal process.

Table 8.3 Rationale for module selection. Enzyme abbreviations are as follows: GOase, glucose oxidase; PanK, pantothenate kinase; DERA, deoxyribose 5-phosphate aldolase; PPM, phosphopentomutase; PNP, purine nucleoside phosphatase; CAT, catalase; HRP, horseradish peroxidase; AcK, acetate kinase; SP, sucrose phosphorylase. Data extracted from Huffman et al. [22]

Module	Primary enzyme(s)	Auxiliary enzyme(s)	Rationale for auxiliary enzyme(s)
1: Oxidation	GOase	CAT	Removal of inhibitory by-product hydrogen peroxide
		HRP	Maintenance of copper oxidation state for effective enzyme operation
2: Phosphorylation	PanK	AcK	Cofactor regeneration
3: Nucleoside assembly	DERA; PPM; PNP	SP	Removal of by-product to drive equilibrium towards product

8.4.2 Modelling Modules

The primary goal of modelling is to assess alternatives and devise suitable operating strategies to afford optimal operation. This has a particularly important role in the development of cascades (see Chap. 6). Specifically each module should be modelled independently, and one of the most important questions is to establish the mass ratio of enzymes to be used in a given module: the so-called enzyme stoichiometry. This is partly determined by the relative activities of the enzymes (k_{cat}), but also by the affinity of the enzymes for the intermediates (K_M). A seven enzyme, three step cascade using known enzymes (esterase, carboxylic acid reductase, and alcohol dehydrogenase as the primary pathway) was reported where some mathematical modelling was used to identify bottlenecks and optimize the system [18]. Enzymes for cofactor regeneration and removal of an inhibitory by-product were added. Optimization is already complex enough, without combining all the modules to make the full cascade and this is another argument for initially sub-dividing the cascade into modules. Process modelling requires the integration of the kinetic models with mass balances, which describe the substrate and product concentrations in an individual module (see also Chap. 7). In a continuous process, these will not change with time, but in a fed-batch process, time is also an important variable to be considered (see also Chap. 7).

Although inspiration can be taken from nature, the inside of cells where in vivo cascades are operating is very different from the environment outside (in vitro). One interesting aspect is that inside cells the proximity of one enzyme to the next in the cascade (usually at very high protein concentration) may result in activity enhancements. In reality, it is hard to distinguish multiple effects and currently we do not have the evidence to suggest design rules for cascades based on nature [60]. Recently, an interesting report has again focused on these effects, indicating that proximity is important with crowding effects, where channeled cascade reactions can be enhanced [26]. Regardless of these discussions, what is clear is that the stoichiometric balance of the cascade kinetics is of great importance. The ratio of the enzymes can be determined experimentally [27, 54] or via kinetic models (if all the parameters are estimated accurately enough) [15]. Experimental methods are also dependent upon suitable analytical techniques (see Chap. 9). Evaluation of the enzyme ratio has also been very well illustrated in a report of a biocatalytic whole-cell system for the conversion of alcohol to lactone [31]. Nevertheless, the scale of the problem increases enormously with longer cascades (and bigger networks) and likewise is of greater significance to optimization. This too supports the concept of using modules (each optimized).

8.4.3 Modelling of Complete Cascades

At a later stage, the modules can be integrated to develop a complete cascade model. Despite the similar range of pH and temperature of enzymes in general, each module can also operate under different optimal conditions. If all modules are operated at the same temperature and pH that may be an excellent start to the analysis, but later this can be improved upon by optimizing the pH and temperature of each module.

In recent years, tools for improving our understanding of how to interpret kinetic data in the optimal way have also been developed [34]. In particular, estimation of kinetic parameters is of great importance and standardized methods to understand the sensitivities are particularly useful (see Chap. 6). Recent progress also includes a strategy for data collection as well as a database to store and archive data in a structured way [8]. In the longer-term this will prove of great value, potentially linked to tools for retrosynthetic analysis.

8.5 Remaining Challenges

The new concepts of combining enzymes in cascades to catalyze synthetic schemes have also been referred to as 'systems biocatalysis,' and indeed systematization is exactly what is required to drive the field forward, but here too the isolation and purification of the individual enzymes should be highlighted as a major challenge [17]. Indeed the purity requirements in specific systems are still largely unexplored, although in the end may prove critical in determining the lowest cost of product upon which such systems are applicable. Solutions to the current limitations of enzymes in synthetic organic chemistry have also been discussed by Reetz [36]. Here too the use of cascades is discussed and differences between artificial cascades and using cells containing modified cascades are debated. In particular, the challenge of handling sensitive (labile) enzymes is highlighted. In these cases, using cells of one sort or another may still prove beneficial. This remains an unexplored area.

A second major challenge remains the need to expand the retrosynthetic toolbox by including a greater number of enzymes to enable entire synthetic routes to be biocatalytic, rather than solely parts inside a much larger chemical synthesis.

Finally, the most important challenge for the future is to implement a systematic methodology such as the one outlined here. Examples to test such a workflow, together with full experimental procedures are still required in order to standardize process design and scale-up. This should include automated methods for testing cross-reactivity.

8.6 Future Perspectives

While it is clear that enzymatic cascades allow the possibility of entirely new pathways to be engineered, metabolic engineering using in vivo pathways also develops. Indeed one future vision which has been presented integrates systems engineering and synthetic biology with metabolic engineering. The aim of such an approach is to reduce the time and effort which metabolic engineering takes [10]. The use of in vitro data to guide models applied in vivo has also been proposed. An interesting example concerns a redox cascade in *Escherichia coli* in which an alcohol dehydrogenase, an enoate reductase, and a Baeyer–Villiger monooxygenase were used together to synthesize optically pure lactones from a secondary alcohol [32]. A model was used to explore various parameters such as enzyme concentration, but revealed also that cofactors were limiting. Experimental data was used to confirm and validate predictions. For some chemical products this may prove a successful biological route to production, but in the case natural products, as well as novel pharmaceutically active compounds, knowledge of the pathways and enzymes remains a bottleneck. Here too automated methods of analysis (Chap. 9) of potential synthetic cascades may prove more timely, especially given the time pressures required for the development of new pharmaceutical processes [50].

Inspiration from nature also informs another future direction. An excellent example overcoming the challenges of cellular constraints such as competing metabolism or issues related with inhibition and regulation uses six enzymes in a cycle [48]. The cyclic nature of the solution means one-pot operation (or a single module) is the best route. The cycle is constructed in such a way that various entry and exit points can be used in an "open" cycle mode. Although complex, this is a direct mimic of cellular metabolism where cycles are used to consume an excess of a compound or to 'release' an excess out of the cycle. In essence, it is a type of control for the cascade.

Another development for the future concerns the use of continuous flow systems [46], as more described in Chap. 7 of this book. One advantage is the ability to spatially separate enzymes in a cascade in a modular way in flow. A simple example of such an approach was described already in 2012 by Wever and co-workers [6], where the synthesis of carbohydrates was undertaken with an immobilized phosphatase

and an aldolase. A packed bed two-module system was operated successfully. The optimization of such a system is complex, especially when constrained by the flowrates and enzyme concentrations in the immobilized packed bed system, but enables excellent spatial control. Indeed, today several biocatalytic cascades have been demonstrated not only using (fed-)batch one-pot reactors or modules, but also in continuous flow reactors. This will be a growing field in the future.

An alternative to metabolic engineering or synthetic biology to create new cascades and pathways might be cell-free synthetic biology [20]. The principle here is to open the cell, after growth to allow reactions and pathways of interest to take place without the limitations imposed by cell walls and genetic regulation. It is still unclear if such approaches will be used for manufacture, or rather as a means of rapidly testing different engineered variants. In any case, the use of "crude" cell lysates is important in industrial biocatalysis. With cell-free approaches, it is clear that both the concentration and the purity of particular enzymes will be of great importance. A recent example of cell-free synthesis was used in the biosynthesis of isoprenoid from isopentenol, with a 3-fold higher productivity than observed in cellular systems [55]. Again, the benefit of overcoming cellular constraints is clear.

Finally, while most of the examples discussed here concern higher-priced (low-volume) products, where selectivity is the driver, it is interesting to see a recent example of a much lower-priced (high-volume) product being made by a biocatalytic cascade. Here a cascade was used to synthesize 2,5-furandicarboxylic acid from 5-methoxymethylfurfural using three oxidoreductases. Almost complete conversion was achieved (98% yield) by driving the reactions through the addition of exogenous methanol. Although such a system is not ready to scale-up, the potential is already clear [9, 59] that such an approach can also inspire reaction schemes where high process yield is a necessity for implementation at an industrial scale.

8.7 Concluding Remarks

Biocatalytic cascades exploit some of the best features of enzymes as catalysts and reports of such cascades for complex chemical product synthesis are becoming increasingly common in the scientific literature. In order to translate more of these laboratory proof-of-concept studies in to industrial processes, a systematic (and standardized) approach to design will be required. A first attempt at developing a suitable methodology has been outlined here. Tools such as retrosynthetic analysis and in particular process modelling will prove essential to implementation, alongside automated methods of data collection.

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Enzyme Cascade Reaction Monitoring and Control

9

Robert Hiessl, Joscha Kleber, and Andreas Liese

Abstract

Enzymatic reaction cascades often consist of various starting materials, intermediates, products, and possible side products. To establish a highly efficient cascade reaction it is necessary to monitor these components, which enables an optimization of the overall system. The scope of this chapter is to provide knowledge about the basic principles of inline analytical technologies. Different definitions about the position and the type of measurement technique are introduced. Additionally, information on the accuracy and the limit of detection are given. Since for most inline methods a chemometric model for the calculation of concentration data from inline determined measurements is needed, a short introduction on chemometrics is provided. Furthermore, inline analytical tools which are suitable for the application in enzymatic cascade reactions are discussed and examples are given to highlight the possibilities arising by the use of such technologies. The analytical devices are critically assessed in terms of their applicability and a general workflow is given on the procedure of setting up inline or online analytical methods. At the end of the chapter, the impact of inline analytics used for monitoring and controlling enzymatic reaction cascades on the design of (industrial) processes is discussed and shortly the concepts of "quality by design" and "quality by control" are introduced.

Keywords

Process analytical technologies · Inline/online analytics · Process monitoring · Process control

9.1 Introduction

9.1.1 Differentiation of Off-, At-, On-, and Inline Analytics and Chemometric Modelling

During the last decades, the use of online analytical methods for process monitoring and control has become popular, with a rising number of publications available every year. Until recently, many measurement techniques have been labeled as "online." Nowadays there is a well-accepted definition available, which is shown in Fig. 9.1. This differentiation is made according to the position of the analytical devices within the reactor setup. If a probe or sensor is integrated directly into the reactor, it is defined as an "*inline*" measurement technique. If a bypass lane is necessary for implementation, e.g. a flow cell, the principle is classified as an "*online*" method. An automated

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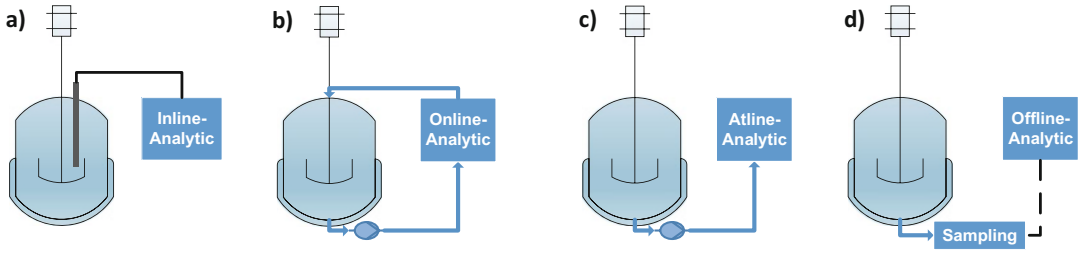

Fig. 9.1 Differentiation of different measurement principles: (**a**) Inline: A probe or measurement device is directly integrated into the reactor vessel and a direct response is measured in real-time. (**b**) Online: The measurement takes place in a bypass section in appropriate short measurement times. (**c**) Atline: The analytical device is in close proximity to the reactor vessel; sample is automatically transported to the measurement. (**d**) Offline: Manually or automatically a sample is withdrawn from the vessel, worked up, and transported to the analytic machinery where the measurement takes place with a time delay

sampling, followed up by analysis in close proximity to the reactor setup, is labeled as "*atline*" and the classical approach of taking a sample workup and analysis is called "*offline*" [35]. Traditional offline methods like gas or liquid chromatography nowadays can be integrated using automated sampling lines and advanced control techniques [45].

Besides this classification, the measurement techniques are also divided into *invasive* and *non-invasive*, sometimes also named destructive and non-destructive. An invasive measurement technique means that the measurement principle is affecting the composition of the reaction media or the analytes in a certain manner. This effect is not necessarily affecting the outcome of the measurement. An example for an invasive measurement is the colorimetric or conductivity measurement of, e.g. residence time distribution using a color or salt additive. Another is the acid titration to determine concentrations in samples taken from reactor vessels. Examples of non-invasive or non-destructive measurement techniques are the application of spectroscopic methods established using a probe for measuring concentrations directly inside the reactor.

To derive concentration data from the measured data, the application of chemometric methods and models is needed. Since the focus of this chapter is to provide an overview about the topic of inline analytics in enzymatic reaction cascades, further detailed reading about chemometric modeling can be found in various publications in this research field [2, 11, 27]. In the following, only selected information about the principle of chemometrics is given.

Analogous to the *calibration* of an offline measured signal from chromatographic analyses with the corresponding (calculated) concentrations, a similar approach is needed for the analysis of spectra. In a traditional calibration, only one signal (e.g. absorption at a certain wavelength) is correlated to the measured variable, which is then called a *univariate* method. In contrast to univariate methods, for analyzing spectra from complex reaction mixtures, *multivariate* data analysis methodologies are necessary. In these approaches, which are shown in Fig. 9.2, the (calculated or offline measured) concentration data is correlated to a set of variables, e.g. the absorption at several wavenumbers within the measured spectra.

A variety of different methods is available for the model construction: peak integration, partial least square [50], multivariate curve resolution [24, 43], or indirect hard modeling [33] are some examples to name. In some cases, during the setup of the chemometric model an internal or cross-validation is done to investigate the model's performance. To ensure a robust and precise model, an external validation is also carried out (see Fig. 9.2).

By the need of a chemometric model, the workload for setting up such systems is usually

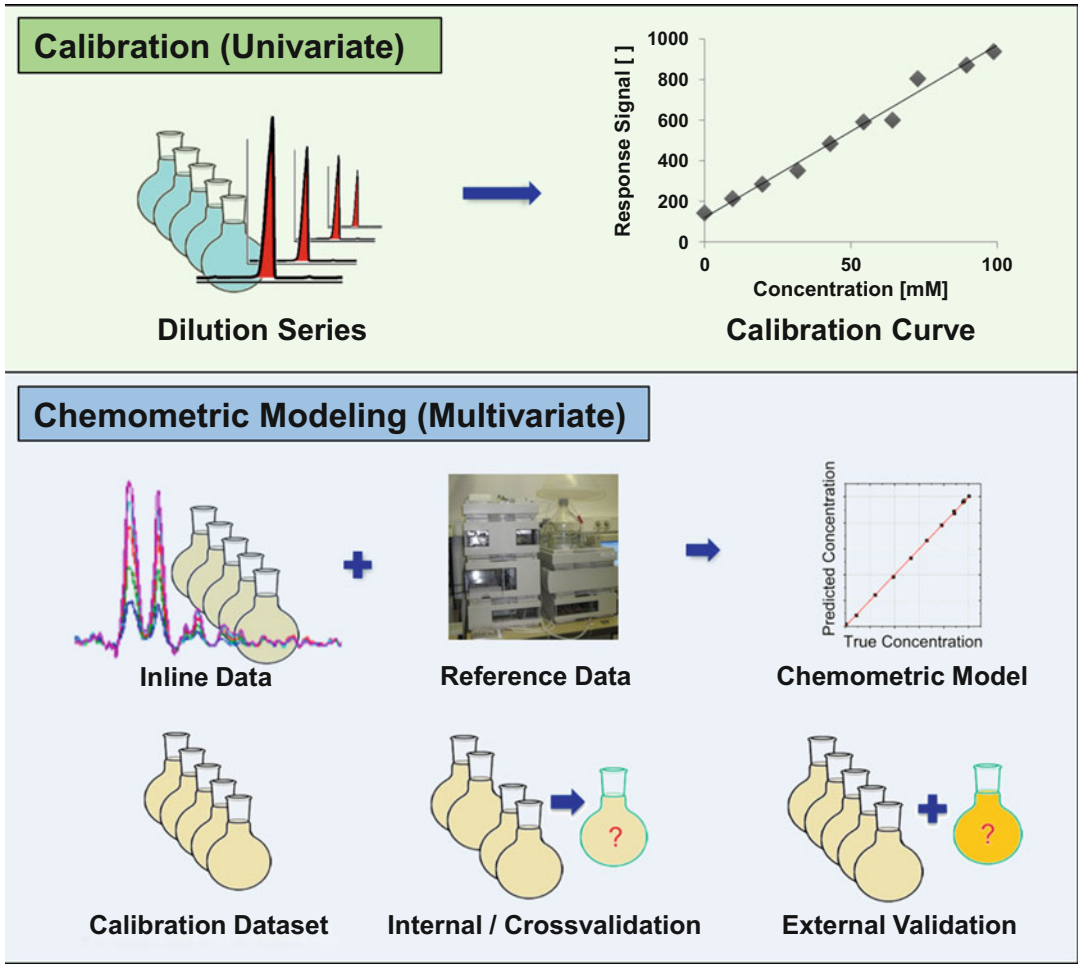

Fig. 9.2 Univariate calibration in comparison to chemometric model construction and calibration, internal and external validation (multivariate)

higher than compared to classical offline approaches. Besides this, inline analytical methods also have some further limitations or drawbacks in comparison to standard offline measurement devices:

- A mathematical model for chemometrics is needed
- Complex methods, difficult to implement
- Often highly specific for a certain case
- Relative high price compared to standard offline analytics

A variety of different inline analytical methods are available, several of which are well suited for the analytics of an enzymatic reaction cascade. A detailed introduction, their classification, and different aspects of their implementation such as precision and time scale are discussed. The aim is to supply basic knowledge about the available inline analytical methods, their advantages, disadvantages, or limitations. Additional goals of this chapter are to highlight future possibilities and encourage a deeper understanding of the methods. Finally, advanced literature references

are summed up to distinct topics, which are discussed within the scope of this chapter.

9.1.2 Challenges in Monitoring Sequential Reactions, Especially Enzyme Cascades

Important challenges enabling optimization of enzymatic/chemical reaction cascades are suitable analytical methods and tools giving rise to a detailed process understanding. Inline or online analytics facilitate monitoring of intermediates within a certain reaction step or cascade and, therefore, give information about possible enzyme inhibition by these transitional compounds. The information gathered using such methods is useful for the full determination of reaction kinetics (see Chap. 6) and thermodynamics, not only by means of starting materials and products, but also in terms of side products. Furthermore, the inline data can be used for wholistic modelling of processes (see Chap. 8). In consequence, insights into the selectivity of certain reaction steps within a reaction cascade are possible. Application of inline analytics to a single reactor or a cascade of reactors helps to determine optimized operation points to avoid accumulation of intermediates within the process. Additionally, inline monitoring gives an insight into the status of the enzymes applied with respect to their activity and stability. Overall, the knowledge gathered via inline analytics supports engineers and scientists in improving biocatalytic reaction cascades resulting in higher space-time yields and a minimization of side reactions and therefore waste produced. The application of offline analytical methods for the previously mentioned aspects demonstrates some disadvantages:

- Time delay from sampling to the result of the analysis, complicating closed-loop control of processes
- Error-prone from operator to operator
- Risk of contamination during sample workup

By using inline measurement methods, these limitations can be overcome:

- Result of measurements with no time delay
- No sample workup needed (less error-prone)
- No or less risk of contamination

So far, most enzymatic reaction sequences are carried out in aqueous media, often with low substrate and product concentrations. Therefore, measurement techniques are needed, which are not disturbed by water and show a high sensitivity towards the analytes. Enzyme cascade reactions are often multiphasic reaction systems in the perspective of reaction media, which consists of buffer salts and gas bubbles. Additionally, a second liquid organic phase or a solid phase like immobilized enzymes or even whole cells can be present in the reaction mixture [32]. The same needs to be taken into account while establishing an inline analytical method in non-conventional media (see Chap. 10). Therefore, the chosen analytical method must not be interfered by solids, gas, or second liquid phases and has to reliably measure the liquid phase with sufficient accuracy at low concentrations. An organic phase can be used as a second phase to establish an in situ product removal or the supply of substrates with low solubility in the aqueous phase. By this approach, also substrate or product inhibition of enzymes can be avoided [21].

A large toolbox of different analytical methods is available, alongside a variety of spectroscopic measurement techniques, classical devices for measuring conductivity, pH, and temperature can be directly integrated inline into reaction vessels. During the last years, optical methods to analyze the dissolved oxygen or carbon dioxide concentrations have also become popular [48]. Moreover, recently, a trend to using miniaturized analytical devices to focus on certain process parameters within a reaction cascade is noticeable [23].

Organizations like the International Association for Process Automation, in short NAMUR, name the use of online and inline measurement technologies as a base for the recently upcoming industry 4.0, focusing on digitalization and inline process optimization using artificial intelligence (AI). Additionally, the Food and Drug

Administration of the USA (FDA) defined a framework "Guidance for Industry PAT (Process Analytical Technologies)", which is giving a set of nonbinding recommendations regarding process monitoring with the aim to ensure a continuous and entire quality assessment of (pharmaceutical) processes and their products [14]. The use of inline measurement paves the way for a direct and real-time process control enabling the operator to optimize process parameters immediately after disturbance from, e.g. steady-state in continuous processes [8].

Within this chapter, different available in- or online measurement techniques are discussed and presented with recent examples from scientific publications. However, the number of publications discussing inline analytics of enzymatic reaction sequences is still limited. Therefore, literature from chemical reactions is also included. The aim of this chapter is to introduce appropriate methodologies to enable inline process monitoring and control, as well as to motivate for their application.

9.1.3 Resolution and Precision

In this section, different terminology in view of time resolution and accuracy of an analytical technique is presented. The signal-to-noise ratio and the limit of detection are introduced, which are needed for the successful qualification of any analytical method.

The *signal-to-noise ratio (SNR)* is an important value for the determination of the sensitivity and gives an indication of the quality of a certain analytical technique. Noise describes the variation of a measured value around its average. The SNR is defined as the averaged signal $\overline{S}$ divided by the noise, which is the root mean square of the standard variation $\sqrt{\sigma^2}$ (= standard deviation) over the course of the measurements (Eq. 9.1).

$$\mathrm{SNR} = \frac{\overline{S}}{\sqrt{\sigma^2}} \tag{9.1}$$

For a concise evaluation of the SNR, the same sample or measurement is performed with fixed conditions numerous times (between 25 and 50 repetitions) to ensure that the sample composition or properties are not changed by variation in any controllable variable over the time of the measurement.

The definition of the real-time measurement of a reaction should be interpreted in context to the reaction velocity and kinetics; therefore, a measurement technique only needs to record data in suitable time steps. For slow reaction kinetics in the time scale of several hours or days, only a low measurement frequency is needed. For a fast reaction velocity of minutes or below, it is important to record inline or online data in sufficient short time intervals. To lower the noise effects of an analytical method, it is common practice to average several measurements or scans to ensure a more accurate result.

For spectroscopic methods, a spectrum or signal is measured by averaging a number of scans, which leads to a classical optimization problem between maintaining a high SNR and a suitable fast measurement time. In an iterative approach, the SNR can be calculated after each measurement and compared to the aimed value of SNR or its effect of accuracy for concentration determination. In the next steps, the scan number/acquisition time is adjusted by a factor determined SNR and target SNR. By this approach, the measurement frequency or time resolution of the measurement is maximized while keeping the SNR above a distinct limit [34].

For an assessment of an inline measurement technique, it is necessary to determine the *limit of detection (LOD)* with respect to the analytes investigated. The LOD describes the concentration at which a signal can be clearly distinguished from the background noise or in a signal of the surrounding matrix. In Eq. 9.2, the calculation for x_{LOD}, the smallest value measurable, is shown. $\overline{x}_{\mathrm{blank}}$ corresponds to the mean and σ_{blank} is the standard deviation of the blank measurements.

$$x_{\mathrm{LOD}} = \overline{x}_{\mathrm{blank}} + k * \sigma_{\mathrm{blank}} \tag{9.2}$$

The International Union of Pure and Applied Chemistry (IUPAC) recommends using a factor $k = 3$ [44].

An important value for determining the precision of inline analytics, and thereby the quality of the methods, is the *root mean square error of prediction* (RMSEP) [31]. The RMSEP (Eq. 9.3) is a value describing the discrepancy between the concentration measured by the (offline) reference analytical method and the predicted values obtained by chemometric models used for the analysis of inline-measured data.

$$\mathrm{RMSEP} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(\widehat{y}_i - y_i\right)^2} \qquad (9.3)$$

All inline measured and predicted values $\widehat{y}_i$ gathered over the course (N) of a reaction are compared to the reference offline analytic y_i. This value needs to be determined prior to the application of the corresponding chemometric model for the characterization of multistep reaction systems or their inline monitoring and control.

When setting up and applying an inline analytical method, the calibrated range should be taken into account. Additionally, a critical evaluation of the methods' accuracy and precision in view of the previously discussed parameters is highly important.

9.2 Measurement Techniques Being Applied in Biotransformations

In this section, the different measurement techniques, which are often used for bioprocess analytics, are presented and fundamentally explained with theory and scientific examples. In Fig. 9.3, as well as introduced in Sect. 9.1.1, the different implementations of analytical tools are demonstrated. In the next section, the measurement tools are classified according to their way of implementation into the bioprocess.

Firstly, the inline measurement method, such as spectroscopic tools, implemented using a probe will be described. Secondly, tools which are often implementable via a bypass system are presented. The methods coming from the traditional offline measurement, like gas and liquid chromatography, will thirdly be described. These analytical devices can be connected to a reactor using an automated sampling line with an automated workup, the sample being analyzed atline.

This classification is done according to common scientific practice from the viewpoint of process analytic technologies and, therefore, does not give a complete picture of any possible implementation of the respective measurement techniques.

As mentioned above for real-time monitoring, an important class of analytical methods is spectroscopy since this can be easily integrated inline or online into a reaction vessel. All spectroscopic methods use a distinct range of the electromagnetic spectrum, which is shown in Fig. 9.4. Starting in the energy-rich UV range, with increasing wavelength, the visible and infrared light and its spectroscopy can be applied. With even lower frequency, radio waves at low energy levels are used for nuclear magnetic resonance measurements.

For all measurement methods, the basis of the underlying physicochemical principle is briefly explained; advantages and known limitations are discussed with respect to the individual technique.

Applying spectroscopic tools as an in- or online analytic method is advantageous since these non-invasive tools are not obstructing the reactions' outcome. Depending on the wavelengths used, certain characteristic properties or functional groups of a chemical compound are measured. This leads to high flexibility of the different spectroscopic methods, often with several solutions for a distinct analytical problem. Additionally, the measurement time required in spectroscopy is usually low, ranging from milliseconds to a few minutes.

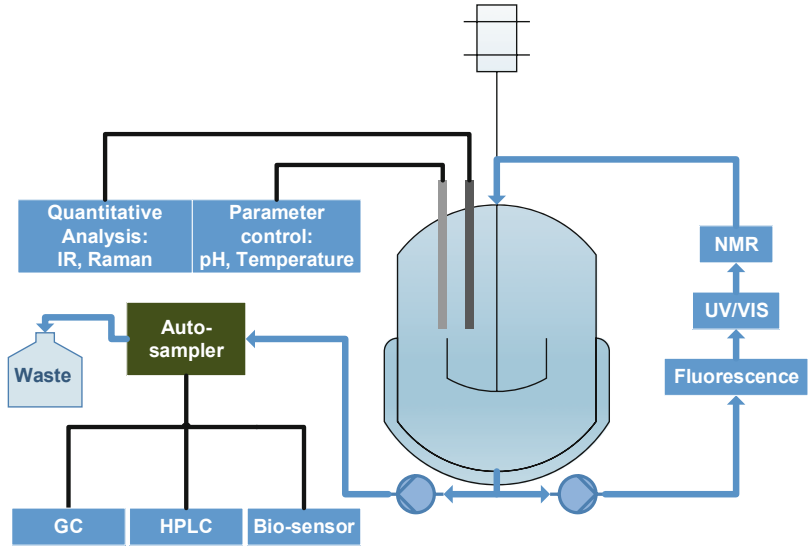

Fig. 9.3 Different measurement techniques and their possibilities for process integration. Spectroscopic methods like infrared (IR) spectroscopy and Raman can be applied inline using probes, in the same way as traditional measurement devices like temperature or pH probes. Often a closed loop, measurement- or flow cells can be used to establish analytical methods like nuclear magnetic resonance (NMR), UV/VIS , or fluorescence. More traditional offline analytics like gas or liquid chromatography (GC or HPLC) can be attached atline to a process or reactor using automated sampling devices

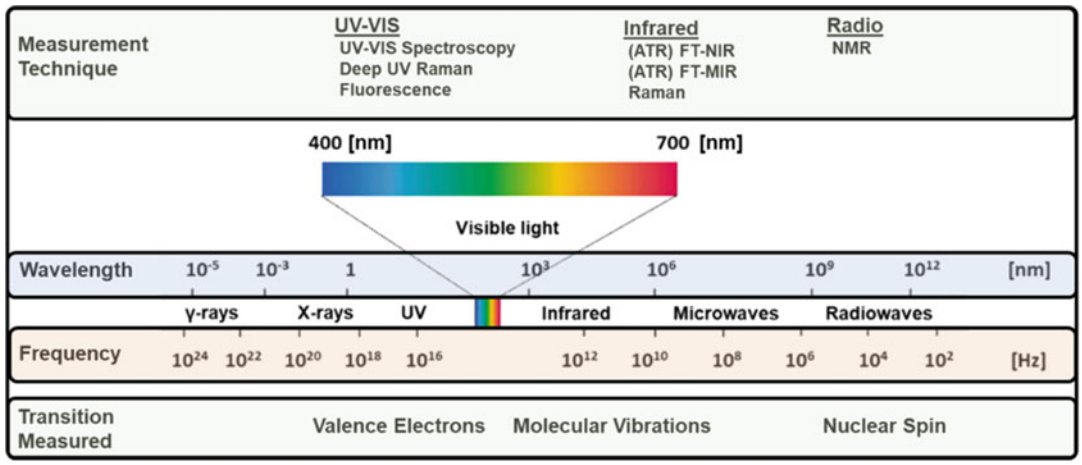

Fig. 9.4 Electromagnetic spectrum, measurement technologies, and the transition measured in the respective spectroscopic method. UV: Ultraviolet, VIS: Visible, FTIR: Fourier-transform infrared spectroscopy, MIR: Mid-infrared, NIR: Near infrared, NMR: Nuclear magnetic resonance

9.2.1 Inline Measurement Techniques

9.2.1.1 Infrared Spectroscopy (NIR and MIR)

Infrared (IR) spectroscopy is a versatile tool for monitoring enzymatic reaction sequences in organic media as well as in aqueous solutions depending on detection limits. IR spectroscopy is based on absorption of IR photons which lead to the excitation of molecular bond vibrations, resulting in a dipole change of the specific molecular bond. Therefore, symmetric molecules like oxygen and nitrogen cannot be measured [7].

The *Fourier-transform IR (FTIR)* enables many applications with short measurement times since different wavelengths do not need to be measured sequentially. They are measured simultaneously. From the resulting interferogram, generated by an accurate moving mirror, the absorption spectrum is obtained by Fourier transformation from the time to the frequency domain. The time needed for acquiring one scan depends on the velocity of the moving mirror in the Michelson interferometer and the desired spectral resolution on the distance for moving. Typically, the measurement duration for one scan is in the range of milliseconds, which enables the analysis of fast catalytic reactions. Since most reactions or transport phenomena are in a time scale one magnitude higher, it is common practice to average several scans for achieving an improved signal-to-noise ratio (SNR).

In IR spectroscopy it is widely common to use wavenumbers, which is the inverse of wavelength, to visualize the absorption or transmission spectra. According to the wavelengths of the photons, IR spectroscopy is divided into near-infrared (NIR) and mid-infrared (MIR) spectroscopy. In NIR, the more energy-rich wavelengths of 0.7–2.5 μm are applied, which correspond to wavenumbers of 14,000–4000 cm^{-1}. For MIR in contrast, light of 2.5–25 μm (4000–400 cm^{-1}) is used for the excitation of molecule bond vibrations.

Unique IR absorption by specific atom–atom bonds enables the identification of the structural or functional groups of the molecule. This even enables the detection and structural identification of the formed intermediates of a reaction sequence that cannot be isolated. Every organic molecule exhibits a so-called unique fingerprint, with a lot of sharp absorption bands in the specific MIR range below 1500 cm^{-1}. Above this fingerprint region, functional groups show distinguishable absorption bands.

For the application of IR spectroscopy as an inline analytical tool, the combination with the *attenuated total reflection (ATR)* effect makes the applicability easier. This effect, depicted in Fig. 9.5, is based on the internal reflection of light beams between two media, with differences in

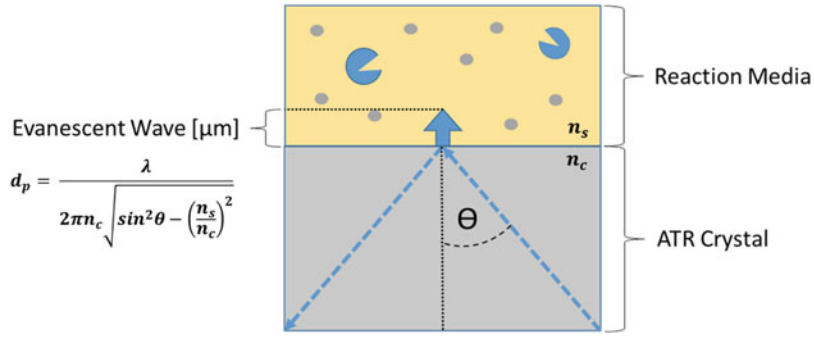

Fig. 9.5 Principle of attenuated total reflection (ATR) based on the difference in the refractive indices ($n_c > n_s$) of the media/sample (n_s) and the ATR crystal (n_c). Light beams exceeding the critical angle (theta) are reflected internally, while an evanescent wave is formed at the interface of ATR crystal penetrating into the sample. The penetration depth depends on the wavelength of the photons

their refractive indices (n_s and n_c). If an incident light beam exceeds a critical angle Θ, the light beam will reflect internally. During the reflection at the interface, an evanescent wave is formed, which penetrates into the less optical dense media with a wavelength dependent penetration depth (d_p).

Typically, the penetration depth is in the magnitude of some micrometers (<10 μm). Using inline ATR FTIR probes connected to optical fibers, the non-invasive measurement inside reactors containing enzymes [3], mammalian cell cultures [39], or downstream processing [19] units is possible. Because the absorption is taking place only in some micrometers above the surface of the ATR crystal, this technique is not disturbed by solids (immobilized enzymes) or gas bubbles, making it suitable for multiphasic reaction mixtures [36]. Using these inline probes, no need for sample drawing is given and no bypass with a measurement cell is needed. This lowers the risk of contaminations and no additional equipment such as a pump is needed. However, in a multiphase reaction system, only the bulk phase (where the probe is emerged in) can be analyzed. To analyze the dispersed phase, a different technique needs to be applied [26].

The measurement of the esterification of glycerol with lauric acid was obtained for the respective mono-, di-, or triester molecule. All components present in the reaction mixture could be detected with a predictive error between 1.8 and 12.5 wt.% with only little calibration data (Fig. 9.6). From the inline-measured data, conclusions to the reaction mechanism are possible. The *Candida antarctica* lipase B (commercially available under the trade name of Novozym 435, Novozymes A/S, Denmark) used in this process first is esterifying the primary alcohol functions before, in the last step, the secondary alcohol is esterified [36].

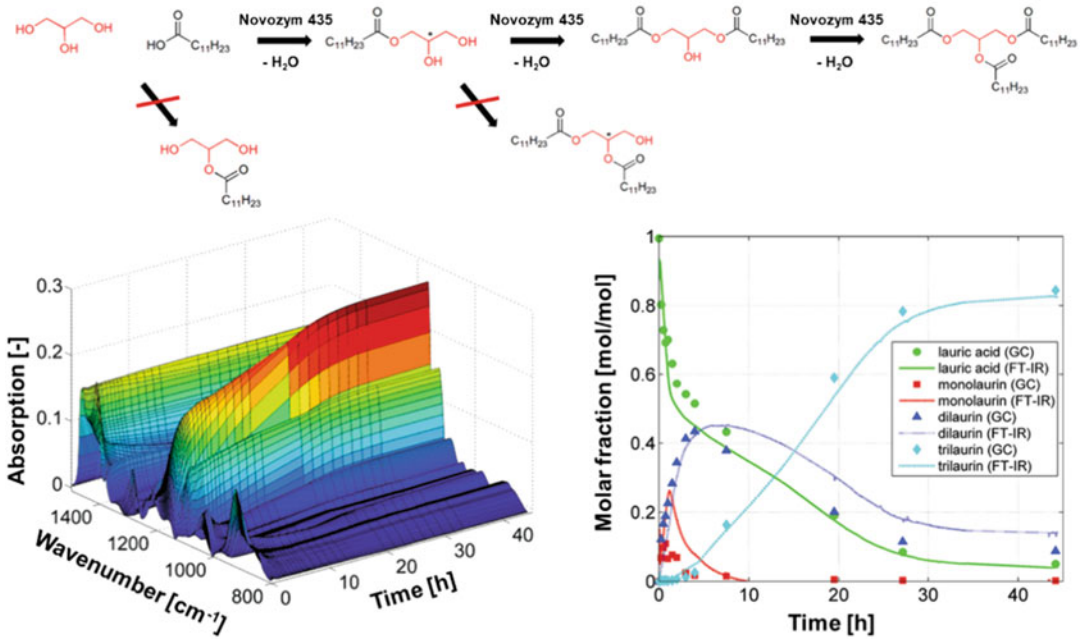

Fig. 9.6 Bottom: Multistep esterification of glycerol with lauric acid measured inline using ATR FTIR and offline using gas chromatography. Top: Reaction mechanism of an enzymatic multistep esterification using Novozym 435. Prior esterification of both primary alcohol groups before esterification of the secondary alcohol in the third reaction step. Experiments were conducted in bubble column reactor equipped with an ATR probe connected to a MIR spectrometer [36]

In a hydrofluoric acid alkylation plant, NIR spectroscopy was used to measure concentrations inline. This enables higher flexibility in controlling the reactor from a safe distance. For MIR, silver halogenide fibers are limited to a relatively short length of some meters, while for NIR glass fibers with a length of up to 100 m can be used [40].

When working with aqueous solutions or buffers containing a high amount of salts, the applicability of IR is limited since the water absorption bands are likely to mask or hide substrate and product signals. For these cases, Raman spectroscopy is usually more suited [1].

9.2.1.2 Raman Spectroscopy

In contrast to IR, *Raman spectroscopy* is based on the change of the polarizability of the vibrations in a molecule, or, in other terms, on the effect of Raman scattering of monochromatic light. From a light source, typically a laser, photons are absorbed by the reaction mixture causing a vibrational excitation. Subsequently, these photons are emitted either at a lower or higher energy level. For traditional Raman spectroscopy, monochromatic lasers usually in the VIS, NIR, or near UV are used. When applying a laser in the deep UV region, the method is called *deep UV resonance Raman spectroscopy*.

Raman spectroscopy is used in different fields of application and is a versatile tool for real-time monitoring of chemical reactions. Also, enzymatic examples for implementing Raman as an inline analytic tool are available. Multiple reaction systems including a nitrile hydratase, as well as different ones with xanthine oxidase were measured with deep UV resonance Raman spectroscopy. By application of multivariate curve resolution, the concentrations could be derived in real-time [49].

In another approach, UV resonance Raman spectroscopy was used to monitor biotransformation using purified enzymes and whole cells as well. Two different nitrile metabolic pathways, shown in Fig. 9.7, are monitored using a flow cell setup (online). As reference analytic, offline samples were withdrawn and subsequently analyzed using HPLC.

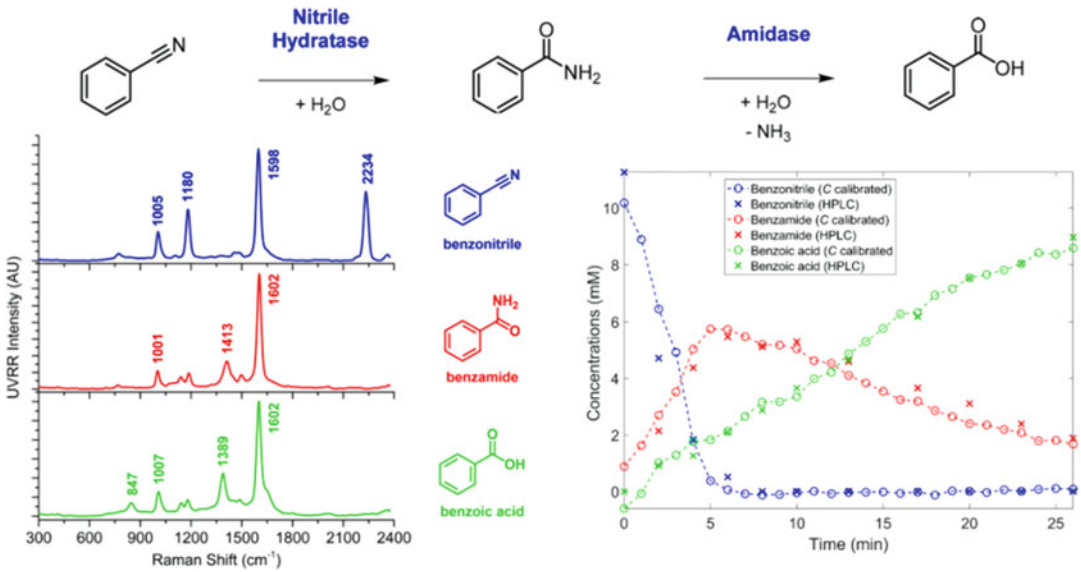

Fig. 9.7 Enzymatic cascade starting from the nitrile leading to the corresponding acid. Ramen spectra and concentration–time plot for the enzyme catalyzed conversion of benzonitrile (via benzamide) to benzoic acid followed online using UV Raman spectroscopy [13]

Using the online analytical method, it is possible to determine the concentration of the starting material benzonitrile, the intermediate benzoamide, and the final product benzoic acid. This is applicable in the case of suspended whole cell catalysis as well as in the case of homogeneously soluble enzyme. The authors successfully differentiated between the two metabolic pathways based on the intermediate benzoamide in whole cell experiments. Additionally, kinetic information could be obtained from the online data [13].

9.2.1.3 Measurement of pH

A customary and state-of-the-art measurement technology in fermentation, as well as in biotransformations, is the *pH value*. The pH value describes how basic or acidic a reaction solution is and is dependent on how many H^+/H_3O^+ ions are available within the solution. The formula for the pH value is shown in the following:

$$\mathrm{pH} = -\log_{10}(\mathrm{H}^+)$$

Since in many reactions occurring in an aqueous medium, protons are released or depleted, the pH value is an easy method to establish an inline reaction monitoring or control. Aside from the pH, the acid number can also be used for determining the conversion of an acid during a reaction. Using a pH meter, a sample from the reaction media is titrated using a base, until the pH reaches the neutral point. From the amount of base used, the acid number (Eq. 9.4) can be calculated, and the conversion can be calculated. The same principle can be applied using an acid for the determination of the base number by titration.

$$\text{Acid Number} = \frac{V_{\text{Base}} * c_{\text{Base}}}{m_{\text{Sample}}} \tag{9.4}$$

Recently, the possibility of reactor control using an inline pH probe was shown for microreactors. Two enzymatic reactions could be maintained at optimum pH values and thereby a more efficient process enabled [20]. A μ-bubble column reactor system was developed for screening purpose. The reactor is equipped in with inline measurement of pH, optical density, and dissolved oxygen, enabling the efficient monitoring of processes using whole cell catalysts. Additionally, glucose can be measured using an online microfluidic flow chip [29].

9.2.2 Online Measurement Techniques

Different analytical devices can be integrated online by using a bypass loop to the reactor like shown in Fig. 9.3. Due to enzymes retained within the reaction vessel or different mixing behaviors inside the bypass loop, deviations between the measurement port and the real reactor can be present. Therefore, knowing the hydrodynamic residence time as well as the duration of a single inline measurement is necessary to conclude the actual conditions within the reactor unit.

9.2.2.1 UV/VIS Spectroscopy

UV/VIS spectroscopy is one of the oldest spectroscopic technique and is widely used in many scientific areas. This method is based on the absorption of photons with a wavelength of 200–400 nm (ultraviolet range) or 400–780 nm (visible range), which are absorbed by the

Table 9.1 Chromophores, their respective absorption maximum in the UV/VIS range and an example molecule

Chromophore	Chemical	UV/VIS Absorption Maximum [nm]	Example Molecule
Carbonyl (ketone)	RR′C=O	271	Acetone
Carbonyl (aldehyde)	RHC=O	293	Acetaldehyde
Carboxyl	RCOOH	204	Acetic acid
Amido-	$RCONH_2$	208	Acetamide
Azo-	–N=N–	339	Diazomethane
Nitro-	$–NO_2$	280	Nitromethane

molecules valence electrons [9]. Different functional groups (chromophores) absorb at certain wavelengths, therefore enabling a detailed differentiation and quantification of components within the reaction mixture [18]. An overview of selected functional groups is shown in Table 9.1.

The measurement principle is widely used to resolve substrate conversion in biotransformations and for the determination of kinetic parameters in small scale experiments using cuvettes. When applying this measurement technique in a flow cell, it can easily be transferred to an online analysis method by installing the spectrometer with a suitable pump in a bypass to the reactor system. Nowadays, probes are also available enabling the implementation of UV/VIS as an inline measurement technique.

One advantage of this method is the relatively low price of the technique. There is no need for expensive maintenance, just the light source needs to be exchanged since the intensity changes after a certain lifetime.

This measurement technique is limited by the Lambert-Beer-Law to relatively low concentrations since the absorption exceeds the linear range for solutions higher concentrated than 0.01 M.

Coenzymes, like NADH and NADPH ($\lambda = 340$ nm), or FAD ($\lambda = 450$ nm), can easily be detected and quantified using UV spectroscopy.

UV/VIS spectroscopy is a technique which has already been suitable for inline, online, or atline measurements in the last decades. For example, UV/VIS was used to monitor a lipase-catalyzed kinetic resolution in supercritical carbon dioxide by using a high pressure flow cell. It could be shown that the solubility of the substrates changes with the pressure of the supercritical carbon dioxide. Therefore, the pressure was kept constant to 110 bar, and the concentration of the acetic aldehyde was determined online [4].

9.2.2.2 Fluorescence Spectroscopy

The principle of *fluorescence spectroscopy* is the absorption of photons by molecules, causing an excitation from the electronic ground state into electronic excited state with a higher vibrational level. The excited species collide with other molecules causing a nonradiative vibrational relaxation to the lowest vibrational level and therefore the excited molecule loses excitation energy. Out of this lowest vibrational level of excited electronic state the emission of a photon takes place. Since the energy of the emitted photons is lower, the result is a emission spectrum of redshifted light, which can then be measured with a detector in a 90° or reflection setup. The emitted photons usually have a higher wavelength compared to the excited ones and therefore the scattered excitation light seperated by filters.

Fluorescence spectroscopy is a useful technology for the detection and quantification of various organic compounds. Molecules are detected based on their valence electrons, like in the UV/VIS range. However, the area in which the response signal is in the linear range is broader, compared to the UV/VIS spectroscopy, enabling the measurement of higher concentrations.

Using the fluorescent effect of tryptophan, cell count was established as an online measurement technique in real-time, which shows a higher sensitivity compared to classical turbidity measurements. By using this technique, biotransformations using whole cell catalyst can be monitored in view of active cell concentrations [41].

As an example for a biocatalytic C–C bond formation, the synthesis of chiral 2-hydroxypropiophenone was successfully monitored using 2D fluorescence spectroscopy applied inline *via* a probe. This measurement technique yields high quality spectra in aqueous solutions at low concentrations of substrates and products, which makes it highly useful for many biocatalytic reactions. Using chemometric methods, like principle component analysis and the partial least square algorithm, it was possible to determine concentration data from the online-measured spectra. In the next step these data can be used to determine kinetic parameters as well as to optimize process conditions [25].

In whole cell reactions, as well in biotransformations using purified enzymes, fluorescence sensors are applied for monitoring intra- and extracellular levels of NADH and NAD^+

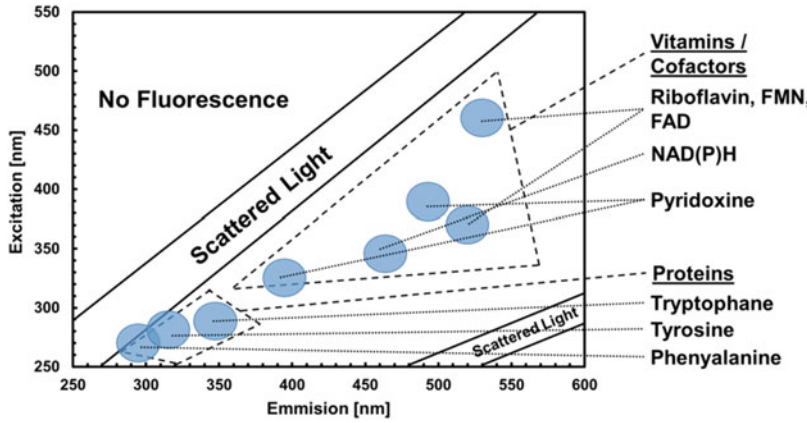

Fig. 9.8 Excitation and emission spectra of several vitamins, cofactors, and proteins detectable using fluorimetry [38]

[38]. Both forms are important cofactors in various enzymatic reactions acting as proton donors or acceptors. Its absorption and emission wavelength can be found in Fig. 9.8. This technique can be extended to 2D fluorimetry, which enables the simultaneous measurement of several components by measuring different wavelengths at the same time [46]. Besides NAD, also FAD, NADP(H), and different amino acids or proteins can be detected.

9.2.2.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is the most important analytical technique applied in chemistry for structure elucidation. Using magnetic fields, the atoms spin is changed and transitions between different energy levels occur. In principle, every atom with a magnetic dipole moment not equal to zero can be measured. This is the case for an atom containing an odd number of protons or neutrons within the nucleus. Therefore, ^{13}C nuclei can be measured, as well as ^{1}H, ^{19}F, and ^{31}P.

The magnetic fields required for the induction of these changes in spins are dependent on the resolution of the desired magnetic shift. A common quality differentiation in NMR techniques is low and high field NMR. For a detailed analysis with a high resolution, high field NMR spectroscopes are used, which nowadays work with a maximum radio frequency of 900 MHz. This corresponds to a field strength of 21 Tesla and is limited by the materials used for the construction of superconducting magnets needed for these devices. A drawback of these highly precise machines helping to elucidate chemical structures is the high maintenance required. The superconducting electromagnets need to be cooled using liquid nitrogen and helium, making the service relatively expensive.

Since a few years, benchtop low field NMR devices are available. The space needed for a low field NMR spectroscope is relatively low and it can be easily placed on a lab bench and connected to the reactor using a bypass. In contrast to the high field machines, they work at a frequency of about 60 MHz and resulting field strength of 1–2 Tesla. No superconducting materials are needed for the generation of such magnetic fields, which leads to significant lower maintenance costs required since no cooling of the coils is necessary. However, the resolution achieved with these devices is lower compared to high-field application. In recent low field NMR devices, a spectral resolution of 0.1 ppm has been realized, which can be successfully applied for precise reaction monitoring [10].

The use of NMR probes gives the possibility to implement NMR measurements in situ or inline into a reactor system. Different designs were developed in the last decades [30].

For analyzing samples in view of proton NMR (^{1}H), deuterated solvents are applied. In these solvents, all protons contained are exchanged with deuterium, leading to a clear differentiation

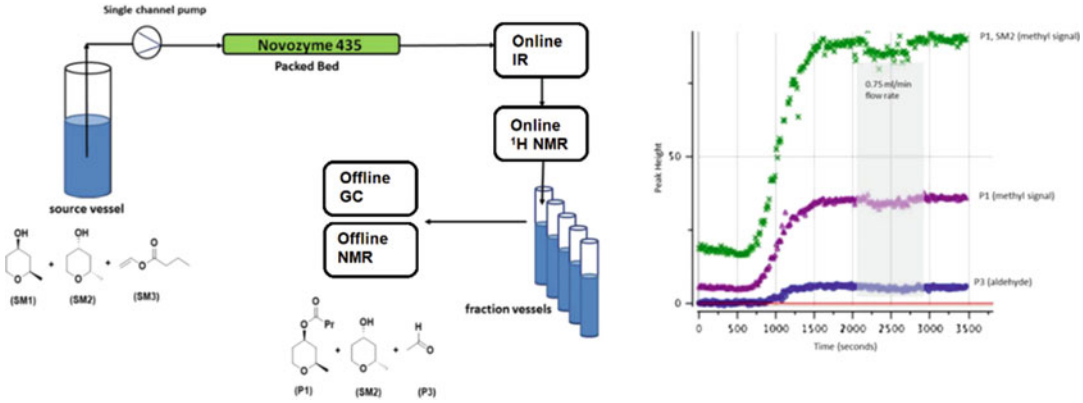

Fig. 9.9 Reaction scheme of the kinetic resolution of racemic cis-pyranol and vinyl butyrate and the respective reaction setup consisting of a packed bed reactor, online NMR and IR, as well as subsequent offline analysis using high frequency NMR and GC analysis, Online determined peak heights versus reaction time of substrates and products of the kinetic resolution of racemic cis-pyranol and vinyl butyrate using Novozym 435 [12]

of the sample and the respective solvent used for the analysis. NMR spectroscopy is a relative method since all atoms contained in the sample are measured. For an improved quantification, an internal or external standard is needed. The standards should show clearly distinguishable chemical shifts in contrast to the analytes from the reaction cascade.

Enzymatic acetylene bond degradation by biotransformation using cells from *Pseudomonas putida,* isolated from rotten fruit, was monitored using an NMR probe. The two substrates investigated were 2-butynedioate (no proton) and propynoate (one proton). The (low) number of protons makes the substrates either invisible or quite difficult to monitor. However, by the biotransformation, protons are added to the triple bond resulting in a clearly visible signal. Using deuterated water, it was possible to trace the metabolites in the degradation pathway. The researchers determined a detection limit of 0.2 mM [6].

The application of inline NMR measurements was published for a fixed bed chromatographic reactor in which the synthesis of formate and methyl acetate was performed. Both reactions could be monitored and quantified with high accuracy, even with an overlap of the reaction compounds in the NMR spectra. The results were validated using a refractive index detector [5].

For monitoring the enzyme catalyzed kinetic resolution of racemic *cis*-pyranol with vinyl butyrate using a lipase (Novozym 435), online NMR and IR were implemented via a flow cell. The flow rate was adjusted according to a maximized signal-to-noise ratio. After the reaction broth was transferred through the IR and NMR flow cell, fractions were collected and analyzed offline afterwards. From the NMR peak heights, conclusions to the conversion were drawn and no chemometric modeling was needed (Fig. 9.9). The results were compared to samples analyzed using a chiral GC calibration as well as a high frequency NMR device and were in good accordance [12].

9.2.3 Atline Measurement Techniques

Traditional offline analytics like *gas chromatography (GC)* or *high performance liquid chromatography (HPLC),* formerly also called high pressure liquid chromatography, can be used as

a suitable atline analysis using automated sampling devices.

The separation principle of gas chromatography is the difference in volatility of the different components as well as the absorption and desorption velocity at the stationary phase (GC column) and the mobile phase (carrier gas). In GC systems, a large variety of organic compound with a low or medium molecular weight can be detected using a flame ionization detector (FID) since the chemical is combusted with hydrogen gas yielding a certain signal.

For HPLC, the separation is done based on the differences in the absorptive behavior between stationary and mobile phase. In normal phase HPLC, the stationary phase is polar and the mobile phase a non-polar solvent, while in reverse phase HPLC the stationary phase is non-polar, and the mobile phase consists of polar solvents. For GC also, different columns are available: depending on the materials used, these are either well suited for polar or non-polar components. A recent development is *ultra-high performance liquid chromatography (UHPLC)*. In this technique the particle size is at 2 μm, leading to a higher surface for adsorption, thus improving the separation and decreasing the time needed for an efficient separation. However, the pressure drop also increases, which requires higher pressures which must be applied by the pump.

Both analytical devices are primary used for the separation of a (reaction) mixture. For the detection of single components and their quantification subsequent to their partition, suitable detector systems like FID, mass spectroscopy (MS), diode array detector (DAD), or refractive index (RI) are used. These are not discussed in detail within the scope of this chapter. Depending on the analytes in the liquid phase, several detector systems can be integrated sequentially.

In literature, a method was published, which makes atline measurement of fast catalytic reactions possible using a GC device. Using the principle of multiplexing GC together with column switching mode, a fast sampling interval of some minutes with only short measurement duration could be achieved. Using these column switching technologies together with backflushing, the signal-to-noise ratio and the throughput of samples could be enhanced making it a suitable method for the analysis of fast catalytic reactions. The authors investigated a process to synthesize olefins from methanol and monitored the reaction selectivity as function of time [52].

In bioprocesses, an automated sampling device was presented, which is suitable for the HPLC analysis of amino acids. The sample processing can be adjusted for a certain case and the respective conditions of the fermentation or reaction process. For example, a certain dilution can be set, or a filtration can be done prior to the analysis, ensuring that no cells are flushed into the HPLC system. Using this atline analysis, a feeding strategy for fermentations can be achieved when the duration of the method is fast enough

Table 9.2 Suitability of different analytical methods as in-, on-, and atline analytics as well as their ability for structural investigations

	Concentration determination			
Method	Inline	Online	Atline	Structural information
UV/VIS	+	++	–	–
FTIR	++	+	–	+
Raman	++	+	–	+
NMR	+	++	–	++
GC-FID	–	–	+	–
GC-MS	–	–	+	++
HPLC-RI	–	–	+	–
HPLC-UV	–	–	+	–
HPLC-MS	–	–	+	++

with respect to the time scale within the reactor or fermenter [22].

9.2.4 Suitability of Different Measurement Techniques

Different analytical methods can be applied in different ways into a reactor setup. In addition to the way of implementation of the respective measurement techniques presented in the previous section, additional setups are possible, which are not discussed in detail within this book chapter. Furthermore, as shown in Table 9.2, some measurements provide structural information helping to identify side products.

Raman spectroscopy is usually better suitable for aqueous solutions in comparison to IR spectroscopy. Absorption bands of water are stronger in the IR range and therefore a covering up of the IR signals of the compound of interest in a low concentration range could appear [1]. In contrast, IR is very sensitive in organic solvents, making it the first choice in these systems. In multiphase systems, ATR FTIR can be applied, since this measurement technique is not disturbed by modereate concentrations of solids, cells, or gas bubbles [36]. IR and Raman spectroscopy can be applied in a bypass section. This is advantageous, if, for example, a film formation takes place at the ATR crystal. Using a bypass section, this film can be removed by flushing with a washing solution.

UV spectroscopy can be applied with high sensitivity for the measurement of substances containing double bond or aromatic systems under the consideration of the wavelengths of interest and solvent signals or its cut-off. For reaction systems with additional solid or gas phase, the common used UV spectroscopy is not suitable due to scattering effects. For UV spectroscopy some probes are also available, which makes this method inline applicable. However, this is not state of the art. For NMR it is similar: NMR probes are available on the market, but most applications for inline measurement are realized using a closed loop.

For the analysis of unknown intermediates or side products, structural information is highly important. Here methods like NMR, LC-MS, or GC-MS are the most suited methods, which are used for the elucidation of unknown structures. By application of UV, functional groups can be identified; however, here Raman or IR yields more detailed information in a broader spectrum. GC-FID and HPLC-RI are not suitable for any resolution of structural details.

9.2.5 General Workflow Generating Inline Analytics

In this subchapter, the general workflow needed for the successful setup of an inline analytic used for the monitoring of an enzymatic reaction cascade is depicted. Since in a reaction cascade several components are present which interact with each other, e.g. with hydrogen bonding, it is important to consider the whole reaction mixture including solvents, heterogeneous phases, and catalysts.

In Fig. 9.10, the workflow for the setup of a suitable inline analytic used for monitoring an enzymatic reaction cascade is shown. In the beginning, the critical reaction steps, which need to be continuously monitored, need to be identified. For this reason, some previous knowledge about the reaction is necessary in this early stage, e.g. if there is a certain product inhibition of a certain enzyme within the cascade. If one or several critical steps in the reaction cascade have been recognized, these steps will be analyzed in detail. Based on knowledge generated by inline data, a suitable reactor type or cascade can be chosen to address product inhibition of a certain enzyme. For detailed information about different reactor setups and operation modes, see Chap. 7. The physicochemical properties of the compounds are evaluated and, for example, electromagnetic spectra are recorded. If the intermediates are not available in pure form, they should be synthesized and purified for doing this first analysis. Additionally, process information like substrates and products of the cascade, used solvent, salts, and additives or

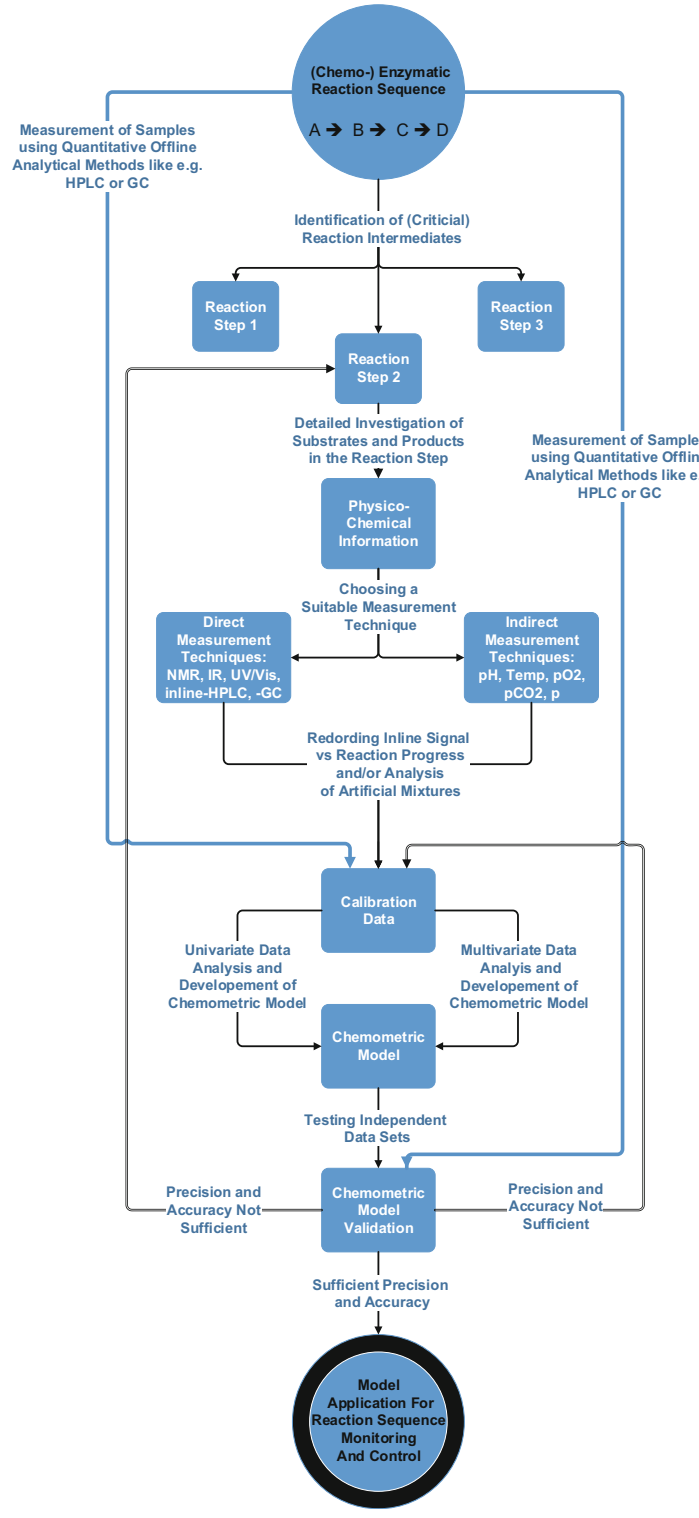

Fig. 9.10 General workflow for designing/establishing a suitable inline analytic for (chemo-enzymatic/enzymatic) reaction cascade

multiphasic components should be taken into account since these can interact with the compound to be analyzed inline or online.

On this basis, one can decide which measurement techniques are suitable depending on the knowledge gathered in the previous step. The analytical methods can be differentiated between indirect and direct measurement methods. Indirect methods are physical parameters, which are influenced by the intermediate or the reaction step itself, for example, the temperature in an exothermic reaction step or the pH shift in an enzymatic hydrolysis reaction. Direct measurement techniques are directly related to the component of interest like their absorption spectra (independent from the wavelength used) or a certain signal of the intermediate in a GC or HPLC connected detector system.

In the next step, data needs to be recorded using the analytical tool chosen before. For this reason, reactions and artificial mixtures are analyzed using the inline system. While for reactions offline samples are taken and analyzed using a suitable reference analytical tool like GC or HPLC, for artificial mixtures the weigh-in data can be used as a reference for the inline- or online-measured data. For a successful application of the model afterwards, it is important that the concentration range of the calibration data is similar to the range present in the reaction cascade later on.

Using the inline data and the collected reference values, the data analysis can be done. It is advisable to check the recorded data for coherences and relations with statistical methods like a principal component analysis (PCA). For example, from a PCA, wavelengths with high influence on the spectra can be extracted and, by doing this, model relevant variables are extracted. Using multivariate methods, a chemometric model can be set up integrating inline and offline data. In the case of simple correlations, e.g. a concentration following the pH with a linear trend, a univariate model such as a simple regression curve is sufficient.

Subsequent to setting up a chemometric model, it is highly important that the model is externally validated using independent data sets. An independent data set means, these data were not part of model construction. This external validation is extremely important, and the user must not only rely on the internal validation procedures done by the chemometric or statistical software when constructing the model. Using these independent data sets, the RMSEP (Eq. 9.3 in Sect. 9.1.3) in relation to the reference analytical system is determined.

9.3 Aim of Inline Analytics in Reaction Cascades

9.3.1 Real-Time Analysis Enabling Reaction Control

After the successful validation and determination of the accuracy of an inline analytic method using independent data sets in the range used for reaction monitoring, the measurement technique can further be applied for reaction or process control. When all components of a (chemo)-enzymatic reaction sequence are analytically accessible, the information gathered is sufficient to establish detailed kinetic models. These kinetic models then guide to implement a scale-up from the lab-scale, including the possibility of an inline monitoring during the later process.

Monitoring and control of a reaction progress in a specific setup can be done using direct or indirect measurements and also with direct or indirect control variables. The concentration of a certain component in the reaction cascade can be determined directly from spectroscopic analysis and suitable chemometric methods or indirectly from, e.g. a pH shift. The velocity of a reaction can either be controlled directly via the substrate feed to the reactor or indirectly by adjusting the temperature of the reactor setup (in a certain range).

The reactor control based on inline analytics can be achieved in several ways. Some of these possibilities are shown in Fig. 9.11. For example, in a continuous stirred tank reactor (CSTR), the inline measurement signal can be used for controlling the feed pump. Such an inline measurement-based feeding strategy increases the space-time yield of a substrate inhibited enzyme catalyzed reaction [37] since the

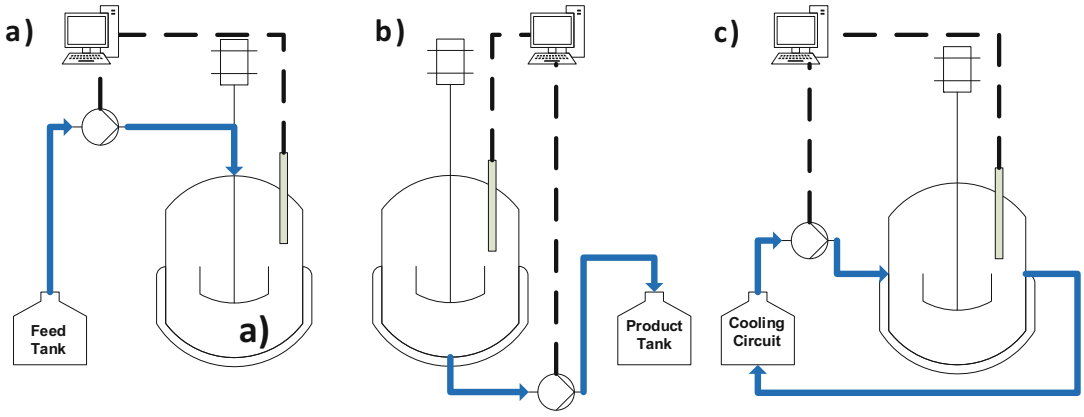

Fig. 9.11 Inline measurements give rise to certain control strategies in multistep reaction systems: (**a**) the inline-measured concentrations are used to control a feed to, e.g. keep the substrate concentration low; (**b**) the concentration profiles are used to control the product flow of a continuous stirred tank reactor at optimal outflow conditions; (**c**) the reaction velocity and selectivity determined by inline data can be adjusted indirectly by controlling, e.g. the cooling/heating circuit of the reaction vessel at an optimum

concentration of a substrate can be controlled to low levels, which enhances the reaction velocity of the enzymes.

For enzymatic reactions exhibiting product inhibition, a series of CSTR can be used to achieve plug flow behavior [28]. Monitoring the product concentration via inline measurement can help to maintain the product concentration at a lower level by controlling the residence time in the reactor. This leads to significantly higher activities of the enzyme affected by product inhibition. See Chap. 7 for more details on design of reactors in series based on enzyme kinetics.

The data acquired needs to be in a suitable time resolution to enable a real-time process monitoring, which then can be successfully used for the purpose of reaction control. Therefore, the time resolution of the analytics should be in the same range as the reaction velocity.

When analytics are implemented online using a bypass, it is important to determine the hydrodynamic residence time to gain knowledge about the difference between the bypass and the remaining reactor volume, in which, for example, the enzymes are retained and which therefore have a difference in concentrations. The temperature in the bypass could also differ from that of the reactor, which has a double jacked cooling or heating circuit. Therefore again a difference in the reaction velocity could be present.

For spectroscopic measurements, where averaging of spectra or scans is a common procedure, an optimum number of scans can be determined in terms of an optimum SNR [34]. Another basis for the optimization of the scan number used for the average of one spectrum is the reaction velocity.

9.3.2 Optimizing Productivity and Quality

The overall aim of an inline analytic in a production scale is maintaining a high quality of the desired product. At the same time, the productivity should be kept at a high level, meaning a high selectivity towards the product, and therefore low or no side products produced within the process. Aside from a high efficiency of the materials used for the conversion, the amount of energy used for the production in the enzymatic reaction cascade can be optimized, leading to a sustainable and economically feasible process.

In continuous processes, inline analytical methods can help to maintain the process at high productivity. Deactivation of the enzymes used

can be detected early and a replacement of the biocatalyst can be done immediately. Closed process loops help to recognize these deviations in an early stage, making a direct correction possible, helping to save resources, time, and energy.

Deviations in the starting material can already be detected at the beginning of an enzymatic reaction cascade. In case of variable raw materials used within a production process, a consistent output can be realized by continuously monitoring all steps of a reaction cascade. Therefore, single steps can be run using variable reaction parameters achieving a stable product concentration [8].

The "quality by design" approach describes a systematic concept for the development of new processes for the production of drugs and chemicals, emphasizing process understanding and control [47]. Inline analytics play a significant role in this approach since these can already offer the needed process understanding in an early stage of process development. Using such methods, higher flexibility and a more robust process design can be achieved.

Inline analytical methods used in batch processes can help to decrease the reaction time needed, as well as the "time to market" for launching a product. Inline measured parameters are almost evaluated in real-time and can be compared to other batch data and confidence intervals set for the product specifications like the purity or limits for concentration of distinct contaminations/side products. This approach is called "quality by control" in industry [42] and is highly simplified using, e.g. spectroscopic measurement methods combined with certified chemometric models.

Aside from the impact on productivity and efficiency, the use of inline analytics already in the stage of process development is advantageous. The inline measurements can not only help to decrease the time needed for screening catalysts for a certain reaction step, but additionally they can be used for a fast determination of a process window and optimum process parameters.

For a reaction system, where characterization is not accessible using offline samples, the development of inline analytics is advantageous. In this case, it is important to have reliable reference analytics, which are then connected with the inline data gathered from the reaction step later on. Here, for example, reference values using a relative analytical method like NMR can help to monitor the reaction without manual sampling and offline analysis.

In the future, machine learning in combination with inline analytics applied in continuously operated reactors can provide new methods for the optimization of enzymatic reaction sequences. The first research reports are already available in the case of protein optimization by machine learning in combination with directed evolution [51]. This means, on the basis of online analytical data, the radical implementation of artificial intelligence by learning algorithms will enable self-optimization of processes. Here a totally new field of research will open up in the future. In the field of pure chemo-catalysis, inline-analysis-based approaches are already available and can be used for the continuous and automated optimization of reaction parameters. In the polymerization reaction of vinyl acetate, inline Raman sensors are used and the obtained process data are evaluated using self-learning algorithms to optimize the whole process [16]. Recently, a tool for the prediction of chemical reactions was published: using a data set of 11,000 reactions with deep learning algorithms enabling the prediction of reaction pathways and possible intermediates, products, and byproducts [15]. In another approach, a data set of 10 million reactions was used to develop a neural network, which is able to predict reaction conditions. Experimental design can be improved and therefore, the time needed for implementation of new reaction sequences decreased [17]. By using such computer-aided approaches, the experimental

effort for developing new reaction cascades can be reduced and in consequence the time needed for their establishment of new and optimized processes decreased.

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Enzymatic Cascade Reactions in Non-Conventional Media

10

Javier González-Sabín

Abstract

The amazing challenge of orchestrate enzymatic cascades (multi-enzymatic or chemo-enzymatic) usually faces numerous issues to be addressed. Together with the advances in synthetic biology, materials science and protein engineering, the discovery of new reaction media represents a valuable tool in the search towards cost-efficient and sustainable enzymatic cascades in an industrial setting. This chapter showcases the recent developments on the implementation of the so-called non-conventional media in such processes.

Keywords

Non-conventional media · Deep eutectic solvent · Ionic liquid · Chemo-enzymatic cascade · Sustainable media · Biocatalysis

10.1 Introduction

Challenges coped by synthetic chemists when trying to access highly-functionalised molecules are evolving rapidly due to the new environmental and economic needs imposed by modern society [1]. As stated in the 'Twelve Principles of Green Chemistry' established by Anastas and Warner, a new awareness arose to produce chemicals and materials in a safer and more sustainable manner [2]. This chapter sets up the guidelines of what would make a greener chemical process:

- Be safe for the human being and the environment,
- Take place under mild reaction conditions,
- Be catalytic [3],
- Be performed using inexpensive and sustainable solvents [4].

At this point, the inherently green enzyme catalysis fits ten of the previous 12 principles and it has experimented a spectacular growth for the synthesis of chiral molecules [5, 6]. Nature's catalysts are usually non-toxic, biodegradable and work under physiological conditions, thus achieving numerous safety and economic benefits.

Deepening in the 12 Principles, a special emphasis is placed in '*Safer Solvents and Auxiliaries*' with the prospect of replacing the classical petroleum-based *volatile organic solvents (VOCs)*, which suffer from toxic/carcinogenic risks, with alternative greener solvents. While water (considered the ideal solvent) is the physiological medium of enzymes, a concomitant challenge for biocatalysis is the low solubility of hydrophobic substrates and products which limits the volumetric productivity demanded at industrial settings [7]. In this context, the staging of

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non-conventional media as biorenewable reaction media has brought new paradigms in many research fields. The term '*non-conventional media*' highlighted in the title of the present chapter compiles the following non-aqueous environments [8]:

- Solvent-free processes
- Supercritical fluids (SCFs)
- Biomass-derived solvents
- Fluorinated solvents
- Ionic liquids (ILs)
- Deep eutectic solvents (DESs)

A shallow analysis reveals attractive advantages but also limitations for all of them: Technology for CO_2 and water in supercritical conditions involves high cost; fluorinated solvents are non-toxic but very expensive and prevalent in the atmosphere; biomass-derived solvents are cheap, safe and biorenewable although non-susceptible to being tuned; ILs offer tunability but suffer from toxicity and low biodegradability; the more recent DESs, intimately related to ILs, meet most of the pursued properties.

Defined as salts with low melting point ($<100\ ^\circ C$), ILs are composed of an organic cation and an organic or inorganic anion (Fig. 10.1). Interestingly, the appropriate combination of such components allows modifying the physical properties (viscosity, hydrophobicity, polarity and hydrogen bond basicity). Ultimately, the so-called *task specific ionic liquids (TSILs)* go beyond a mere solvent to display key roles in a myriad of areas such as catalysis, electrochemistry, spectroscopy or material science [9]. ILs were the first non-conventional medium exploited in biocatalysis, the last two decades witnessing a plethora of biotransformations [10]. ILs stabilise some kinds of enzymes through the microenvironment generated and acting such a liquid support (interactions between enzyme and IL). Thus, although lipases and proteases were the most extensively used enzymes, C-C bond formation and redox reactions have also been conducted in such reaction media.

DESs are mixtures of low-cost biodegradable components such as hydrogen bond acceptors (HBA; ammonium salts) and uncharged hydrogen bond donors (HBD; urea, carboxylic acids or polyols) with a lower melting point than either of their components (Fig. 10.2). Compared to ILs, DESs are cheaper, readily available, do not require further purification and are considered to be less toxic given the nature of its components [11, 12]. In particular, those DES consisting of primary metabolites, namely, amino acids, organic acids, sugars or choline derivatives, are defined as *natural deep eutectic solvents (NADESs)* [13], and their use is allowed in food and pharmaceutical formulations [14]. Although research on DESs runs still in its infancy, the amazing properties of these neoteric solvents have permitted the flourishment of applications in research fields such as electrochemistry and metal processing, material chemistry, nanotechnology, photosynthesis and energy technology, and separation processes. With regard to synthetic applications, DESs have provided examples of improved activity and selectivity in: (1) organometallic-mediated stoichiometric transformations and (2) metal- and organo-catalysed reactions. Likewise, they offer an ideal reaction medium for both isolated enzymes (lipases, proteases, epoxide hydrolases, peroxidases, ketoreductases (KREDs), lyases and transaminases (TAs)) and whole cells [15, 16].

The preceding chapters have evidenced fundamental principles and impressive advances in multi-enzymatic processes and their monitoring during the last decade, enabling the construction of enantiopure high added-value chemicals under mild reaction conditions. To increase the sustainability of chemical manufacturing, these systems emulate the biosynthetic pathways where the living cells work as a perfect machinery of enzymatic networks. Nevertheless, when mimicking Nature things are more complicated than it seems. Once planned a synthetic cascade, a number of concerns must be circumvented to reach an efficient implementation of such a process. Incompatibility of catalysts and their preferred reaction profiles are commonly found as well as problems arising from inhibition and instability.

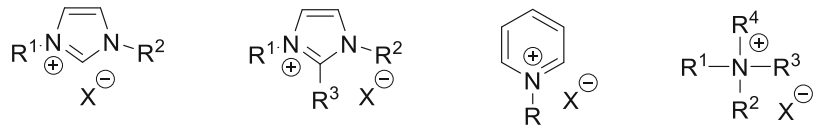


Fig. 10.1 Chemical structure of commonly used ILs

As a result, chemists have developed a battery of ingenious approaches aimed at circumventing the multiple drawbacks inherent to these processes [17]. The object of this chapter is to showcase non-conventional media as a new platform to perform enzyme cascade reactions (multi-enzymatic or chemo-enzymatic) in a more efficient manner. Along with advances in synthetic biology, materials science or protein engineering, medium engineering can be a practical solution for filling remaining gaps in some examples disseminated in the book as well as a valuable tool for future biocatalytic processes.

10.2 Chemo-enzymatic Cascades in Non-conventional Media

As reflected in Chap. 5, the wide interest in chemo- and biocatalysis has spurred smart methodologies to merge the synthetic potential of these pivotal areas of catalysis [18]. Historically, chemists combined reactions belonging to a single type of catalysis. Metal-catalysed reactions usually rely on the use of VOCs to avoid catalyst degradation in water, and are accomplished at high loadings of substrate. Conversely, enzyme catalysis is performed in aqueous medium and suffers from low substrate concentrations due to the poor water solubility of the reaction partners. Likewise, most biocatalysts are readily inactivated in organic solvents. Bearing all these facts in mind, the implementation of hybrid catalytic systems demands a compatibility window where both catalysts coexist and exert their activity. Interestingly, the pool of metal catalysts operating in water has expanded enormously in the last years, and the advances in protein engineering and immobilisation have boosted the combination of several metal-catalysed reactions (Pd-catalysed C−C coupling, Wacker oxidation, and C−H activation, Cu(I)-catalysed click chemistry, Ru-catalysed olefin metathesis or Au-catalysed cyclisations) and

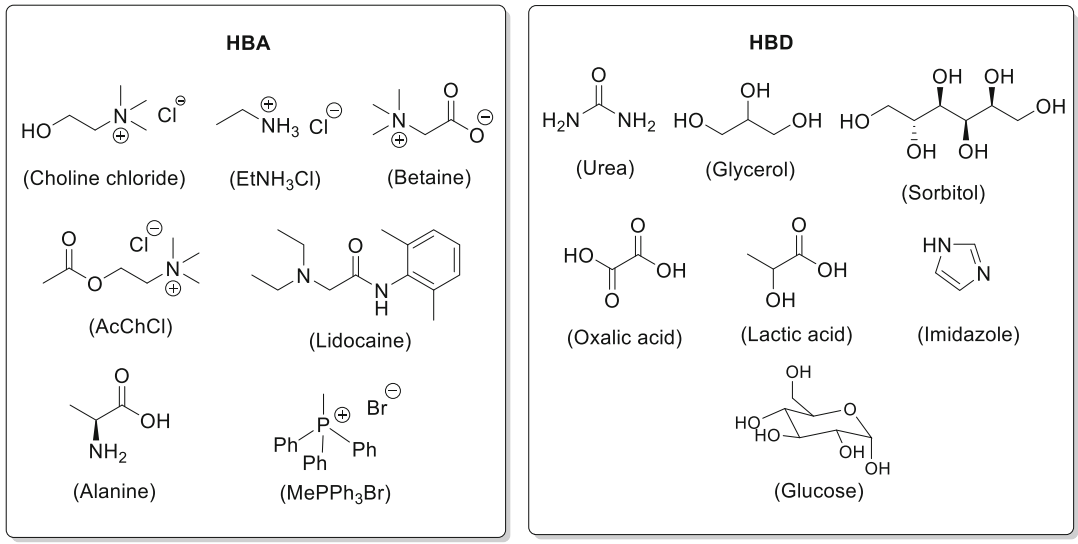


Fig. 10.2 Typical HBA and HBD components in DES mixtures; the formed DES is named as HBA:HBD (mol:mol)

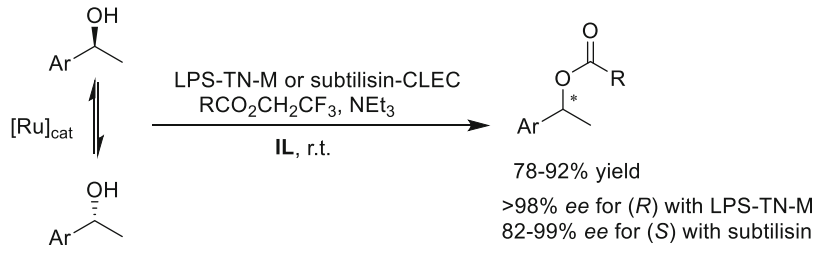
biotransformations such as reductions, halogenations, hydrolyses or epoxidations [19]. The emergence of ILs and DESs, bordering with organic solvents and water, offers a new scenario to interface metal and enzymes in a common reaction medium.

The case for combining enzymes and metal catalysts in a one-pot fashion reached relevance in the 1990s throughout *dynamic kinetic resolution (DKR)* processes mediated by a lipase and a Pd or Rh racemisation catalyst [20, 21]. These early studies were conducted in organic solvent and overcame the inherent 50% maximum yield of classical kinetic resolutions. In 2004, Kim and Park practiced the same process, namely a DKR of secondary alcohols by a combo metal-enzyme catalytic system, in a neat ionic liquid (Scheme 10.1) [22]. Together with the avoidance of VOCs, the metal catalyst turned out to be much more active in ILs and allowed the reaction to proceed at room temperature. The combination of a cymene-ruthenium complex either with LPS-TN-M lipase and subtilisin rendered (*R*)- or (*S*)-alcohols, respectively, in high yield and optical purity.

One of the pioneering examples combining metals and enzymes in aqueous medium consisted of a Suzuki cross-coupling of halogenated acetophenone followed by an alcohol dehydrogenase (ADH)-mediated reduction of the transiently formed ketone. The original report in 2008 described the coupling step at 33 mM loading of substrate and 70 °C and the bioreduction at 25 mM (4.9 g/L) and room temperature [23]. Later, the employment of water-soluble palladium catalysts enabled the first step at room temperature although the loading of substrate remained challenging (40 mM) [24]. Owing to the good tolerance exhibited by palladium catalysts and ketoreductases towards ILs, the employment of IL-buffer mixtures as biphasic media was envisaged to tackle the solubility hurdles (Scheme 10.2) [25]. Accordingly, the Suzuki-coupling was catalysed by $Pd(PPh_3)_4$ (1.2 mol%) in IL:H_2O (IL: [bmim][NTf_2]), at 100 °C and 210 mM (41 g/L). For the second-stage enzymatic reduction the reaction mixture was diluted to 125 mM with a buffer solution containing *E. coli*/ADH-A cells, NADH as cofactor and *i*-PrOH for cofactor recycling. The biphasic medium enabled to recycle both catalytic species four times. On the one hand, the supernatant aqueous phase harbouring all the components of the biotransformation was directly separated and ready for re-use. On the other hand, once extracted the target biaryl alcohol from the lower IL-phase with an organic solvent, the palladium (Pd) catalyst remained active in the IL. The process exhibited broad substrate scope and a variety of enantiomerically pure (*S*)-biaryl alcohols were obtained in high yields, being in fact the first example of chemoenzymatic cascade in IL-buffer biphasic system.

Some years later, the burgeoning interest in DESs motivated the study of the above cascade in these media (Scheme 10.3) [26]. Indeed, both Pd-catalysed coupling reactions (Suzuki–Miyaura, Sonogashira and Heck couplings) and bioreductions had been efficiently established in neat DESs and DES–buffer mixtures [27]. Through parametrisation, the Suzuki-coupling was set up by the water-soluble system $PdCl_2$/TPPTS (1 mol%/3 mol%) in DES–buffer 4:1 [DES = choline chloride (ChCl)/glycerol (Gly) 1:2 mol:mol] at 100 °C. The reaction was highly influenced by both aryl halide and boronic acid, with a limiting substrate concentration of 200 mM. Then, the reaction mixture was diluted to 75 mM (DES–buffer ~1:1 v/v) with a solution containing *i*-PrOH, KRED and cofactor. The general applicability of the process was demonstrated with unsubstituted, fluorinated and pyridyl derivatives, and the employment of stereocomplementary ADHs from *Lactobacillus kefir* ($NADP^+$ dependent) [28] and *Rhodococcus ruber* (NAD^+ dependent) [29, 30] gave access to (*R*)- and (*S*)-enantiopure biaryl alcohols, respectively.

The first cascade involving organocatalysts and enzymes in DESs was described in 2014 and consisted of the enzymatic production of acetaldehyde and its further cross-aldol reaction on aromatic aldehydes (Scheme 10.4) [31]. Regarding such enzymatic reaction, a previous study on lipase-catalysed transesterification of alcohols ascertained that, under some reaction

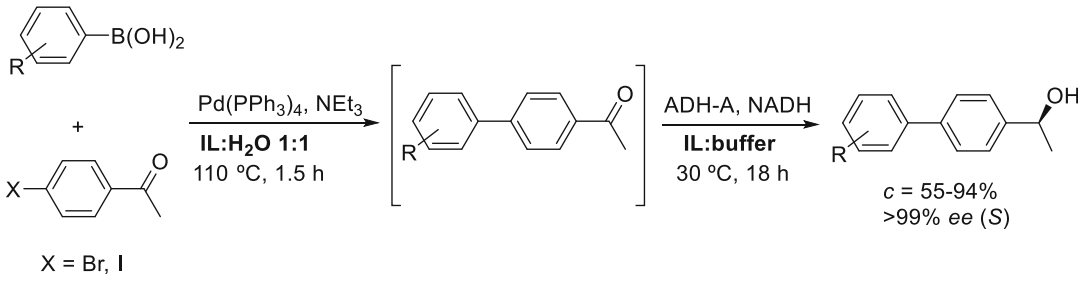


Scheme 10.1 DKR of secondary alcohols by tandem chemoenzymatic catalysis

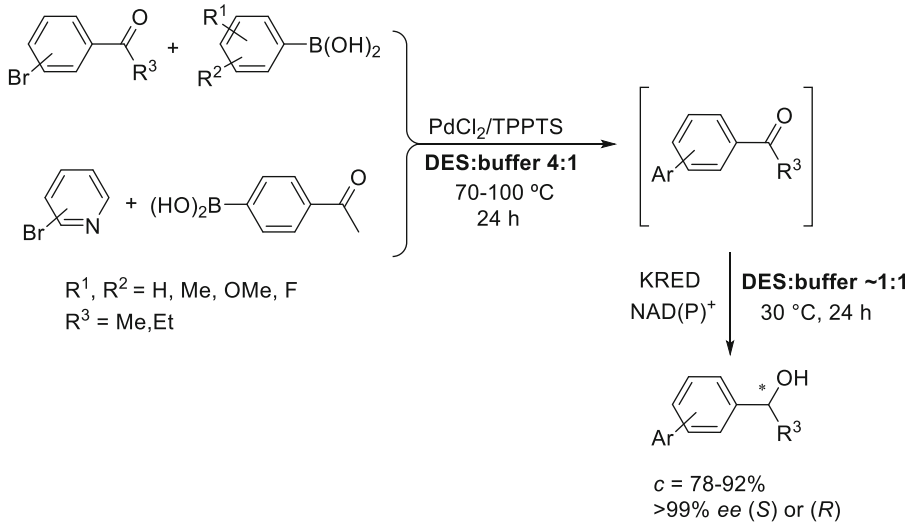
conditions, the hydrolysis of vinyl acetate predominates over the expected acylation. As a result, acetaldehyde and acetic acid are released at the expense of water consumption. This side reaction also produces hemiacetal derivatives which are responsible for disappointing enantioselectivities [32, 33]. This concept was exploited to couple the enzymatic acetaldehyde production (CAL-B catalysed transesterification of vinyl acetate with *i*-PrOH) with an enantioselective C-C aldol reaction mediated by proline-based organocatalysts. A DES turned out to be the optimal reaction medium, namely ChCl/Gly (1:2 mol:mol), and some aromatic 1,3-diols were obtained in good yield and enantioselectivity. Interestingly, both catalysts and DES could be reused for several cycles.

The first chemoenzymatic cascade in DESs–buffer mixtures connected a metal-catalysed isomerisation reaction of allylic alcohols with an enantioselective bioreduction promoted by KREDs (Scheme 10.5) [34]. The process had been previously set up in aqueous medium in both sequential and concurrent mode, establishing a practical approach to convert a racemic mixture of allylic alcohols into saturated enantiopure alcohols without isolation/purification steps [35]. The overall transformation occurred through three steps, namely reduction of the allylic C–C double bond, oxidation of the secondary carbinol moiety and asymmetric bioreduction of the generated prochiral ketone.

The reported process in aqueous medium was robust and operationally simple in the sequential fashion (one-pot two steps); once the isomerisation was complete at 50 °C, the only adjustment before adding the pair enzyme/cofactor consisted of a slight decrease on temperature. The implementation of DESs resulted in a beneficial effect on the enantioselectivity exerted by the KRED, particularly in the case of substrates unresponsive to be selectively reduced in aqueous medium. ChCl/Gly (1:2 mol:mol)-buffer and ChCl/sorbitol (Sorb, 1:1 mol:mol)-buffer mixtures led to substantial enhancement of the optical purity of the resulting alcohol at high percents of DES. With regard to the concurrent process (one-pot one-step), an open issue was the stability of the KRED in aqueous medium. In fact, the enzyme underwent rapid deactivation in the buffer which impacted negatively in the overall conversion when starting from allylic alcohols with slow isomerisation rate. With these premises, ChCl/Gly (1:2 mol:mol)-buffer 4:1 (w/w) was tested for a set of allylic alcohols combining a commercial KRED (KRED-P2-



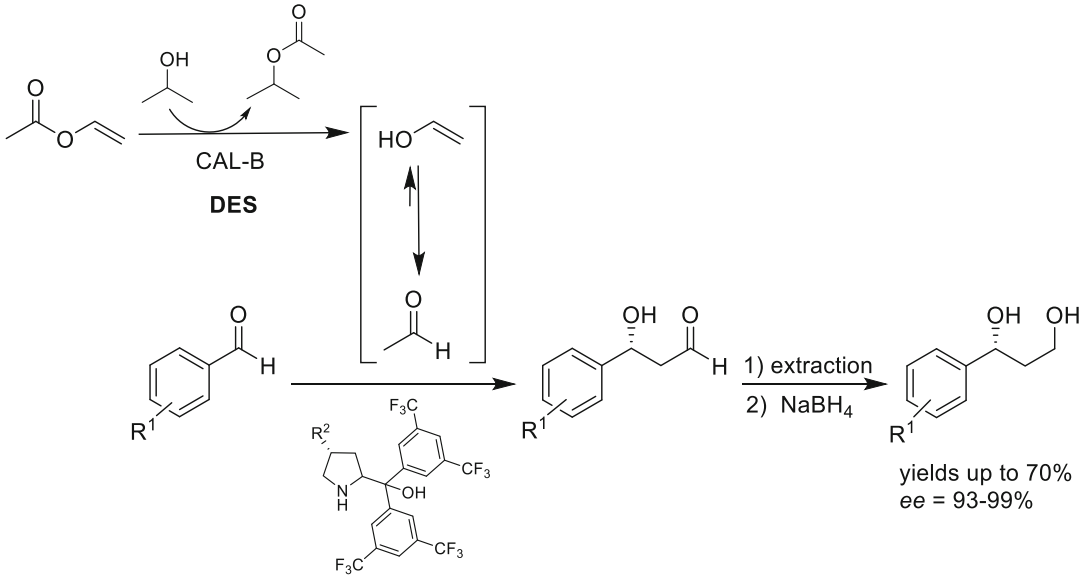
Scheme 10.2 Cascade synthesis of enantiomerically pure biaryl alcohols in IL-buffer mixtures



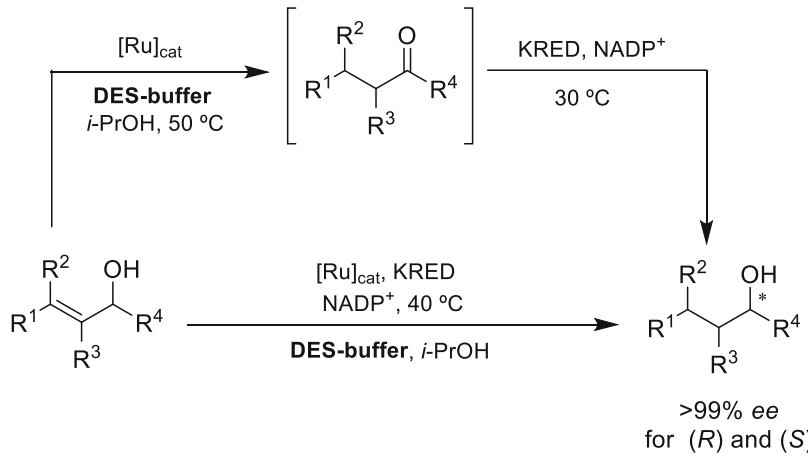
Scheme 10.3 Cascade synthesis of enantiomerically pure biaryl alcohols in DES–buffer mixtures

C11) and a ruthenium complex. Working at 40 °C and 10% mol of catalyst loading, the saturated chiral alcohols were obtained in enantiopure form with overall conversions ranging from 68 to 96% [(*R*)-enantiomer]. In particular, 1-(4-bromophenyl)prop-2-en-1-ol rendered the saturated analogue in 96% overall conversion, the biggest so far.

The potential and versatility of DESs were featured by means of an enzymatic cascade to convert limonene from orange peel wastes into epoxide derivatives (Scheme 10.6) [36]. Typically, the enzymatic version of the Prilezhaev reaction (epoxidation of an alkene with peracid to give an oxirane) is performed by a hydrolase which catalyses the in situ formation of peracid from an acid and H_2O_2. In an innovative concept,



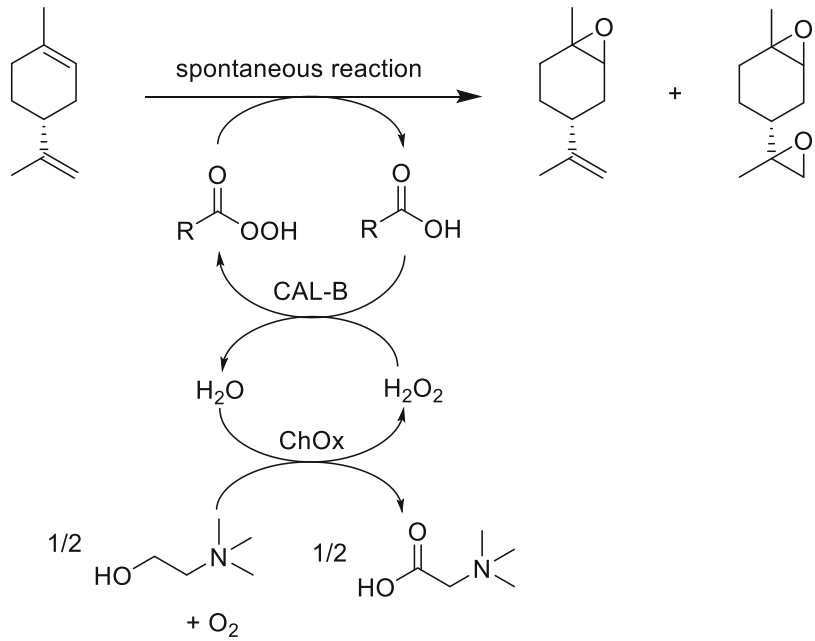
Scheme 10.4 Tandem catalysis of enzymes and organocatalysts in DES towards optically active 1,3-diols



Scheme 10.5 Cascade synthesis of optically active alcohols by Ru-catalysed isomerisation/enzymatic reduction in DES–buffer medium in both sequential and concurrent mode

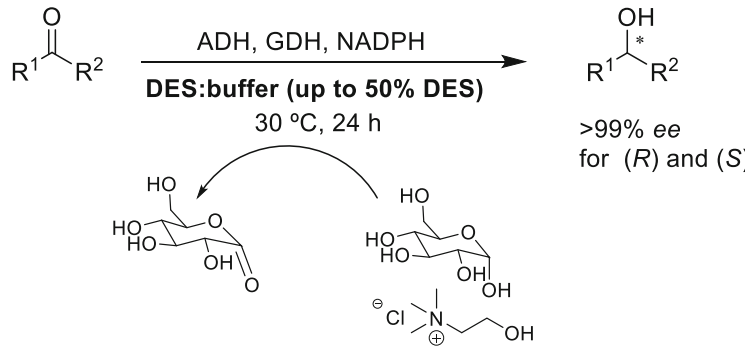
a DES displayed a triple role as extracting solvent, reaction medium for the biotransformation and reagent for cofactor recycling. In first instance, two DESs, namely ChCl/propane-1,2-diol/H_2O (1:1:1 mol:mol:mol) and ChCl:ethylene glycol (EG, 1:1 mol:mol) showed comparable efficacy to ILs and organic solvents to recover limonene from the agricultural waste. On the other hand, the choline oxidase (ChOx) from *Arthrobacter nicotianae* was able to produce H_2O_2 from the previous ChCl-based DESs. Meeting both facts, the coupled catalytic system consisted of orange peels, octanoic acid, hydrolase Novozym 435 (CAL-B) and ChOx in a mixture DES:buffer 1:1. The reaction mixture was heated at 40 °C and 500 rpm with an O_2 atmosphere. The overall yield was 33%, for a mixture mono/diepoxide (70:30).

Similarly, a rationally designed NADES served as the reaction medium for the bioreduction of prochiral ketones and acted as co-substrate for the recycling of the required cofactor (Scheme 10.7) [37]. The ADHs considered in the study operate with the assistance of



Scheme 10.6 Enzymatic cascade epoxidation of limonene employing DES as multifunctional solvent

Scheme 10.7
Bioreduction of prochiral ketones in a rationally designed DES exerting a dual function as solvent and co-substrate for cofactor recycling



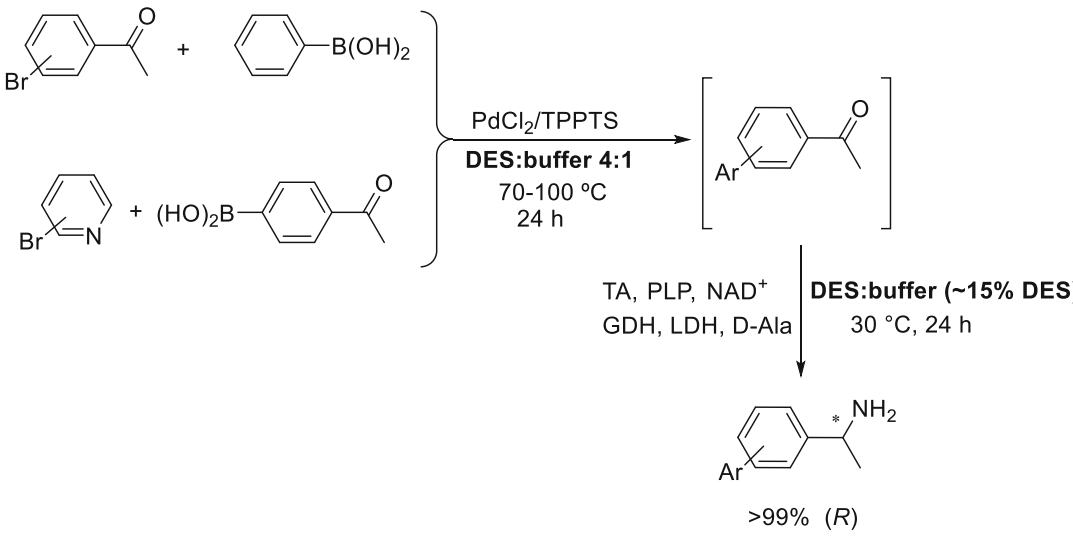
NADPH which is typically recycled by a parallel enzymatic reaction mediated by GDH or FDH. Seeing as GDH takes advantage of glucose as sacrificial co-substrate, the authors conceived a DES containing such sugar to perform that role. Thus, the mixtures of ChCl:glucose (Glu, 1.5:1 mol:mol) and an aqueous buffer with up to 50% (v/v) of DES were ideal media for the five overexpressed ADHs, namely *Lactobacillus brevis* (LbADH) [38], *Thermoanaerobacter ethanolicus* (TeSADH) [39], *Thermoanaerobacter* sp. (ADH-T) [40], *Sphingobium yanoikuyae* (SyADH) [41] and *Ralstonia* sp. [42]. Interestingly, since D-gluconic acid is released as by-product, the presence of a buffered aqueous solution was necessary to avoid a drastic drop of pH which would damage the enzyme. As a result, the enantioselective reduction of several ketones was accomplished in very high enantiomeric excess and yields, enabling higher loadings of substrate than those reported by the solubilising effect of the DES.

Very recently the good tolerance of transaminases towards DESs was unveiled, and by extension the asymmetric bioamination of ketones within these solvents [43]. Remarkably, some TAs turned out to be stable in DES–buffer mixtures containing up to 75% (w/w) of DES. Among the biocatalysts studied was included the (*R*)-selective transaminase *from Exophiala xenobiotica* (EX-ωTA), a biocatalyst found by data mining which is able to accept differently bulky biaryl ketones [44]. While EX-ωTA only accepted 15% (w/w) of neoteric solvent, it was the least harmful co-solvent of those essayed.

On the other hand, the discovery of EX-ωTA in parallel to that of TA from *Aspergillus fumigatus* (4CHI-TA) paved the way to establish a chemoenzymatic cascade toward chiral biaryl amines by combining a Suzuki-coupling with an enantioselective bioamination. In first instance, Bornscheuer's group developed such a sequential process in aqueous medium employing 4CHI-TA at low substrate concentration, namely 2 mM and 1 mM for each step [45]. Soon after, an identical cascade with EX-ωTA was reported in a reaction medium consisting of a DES–buffer mixture (Scheme 10.8). As a result, the metal-catalysed step was accomplished at 200 mM loading of substrate and the subsequent biotransformation at 25 mM. It is worth noting that ChCl/Gly (1:2, mol:mol) emerged as the only co-solvent compatible for both steps since DMSO inhibited the Pd catalyst and THF and *i*-PrOH were harmful for the TA. The methodology was extended to *meta*- and *para*-biaryl ketones and pyridylphenyl ketones as well, rendering the corresponding (*R*)-biaryl amines with >99% *ee*.

10.3 Enzymatic Cascades in Non-conventional Media

The inherent biocompatibility and solubilising properties of DESs crystallised in the application for one-pot biomass processing (Fig. 10.3) [46]. First, ChCl-based DESs turned out to be suitable solvents for the pretreatment step of crude biomass, the levels of degradation products such as furfural (polysaccharides) and ferulic acid (lignin) being low enough for the growth of yeast



Scheme 10.8 Cascade synthesis of enantiomerically pure biaryl amines in DES–buffer mixtures

Saccharomyces cerevisiae. Likewise, DESs were also biocompatible with the hydrolytic enzymes involved in the process. As a result, saccharification and fermentation steps were effectively established in one-pot fashion resulting in an ethanol production of 77.5% theoretical yield in ChCl/Gly (1:2 mol:mol) pH 5.8 (10 wt% aqueous solution). Compared to previous approaches relying on ILs, the implementation of DESs avoided any pH adjustment and solid/liquid separation steps throughout the above process.

Similarly, DESs served as the reaction medium for a one-pot two-step enzymatic process to obtain biodiesel from waste cooking oils as feedstock (Fig. 10.4) [47]. A first enzymatic esterification in aqueous medium and 30 °C catalysed by *Thermomyces lanuginosus* lipase was selective on triglycerides; further sequential addition of *Pseudozyma antarctica* lipase B and ChCl/Gly (1:2 mol:mol) completed the transesterification of the remaining glycerides and fatty acids at 45 °C. The resulting two-phase system delivered lipids to the upper phase and a glycerol-DES mixture to the lower one. Conventional alkaline refinement delivered the product with an ethyl ester content of 97.6% and free of glycerol and acid, which meets the requirements for Biodiesel standard EN 14214 in Europe. An extra benefit of using DESs lies in the easy purification of glycerol from the hydrophilic phase by

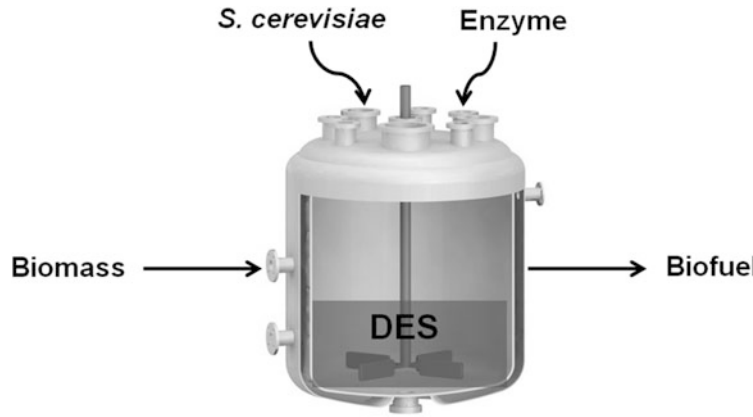


Fig. 10.3 Enzymatic one-pot production of bioethanol in DESs

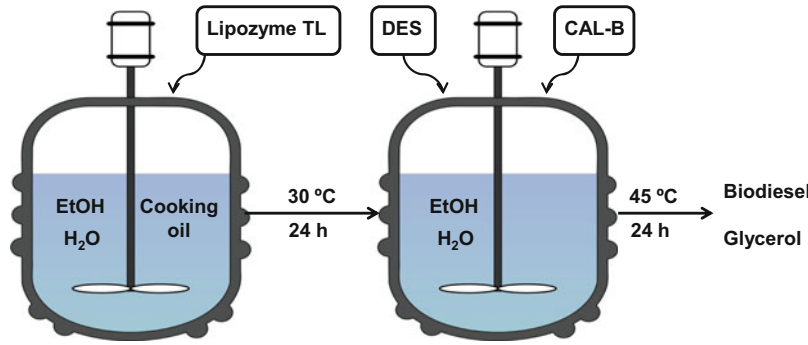


Fig. 10.4 One-pot-two-step enzymatic production of biodiesel from used cooking oil in a DES–water medium

distillation of the DES component. Likewise, Lipozyme CAL-B was recovered from such phase and reused in five cycles.

The capture, storage and processing of carbon dioxide (CO_2) represent a capital challenge today due to the threat of greenhouse effect. As a sustainable solution, the enzymatic conversion of CO_2 into methanol in the presence of ILs was investigated (Fig. 10.5) [48]. This multi-enzymatic cascade process had been described in aqueous medium, though the production of methanol reached a yield of 44% due to unfavourable kinetics in the first step, namely the conversion of CO_2 into formic acid mediated by formate dehydrogenase (FDH) [49]. The incorporation as co-solvent of biocompatible ILs based on ChCl and amino acids enabled a higher solubilisation of CO_2 and also had a stabilising effect on FDH. The four enzymes involved in the biotransformation (FDH, FaldDH, ADH and GDH) were immobilised in a cellulose membrane and a separation system platform enabled the recycling of biocatalysts and the removal of methanol. Under the optimised reaction conditions, an aqueous solution containing 20% of [choline][L-glutamic acid], the production of methanol was improved fivefold with regard to the aqueous medium used as control.

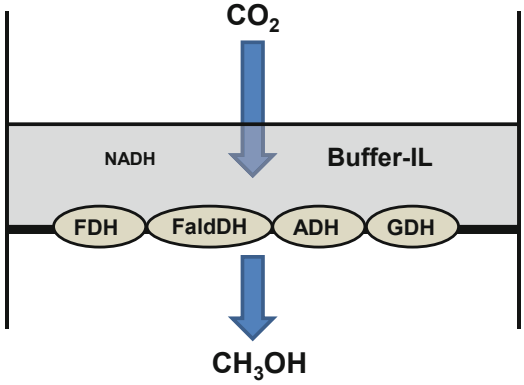


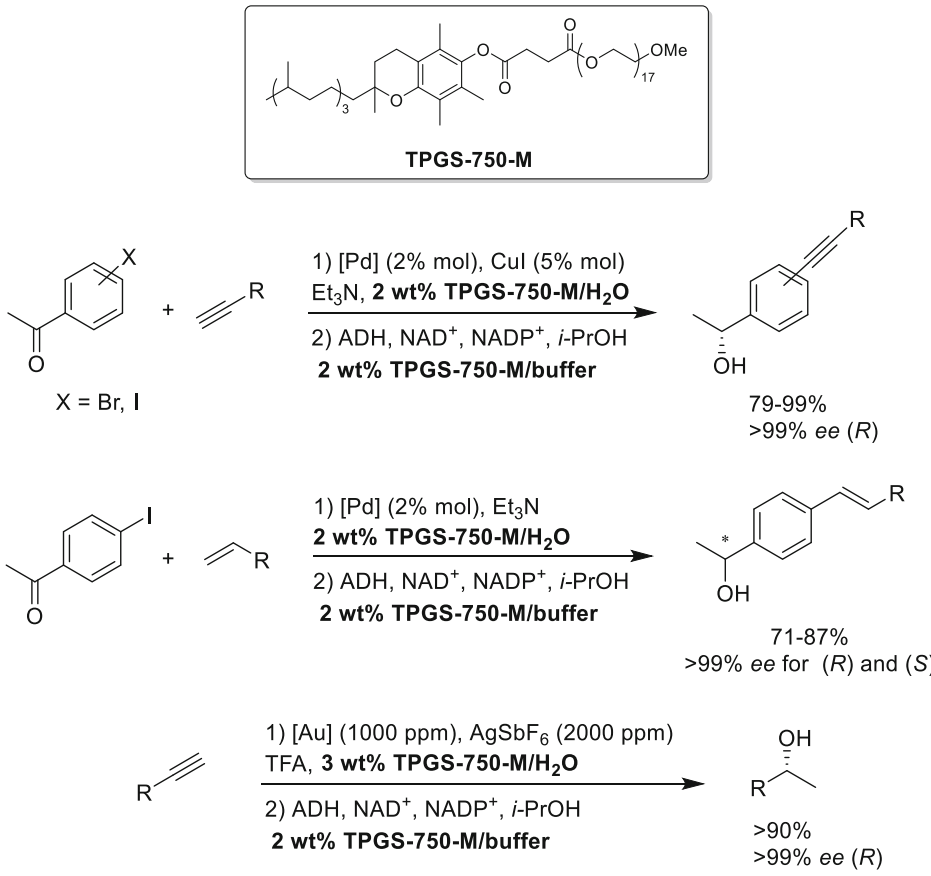
Fig. 10.5 Enzymatic cascade conversion of carbon dioxide to methanol in buffer-IL mixtures

10.4 Recent Developments

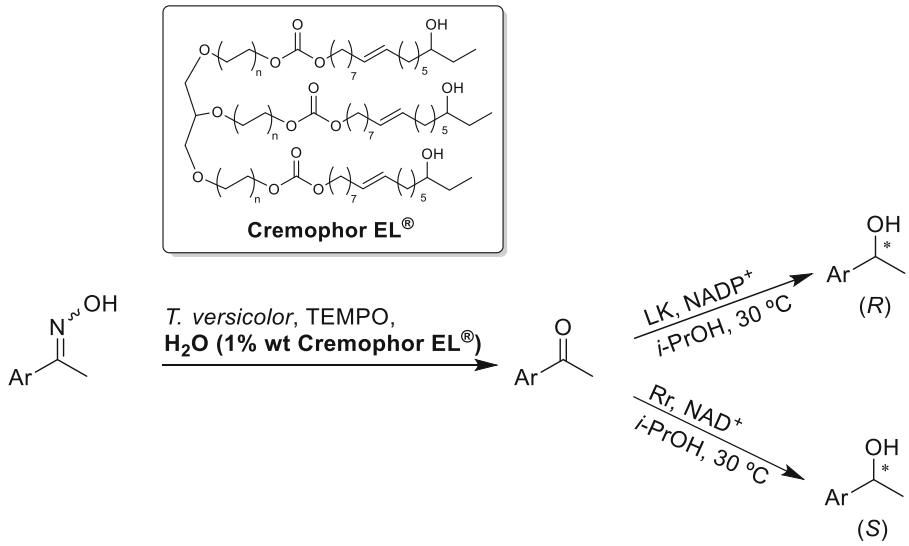
Although outside the established classification of non-conventional media, recently it has appeared an innovative reaction medium based on aqueous micellar solutions. The concept was originally conceived with the aim of making synthetic chemistry in water upon the assistance of designer surfactants [50]. Now, these would act not as mere solubilisers of catalysts and reagents but as key co-solvents forming such smart nanoreactors for specific transformations. After many successful examples in which the chemical reaction occurs in the inner hydrophobic core of the micelles, Lipshutz and co-workers went one step further and envisaged to meet enzymes and metal catalysts through this so-called micellar catalysis. Most logically, the enzymes would remain in the aqueous solution and the micelle host both organic substrates and chemo-catalysts to minimise the expected metal-enzyme inhibition. Accordingly, TPGS-750-M, a surfactant bearing a vitamin E as hydrophobic moiety, was introduced with the prospect of suit apolar substrates and metal species. Once demonstrated the perfect tolerance of alcohol dehydrogenases

towards TPGS-750-M, this pioneering report showed the versatility of the methodology through several hybrid catalytic systems combining metal-catalysed processes such as Sonogashira and Heck couplings (Pd catalysts) or alkyne hydrations (Au and Ag catalysts) with a further bioreduction (Scheme 10.9) [51]. On the one hand, the micellar medium impacted significantly on the enzyme activity, leading to improved productivities. On the other hand, once suppressed the inhibition of the metal catalyst on the enzyme the chemoenzymatic one-pot sequential processes were successfully accomplished leading to products in high overall yield and enantiomeric excess.

Similarly, the beneficial impact of surfactants on enzymatic activity was exploited in an enzymatic cascade in aqueous medium to convert prochiral ketoximes into optically active alcohols by sequential laccase-catalysed deoximation and further bioreduction of the triggered ketone (Scheme 10.10) [52]. Owing to the employment of exclusively 1% (w/w) of Cremophor®, a polyethoxylated castor oil typically used as a formulation vehicle for poorly-water soluble drugs, a high enzymatic performance was achieved. Thus, both laccases and ketoreductases showed perfect tolerance towards the surfactant and such medium free of organic co-solvents enabled to increase the substrate concentration up to 200 mM in the initial biodeoximation and 100 mM in the later bioreduction. As a result, and depending of the KRED employed, the (*R*)-or (*S*)-enantiomer of the corresponding alcohol was isolated in good overall yield and >99% *ee*.



Scheme 10.9 Representative examples of chemoenzymatic cascades performed in micellar aqueous medium



Scheme 10.10 One-pot sequential enzymatic transformation of ketoximes into optically active alcohols in an aqueous medium supplemented with surfactant

10.5 Conclusions and Future Prospects

Enzyme-enabled cascade processes have boosted incredibly in the recent years. Together with the combination between a series of enzymes, the assembly of biocatalysis with other disciplines such as metal- or organo-catalysis has endowed these processes with great synthetic potential. In this context, recent research in medium engineering has brought non-conventional media to the forefront of organic synthesis. As highlighted along this chapter, these reaction media have gone from mere spectators to display key roles in the synthetic transformation. Some of the disclosed examples revealed a critical impact on enantioselectivity, loading of substrate or yield of different cascades processes. Likewise, the implementation of non-conventional media in some selected processes enabled to alleviate the inhibition between metals and enzymes. Once the first seeds have been planted and seeing as today's world claims for a more sustainable chemical industry, we anticipate that the necessary symbiosis between enzymatic cascades and non-conventional media will result in astonishing breakthroughs in a near future.

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Perspectives

11

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Abstract

Biocatalysis is a key emerging field in Industrial Biotechnology towards synthesis of (complex) chemicals for our daily use. The enormous potential of nature's catalysts can be boosted by combining them either with other enzymes, i.e. multi-enzymatic cascades or with chemo-catalysts, i.e. chemo-enzymatic cascades. The early developments in the field of enzymatic cascade reactions have led to a new phase in which multi-catalytic systems can now be specifically designed and modelled in order to allow efficient cascade reactions that may even suppress side reactions. Recent advances in real-time monitoring and control of catalytic systems allow quality assurance for target product specifications in industry. The online concentration data obtained can be implemented in kinetic modelling, providing more insights in the progress of individual compounds within a complex reacting system. To reach technical benchmarks for volumetric productivities—especially for hydrophobic compounds—medium engineering, i.e. the choice of alternative reaction media opens new possibilities. Also, efficient downstream processing can strongly benefit therefrom. The upcoming advances in enzymatic cascade reactions will profit from digitalization, miniaturization, and automatization and will enable design and optimization of multi-catalytic systems in an economically feasible way. Not only science, but also education and training may shift more towards cross-disciplinary curricula that bring together natural and technical sciences to train the new generations for technological developments.

In the past decades, biocatalysis moved from single-step transformations towards more complex and challenging cascade type reactions. The progress in bioinformatics, molecular biology and genetics led to the possibility to use biocatalytic reactions for the synthesis of highly complex molecules. The recent developments in the field of enzyme discovery and protein engineering gave rise to a pool of about 100 different biocatalytic transformations. In general, the main areas of metal-, organo- and bio-catalysis can be considered the modern catalytic toolboxes in synthesis. Irrespective of which toolbox (or combination thereof) to be used for the synthesis, 'retrosynthesis', a concept introduced by E.J. Corey in the 1960s, has nowadays become the way to go. Thereby, target molecules are disconnected stepwise into simpler structures by

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logic breaks of key bonds, until a commercially available starting material is accessible. In a second step, the forward planning by (bio)-chemical transformations is performed to synthesize the target molecule in the lab. This 'disconnection concept' was now extended by biocatalytic transformations and gives access to a vast number of novel synthetic routes. In the early days of retrosynthesis, the number of possible transformations was limited, and could be recalled by a well-educated organic chemist from memory.

In the past 50 years the development of new organic reactions, new reagents and the demand of stereoselective syntheses resulted in the attempt to apply computational methods to assist with both retro- and forward synthesis (Chap. 2). The recognition of biocatalysis by the chemistry community together with the developments in the field of organic synthesis will change the education curriculum of studies towards more interdisciplinary fields. In the past decade, increasing efforts have been made to design and model enzymatic cascades reactions for the synthesis of (complex) chemical structures of our daily use. In the early days of 'biocatalytic cascade reactions', the major aim was to show proof-of-principle studies and demonstrate the possibilities of the combinations of different catalysts in a one-pot fashion. Now, the field enters a second phase, where the optimization and the biotechnological application of such cascades are becoming more and more important. Recent examples from companies corroborate this and strengthen scientists in the field to proceed in the development of new and more complex cascade systems.

To date, different classifications of cascade reactions, depending on the process constraints are known. The design of an artificial enzymatic cascade is based on using either isolated enzymes (Chap. 3), whole cells (Chap. 4) or by the combination of chemo- and biocatalysts (Chap. 5). A major challenge, still, is that enzymes do not necessarily always work under the same reaction conditions as non-biocatalysts. In principle, the parameter space for biocatalytic transformations is much smaller than for chemical reactions. Nonetheless changes in pH, media/buffer, temperature and substrate/product inhibition have a tremendous effect on the activity of the catalysts and therefore on the overall cascade performance. The symbiosis of cascade development (enzyme discovery, protein engineering) and its optimization (flux improvement) is crucial to bring the field on to the next level for real industrial applications.

Kinetic modelling of multi-enzymatic cascade reactions (Chap. 6) is a rapidly growing field; however, it is still in its early stages. It is of key importance to know the basic concepts, scope, characteristics and methodologies of kinetic modelling. Knowing kinetics of cascade reactions provides key insights to understand multi-catalytic reaction systems and ideally to accelerate catalysis, to suppress by-product formations and cross-reactions (if any exist) and to increase the yield of the target product. Most importantly, enzyme kinetic modelling does not only serve the fundamental scientific interest, but it is the basis that engineers need to design and dimension reactors (Chap. 7) and in this sense, kinetics are interrelated with economics too. The potential of mathematical modelling of multi-enzymatic reactions is enormous as it provides valuable information with a minimum amount of experimental data and resources used. Once a reliable kinetic model is developed, the progress of a cascade reaction running under different conditions can be simulated and the best operational conditions can be predicted.

Reactor engineering based on enzyme kinetics modelling (Chap. 7) is a systematic approach to implement enzymatic cascade reactions in the most suitable reactor type and to run them under the best operational conditions. Modern reaction engineering takes advantage of different individual reactor designs, but also their combinations with each other to enhance the productivity of cascade reactions. The challenges related to the implementation of biocatalytic cascade processes mainly arise from aforementioned cross-reactions, cross-inhibitions and operational stability issues that may suggest not to combine individual catalytic steps in a one-pot. Instead, 'modularization' (or so-called compartmentalization) is a strategy to follow whereby each reaction step can run at its best

operational conditions with minimum to no disturbance by the components for another reaction step (Chap. 8). These spatial arrangements via modularization will be also useful in continuous flow reactors, whereby one flow reactor can occupy one immobilized enzyme combined with another one in series. Continuous flow biocatalysis has been a recently emerging field and will grow further in the future.

Regarding process monitoring and control (Chap. 9) the 'Quality by Design' approach stresses the importance of process understanding and control, which reduces the time needed from proof-of-concepts to applications. In this context, the use of process analytical technologies (PAT) is the key to understand a process in an early stage of its development and thus PAT will further play a significant role for the time efficient design of robust processes. The precision in real-time monitoring done with modern analytical tools combined with experimental *offline* data and chemometrics analysis tools will provide the desired product specifications, the so-called Quality by Control strategy used in industry.

Medium engineering (Chap. 10) has been of great interest for enzymatic synthesis where buffer systems are replaced with non-conventional reaction media. The systematic analysis of the effects of organic media on selectivity, activity and stability of enzymes will enable tailored solvent design for the specific enzyme and reaction of interest. The necessary symbiosis between enzymatic cascades and non-conventional media will result in astonishing breakthroughs in a near future for reaching industrially relevant substrate loadings and product titers.

Mathematical kinetics modelling (Chap. 6), reactor engineering (Chap. 7), process design (Chap. 8) and modern process monitoring tools (Chap. 9) offer a multitude of synergistic possibilities to run multi-enzymatic or chemo-enzymatic cascade reactions in suitable media (Chap. 10) at high productivities. Digitalization, miniaturization and automatization will be the keys to transform biocatalytic cascade reactions from proof-of-concept studies into technical applications using industrially relevant concentrations. Novel strategies for data collection as well as for databases to store and archive data will enable us in the future to accelerate this transformation. With these approaches, enzymatic cascade reactions towards the synthesis of complex chemical structures with high space–time yields would be possible, making the use of enzymes competitive to conventional purely chemical based methods. It will be a drastic change in chemical production when the efficiency of enzymatic cascade reactions will be increased, especially with regard to lower-priced (high-volume) products.