Biocatalysis employs enzymes for catalysing chemical reactions. Replacement of traditional chemical methods by the use of enzymes can be advantageous for many reasons. First, enzymes often show great selectivity. In the active site of an enzyme, the local protein environment dictates interactions of the enzyme with a substrate and thus the structure of a product. For this reason, enzymes can functionalise complex molecules at very specific sites. They also promote formation of a specific product over the alternative products or stereoisomers. Therefore, biocatalytic processes may result in the manufacture of highly pure products, which simplifies downstream processing. This is crucial in many cases, for example, in pharmaceutical industry, where high standards for product purity have to be maintained. Next, enzymes operate mostly in water solutions, at moderate temperatures and pH, and under atmospheric pressure. Also, application of enzymes often eliminates the need for the use of heavy-metal-containing catalysts, organic solvents, and toxic or dangerous compounds. Thus, it can help to increase process safety and reduce waste production. Notably, if needed, enzymes resistant to high temperatures, extreme pH values, or organic solvents can be found in nature or engineered. Therefore, enzymes show great versatility of operating conditions. Overall, application of biocatalysts may lead to more efficient and environmentally friendly processes.

In order to find a suitable enzyme for a given process, one can search in natural diversity or engineer available enzymes. Enzyme engineering has proven to be a powerful technology that allows modifications of enzymes in many ways: introducing new reactivities, improving activity, changing substrate acceptance, increasing chemo- and stereoselectivity, improving protein stability, increasing expression levels and solubility, or adjusting to certain operating conditions such as: temperature, pH, or presence of organic solvents. In the past, protein engineering split into two directions: rational engineering and directed evolution, which nowadays are often combined in various ways in order to maximise the efficiency of engineering projects.

Baeyer–Villiger monooxygenases (BVMOs) catalyse Baeyer–Villiger oxidation using flavin cofactors, nicotinamide coenzymes, and molecular oxygen, which is a cheap and clean oxidant. Due to the ability to perform this reaction and other types of oxidations as well as their high selectivity, BVMOs attract increasing attention from both academic and industrial point of view. However, before these enzymes can be applied on large scale, a number of their features must be improved. The research described in this thesis aimed at engineering phenylacetone monooxygenase (PAMO) into an efficient catalyst, at advancement of methods for engineering of BVMOs, and at increasing the knowledge on these enzymes. In the first part of the thesis (Chapters 3 and 4), the results of rational engineering of PAMO are presented. First, we attempted to change the coenzyme
specificity of PAMO, which strongly prefers NADPH over NADH (Chapter 3). The approach was based on the analyses of the structures and the sequences of PAMO and related enzymes. By testing several site-specific mutants, we found variants for which the preference for NADPH was relaxed as compared to the wild-type enzyme. However, these mutants still performed better with NADPH than with NADH. Moreover, their catalytic efficiency was low. Nevertheless, this study confirmed the role of the proposed amino acid residues in the interactions with the coenzyme. Second, in Chapter 4, we thoroughly compared the structure of PAMO with a model of cyclopentanone monoxygenase (CPMO), a related enzyme with remarkably distinct and much broader substrate range. This structural analysis suggested which residues determine the substrate specificity in BVMOs. We verified this hypothesis by preparing a series of single and multiple mutants and testing them for activity and selectivity with different substrates. We found several mutants with increased activity and significantly altered regio- or enantio-selectivity. Although the substrate specificity of CPMO could not be introduced in PAMO, we identified residues that affect the selectivity of PAMO, and we concluded that these residues might be involved in the interactions with the substrate.

By these comprehensive mutagenesis studies, we gained valuable insights into coenzyme and substrate specificity of PAMO. On the other hand, the limited success of our attempts to modify coenzyme/substrate specificity of PAMO by rational engineering underscored the need for alternative approaches. Therefore, we put efforts in developing an activity assay for screening libraries of PAMO mutants. The new method presented in Chapter 5 employs the regeneration of NADPH by phosphite dehydrogenase for measuring the BVMO activity. In this system, activity of PAMO is coupled to the formation of phosphate, and we have shown that phosphate can be used as a reporter of the BVMO activity. The method is generic because it can be used in screening for activity with any substrate. It is a great advantage since PAMO and other BVMOs can accept different substrates and catalyse various reaction types. Thus, the phosphate-based method can be used for screening PAMO, other BVMOs, or other NAD(P)H-consuming monoxygenases. Furthermore, the assay is reliable: the amount of phosphate measured under the assay conditions corresponds to the amount of the oxidised product being generated. By expressing PAMO in the periplasm of Escherichia coli, we separated the enzyme from cellular NADPH-consuming enzymes and, thereby, reduced the background. No false positive clones are isolated thanks to the detection of hydrogen peroxide, the product of uncoupling. Lastly, the activity is tested using whole cells, without the need for preparing cell extracts, and it is performed in 96-well plate format. Therefore, it allows relatively high-throughput screening of mutant libraries.

Equipped with the knowledge on the active site of PAMO and the efficient screening method, we proceeded with design, preparation, and screening of
a library of PAMO mutants (*Chapter 6*). As previous studies showed that introducing single mutations usually brings only small changes in the catalytic properties of PAMO, we simultaneously modified 11 positions in the active site of the enzyme. Screening of the library of 1,500 clones yielded a quadruple mutant PAMO$_{15\text{-}F5}$ active on cyclopentanone. Further characterisation of this mutant revealed that the biocatalytic properties of PAMO$_{15\text{-}F5}$ place it in between PAMO and cyclopentanone/cyclohexanone monoxygenases. Remarkably, the stability of the mutant was the same as that of wild-type PAMO.

The research described in this thesis comprises a representative case of a protein engineering cycle, which includes enzyme characterisation, development of suitable screening methods, design and screening of libraries, ultimately leading to the isolation of variants with improved properties. Detailed knowledge of the active-site architecture and the enzyme mechanism, coming from structural studies, modelling, kinetic analyses, and characterisation of mutants allows design of high-quality libraries while efficient screening methods enable successful fishing of mutants with improved properties. Most importantly, the presented approach, which was shown successful for PAMO, can be applied in engineering of other (Baeyer–Villiger) monoxygenases thanks to the generic nature of the developed screening method.