This thesis provides methodological progress in directed evolution, which is illustrated using two different enzymes as catalyst in organic chemistry: an enoate-reductase and a P450 enzyme. The respective goal was to test iterative saturation mutagenesis (ISM) in the form of CASTing, which the group had previously developed for efficient evolution of stereoselective and thermostable enzymes.

In the directed evolution process of YqjM, an Old Yellow Enzyme family member, catalyzing the trans asymmetric reduction of the C=C bonds in enones, all possible evolutionary pathways deriving from three chosen library sites were explored for the model reaction $1a \rightarrow 2a$. Two iterative CASTing strategies were followed, one on basis of 'pick always the best hit' as a parent for the next generation as well as the strategy 'pick either the 1st or 2nd best' hit as parent. By doing so, it could be demonstrated that the choice of the parent is a critical parameter. As a further consequence, it was found that the library quality is essential for the success of the iterative CASTing strategy, especially when the 1st generation is created. And it also emphasizes the importance of a library quality control. Any missed amino acid combination could lead to a 'wrong' parent choice and would direct the pathway on a less favored trajectory. In the case of YqjM this still leads to an improvement of the biocatalyst, if only with medium success. It could also be demonstrated that backtracking easily allows the correction of such a 'wrong' decision and dead ends can be circumvented.

The obtained insights to the fitness landscape of YqjM revealed that two major trajectories exist when 'always the best' mutant is chosen as a parent. One trajectory from the ancestor site C, that leads to the highest success with mutant D_T, and one that is accessible from all ancestor sites and leads to acceptable success, namely mutant WAT. It was also demonstrated that these two trajectories are accessible, too, when the 2nd best mutant is chosen as a parent and either the 1st or 2nd best performing site is addressed.

Extremely interesting was the observation of recurrent amino acids, which have already been identified in the pool of 1st generation mutants; a phenomenon that was for YqjM and in the other project regarding P450BM3. This observation led to the generation of a knowledge guided 'combined diversity of 1st generation' library, which leads in one step to mutants D_T and WAT. With this approach, man-made mistakes of a 'wrong' ancestor or 'wrong' uphill pathway choice can be ruled out. Moreover, this strategy reduces the screening effort for CASTing projects which consist of only a couple of sites. Therefore, evidences accumulate that there is highly valuable knowledge in the pool of mutants that are obtained after the 1st round of evolution. These pools of mutants obtained in every site and generation reminds of the concept of 'neutral networks'\cite{189,190} and of the Eigen/Schuster concept of 'quasi-species'\cite{191,192}, which has been applied by Mannervik\cite{293,294} on directed evolution. These two concepts describe a drift of the protein which creates a 'cloud' of sequences around an existing sequence. This cloud retained its original function, but has accumulated 'neutral' mutations. These mutations give these enzymes an advantage, if evolutionary selection pressure is applied.

Overall, application of iterative CASTing to YqjM led to a set of highly active (R)- and (S)-enantiomer producing variants for the stereoselective reduction of a variety of cyclic enones (Figure 78). The best three obtained variants in this study, A_T, D_V and especially D_T were found to fulfill the requirements for industrial biocatalysis, too.
In summary, YqjM can be quickly evolved when the spatial positions X1-3 as outlined in Chap. 6.4 are addressed. The author also assumes that this applies to all OYEs, because these residues seem to play a similar role in all of them and directed evolution following the presented strategy would lead therefore to success. In addition, a ‘combination library’ including amino acids from positions X1-3 identified from the 1st generation for residues C26, I69 (both from this study), A60 and A104 (study of a colleague) may further improve the catalytic abilities of YqjM variants, providing a promising perspective. Monocarboxylic acids and esters as well as amides are so far no substrates for members of the Old Yellow Enzyme family, but the successful substrate scope and acceptance broadening by directed evolution of YqjM provides a promising outlook: it is highly probable that engineering towards their acceptance can be achieved in the future with the iterative CASTing approach.

Before P450BM3 could be subjected to directed evolution, it had to be cloned and mutant F87A had to be constructed. After facing difficulties with the normal library diversity creating PCR techniques, a new approach for difficult-to-amplify templates was implemented and further improved. It also based on the use of megaprimer and works well when traditional methods for saturation mutagenesis such as QuickChange fail. During the course of the library creation experiments the importance of a library quality control could be once more demonstrated. Furthermore, a comparison between synthesized and PCR generated libraries was performed to study the influence of the PCR bias, the parent contamination and the activity distribution on the outcome of a directed evolution process. First of all, it could be clearly proven that the introduced quick library quality control is a reliable, simple and cost efficient way to evaluate a generated library for directed evolution. Second, it was found that the synthesized libraries show a higher density of improved catalysts and in general are characterized by a clearer background to mutant signal ratio, proving them especially useful for directed evolution goals such as activity enhancement.

In addition to a successful library creation, at least two screening platforms were established. Both are based on the screening of resting cells to reduce the need for expensive cofactor and cofactor recycling enzymes. The first platform consists of a colorimetric detection system for aromatic α-hydroxyketones by tetrazolium salts (TTC-Assay) followed by precise characterization of positives by GC analysis, which however did not work so far due to hydrolysis problems with 6a and solubility problems with compound
The second platform contains a medium throughput HPLC screen for the detection of hydroxylated steroids such as testosterone, progesterone or \( \beta \)-estradiol. In the time frame of this thesis, screening of three out of nine defined CASTing sites using P450\(_{BM3}\)-F87A was performed. It was found that these three sites already contain highly regio- and stereoselective variants with improved activity (Figure 79). Highly active but non-selective variants were also obtained for the hydroxylation of testosterone. These would possibly give access to the dihydroxylated testosterones. In a following round of evolution, the 15\( \beta \)-regioselectivity could be further improved. The regioselectivity of the 16\( \beta \)-testosterone hydroxylation with obtained variants is still low (74%) and could not be further increased on the pathway that was chosen for the second round of evolution. Screening of the remaining library sites will hopefully provide a solution for this issue. In addition to the CASTing sites, three single residues were separately saturated and position A330 turned out to have a pronounced effect on regioselectivity (Figure 79). From a synthetic organic viewpoint, the addressed goal was reached.

Overall, iterative CASTing efficiently evolves P450\(_{BM3}\) into a regio- and stereoselective steroid hydroxylase, yielding a toolbox of enzymes able to hydroxylate testosterone selectively in either 2\( \beta \), 15\( \beta \) or with medium selectivity at 16\( \beta \) position. With this toolbox, 2\( \beta \)- or 16\( \beta \)-hydroxylated progesterones are accessible, too. Activity with estradiol was also observed, although products have not been fully characterized yet. The observed substrate scope indicates that other similar steroids could be accepted, too. Furthermore, the occurrence of dihydroxylation indicates that also other hydroxylated steroids can be potential substrates. In addition, initial preparative scale biotransformation with progesterone indicates the usefulness of this toolbox for industrial application and further evaluation of the toolbox variants is in progress.

Even though static docking of the steroids into a ‘substate-closed’ conformer of P450\(_{BM3}\)-F87A reflects the positions where hydroxylation was found to occur, the origin of steroid hydroxylation selectivity observed during the course of this study is not fully explainable by the model presented in Chap. 7.5.9. Detailed molecular dynamic (MD) simulations will hopefully shed some light on this process performed by this highly flexible enzyme that passes through a couple of conformational rearrangements upon substrate binding and substrate hydroxylation.