DyP-type peroxidases are a relatively recently discovered family of heme-containing peroxidases. About forty DyPs were characterized to some extent in the last two decades, rapidly increasing the knowledge of this enzyme family. DyP-type peroxidases are different in fold and function from other peroxidases; they show a ferredoxin-like fold, are active at an acidic pH and oxidize/decolorize recalcitrant synthetic dyes. Based on sequence homology the family of DyP-type peroxidases can be divided in four subclasses (A-D). The crystal structures of sixteen DyPs have been elucidated, including members from every subclass.

The aim of this thesis was to broaden the knowledge on class A DyP-type peroxidases. Enzymes from this subclass are from bacterial origin and Tat-dependently secreted to the periplasm. TfuDyP from *Thermobifida fusca* YX and SviDyP from *Saccharomonospora viridis* DSM 43017 were selected as model enzymes. TfuDyP is a thermostable dimeric DyP-type peroxidase which is active on various dyes, monophenolic compounds and performs the enantioselective sulfoxidation of small aromatic sulfides. SviDyP, another class A DyP-type peroxidase, shares 42% sequence identity with TfuDyP and is active at a slightly higher pH range.

**Dye decolorization and divanillin production**

The family of dye decolorizing peroxidases (DyP) got its name due to the common activity of its members on synthetic (anthraquinone and azo) dyes. To investigate the dye decolorizing potential of this enzyme family further, we studied the ability of TfuDyP to degrade a more diverse palette of synthetic dyes and natural pigments in chapter 2. Thirty compounds from seven classes of dyes were studied together with three natural carotenoids. TfuDyP showed to be a true dye decolorizing enzyme, as it showed activity towards most studied dyes. The highest initial activities and overall conversions were observed for a xanthene dye, a copper phthalocyanine dye and dyes from the most frequently studied class of dyes, the anthraquinone dyes. Many synthetic dyes are not easily biodegraded. Although DyP-type peroxidases do not degrade these dyes to primary metabolites, they could form a good first step in the full biodegradation as the formed products might be more easily degraded in microbial catabolic routes.

DyP-type peroxidases are not only active on dyes but show a much broader substrate scope, including monophenolic and lignin model compounds, Kraft lignin, β-carotene and aromatic sulfides. To explore the biocatalytic potential of these enzymes further, the research in chapter 2 was extended to a broader range of substrates. TfuDyP was found to show activity towards the six tested phenolic
compounds, hinting at a potential role of the bacterial peroxidase in biomass (lignin) degradation. Phenolic compounds are known to be natural mediators used by other peroxidases in the degradation of lignin. Additionally, TfuDyP showed activity towards the phenolic lignin model compound guaiacylglycerol-β-guaiacyl ether, but not towards the nonphenolic veratrylglycerol-β-guaiacyl ether. The products formed upon conversion of phenolic compounds (vanillin, vanillyl alcohol, vanillylaceton and the phenolic lignin model compound) were analyzed by HPLC and LC-MS and consisted of dimers and higher oligomers. Interestingly, the formed dimer of vanillin, divanillin, is in demand as taste enhancer.

Exploring the catalytic properties of TfuDyP
To gain a better understanding of the catalytic machinery of TfuDyP, various point mutations were studied in chapter 3. Mutations were made around the heme cofactor, in the proposed hydrogen peroxide tunnel, to shift the pH optimum for activity and to study the dependence of TfuDyP activity on long-range electron transfer (LRET). The importance of the catalytically active arginine and the aspartate from the GXXDG-motif, which is the proposed acid-base catalyst, were reported in earlier work. In this work, the heme ligand, a histidine, was mutated to a cysteine to convert TfuDyP into a heme-thiolate enzyme with potentially improved peroxygenase activity. This histidine is however crucial for TfuDyP: mutating this residue yielded an inactive yellow-colored enzyme. DyP-type peroxidases contain a highly conserved GXXDG-motif. Mutant G243A (GXXDＧ), designed to narrow the proposed hydrogen peroxide tunnel, reduced the activity towards Reactive Blue 19 to 15%. Furthermore, the area around the heme propionate and the catalytically active arginine was found to be important for the pH optimum for activity. Mutations A245R and N246L in this area broadened the pH range for activity towards Reactive Blue 19 by one pH unit. For the oxidation of substrates that are too large to enter the active site, long-range electron transfer pathways from the protein surface to the heme cofactor were reported for multiple (DyP-type) peroxidases. To test whether TfuDyP is dependent on such a mechanism all eight surface exposed tyrosines and tryptophans within 20 Å from the heme cofactor were mutated to phenylalanine individually. The obtained mutants showed however a comparable activity as the wild-type enzyme, indicating that TfuDyP is either not dependent on LRET or able to use multiple pathways.
High overexpression of TfuDyP leads to the incorporation of heme precursor PPIX

For the industrial applicability of enzymes a high overexpression level is desired. In contrast to eukaryotic peroxidases are bacterial DyPs relatively easily expressed in a heterologous host, such as Escherichia coli. The heterologous expression of TfuDyP in E. coli was successfully boosted through a SUMO-fusion, increasing the enzyme expression from 25 mg to 200 mg per liter culture broth (chapter 4). The highly overexpressed SUMO-fused enzyme was more purple in color and while it displayed the typical Soret band, it showed five instead of the expected one or two Q-bands in UV-vis absorbance spectroscopy. Moreover, the specific activity decreased to 22%. In an attempt to understand the reason behind the lower activity, different expression conditions, expression hosts and enzyme fusions were studied. The decrease in activity was not due to the fusion to SUMO; highly overexpressed SUMO-TfuDyP, MBP-TfuDyP and TfuDyP (with only an N-terminal His-tag) gave comparable results. Addition of hemin, iron salts or a heme precursor (δ-aminolevulinic acid) to the cultures increased the enzyme specific activity only slightly. Important information was obtained when SUMO-TfuDyP was expressed in different E. coli strains and under varying arabinose concentrations for induction. These experiments clearly showed that the activity of the purified enzyme was dependent on the expression level. Strains which yielded less than 50 mg enzyme per liter culture broth produced fully active enzyme, while strains with an expression level above 150 mg yielded enzyme with a reduced specific activity. Further analysis of the isolated heme cofactors by mass spectrometry revealed a crucial difference between the active and less active enzymes: the active enzyme samples contained the expected heme cofactor while the less active enzyme samples were found to contain a significant amount of an iron deficient heme precursor protoporphyrin IX (PPIX). As a heme enzyme deficient in iron is unable to perform catalysis, this solved the riddle of the relatively low specific activity when the enzyme was highly overexpressed.

To study whether the in vivo binding of heme precursor PPIX is specific to TfuDyP or a more general feature of DyP-type peroxidases, we boosted the expression level of SviDyP from S. viridis to approximately 100 mg per liter culture broth. UV-vis absorbance spectroscopy revealed that SviDyP too binds a significant amount of PPIX, showing the same five PPIX specific Q-bands in UV-vis absorbance spectroscopy. This shows that E. coli has to be tuned when it is used for the overexpression of DyP-type peroxidases. The insufficient conversion of PPIX into iron-containing heme b may be solved by the coexpression of heme ferrochelatase.
Oxidase-peroxidase fusion enzymes: biosensors and cascade reactions
Many enzymes take part in metabolic routes in nature. During evolution some enzymes formed complexes or fused together to reach a higher efficiency. Oxidases and peroxidases form catalytically logical combinations and are often coexpressed, for instance in biomass degradation. The oxidases use molecular oxygen as oxidant and produce hydrogen peroxide as by-product, which in turn fuels the peroxidases. Even though various artificial fusion enzymes were made before, no fusion enzyme between an oxidase and a peroxidase had been reported. Chapter 5 presents the first artificial oxidase-peroxidase fusion enzymes produced as recombinant proteins. In chapter 5, bacterial DyP-type peroxidase \( \textit{SviDyP} \) was fused to four different flavoprotein oxidases: chitooligosaccharide oxidase (ChitO), eugenol oxidase (EugO), HMF oxidase (HMFO) and alditol oxidase (HotAldO). Fusion of these enzymes yielded four well expressed bi-functional enzymes. Two of the artificial fusion enzymes were applied as biosensor for the detection of sugars; P-ChitO and P-HotAldO (in which P stands for peroxidase \( \textit{SviDyP} \)). With such fusion enzymes, the model substrates cellobiose and xylitol could be detected with a detection limit in the low μM range. The coupled assay fundamental to these biosensors is closely related to the well-established HRP-assay in which the activity of an oxidase is coupled to the activity of plant peroxidase horseradish peroxidase (HRP). \( \textit{SviDyP} \) proved to be a good substitution for HRP in this assay; it is well expressed in a bacterial host, performs the same reaction, and can be applied at a more acidic pH range.

The other two oxidases, EugO and HMFO, show a partially overlapping substrate scope with peroxidase \( \textit{SviDyP} \). These three enzymes are all active on phenolic compounds. This overlap in substrate scope is perfectly suited for molecular oxygen driven one-pot two-step cascade reactions. P-HMFO and P-EugO produced the taste enhancer divanillin in a two-step reaction from vanillyl alcohol. P-EugO converted eugenol in lignin-like dimers and oligomers.

Future perspectives
DyP-type peroxidases are named after their activity on synthetic dyes. The physiological substrate is however still unknown. Until now, most evidence points towards a role in biomass degradation. DyP-type peroxidases from class A and D are (Tat-dependently) secreted, which supports this hypothesis. Identification of the physiological substrates of (intra/extracellular) DyPs would be very informative. Besides the unknown physiological substrates is the catalytic mechanism of DyP-type peroxidases not well understood. DyP-type peroxidases rely on a heme cofactor for their activity. Different oxidation sites were observed
Conclusions and future perspectives

or proposed for different members of this family. AauDyP, TcDyP and VcDyP showed long-range electron transfer (LRET) from the heme cofactor to a Tyr/Trp on the protein surface, implying that this is the oxidation site for (bulky) substrates.\(^1\)\(^–\)\(^3\) Whether all DyP-type peroxidases use LRET is unknown. DyP from *Bjerkandera adusta* Dec 1 was co-crystallized with two substrates, each bound to the surface exposed heme propionate, suggesting that this is the oxidation site of DyP.\(^4\) In future work, oxidation sites and reaction pathways could be studied further through site-directed mutagenesis and enzyme crystallization in the presence and absence of small and bulky substrates. Small substrates might react at the heme-iron directly while large substrates get oxidized at the surface exposed heme propionate or via LRET.

For the industrial applicability of enzymes a high overexpression level in a heterologous host is desired. Both bacterial and fungal DyPs are easily heterologously overexpressed in a bacterial host which is in stark contrast to peroxidases from other enzyme families. Enzymes from class D seem to be more effective catalysts, as they show a higher catalytic efficiency towards anthraquinone dyes \((k_{\text{cat}}/K_M = 10^6-10^7 \text{ s}^{-1} \text{ M}^{-1})\) when compared to enzymes from classes A and B \((10^4-10^5 \text{ s}^{-1} \text{ M}^{-1})\).\(^5\) The sequence identity between DyPs from bacterial classes A and B and fungal class D is low, <20%. The higher catalytic efficiency and the possibility to express fungal DyPs from class D heterologously in a bacterial host makes them interesting candidates for future research. D-type DyPs could potentially substitute plant and fungal peroxidases, which are not easily heterologously produced.\(^6\)

Most characterized DyPs were heterologously overexpressed in *E. coli*. Chapter 4 showed however that the high overexpression of *Tfu*DyP and *Svi*DyP resulted in partially inactive enzymes due to the incorporation of the iron deficient heme precursor protoporphyrin IX. To circumvent this problem, the expression level was lowered to approximately 50 mg enzyme per liter culture broth, at which the enzyme was still fully loaded with heme. In future work this problem could be solved by increasing the heme amount in the cell by co-expression of heme ferrochelatase, an enzyme which incorporates iron in heme.\(^7\)

Furthermore, the low selectivity of some DyP-type peroxidases between heme and heme precursor protoporphyrin IX, and the expression of other DyP-type peroxidases as apo-enzyme offers some interesting possibilities. Key et al. (2016) showed that the reconstitution of apo heme \(b\) (Fe-porphyrin IX, Fe-PIX) containing enzymes with unnatural metalloporphyrins (M-PIX) resulted in abiological catalysis.\(^8\) It would be very interesting to load DyP-type peroxidases with e.g. Mn, Co, Cu, Zn or Ru-based porphyrins and probe such engineered enzymes for novel activities.
DyP-type peroxidases show a pH optimum for activity in the acidic range, while oxidases show a pH optimum in the neutral to mild alkaline range. For the applicability of DyPs in cascade reactions with oxidases, it would be beneficial to shift the pH optimum towards a more neutral pH range. The pH optimum of other enzymes, including *PpDyP*, were shifted by site-directed mutagenesis in the past. Yet, as shown in chapter 3, analogous mutations in *TfuDyP* did not result in a major change of the pH optimum, which suggests that changing the pH optimum of a DyP is not always straightforward. Alternatively, DyPs with a higher pH optimum for activity could be isolated from alkaliphilic or alkali-tolerant microorganisms. The Gram-positive alkaliphilic bacteria *Cellulomonas bogoriensis* and *Dietzia natronolimnaea* contain one and two DyP-type peroxidases respectively. These DyPs are most related to the Tat-dependently secreted DyPs from class A. The DyP from *C. bogoriensis* shows an interesting feature; the aspartate of the conserved GXXDG-motif is substituted for a glutamate. Another highly promising DyP was found in the genome of the biomass degrading thermophilic and alkali-tolerant *Aspergillus fumigatus Z5*. This enzyme is predicted to belong to class D but no secretion sequence could be identified. In the genome of fungus *Aspergillus versicolor* five genes were found that resemble to DyPs; three DyPs D, a DyP C and one DyP that surprisingly resembled to the bacterial DyPs from class B. Small modifications in the GXXDG-motif were also observed for some of these DyPs; the last glycine of the GXXDG-motif is substituted for a Leu, Thr or His in the DyP from *A. fumigatus*, and two DyPs D and the DyP C from *A. versicolor*.

Besides shifting the pH optimum, DyP-type peroxidases could be engineered towards an improved thermostability and/or an improved tolerance towards hydrogen peroxide or organic solvents. To improve the biomass degrading capacity of DyPs, it would be desirable to increase the specific activity towards natural mediators such as phenols and Mn$^{2+}$. Next to improving the activity or the robustness of these enzymes, DyPs could be engineered towards non-natural activities; for instance towards (an improved) peroxygenase activity or to become an oxygen carrier as hemoglobin and myoglobin. Exchanging the activities of heme $b$ containing peroxidases, peroxygenases and oxygen carriers was done before.

The research presented in this thesis showed that the dye decolorizing peroxidase *TfuDyP* is a true dye decolorizing enzyme. In future work, DyP-type peroxidases could be applied in the bioremediation of synthetic dye contaminated waste water. Even though DyP-type peroxidases are not capable of the full biodegradation of these dyes to regular metabolites, they form a good first step in this process. To go one step further, a whole cell based system could
be created for this purpose through the extracellular coexpression of a DyP-type peroxidase with other oxidative enzymes. Upon coexpression of other required catabolic enzymes the dyes could be fully mineralized and detoxified.

Besides waste water treatment, in-textile bleaching for the creation of a pattern or for the production of a stonewashed-look could be an interesting application area for DyPs. Bleaching is also of interest to the food industry. In fact, a fungal DyP was patented and marketed under the name MaxiBright for the enzymatic bleaching of carotenoids in whey containing food and beverages. Next to bleaching it is worth noting that DyPs can be used for the dimerization and polymerization of phenolic compounds. Both DyPs that were studied as part of this thesis were found to catalyze the dimerization of the phenolic compound vanillin to the taste enhancer divanillin and higher oligomers. Divanillin gives an impression of creaminess to food and masks the sense of bitterness, and is therefore attractive for the food industry. Polymerization of vanillin is considered for the production of bio-based plastics.\textsuperscript{16}

Lastly, biosensors form an interesting field of application for DyP-type peroxidases. Oxidase substrates could be detected through a coupled reaction between an oxidase and a peroxidase, during which a chromogenic compound is formed. In this work, sugars cellobiose and xylitol were detected with a μM detection limit by P-ChitO and P-HotAldO, respectively. Future enzyme discovery and enzyme engineering efforts will reveal the full potential of DyP-type peroxidases.
References


