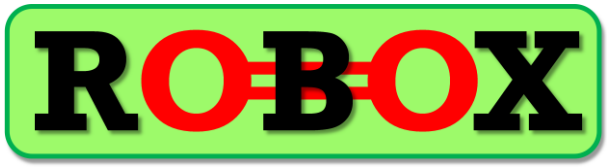

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Deliverable report 1.2: Set of P450 enzymes with improved performance for hydroxylation of aromatics, alkenes and APIs and a set of API-sensors to enable P450 improvement

Summary

The ROBOX consortium aims at developing biocatalytic routes and techniques enabling their development for industrial production or conversion of hydroxylated substances employing Baeyer-Villiger monooxygenases (BVMOs), P450 monooxygenases, alcohol dehydrogenases (ADHs) and alcohol oxidases (AOXs) as catalysts. The key enzyme subclass for regioselective hydroxylation is that of P450 monooxygenases. Within the past 24 months, our work has been mainly focused on the identification and engineering of P450 monooxygenases capable of converting pseudocumene (an aromatic substance), isophorone (an alkene), and diclofenac (an active pharmaceutical ingredient, API). Functional and improved P450s could be identified for all of these substrates and a first pilot scale hydroxylation process of the API has been established. This report provides an overview of the selected biocatalysts, a short description of the current knowledge about each enzyme target and a summary of the key findings of the individual ROBOX partners. The identification and engineering of better P450s for the demonstration of this technology on pilot scale is still ongoing and will continue in the second half of the project period. We therefore expect that further improvements on the current-state-of-the-art P450 catalysts are conceivable.

Introduction

Cytochrome P450 monooxygenases (P450s) are versatile biocatalysts capable of regio- and stereospecific oxidation of non-activated hydrocarbons under mild reaction conditions[1–3]. The reaction requires nicotinamide cofactors (NADH or NADPH) and is therefore preferably performed with whole cell biocatalysts capable of providing and regenerating this cofactor from inexpensive carbon and energy sources (e.g. glucose). Alternatively *in situ* cofactor regeneration strategies need to be applied as they have been developed for efficient reductive ADH reactions. Efficient electron transfer to the catalytic heme center and the control of this transfer are required in order to avoid unproductive oxidation of NADH or NADPH and hydrogen peroxide formation eventually leading to deactivation of the P450 or even to cell death. For demonstration of the catalytic performance of P450s, we focused on the synthesis of precursors for a specialty chemical used by the food/feed industry and drug metabolites formed from APIs.

Several P450 monooxygenases have been selected as starting point for biocatalyst development. Owing to its molecular architecture (a natural fusion of the reductase and a catalytic heme domain), the detailed knowledge of the enzyme and its high promiscuity and amenability for engineering, the bacterial P450 monooxygenase from *Bacillus megaterium* (P450 BM3)[4] represents a chief biocatalyst target for the ROBOX consortium. Three parties (i.e. **DSM**, **RWTH** and **ETH**) focus on the application or engineering of P450 BM3 for hydroxylation of aromatic substrates (pseudocumene and xylene), alkenes (isophorone), and APIs (diclofenac), respectively. The conversion of alkenes is also addressed by **UNIMAN** which takes advantage of the artificial fusion protein P450 Cam-RhFRed (heme domain from *Pseudomonas putida*, reductase domain from *Rhodococcus* sp.)[5] for catalysis of the hydroxylation of α-isophorone to keto-isophorone. Furthermore, **TUG** employs a human P450 (CYP2C9)[6] recombinantly expressed in yeast in order to achieve regioselective hydroxylation of diclofenac. Last but not least, in addition, **RUG** has recently identified a gene in *Myceliophthora thermophila* predicted to encode a novel cytochrome P450 similar to P450 BM3, comprised of a single polypeptide carrying both the reductase and the monooxygenase domains. The latter is currently characterized further with respect to its suitability for conversion of the ROBOX substrates.

All of the abovementioned P450 enzymes have been successfully expressed in microbial hosts and the target activities have been identified. Protein engineering studies are being carried out in order to fine-tune the catalysts' specificities and boost their activities while minimizing futile consumption of reducing equivalents for the formation of hydrogen peroxide. The set of P450 monooxygenases available to all ROBOX partners is as follows:

- P450 BM3 from *B. megaterium*
- P450 Cam-RhFRed (heme from *P. putida*, reductase from *Rhodococcus sp.*)
- CYP2C9 from *H. sapiens*
- P450 from *M. thermophila* (now reclassified as *Thermothelomyces thermophile*)

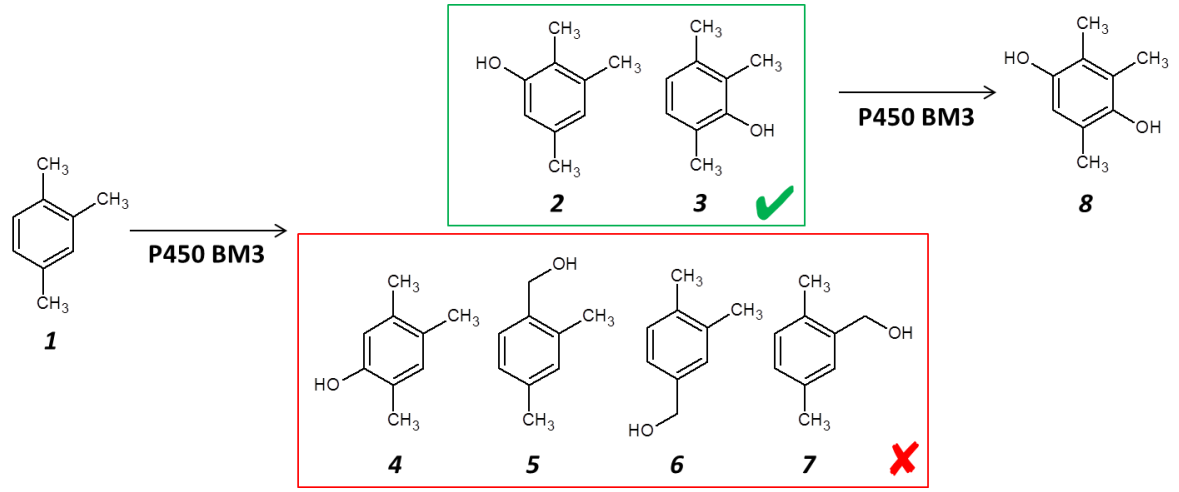
Results

P450 monooxygenase catalyzed hydroxylation of aromatics

The hydroxylation of pseudocumene **1** to trimethylhydroquinone (1,4-hydroxy-2,3,5-trimethylbenzene, **8**) requires two consecutive hydroxylations in order to yield the end-product which is an intermediate for the synthesis of feed/food supplements. P450 BM3 wildtype catalyzes the desired 1,4-hydroxylation of pseudocumene but especially the first hydroxylation proceeds at low rates and with poor regioselectivity[7,8]. In order to improve on this, P450 BM3 engineering relying on semi-rational methods has been performed by **RWTH**.

Screening of one site-saturation mutagenesis libraries (1200 clones) targeting simultaneously 2 amino acid positions located in the substrate entrance channel (R47/Y51) as well as 2 libraries (each 400 clones) targeting amino acids in the active site in close proximity to the heme (I401/A330) lead to the identification of the variant P450 BM3 M3 (R47S, Y51W, I401M, A330F). Screening was performed in 96-well plates with the NADPH consumption assay in combination with the 4-AAP assay which is specific for the detection of phenols. Table 1 summarizes the catalytic data for conversion of pseudocumene **1** with purified P450 BM3 enzymes[9]. Subsequently, five different saturation mutagenesis libraries targeting key residues (I263/E267, A264/T268, A328, A330, L437/T438) in the active site of P450 BM3 were generated and in total 3500 variants were screened by the NADPH consumption assay. Activity improved variants were identified and selected hits were characterized. Finally, 2 improved mutants were isolated (named A6 and B11, due to a not finalized manuscript the obtained substitutions are not stated in this public report). P450 BM3 variant B11 showed 50-fold activity improvement over the wildtype [NADPH oxidation rate ($mol_{cofactor}$ $mol_{P450\text{-}1}$ min^{-1}) of wildtype for pseudocumene is 22 ± 4, TMHQ formation <0.01 g L^{-1}] and a 1.8-fold improvement over the previously identified state-of-the-art variant M3 (R47S, Y51W, I401M, A330F, TMHQ formation 0.1 to 0.18 g L^{-1}). Up to 2.5-fold less by-product formation (unwanted side products) was observed for both variants (decreased formation of 2,4 DMBA and 2,4,5-TMP) compared to variant M3 and the wildtype (WT: 27 % 2,4-DMBA , 34 % 2,4,5-TMP; M3: 17 % 2,4-DMBA , 29 % 2,4,5-TMP, table 2). The variant with the highest TMHQ formation (P450 BM3 variant B11) will be used as template for a first round of directed evolution to obtain further improvements. In order to efficiently screen larger numbers of candidates, the partners can rely on a flow cytometry based screening system developed by **ETH** (see deliverable report 1.4).

In summary, a first significant step in Deliverable 1.2 is achieved by providing P450 BM3 monooxygenase variants for hydroxylation of aromatics.



Hydroxylation of pseudocumene by P450 BM3 monooxygenase

*The desired hydroxylation product 1,4-dihydroxy-2,3,5-trimethylbenzene (**8**) can be formed via two different monohydroxylates (**2, 3**). All other hydroxylation reactions lead to unwanted dead-end products (**4,5,6,7**).*

Table 1. Catalytic data for conversion of pseudocumene 1 with purified P450 BM3 enzymes.

Entry	Substrate	Catalyst	NADPH ox. rate [min^{-1}][a]	Coupling [%][b]	TOF [min^{-1}][c]
1	**1**	WT	22 ± 4	15 ± 2	3.2
2	**1**	M2	87 ± 3	19 ± 2	17
3	**1**	M3	499 ± 91	45 ± 6	226

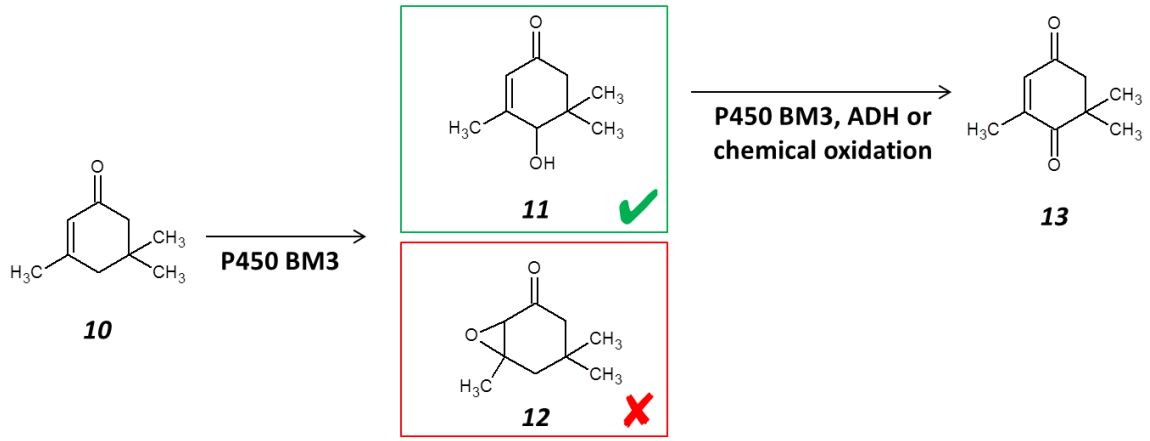
[a] NADPH oxidation rate ($mol_{cofactor}$ mol_{P450}^{-1} min^{-1}) was determined spectrophotometrically at 340 nm absorbance; [b] Coupling efficiency (%) = ratio between product formation [µM] and oxidized cofactor [µM]; [c] Turnover frequency ($mol_{product}$ mol_{P450}^{-1} min^{-1}). **WT** = P450 BM3 wild type; **M2** = P450 BM3 mutant (R47S, Y51W, I401M)[2a]; **M3** = P450 BM3 mutant (R47S, Y51W, I401M, A330F) (obtained in this study). Conversions were done using purified P450 BM3 protein (0.3 µM WT enzyme or 0.1 µM for the P450 BM3 variants), 10 mM substrate, 1.5% DMSO as co-solvent in a final volume of 5 mL. 200 µM NADPH were added and the activity of P450 BM3 was measured as initial NADPH oxidation rates at 340 nm. Products were quantified using GC-FID and commercial standards for **2-8**.

Table 2. Regioselectivity of the P450 BM3-catalyzed hydroxylation of pseudocumene

variant	2,5-DMBA	2,4-DMBA	2,3,6-TMP	2,3,5-TMP	3,4-DMBA	2,4,5-TMP	TMHQ	TMHQ [mg/L]
WT	14 %	27 %	9 %	8 %	5 %	34 %	3 %	< 10
M3	6 %	17 %	7 %	4 %	2 %	29 %	35 %	180

P450 monooxygenase catalyzed hydroxylation of alkenes

Hydroxylation of the alkene α-isophorone **10** by P450 monooxygenases can render ketoisophorone **13** which is an alternative chemical precursor for synthesis of feed/food supplements. Prior to the ROBOX project **DSM** had successfully scaled up the P450 BM3 catalysed hydroxylation reaction α-isophorone to 4-hydroxy-isophorone to 100 L reaction or 1 kg product scale[10]. The enzymatic production of ketoisophorone is being investigated by ROBOX partners **RWTH** and **UNIMAN**. The former party employs P450 BM3 while the latter exploits a hybrid P450 enzyme with an alcohol dehydrogenase for biocatalytic synthesis of ketoisophorone **13**.



Hydroxylation of α-isophorone by P450 BM3 monooxygenase

*P450 BM3 wildtype hydroxylates α-isophorone either at the desaturated carbon leading to the formation of an unwanted epoxide (**12**) or at the desired site leading to the formation of 4-hydroxy-isophorone (**11**) which can then be easily (bio)chemically oxidized to the end-product (**13**).*

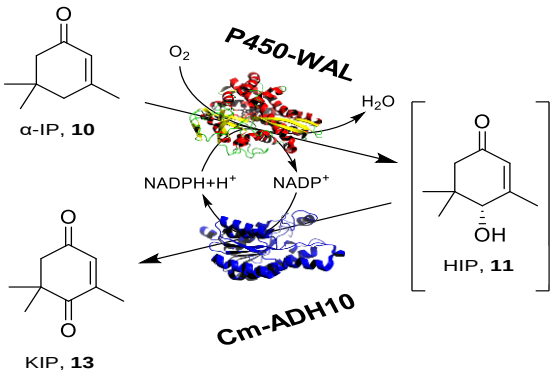
P450 BM3 wildtype has been shown to catalyze the hydroxylation of α-isophorone **10** to 4-hydroxy-isophorone **11** and isophorone oxide **12**, albeit with low catalytic efficiency[10]. Subsequently, a protein engineering campaign relying on a semi-rational approach was initiated in order to increase the activity and specificity of P450 BM3. Screening of eight site-saturation mutagenesis libraries (SSM) with in total 4140 variants targeting 11 amino acid positions (R47, Y51, V78, F87, A184, I263, A264, E267, T268, A328, I401) via an NADPH depletion assay brought 18 hits putatively displaying elevated activity. Three of these variants (named AG1, AG2, AG3) showed during small scale conversion of α-isophorone an increased product formation of up to 12.3 mM 4-hydroxy-isophorone (**11**) / µM enzyme. In relative terms, this corresponds to an approximately 3-fold improvement compared to P450 BM3 wildtype. Variants AG2 and AG3 showed 2.25 fold and 2.18 fold increased 4-OH-isophorone formation, respectively. For AG1 and AG3, the amount of the side-product isophorone oxide **12** increased proportionally and variant AG2 displayed a 50% reduced side-product formation. The characterization of the remaining variants discovered in the initial screen showed minor increases of product formation (maximal 1.5-fold) or strong increase (more than 30%) in side product formation. Additionally, an OmniChange library for simultaneously targeting four mutagenic **hotspots** (V78/F87/A184/I263) was generated and is currently being screened. Similarly, the combination of already identified beneficial mutations into a single variant is in process.

In order to exploit an alternative to P450 BM3 and its variants, **UNIMAN** screened a P450cam-RhFRed mutant library and identified α-isophorone converting variants.

Starting from P450cam-RhFRed Y96F, 96 P450cam-RhFRed active site variants were previously generated at **UNIMAN** by site-directed mutagenesis following the CASTing approach[11] and screened for indigo production using a colony-based high-throughput screen format. Since, it was shown that neutral mutations might improve activity towards structurally different substrates[12], we have screened the developed libraries to find variants for the allylic oxidation of α-IP. A total of 96 P450cam-RhFRed variants were clustered according to the mutated residues in five pooled libraries (A-E) and screened for isophorone allylic oxidation activity by GC-analysis. The biocatalytic performance was measured as total turnover numbers (TTNs), that is the ratio between total product concentration and catalyst concentration, calculated over 24 h. Best results were obtained with library D (17 variants, mutations at residues L244-V247) which was subsequently analysed at the single-clone level. 13 out of 17 variants analysed showed TTNs ranging from 4 to 70. A positive effect of the substitution of L244 with small or apolar residues was observed and therefore the mutation L244A was introduced in the wild-type background or along with V247L, which occurred in many positive hits. The two mutants L244A and L244A-V247L showed the best TTN values (94 ± 9 and 83 ± 11, respectively) and were selected for further engineering. In order to try to improve enzyme-substrate fit, the bulky tryptophan was introduced at position 87, known to be involved in substrate access and recognition. Eventually, variant Y96F-F87W-L244A-V247L (termed P450-WAL) showed more than a 6-fold improvement of TTNs values with respect to L244A-V247L mutant with no by-products detected and an *e.e.* of 99% (*R*)-**11**. P450-WAL was selected for subsequent optimization experiments for the designed cascade reaction.

A collection of 116 alcohol dehydrogenases (ADH, provided by ROBOX partner **CLE**) for the oxidation of 4-hydroxy-isophorone **11** to the desired ketoisophorone product **13** were screened. Several enzymes (>80% of the panel) showed almost no activity among the positive hits and two were selected for having the highest activity: Cm-ADH10 from *Candida magnoliae* (GenBank AGA42262.1) and the NADPH-dependent carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor* (UniProt Q9UUN9). Both ADHs accept NADP(H) as cofactor, which is desirable to create a redox-self-sufficient cascade. The corresponding wild-type genes were cloned into a pET28a vector to carry out expression trials, which revealed good expression levels for Cm-ADH10, as opposed to SSCR. Thus, Cm-ADH10 was selected for subsequent combination with the P450. After optimisation of reaction conditions (described in WP3), the oxidation of 4-hydroxy-isophorone **11** to ketoisophorone **13** was accomplished using a concentrated cell-free extract (CFE, corresponding to 1 mg/mL final protein concentration) of *E. coli* overexpressing Cm-ADH10. Moreover, the co-expression of P450-WAL variant with Cm-ADH10 was also attempted in the same *E. coli* host but conversion values were approximately 30% lower than for the one-pot two-step process carried out at screening scale.

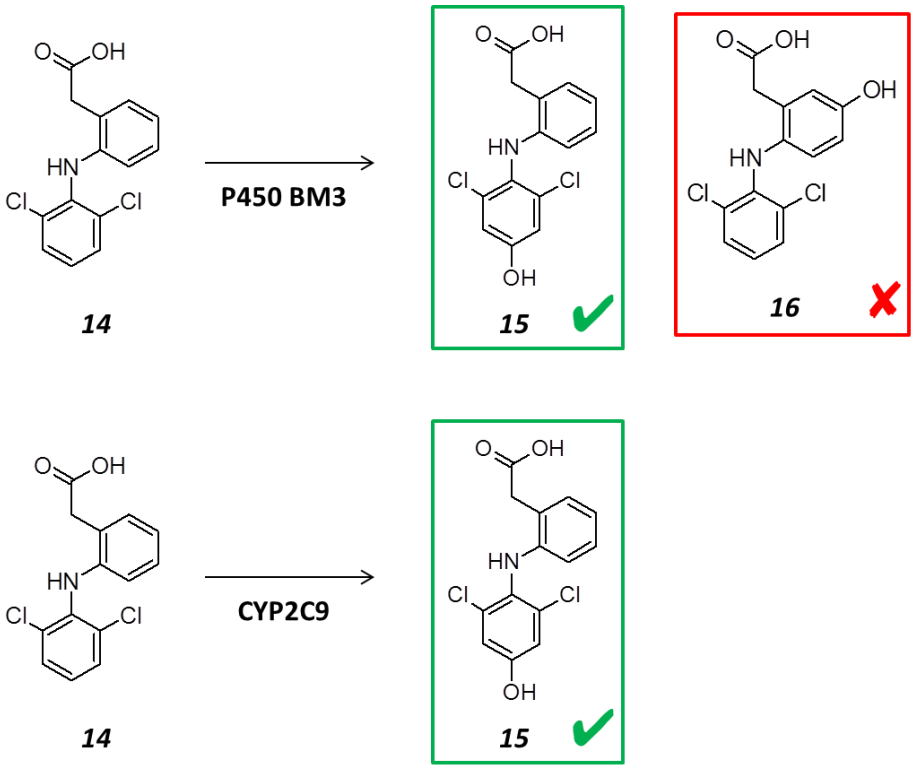
Eventually, conversion of α-isophorone **10** yielded up to 1.4 g L^{-1} d^{-1} of ketoisophorone **13**. To the best of our knowledge, this is the first biocatalytic route to ketoisophorone **13** that employs a monooxygenase and an alcohol dehydrogenase.



Cascade double oxidation of α-isophorone to ketoisophorone via 4-OH-isophorone employing P450 and ADH
*P450cam-RhFRed variant Y96F-F87W-L244A-V247L (P450-WAL) was used for hydroxylation of α-isophorone (**10**) to 4-hydroxy-isophorone (**11**) which is in turn further oxidized by Cm-ADH10 to ketoisophorone (**13**) in a redox-neutral enzymatic cascade reaction.*

P450 monooxygenase catalyzed hydroxylation of APIs

The hydroxylation of diclofenac **14** (an API) by human monooxygenases yields 4'-OH- and/or 5-OH-diclofenac (**15** and **16**, respectively) as the main metabolites[6,13]. These hydroxylates are important for pharmacokinetic and toxicological studies for which they are frequently needed in multi-gram amounts. One of the goals of ROBOX is the identification of monooxygenases capable of regiospecifically hydroxylating diclofenac to be able to produce both metabolites. For this purpose both P450 BM3 and the human CYP2C9 were identified to be promising starting points as indicated in previous scientific reports.



P450 catalyzed hydroxylation of diclofenac

*The commercially interesting drug metabolite also formed in humans is compound **15**. P450 BM3 non-specifically catalyzes the formation of both products (**15** and **16**) while CYP2C9 exclusively forms the 4'-hydroxylated product (**15**).*

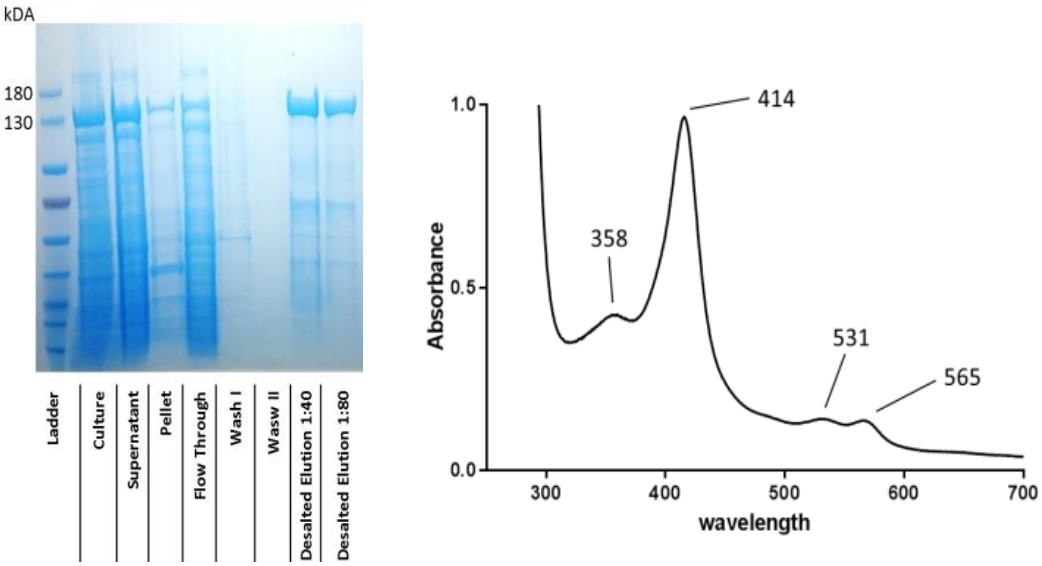
TUG focuses on establishing a diclofenac hydroxylation route using the human CYP2C9 produced in yeast in combination with a compatible reductase domain (CPR). This P450 enzyme exhibits high regioselectivity whereby 4'-OH-diclofenac is exclusively formed. Expression of CYP2C9 (wildtype enzyme) and CPR in yeast was optimized by tuning the expression levels of either of the two enzymes and while relying on new promoters. The bidirectional promoter P_{PDF}/P_{DC} (provided by bisy e.U., Austria) was found to be suited best for expression of the target proteins. Furthermore, the cultivation media was improved whereas increasing the pH, lowering the induction temperature and supplementation of the medium with Δ-aminolevulinic acid proved to have a positive effect on P450 expression in yeast. With the optimized reaction conditions and using a whole cell biocatalyst, conversion of 6 mM diclofenac could be driven to completion (0.97 g L^{-1} 4'-OH-diclofenac **15**). Furthermore, we made the first steps towards improving the activity of CYP2C9 by directed

evolution. For this, two epPCR libraries of CYP2C9 were screened using HPLC/MS: the first library comprised 2000 and the second 2500 clones. Different epPCR conditions were tested and activity-fitness landscapes with 42 transformants were generated using diclofenac as substrate. The chosen condition, which showed 40% active clones in the activity-fitness landscape and a mutation rate of one to three mutations per kb, are as followed: 5 mM $MgCl_2$, 0.2 mM dATP and dGTP and 0.6 mM dCTP and dTTP. However this effort did not divulge more active enzyme variants. In order to cover a larger sequence space in the future, a flow cytometry screening system using a surrogate fluorogenic substrate is currently under development at **TUG** and supported by **ETH**.

DSM also addressed the hydroxylation of diclofenac using P450 BM3 as catalysts. Unlike the human CYP2C9, P450 BM3 also yields the regioisomer 5`-OH-diclofenac (**16)**. Therefore, a combinatorial P450 BM3 mutant library consisting of almost 8000 variants, in which up to 5 amino acid residues are exchanged for 6 different amino acids at the same time, were screened in order to identify more active and selective variants. The best performing mutant 22C02 was afterwards successfully used for the conversion of diclofenac (99% conversion efficiency, space-time yield of up to 13.4 g L^{-1} d^{-1}, 100 L scale). In relative terms, this translates into 1.5-fold improvement of the conversion catalyzed by the wildtype enzyme and a 15-fold improvement of the final product concentration. This represents the first example of P450 catalyzed *in-vitro* API metabolite production performed within the ROBOX consortium at pilot plant scale.

Additional P450 monooxygenase targets

Inspired by the discovered Baeyer-Villiger monooxygenase (PockeMO) from the fungus *M. thermophila* (recently reclassified as *Thermothelomyces thermophila*) and based on sequence homology with known P450 sequences, **RUG** has identified a gene in *M. thermophila* predicted to encode a cytochrome P450. The provided evidence suggests that this protein is a catalytically self-sufficient P450 with the heme reducing NADPH–P450 reductase fused to the heme containing hydroxylase. Similar to P450 BM3, both domains are believed to be part of a single polypeptide. An *E. coli* codon optimized copy of the gene was cloned in a pET expression vector conferring an N-terminal His-tag to the protein sequence. The protein could be well over-expressed and remained soluble in *E. coli* BL21 (DE3) grown in rich medium. The protein was purified and displayed a red color visible by eye and displayed the typical UV/Vis absorption spectrum of protein-bound heme and flavin cofactors (strong absorbance peak at 414 nm and several minor peaks). A carbon monoxide spectrum was also recorded indicating a Soret band with an absorbance peak at 450 nm being indicative for P450 enzymes.



SDS-PAGE analysis (left) and UV/Vis spectrum (right) of purified P450 from Thermothelomyces thermophila. *The enzyme had been well expressed in* E. coli *and displays the typical spectral features of a hemoprotein.*

The purified protein was tested for catalytic activity using lauric acid as substrate and NADPH as the reducing cofactor. By monitoring the absorbance at 340 nm, it was found that addition of the fatty acid triggers NADPH depletion. The stability of the enzyme was also investigated in different conditions by measuring the apparent melting temperature of the protein in a ThermoFluor assay indicating T_{50} melting points between 50-56 °C depending on the applied condition. **RUG** aims to further characterize this enzyme and to investigate its versatility as a biocatalyst by analyzing its substrate and product spectrum and its suitability as a starting point for generation of novel variants generated by directed evolution.

Conclusions

In summary, we see from our point of view D1.2 as achieved as improved P450 Monooxygenases for the hydroxylation of aromatics, alkenes, and APIs could be identified.

Within the past 24 months, we delivered a considerable number of novel robust P450 monooxygenases. The enzymes have been characterized and all initially selected products could be synthesized. In case of the drug metabolite, a pilot plant scale (100 L) process with an engineered P450 BM3 variant has been established.

To facilitate the further exploitation and information sharing, key parameters of all P450 monooxygenases have been summarized in datasheets which are shared among all consortium members.

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