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Deliverable report D2.2: First generation production strains for robust BVMOs (RUG, M18)

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Summary

This report describes the successful production of industrially relevant Baeyer-Villiger monooxygenases (BVMOs) of prokaryotic and eukaryotic origin using *Escherichia coli* as a host. Specifically, RUG has optimized the production of cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO), *Rhodococcus* sp. HI-31 (RhCHMO), and *Thermocrispum municipale* (TmCHMO), as well as that of cyclopentadecanone monooxygenase from *Pseudomonas* sp. HI-70 (PsCPDMO) and polycyclic ketone monooxygenase from *Myceliophthora thermophila* C1 (PockeMO, previously termed C1-BVMO). DSM constructed *E. coli* expression strains for these and 11 additional BVMOs. Cyclododecanone monooxygenase (RrCDMO) from *R. ruber* SC1 was successfully produced in a high cell density fermentation.

AcCHMO, RhCHMO, RrCDMO and PsCPDMO were target enzymes from the project beginning, while the more robust TmCHMO and PockeMO were discovered and added as target enzymes during the project. Production comprised gene cloning in appropriate expression vectors, expression of the proteins in a suitable expression host and preparation of the enzyme in both cell free extract and purified form. These were considered as the required steps to be investigated, in order to make a knowledge-based choice for the final production strains for BVMOs to be applied in other work packages.

Introduction

Baeyer-Villiger monooxygenases, mode of action and practical implications

Baeyer-Villiger monooxygenases (BVMOs, EC 1.14.13.x) are one of the four enzymes classes selected as targets for the ROBOX project. They insert oxygen functionalities in relevant starting materials, yielding value-added compounds as products. Specifically, BVMOs catalyze the insertion of a single oxygen atom stemming from dioxygen next to a carbon atom bearing a keto functionality (Figure 1). The resulting compounds are therefore esters and lactones in case of linear and cyclic substrates, respectively. The BVMOs are dependent on two cofactors: flavin adenine dinucleotide (FAD) which is tightly bound to the protein and responsible for the actual catalysis after reaction with dioxygen, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) which provides the hydride required in the process. Briefly, the catalytic mechanism of BVMOs (Figure 1) comprises reduction of FAD by NADPH, reaction of reduced FAD with dioxygen to form a C4a-peroxyflavin intermediate, nucleophilic attack on the carbonyl carbon of the substrate resulting in the formation of a tetrahedral Criegee intermediate, and bond migration of the alkyl or aryl group from the ketone moiety to form the

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product. Subsequently, the resulting ester or lactone, water and $NADP^+$ are released from the enzyme active site. Alternatively, the C4a-peroxyflavin intermediate spontaneously decomposes yielding hydrogen peroxide; usually this so called uncoupling reaction is, however, only considerable in the absence of a ketone substrate.[1]

From a protein production point of view, these enzyme characteristics play an important role: the expression host needs to be selected in such a way, that it is able to produce the enzyme in high amounts with incorporated FAD, without external addition of this expensive molecule. Furthermore, the selected host has to be able to regenerate NADPH from $NADP^+$, when whole cells are applied in biotransformations. In the case of purified enzyme reactions, a suitable NADPH recycling system must be implemented.[2]

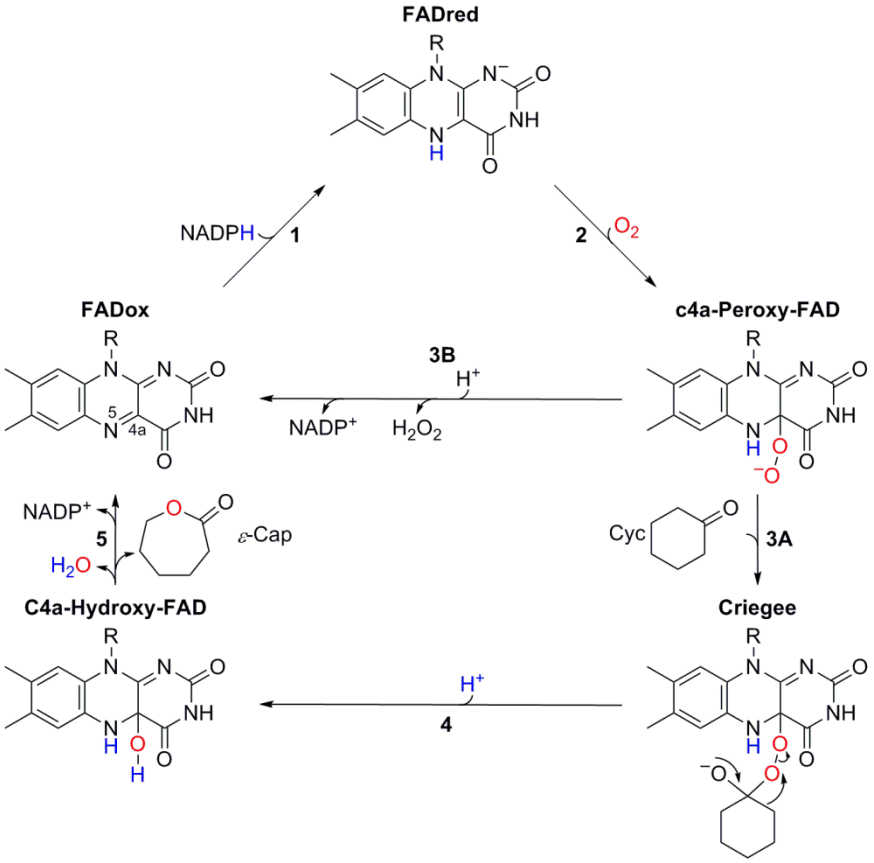

Figure 1. Catalytic mechanism of BVMOs cyclohexanone. R, ribitol adenosine diphosphate.

BVMO variants and sources

To date, the by far best described BVMO is cyclohexanone monooxygenase (EC 1.14.13.22) from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO).[3] Since its discovery, this extremely versatile enzyme was shown to oxidize hundreds of carbonylic compounds and even heteroatom containing compounds. The conversion of its natural substrate cyclohexanone is of particular industrial interest

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since the product ε-caprolactone is a precursor of both adipic acid and ε-caprolactam,[4] which are known polymer building blocks.[5] However, AcCHMO exhibits a poor stability. This fact limits its implementation in industrial processes and it is likely also the reason for the lack of its crystal structure. This in turn contributes to the limited success in engineering efforts, aiming to create a robust enzyme variant as an industrial biocatalyst.[6]

Only some years ago the crystal structure of a close homolog of AcCHMO, the CHMO from *Rhodococcus* sp. HI-31 (RhCHMO), was solved. This contributed to understand the catalytic mechanism of BVMOs and opened up new possibilities for rational design strategies. Characterization efforts carried out with RhCHMO indicated that this enzyme exhibits a very similar substrate scope and performance to those of AcCHMO.[7] Since the wealth of literature data available for AcCHMO demonstrating its excellent performance and the crystal structure of RhCHMO is available, these two enzymes have been chosen as promising candidates for biocatalysts to oxidize the ROBOX target molecule cyclohexanone and other small (cyclic) ketones.

For the conversion of macrocyclic ketones, cyclopentadecanone monooxygenase from *Pseudomonas* sp. HI-70 (CPDMO) was selected. This protein represents the best described enzyme with high activity on bulky substrates, but its crystal structure has not been determined so far.[8] As AcCHMO and RhCHMO, CPDMO presents a limited stability towards temperature and organic solvents. WP1 aims to improve the stability of these BVMOs by protein engineering, as well as to discover more robust variants in other sources. The latter aim led already early on, within WP1 of ROBOX, to the discovery and investigation of two new enzymes: CHMO from *Thermocrispum municipale* (TmCHMO) and polycyclic ketone monooxygenase from *Myceliophthora thermophila* C1 (PockeMO). RUG has demonstrated that these BVMOs are robust and active on small and bulky ketones, respectively.

We describe in this report the investigation and the establishing of an efficient and reliable production system for the above BVMOs. High enzyme yields and activity have been achieved with our production systems. In addition, it is simple, cheap and fast.

Results

NADPH regeneration

In enzyme-catalyzed Baeyer-Villiger reactions, one molecule of NADPH is consumed to provide the electrons for the reduction of the flavin cofactor. When using whole cells for the bioconversions, this has no further consequences that need to be considered, since the cell itself is able to regenerate the NADPH. When using cell free extract or purified enzymes, however, instead of adding this costly cofactor, it is usually regenerated by other means during the conversion. This can for example be done

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by using the oxidized variant $NADP^+$ as a cofactor in a coupled enzymatic reaction which yields NADPH again. This secondary enzymatic reaction can be a previous or later step in the production of the desired compound, or it can be the conversion of a cheap sacrificial substrate. Usually, $NADP^+$ dependent dehydrogenases are used for this purpose, and one example is glucose-6-phosphate dehydrogenase. Potential problems can occur from the reactivity of the respective substrate and/or formed products.[9]

Phosphite dehydrogenase (PTDH) has been shown to be efficient in biocatalytic settings, due to the cheap availability of its substrate, phosphite, the favorable thermodynamic equilibrium of the reaction, and its (engineered) thermostability. Furthermore, the formed product, phosphate is an unreactive and commonly used buffer component. At RUG, several BVMOs have previously been fused to PTDH, creating self-sufficient redox biocatalysts that show the same or higher activity than that of the non-fused BVMOs. This fusion enzyme can generate the NAPDH molecule by the action of PTDH in situ which is then used for the reduction of the BVMO (Figure 2).[9-10]

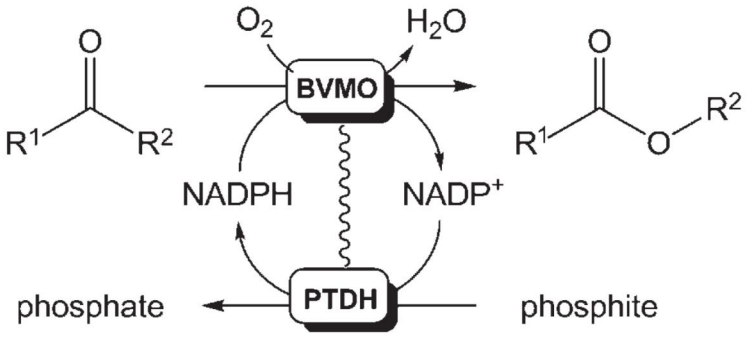

Figure 2. NADPH regeneration catalyzed by PTDH-BVMO fusion enzymes.[9]

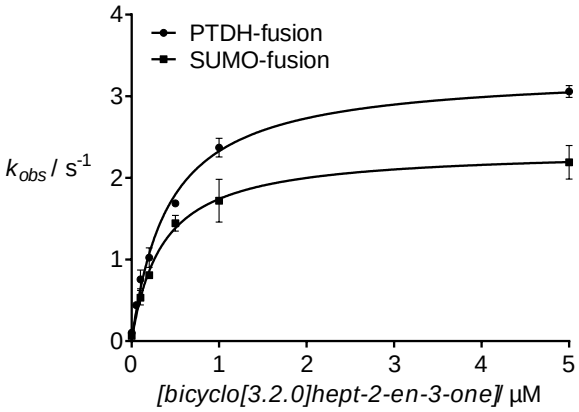

Figure 3. Activity of PockeMO fused to either SUMO or PTDH using increasing ketone concentrations.

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RUG has previously developed a pBAD based vector that facilitates the cloning of any desired BVMO gene to generate fusion enzymes (see Material and Methods below). In the scope of ROBOX, RUG has used existing constructs and created new fusion enzymes containing either TmCHMO or PockeMO. A comparison of single enzyme (fused to SUMO; without NADPH regeneration system) and PTDH fusion was performed for PockeMO. Higher activity was observed for the PTDH-fusion enzyme, as shown in Figure 3.

Expression host

A large number of different protein expression systems have been tried and successfully applied to date, covering all kinds of different classes of organisms. Eukaryotic hosts can be plants, fungi – most commonly yeast – and even mammalian cell lines. Prokaryotic hosts can be gram positive bacteria such as *Bacillus subtilis* and gram negative like *Pseudomonas* sp. or *E. coli*. The two by far most frequently used hosts, however, are *E. coli* and yeast, either *Saccharomyces cerevisiae* or *Pichia pastoris*. *E. coli*, is often used because i) handling is save, most simple and well established, ii) it grows very fast due to the very short doubling time of about 20 minutes, iii) it grows in comparably cheap and undefined media, iv) there is an enormous amount of different strains available depending on specific requirements and v) it does not cause post-translational modification heterogeneity. Due to long term experience with BVMOs gathered at RUG, expression of these proteins in *E. coli* can usually be successfully carried out leading to highest yields. Therefore, *E. coli* was chosen as the host for the expression of both previously established BVMOs, as well as the newly identified ones.

The strain *E. coli* NEB 10-*β*, a phage resistant variant of the K-12 type Top10 strain, is a common host for cloning. Comparison with other strains showed that it is also the most suitable strain for BVMO expression. The genotype of this strain is *Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC).*

Expression vector and induction

In order to express a protein of interest, the gene carrying the respective DNA sequence can be introduced into *E. coli* on a plasmid vector optimized for protein expression. This is mainly influenced by the type of promoter that is used to express the gene of interest. It is important to note that the vector of choice in many cases has to be selected under consideration of the specific *E. coli* strain used, or *vice versa*. Since the host strain is a *Δara* genotype which causes the disruption of the arabinose metabolism pathway, an arabinose inducible expression system can be chosen. The pBAD plasmid is a medium-copy plasmid for araBAD promoter (P_{BAD}) controlled, recombinant protein expression in *E. coli*. L-(+)-Arabinose acts as an interaction partner of the constitutively expressed AraC protein. AraC

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acts as a repressor by binding to the respective operator sequence of P_{BAD}, thereby preventing the binding of RNA-polymerase and gene expression. Upon arabinose binding, AraC dissociates from the DNA and expression is induced.[11] L-(+)-Arabinose is a cheaply available sugar with prices roughly 100-fold lower than the allolactose analogue isopropyl *β*-D-1-thiogalactopyranoside (IPTG) commonly used for T7 promoter induction. The ideal concentrations of arabinose as well as the ideal optical density (OD_{600}) of the culture vary greatly and have to be optimized in each system. RUG optimized the expression for the pBAD based pCRE vector system (see above) and determined an optimal concentration of 0.02% (w/v) added directly at the start of the culture. This has the additional advantage to facilitate the handling of the induction procedure, since there is no need for the OD measurements to time the induction.

Expression conditions

E. coli has a temperature optimum of 37 °C and this temperature is used to grow an uninduced starting culture which is used to inoculate the main culture using 2% of that volume. While the starting culture can be grown in lysogeny broth (LB), the main culture is grown in terrific broth (TB) medium, which allows the growth of high cell densities and maximizes protein yields. The temperature for the growth of the main culture is then commonly decreased to prevent the accumulation of the recombinant protein in inclusion bodies and thus produce soluble protein.

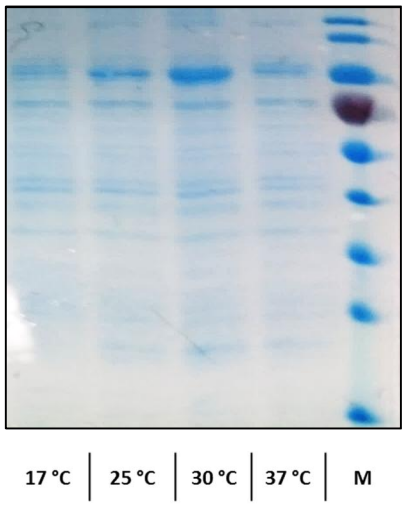

Figure 4. Expression of PockeMO at different temperatures. The marker (M) indicates the size of the protein to be just below 100 kDA which corresponds to the His6-SUMO-fused variant. All lanes are cultures of cells expressing PockeMO at different temperatures. All samples were adjusted to the same OD_{600} before applying on the SDS-PAGE.

Especially in the case of BVMOs with limited stability, temperature is a critical factor in order to produce high yields of active enzyme. Highest rates of soluble enzyme expression were observed at a

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temperature of 24 °C, which leads to about 36 h of time needed for reaching a reasonable OD_{600} of just above 10. This temperature represents the determined optimum between cell growth, protein production and retention of enzymatic activity by keeping the protein in a soluble form. Higher temperatures lead to protein degradation and accumulation in the insoluble fraction, while lower temperatures have no positive effect on expression and considerable lower the growth rate. For thermostable BVMOs, a higher expression temperature may lead to better results. This is the case for PockeMO based on an expression screen carried out at different temperatures (Figure 4).

Enzyme purification

Using purified enzyme on industrial scale is only feasible when a product is very high-added value, or when the purification procedure is very easy and cheap. For most of the potential applications of the bioconversions using BVMOs, these cases are unlikely to apply. However, for investigation purposes, reliable and quantifiable results can only be achieved with purified proteins, in order to exclude the influence of contaminants on the measurements. It is therefore anyway desirable to have a reliable purification method established.

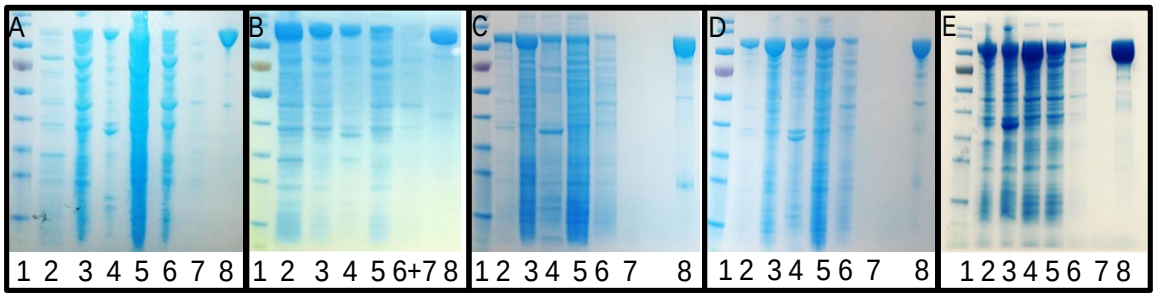

Figure 5. SDS-PAGEs of typical expression and purification of PTDH-BVMO proteins. High amount of BVMO in the cultures and in the soluble fractions can be observed, and the enzyme is typically more than 90% pure. A) CPDMO, B) PockeMO, C) AcCHMO, D) RhCHMO, E) TmCHMO.
1 marker, 2 culture, 3 pellet, 4 CFE, 5 flow through, 6 wash I, 7 wash II, 8 elution

PTDH has an N-terminal His6-tag which was used to purify the enzyme using Ni^{2+}-Sepharose affinity chromatography. In brief, cells were washed in buffer, sonicated and cleared from cell debris to create cell free extract (CFE). This was applied to Ni^{2+}-Sepharose beads, washed and protein in high purity was eventually eluted under high salt conditions and then desalted via size exclusion. RUG has demonstrated that with this method all the ROBOX BVMOs can be reliably and efficiently purified (Figure 5). Typical yields range from 75 to 250 mg pure protein per liter of culture, depending on the enzyme and the specific batch. Due to the unavoidable loss during the purification process, active

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enzyme content in whole cells or CFE is even higher. A detailed protocol for enzyme expression and purification can be found in the Material and Methods section below.

Upscaling

As a first step towards upscaling, RUG has furthermore produced two of the enzymes on a gram scale. AcCHMO and RhCHMO were purified from expression cultures of five liters, representing an upscaling of factor 10 (Figure 6). It was shown that without optimization, protein yields were the same as for smaller scale and the resulting protein was lyophilized and provided to the ROBOX partner UAB, where enzymatic activity after immobilization was demonstrated. Using amongst other input from RUG, DSM has developed its in-house set of *E. coli* based expression systems for all ROBOX BVMOs in order to produce the enzymes at large scale.

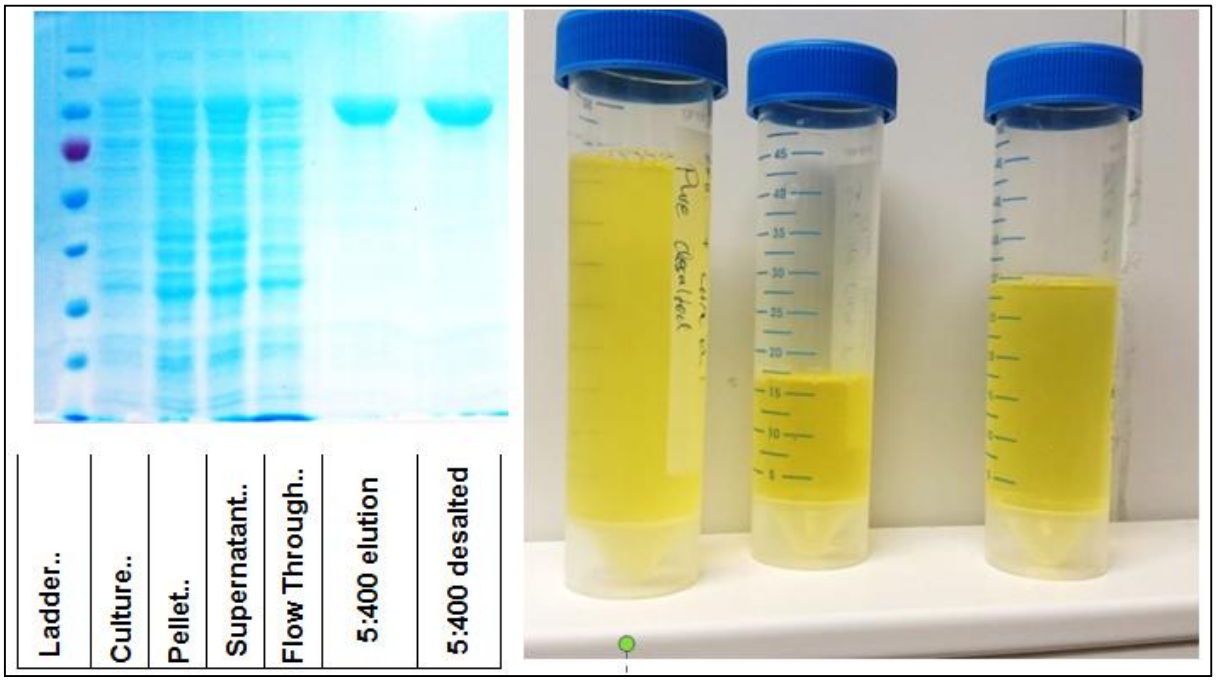

Figure 6. Gram scale production of RhCHMO. A) SDS-PAGE of samples taken during the purification. The eluted and desalted protein fractions show high purity in the upscaled process. B) Photograph of the large amounts of pure protein solution obtained after purification.

BVMO expression systems for fermentative production

To enable the upscaling and industrial implementation of BVMO reactions stable and scalable systems for the fermentative production of these enzymes are required. Based on the input of other consortium members and literature study, 16 BVMO enzymes of high interest for oxygenation of bulky and cyclic ketones were identified (table X).

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Table 1. Overview of BVMOs selected and cloned within the ROBOX project

Name	Origin	Comment / accession number
CoCPMO	*Comamonas* sp. NCIMB 9872	Q8GAW0
AcCHMO	*Acinetobacter* sp. NCIMB 9871	BAA86293
PockeMO	*Myceliophthora thermophila* C1	From Dyadic/Genencor and RUG
PlCHMO	*Parvibaculum lavamentivorans*	A7HU16
ObCHMO	*Oceanicola batsensis*	A3U3H1
MtEtaA	*Mycobacterium tuberculosis H37Rv*	NP_218371
PpOTEMO	*Pseudomonas putida* ATCC 17453	3UOV_A
TfPAMO	*Thermobifida fusca*	1W4X_A
PfHAPMO	*Pseudomonas fluorescens*	Q93TJ5
RrSTMO	*Rhodococcus rhodochrous*	4AP1_A
PsCPDMO	*Pseudomonas* sp. HI-70	BAE93346
RrCDMO	*Rhodococcus ruber* SC1	A7HU16
RhCHMO	*Rhodococcus* sp. HI-31	C0STX7
TmCHMO	*Thermocrispum municipale*	WP_028849141.1
AtCHMO	*Amycolatopsis thermoflava*	From genome inspection
SrCHMO	*Saccharopolyspora rectvirgula*	From genome inspection

The encoding genes were ordered as synthetic DNA and cloned to the up-scalable *E. coli* expression vectors pDSM242 (DSM proprietary pBAD based system) as well as pD871 (pRHA based DNA2.0 system). The second being a freely usable vector system, which also enables stable high-cell density fermentations and will be made available to the consortium members as well.

Transformation of competent *E. coli* expression strains RV308 with the pD871 constructs and RV311 with the pDSM242 plasmids, respectively, were successful and cultivations of the resulting expression strains conducted on small scale shake flask to produce the various BVMOs recombinantly. Induction of gene expression was triggered by addition of 0.02% (w/v) L-arabinose or L-rhamnose for pDSM242 and pD871, respectively. SDS-PAGE analysis revealed soluble expression of 13 out of 16 BVMOs. Activity determination of the BVMOs in the soluble fractions is currently ongoing. For those BVMOs which do not show soluble and active expression, optimization of cultivation and induction conditions will be performed.

The recombinant *E. coli* expression strain RV311 pDSM242-CDMO for Cyclododecanone Monooxygenase from *Rhodococcus ruber* SC1 (RrCDMO)[12-13] was already present at DSM. This strain was fermented in a 10 l-scale high cell density fermentation, which yielded approximately 3.5 kg recombinant *E. coli* cells. The biomass yield was wet weight 357 g wet cell weight or 74 g dry cell weight per kg fermentation broth, respectively. The specific activity for macrocyclic ketone oxidation was 0.34 U/mg total soluble protein. SDS-PAGE analysis revealed high levels of overexpression in the

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soluble fraction (figure 7). RrCDMO cell-free extract is used by DSM and was supplied to DTU and UM in the target reactions of WP3.

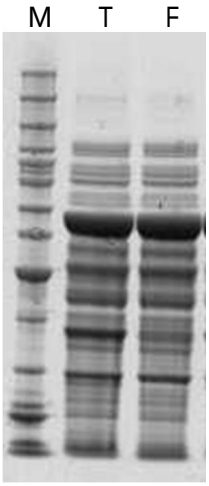

Figure 7. SDS-PAGE analysis of fermentative RrCDMO production. M: molecular weight marker, T: cell-free extract total protein fraction, S: cell-free extract soluble protein fraction.

Conclusions

RUG and DSM have successfully demonstrated the applicability and efficiency of producing ROBOX BVMOs in *E. coli*. As self-sufficient NADPH recycling fusion proteins that sustain full enzymatic activity, high yields of protein could be obtained in soluble form and the activity was confirmed for all enzymes. The newly discovered and more robust BVMOs TmCHMO and PockeMO were produced in the same way as their less stable counterparts which demonstrates the reliability and reproducibility of the production procedure. Nevertheless, these enzymes are also suitable for higher temperature expression, thereby decreasing the time required for production. On larger scale, more investigations on the robustness of these enzymes can be performed and exploited.

A vector backbone for simple cloning of any BVMO to this system and the constructs with all ROBOX BVMOs are available at RUG for the entire consortium and have already been shared with several members. The expression strain of *E. coli* is easily available and constructs transformed in this strain can be – and already were – shared with the consortium, together with protocols for cell growth and induction. Notably, expression conditions such as the use of TB medium are obviously optimized for laboratory scale and should be seen as a starting point for potential optimization on large scale production (second generation). For detailed analytics of enzyme activity and/or stability, purification protocols as well as easy to use enzyme assay protocols are also available and were already shared

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with several members of the consortium. For large scale fermentative production two stable and scalable expression systems are available and fermentatively produced BVMO is being evaluated in work package 3.

Material and Methods

Cloning

PockeMO was amplified from the existing pET-C1-BVMO-SUMO vector (codon optimized, ordered gene, cloned into the pET-SUMO vector), using a forward primer to introduce a C-terminal XhoI restriction site and a vector specific reverse primer covering the 3'-UTR that harbored a HindIII restriction site. The PCR product and a plasmid isolation of pCRE-AcCHMO vector were digested overnight with XhoI and HindIII (New England Biolabs). The vector was then digested overnight with alkaline phosphatase (Roche). The PCR product was purified and the backbone of the digested vector was cut from an agarose gel and also purified. The two fragments were ligated with T4 ligase (Promega) for 6 h and the 5 µl of the ligation were used to transform 100 µl of competent *E. coli* NEB 10-*β* cells. Unless otherwise indicated, all enzymes were used according to manufacturer's instructions. Successful cloning was confirmed by sequencing (GATC-Biotech, Germany).

Protein expression and purification of CRE-BVMO constructs

A baffled Erlenmeyer flask (200 ml – 5 L) was filled up to 10% of the volume with TB medium. 5ml of an overnight culture were used to inoculate the main culture and 0.02% L-arabinose and 50 µg/ml ampicillin were added before the flask was incubated at 24 °C for 36 h with shaking. Cells were harvested by centrifugation (6,000 × g for 15 min at 4 °C, JA-10.5 rotor, Beckman Coulter) and resuspended in 50 mM Tris-HCl buffer, pH 7.5 (5x volume of the cell pellet). Cells were disrupted by sonication (3 s ON, 3 s OFF, 70% amplitude) and subsequently subjected to centrifugation (15,000 × g for 45 min at 4 °C, JA-17 rotor, Beckman Coulter). The cell free extract was filtered and loaded on 2 mL of Ni^{2+}-Sepharose HP (GEHealthcare) pre-equilibrated with the Tris-HCl buffer and incubated for 1 h at 4 °C with rotating. Then, the column was washed with at least three column volumes of 50 mM Tris-HCl pH 7.5, followed by at least three column volumes of 50 mM Tris-HCl pH 7.5 containing 5 mM imidazole. The protein was eluted using 50 mM Tris-HCl pH 7.5 with 500 mM imidazole. Fractions containing yellow protein were applied on a preequilibrated Econo-Pac 10DG desalting column (Bio-Rad). The desalted protein was flash frozen in liquid nitrogen and stored at -80 °C.

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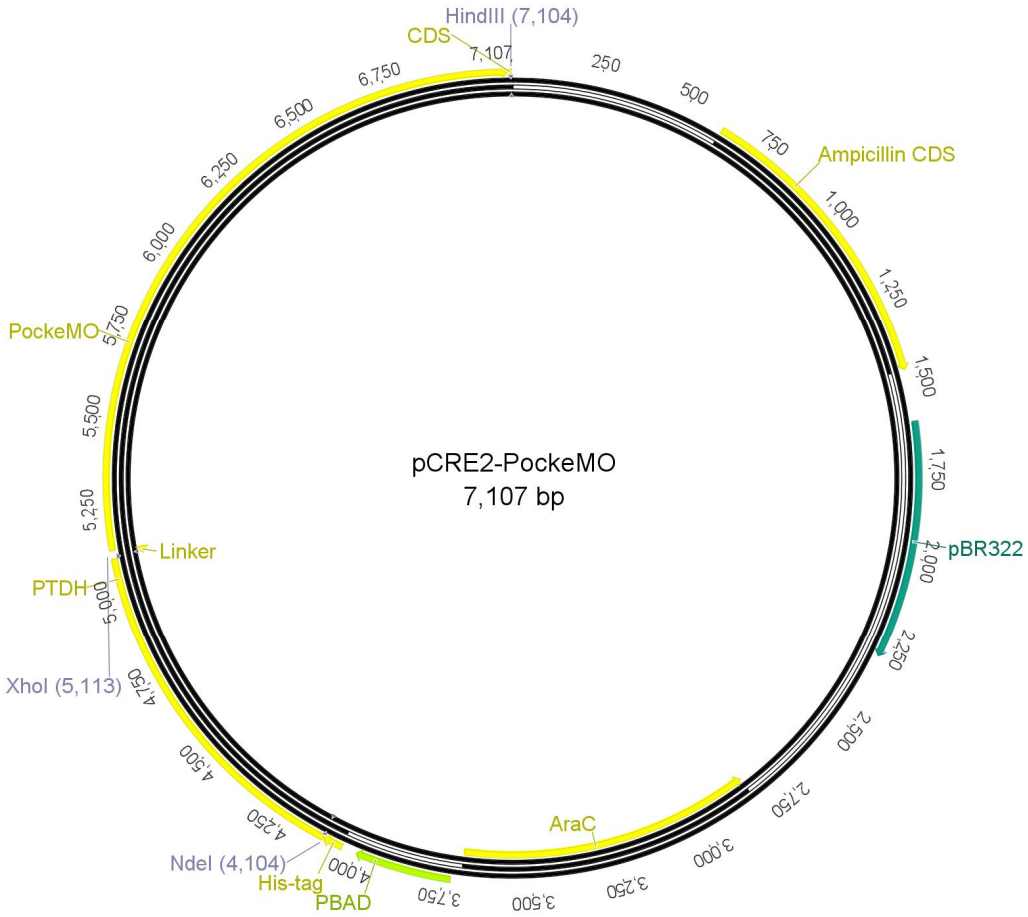

Figure 8. Vector map pCRE-PockeMO illustrating the arrangement of His6-PTDH-Linker-PockeMO coding sequences under control of the P_{BAD} promoter. The ampicillin and araC genes are located separately and the origin of replication is pBR322. All CRE-BVMO vectors were constructed in this way.

Activity, temperature and pH optimum assays

Enzyme activity was measured in 50 mM Tris-HCl pH 7.5 at 25 °C by spectrophotometrically following the decrease in coenzyme absorption at 340 nm ($\varepsilon_{NADPH,\,340}$ = 6.22 $mM^{-1}\,cm^{-1}$). Hydrophobic substrate solutions were solubilized using 1,4-dioxane as a co-solvent. Enzyme and NADPH were kept on ice before adding to the substrate and the measurement was started immediately after mixing.

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