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Membrane Protein Expression in *E. coli*

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ABSTRACT

Membrane-attached cytochrome P450 monooxygenases play a central role in biosynthesis of important natural compounds and are involved in drug metabolism. Therefore, these catalysts are very attractive for industrial application. However, a number of limitations have restricted their use in industrial processes including substrate specificity, the need for a complex system of cofactor regeneration, low activity and poor stability. Codon optimization, the co-expression of a P450-reductase and the availability of co-factors (NAD(P)H) may be prerequisites for functional expression of recombinant P450-enzymes.

E. coli was evaluated as expression system regarding its ability to heterologously express cytochrome P450 enzymes at high yield as well as functionality and to overcome the major limitations including expression level, co-factor recycling and enzyme stability by engineering and optimization strategies. Activities were tested establishing different assays, including in vivo and in vitro approaches.

ZUSAMMENFASSUNG

Membrangebundene Cytochrom P450 Monooxygenasen spielen eine zentrale Rolle in der Biosynthese wichtiger Naturstoffe und sind am Metabolismus unterschiedlicher Arzneimittel beteiligt. Deshalb stellen sie attraktive Katalysatoren für industrielle Anwendungen dar. Eine beträchtliche Zahl von Problemen sorgt jedoch für limitierte Einsatzmöglichkeiten in industriellen Prozessen, wie zum Beispiel die Substratspezifität, die Notwendigkeit eines komplexen Systems zur Cofaktor-Regenerierung, geringe Aktivität und schlechte Stabilität. Codon Optimierung, die Co-Expression einer P450-Reduktase und die Verfügbarkeit von Cofaktoren (NAD(P)H) sind möglicherweise Grundvorrausetzungen funktionaler Expression rekombinanter P450-Enzyme.

Als Expressionssystem wurde *E. coli*, hinsichtlich seiner Fähigkeiten, Cytochrome P450 Enzyme rekombinant sowohl in großen Mengen als auch funktional zu exprimieren, getestet. Hierfür sollten die grundlegenden Limitierungen wie Expressionslevel, Cofactor-Regenerierung und Enzymstabilität durch Engineering Strategien und Prozessoptimierungen überwunden werden. Aktivitäten wurden anhand unterschiedlicher, etablierter Assays gemessen, wobei sowohl in vivo- als auch in vitro-Tests eine Rolle spielten.

INTRODUCTION

Membrane-attached cytochrome P450 monooxygenases (CYPs) belong to the superfamily of heme-containing enzymes and form a large, ubiquitous enzyme family with high sequence diversity. They are involved in the biosynthesis of important natural compounds, but also constribute to detoxification mechanism in primary and secondary metabolic pathways of microorganisms, plants and animals[1].

Due to their ability to regio- and enantioselectively oxidize a wide variety of compounds; CYPs are attractive and versatile biocatalysts for industrial application

Our researches focus on a single cytochrome P450 enzyme, *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO), which naturally catalyses the biosynthesis of a potent phytoalexin in Egyptian henbanes by successively oxidizing the C-2 atom of premnaspirodiene to give solavetivone. Therefore, HPO also seems to be an appealing biocatalyst for the generation of nootkatone, which is the most important and expensive flavour of grapefruitshttp://en.wikipedia.org/wiki/Nootkatone_-_cite_note-0.[2] Valencene in contrast, is a cheap and easily available fragrance of oranges, upon oxidation at the C-2 atom valencene gives the two intermediate stereoisomers cis- and trans-nootkatol, and finally results in nootkatone in a premnaspirodiene-related oxidation mechanism.[3] All substances involved, valencene, cis-, trans-nookatol and nootkatone, are believed to be extremely volatile[4], which in combination with their high hydrophobicity exacerbates conversion conditions.

Figure 1: *Hyoscyamus muticus* (Egyptian henbane)

The active side of all CYPs contains a heme iron center that is tethered to the enzyme via a thiolate ligand derived from a cysteine residue. This region is highly conserved among all cytochrome P450 enzymes. For conversion, the substrate is bound in close proximity to the heme group, which induces a change in the conformation of the active side. This confirmation change triggers a cytochrome P450 reductase to transfer an electron from the required cofactor NADPH. Due to the electron- transfer, the ferric heme iron is reduced to the ferrous state. Then, molecular oxygen binds covalently to the distal axial coordination position of the heme center. A second oxygen is transferred via the electron-transport system from the corresponding reductase, reducing the dioxygen adduct to a negatively charged peroxo group, that is rapidly protonated from surrounding amino acid side chains, releasing a water molecule and forming a highly reactive iron(V)-oxo species. This activated compound causes finally the oxidation of the vicinal bound substrate, which is released in the next step.[5]

To provide HPO with an electron-transfer system, a cytochrome P450 reductase (CPR) derived from *Arabidopsis thaliana* was cloned into a pMS470 expression vector upstream of HPO including an own Shine-Dalgarno sequence.[6]

Heterologous expression of insoluble cytochrome P450 enzymes derived from eukaryotic cells in *E. coli* has proven to be difficult in the past. Although *E. coli* shows indisputable drawbacks in correct protein folding because of its easy cellular machinery, it has always been the system most desirable in terms of genetic tractability, fermentation, well-known characteristics, low costs, ease of use, and amenability to scale up.[7] For these reasons, some improvements were implemented to express CPR and HPO in *E. coli*. Codon optimizations of HPO and CPR were performed for *E. coli* and also *P. pastoris* by T. Wriessnegger and DSM Pharma Chemicals to increase functional expression of the genes in the corresponding host systems.

Membrane proteins are attached to, or associated with the membranes of a cell or an organelle by one or more transmembrane domains, that are highly hydrophobic. Truncation of single transmembrane domains led to high-level expression and activity of microbial P450 enzymes, due to better solubility if heterologously expressed in *E. coli*.[8]

For activity and stability of HPO, correct formation of its disulfide bonds is a prerequisite. This might be problematic in the reducing environment usually present in the cytoplasm of *E. coli*, also lacking native enzymes that catalyse thiol oxidation.[9] Although the sequences expressed carried the transmembrane domain, and are therefore believed to stick to the cell membrane, a specially engineered *E. coli*, namely Origami B (DE), was additionally tested as expression strain. The name Origami B alludes to the Japanese handcraft of folding paper to create different three dimensional objects. *E. coli* Origami B (DE) promotes a more oxidative environment in the cytoplasm because of mutations in the genes for thioredoxin (*trxB*) and

glutathione reductase (*gor*), and, therefore, favours disulfide bond formation of cysteins. This strategy already has proven successful for our group in the past. [10,11]

Another possibility to enable support protein folding is the coexpression of chaperones. Upon removal of its signal sequence, disulfide bond isomerase C (DsbC) is localized to the prokaryotic cytoplasm. DsbC, a V-shaped homodimer, is responsible for breaking incorrectly formed disulfide bonds by performing a nucleophilic attack with its active residue Cys98, which results in an unstable disulfide bond between the chaperone and the protein. When another DsbC molecule or a cystein of the substrate molecule is attacking, DsbC is released and the enzyme can find its functional three dimensional structure.[11] To check for a potentially beneficial effect of DsbC co-expression on HPO activity, the respective gene was positioned on the same vector as HPO and CPR upstream of both sequences and featuring its own Shine-Dalgarno sequence for ribosomal binding.

In most cells, 5-aminolevulinate synthase directly synthesises the heme precursor δ-ALA in the course of citric acid cycle by conversion of succinyl CoA and Glycine. This constitutes the first step of porphyrin synthesis, but is absent in the metabolism of *E. coli*. Media supplementation with the heme precursor δ-aminolevulinic acid in the culture increases heme synthesis by *E. coli* leading to the expression of the recombinant enzymes in their active form[12]. For similar reasons, cell cultures have been supplemented with $FeSO_4$ to encourage heme center formation.**Fehler! Verweisquelle konnte nicht gefunden werden.**[13] Another influencing factor tested was the choice of fermentation media. Cells were grown under otherwise similar conditions in LB- and TB-medium. TB stands for terrific broth and is a much more complex medium mostly preferred in terms of heme-containing protein biosynthesis.

For bioconversion, different in vivo and in vitro assays were developed.

For in vivo assay, a whole cell bioconversion was performed. First, HPO and CPR expression was induced. Then, substrate was added at a certain cell density. A modification of this assay represented the use of resting cells in a buffered system supplied with only traces of nutrients. Another strategy chosen was the implementation of an aqueous-organic two-liquid-phase system, which was reported to represent a powerful tool for biotransformations of toxic organic compounds. This approach is used for high overall concentrations of hydrophobic substrates by regulating substrate and product concentrations in the aqueous phase and enables *in situ* product recovery into the organic phase. [14] Additionally, literature pronounces a reduction of multi-oxygenated by-products.[13] Therefore, dodecane was added during biotransformation, which distinguishes itself from other organic solvents by high biocompatibility with *E. coli*.

The advantage of in vivo over in vitro assays is the natural recycling system of cofactors within the cell. After regenerating HPO by transferring electrons, the reductase CPR needs to be recycled itself, which is facilitated by the cofactor NADPH.

For in vitro assays, cell free extracts of expression strains were used to convert valencene. To protect whole cell lysates from degradation by proteases, phenylmethylsulfonyl fluoride (PMSF) was added, which is a protease inhibitor that reacts with serine residues and generally inhibits trypsin, chymotrypsin, thrombin, papain and similar proteases.

An effect that comes along with cell disruption is a decrease in concentration of required cofactors that are naturally available in the intact cell at any time. To overcome this major limitation of the in vitro assay, Mg^{2+} and NADPH were added to support. For industrial applications, the use of large amounts of expensive cofactors is not appreciated. Therefore, a NADPH-regeneration system was established by adding glucose dehydrogenase (GDH) and its substrate glucose to the in vitro reaction. Reduction and, therefore, regeneration of $NADP^{+}$ takes place due to the transfer of a proton withdrawn from glucose by GDH. As a result, gluconic acid is formed and the system needs to be well buffered for pH stabilization.**Fehler! Verweisquelle konnte nicht gefunden werden.** **Fehler! Verweisquelle konnte nicht gefunden werden.**[3]

MATERIALS AND METHODS

Some of the following materials and methods have been provided by Tamara Wriessnegger from ongoing work.

STRAINS, CELL CULTURES AND SUPPLIERS

TABLE 1: *E. COLI* STRAINS

Strain	Genotype	Source
Top10F'	F'{*lac*I^{q} Tn*10* (Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 *lac*ZΔM 15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U galK *rps*L *end*A1 *nup*G	Invitrogen
Origami B (DE3)	F^{-} *omp*T *hsd*S$_{B}$(r$_{B-}$m$_{B-}$) *gal dcm lacY1 ahpC* (DE3) *gor522*::Tn*10 trxB*	Novagen
BL21 Star™(DE3)	F^{-} *omp*T *hsd*S$_{B}$(r$_{B}^{-}$m$_{B}^{-}$) *gal dcm rne*131 (DE3)	Invitrogen
BL21 Gold (DE)	F^{-} *ompT hsdS*(r$_{B}^{-}$m$_{B}^{-}$) *dcm*$^{+}$ Tet$_{r}$ *gal* λ(DE3) *endA* Hte	Invitrogen

TABLE 2: EXPRESSION PLASMIDS

Plasmid	Features	Source
pMS470	Standard expression vector with *tac* promoter and ampicillin resistance	Reference[15]
pMS470_dsbC_linker	pMS470 containing the sequence coding for truncated *dsbC*	A. Braun

SYNTHETIC GENES

All genes were synthesized by GENEART AG, Germany. Codon optimizations of wild type sequences had been carried out by T. Wriessnegger and DSM.

TABLE 3: SYNTHETIC GENES

Gene	Features
CPR1	native *CPR* sequence, synthetic gene with TMD
CPR2	*CPR* sequence with TMD, codon opimized for *P. pastoris* by DSM
CPR3	CPR sequence with TMD, codon opimized for *P. pastoris*, by T. Wriessnegger
HPO4	native *HPO* sequence, synthetic gene with TMD
HPO5	*HPO* sequence with TMD, codon opimized for *P. pastoris*, by DSM
HPO6	*HPO* sequence with TMD, codon opimized for *P. pastoris*, by T. Wriessnegger
CPR7	native but truncated *CPR* sequence, synthetic gene
CPR8	truncated *CPR* sequence, codon opimized for *E. coli*, by DSM
CPR9	truncated *CPR* sequence, codon opimized for *E. coli*, by T. Wriessnegger
HPO10	native but truncated *HPO* sequence, synthetic gene
HPO11	truncated *HPO* sequence, codon opimized for *E. coli*, by DSM
HPO12	truncated *HPO* sequence, codon opimized for *E. coli*, by T. Wriessnegger

PRIMERS

TABLE 4: SEQUENCING PRIMERS

Primer	Sequence	Primer	Sequence
Fw_pMS470-DsbC	5' AAATGACCAGCGGTAAATAA	FwHPO_DSM3	5' CAATGGTTCAGATGATGCGT 3'
RvHPO1	5' CTGTTCCTTGAACACTTTCC 3'	RvCPR_DSM1	5' CAACGTTGGATTCCATAGAT 3'
FwHPO2	5' GCCGGTTAACTTTACTGAAA 3'	FwCPR_DSM2	5' ACACTGCTGTTATCCCGGAA 3'
RvHPO2	5' TACTTCTGCTTGAGCTTTGG 3'	RvCPR_DSM2	5' TTCATCCAGGTGGAGCAAAC 3'
FwHPO3	5' CTATGGTGCAAATGATGAGA 3'	FwCPR_DSM3	5' CTCGCGTTCACGTAACTTCT 3'
Rv_pMS470-DsbC	5' TTTAATCTGTATCAGGCTGA 3'	RvHPO_TWopt1	5' CCAGGCCGATTACTTCTTTG 3'
FwCPRend	5' AAGAAACTTCAAACCGAAGG 3'	FwHPO_TWopt2	5' GCCAGTGAATTTTACCGAAC 3'
RvCPR1	5' CCACATTTGATTCCATTGAT 3'	RvHPO_TWopt2	5' CTTTGAAGGCTTCGCGAACT 3'
FwCPR2	5' ATACAGCTGTTATTCCTGAA 3'	FwHPO_TWopt3	5' AACCCGACCATCTTAGCTAA 3'
RvCPR2	5' TCATCCACGTAGAACACACA 3'	RvCPR_TWopt1	5' GGTTGCAACACTTTTATCAT 3'
FwCPR3	5' GTAGAGTTCATGTTACATCCG 3'	FwCPR_TWopt2	5' ATGGAAAGAATCGCTGTGGA 3'
RvHPO_DSM1	5' CTGCTGTTTGAATACTTTAC 3'	RvCPR_TWopt2	5' AAGGCTGAGGTCACATGCAC 3'
FwHPO_DSM2	5' ACCGGTTAACTTCACTGAAC 3'	FwCPR_TWopt3	5' GATGAATACAGCCAGTGGAT 3'
RvHPO_DSM2	5' GAACTTCAGCCTGCGCTTTC 3'		

TABLE 5: PRIMERS USED FOR FUSION OF TAGS

Primer	Sequence
CPR4 Fw	5' GAATTC AGCGGTAAATAAGGATCC 3'
CPR4 Rv	5' AAGCTTGCGGCCGCTCGAGTTATTACAAATCCTCTTCAGAAATCAATTTTTGTTCCCAGACATCTCTGAGGTATCT 3'
HPO1 Fw	5' TTCAAACCGAAGGAAGATACC 3'
HPO1 Rv	5' AAGCTTGCGGCCGCTTATTACTTATCGTCGTCATCCTTGTAATCCTCTCGGGAAGGTTGATAAGG 3'
CPR5 Fw	5' GAATTC AGCGGTAAATAAGGATCC 3'
CPR5 Rv	5' AAGCTTGCGGCCGCTCGAGTTATTACAAATCCTCTTCAGAAATCAATTTTTGTTCCCAGACATCTCTCAAGTATCT 3'
HPO2 Fw	5' AAGAAATTGCAAACCGAAGG 3'
HPO2 Rv	5' AAGCTTGCGGCCGCTTATTACTTATCGTCGTCATCCTTGTAATCTTCTCTGGATGGTTGGTATGG 3'
CPR6 Fw	5' GAATTC AGCGGTAAATAAGGATCC 3'
CPR6 Rv	5' AAGCTTGCGGCCGCTCGAGTTATTACAAATCCTCTTCAGAAATCAATTTTTGTTCCCAGACATCTCTCAGATATCT 3'
HPO3 Fw	5' AAGAAGTTGCAAACCGAAGG 3'
HPO3 Rv	5' AAGCTTGCGGCCGCTTATTACTTATCGTCGTCATCCTTGTAATCCTCTCGGGAAGGTTGGTAAGG 3'
P7: FwCPRend	5' AAGAAACTTCAAACCGAAGG 3'
P62: Rv_HPOtrunc_FLAG	5' CTGCAGAAGCTTGCGGCCGCTTTATTATTTATCGTCGTCATCTTTGTAATCCTCTCGGGAAGGTTGATAAGG 3'
P19: FwCPR_DSM3	5' CTCGCGTTCACGTAACTTCT 3'
P63: Rv_HPOtrunc_DSM_FLAG	5' CTGCAGAAGCTTGCGGCCGCTTTATTATTTATCGTCGTCATCTTTGTAATCTTCACGGGACGGCTGGTACGG 3'
P27: FwCPR_TWopt3	5' GATGAATACAGCCAGTGGAT 3'
P64: Rv_HPO_trunc_TW_FLAG	5' CTGCAGAAGCTTGCGGCCGCTTTATTATTTATCGTCGTCATCTTTGTAATCCTCCCGACTCGGCTGGTACGG 3'

INSTRUMENTS AND DEVICES

TABLE 6: USED INSTRUMENTS AND DEVICES

Task	Instrument/Device	Manufacturer
Absorption measurement	Microplate Reader	Molecular Devices, USA
	Microplate, 96 well, PS, U-bottom, MICROLON®	Greiner bio-one GmbH, Germany
Cell harvest	Eppendorf Tabletopcentrifuge 5810R	Eppendorf, Germany
	AvantiTM Centrifuge JA-10 and JA-25.50 rotors	Beckman Coulter™, USA
Electrophoresis	PowerPac™ Basic + Sub-Cell GT	BIO-RAD, USA
	NuPAGE® Novex® Bis-Tris Mini Gels	Invitrogen, USA
Electrotransformation	MicroPulser™	BIO-RAD, USA
	Electroporation Cuvettes (2 mm gap)	Molecular BioProducts Inc., USA
GC-FID	Hewlett Packard 6890 Series with a FID detector	Agilent Technologies, Austria
	Agilent 19091J-141 (Capillary 10.0 m x 0.1 mm x 0.1 µm film)	
GC-MS	Hewlett Packard 5890 Series II with a MS detector	Agilent Technologies, Austria
	HP 5 MS Column (Capillary 28.8 m x 0.25 mm x 0.25 µm film)	
Resuspension of pellets	Homogenizer	Satorius AG, Germany
Mixing (small volumes)	Vortex-Genie 2	Scientific industries Inc, USA
OD measurement	BioPhotometer	Eppendorf, Germany
	Cuvettes (10x4x45mm)	Sarstedt, Germany
PCR reaction	GeneAmp®PCR System 2700	Applied Biosystems, USA
Shaker for small volumes	Thermomixer comfort	Eppendorf, Germany
Shaker for cell cultures	Certomat® BS-1	Sartorius, Germany
Sonication	Sonifier 250	Branson, USA
	Syringe filters 0.2 µm	Sarsted, Germany
Western Blot	XCell *SureLock*™ Mini-Cell	Invitrogen, USA
	Nitrocellulose membrane, Hybond-ECL™	Amersham Biosciences, Sweden
	G:Box HR	Syngene, UK
Incubator (37°C)	BINDER Kühlbrutschränke	Binder GmbH, Germany
Fermentation	50 mL Greiner tube	Greiner bio-one, Germany
Plate shaker	GFL 3013	GFL GmbH, Germany

REAGENTS

TABLE 7: USED REAGENTS AND SUPPLIERS

Reagent	Supplier
5-Aminolevulinic acid hydrochloride (δ-ALA)	Sigma-Aldrich, Germany
Acetic acid (CH_3COOH)	Roth GmbH, Germany
Agar Agar	Roth GmbH, Germany
Agarose LE	Biozyme, Germany
Ampicillin (Amp)	Sigma-Aldrich, Germany
Aqua bidest.	Fresenius Kabi GmbH, Austria
Bacto™ Agar	BD, USA
Bovine Serum Albumine (BSA)	Roth GmbH, Germany
cis-, trans-Nootkatol	DSM Pharma Chemicals, The Netherlands
Dimethylsulfoxide (DMSO)	Roth GmbH, Germany
Dream Taq Polymerase [5 U/µL]	Fermentas GmbH, Germany
Dry milk powder	Low fat milk powder, bought in a local supermarket
Enzymes and adequate buffers, various	Fermentas GmbH, Germany
$FeSO_4$ heptahydrate ($FeSO_4$ * 7 H_2O)	Roth GmbH, Germany
GDH (glucose dehydrogenase, 133 U/mg)	Codexis GmbH, USA
Gene Jet™ Plasmid Miniprep Kit	Fermentas GmbH, Germany
Glucose	Roth GmbH, Germany
Glycerol	Roth GmbH, Germany
Hydrochloric acid (HCl)	Roth GmbH, Germany
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Peqlab Biotechnologie GmbH, Germany
LB (Luria-Bertani) Lennox	Roth GmbH, Germany
Magnesiumchloride heptahydrate ($MgCl_2$*7 H_2O)	Roth GmbH, Germany
Nootkatone	DSM Pharma Chemicals, The Netherlands
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Germany
Ponceau S ($C_{22}H_{16}N_4O_{13}S_4$)	Sigma-Aldrich, USA
Potassium chloride (KCl)	Roth GmbH, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth GmbH, Germany
Potassium hydrogen phosphate (K_2HPO_4)	Roth GmbH, Germany
SuperSignal West Pico Chemoluminescent Kit	Pierce, USA
T4 DNA Ligase [10 U/µl]	Fermentas GmhH, Germany
Tryptone	Roth GmbH, Germany Oxoid, USA Bacto Laboratories Pty Ltd, Australia
Tween 20	Roth GmbH, Germany
Valencene	DSM Pharma Chemicals, The Netherlands
Wizard® SV Gel and PCR Clean Up System	Promega Corporation, USA

Yeast Extract	Roth GmbH, Germany Oxoid, USA Bacto Laboratories Pty Ltd, Australia
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas GmbH, Germany
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, Germany
Primary antibodies (Anti-cMyc-Ab rabbit and Anti-FLAG-Ab mouse)	Sigma-Aldrich, USA
Secondary antibodies (Anti-Rabbit IgG and Anti-Mouse)	Sigma-Aldrich, USA

MEDIA AND BUFFERS

TABLE 8: USED MEDIA AND BUFFERS

Media/Buffer	Composition
10x TBS	30.3 g Tris (0,25 M), 87.6 g NaCl (1.5 M), pH adjusted with 1 M HCl to pH 7.5, to 1 L with ddH_2O
20x Transfer Buffer	14.5 g Tris (24 M), 72 g Glycin (1.92 M), to 500 mL with ddH_2O
Ampicillin Stock (Amp), (100 µg/mL)	100 mg/mL dissolved in ddH_2O
IPTG stock (100 mM)	1.19 g IPTG dissolved in 50 mL ddH_2O
LB-Agar	LB-Media + 20 g/L agar agar
LB-Media	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl
Ponceau S solution	0.1% Ponceau S in 5% acetic acid
Potassium phosphate buffer (KP_i buffer, pH 8.0)	214.54 g KH_2PO_4, 8.17 g K_2HPO_4, to 1 L with ddH_2O
SOC-Media	20 g/L bacto tryptone, 0.58 g/L NaCl, 5 g/L bacto yeast extract, 2 g/L $MgCl_2$, 0.16 g/L KCl, 2.46 g/L $MgSO_4$, 3.46 g/L dextrose
TB-Media	12 g/L Bacto tryptone, 24 g/L yeast extract, 4 mL/L glycerol to 1 L with ddH_2O, After separately autovlaving, addition of 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 in 100 mL ddH_2O.
TBST	999.5 mL 1x TBS, 0.5 mL Tween 20
TBST-milk	5 g dry milk powder dissolved in 100 mL TBST
Transfer solution (Western Blot)	50 mL 20x Transfer buffer, 100 mL Methanol, to 1 L with ddH_2O
Tris-HCl (100 mM, pH 7.0)	12.1 g Tris diluted in ddH_2O, pH adjusted with 1 M HCl

METHODS

GENERAL METHODS

PLASMID PURIFICATION

Isolation and purification of plasmids was done using the Gene Jet™ Plasmid Miniprep Kit based on the method of silica-gel membrane adsorption. DNA was finally eluted with 30 µL ddH_2O.

DNA GEL PURIFICATION

All DNA fragments were separated with standard 1% agarose gels at 90 V and purified according to the manual of the Wizard® SV Gel and PCR Clean Up System. 30 µL of ddH_2O were used for final DNA elution.

DETERMINATION OF DNA CONCENTRATION

Two µL of the purified DNA fragments were supplied with 6 µL ddH_2O and 2 µL 6x loading dye. The whole volume was loaded onto a 1% agarose gel and separated at 120 V. Concentrations were estimated by comparing band intensities to those of a DNA ladder standard.

ELECTROCOMPETENT CELLS

Several colonies of the respective strain were picked from a freshly grown LB-Amp plate to inoculate 30 mL of LB media for overnight cultures at 37°C and 220 rpm. Then, 500 mL of LB media in 2 L baffeled flasks were inoculated with 5 mL of the overnight culture and incubated at 37°C and 170 rpm until an OD_{600} between 0.7 and 0.9 was reached. Before harvesting, cells were chilled on ice for 30 min.

Cells were centrifuged in sterile, pre- chilled 500 mL centrifuge bottles at 2,000xg and 4°C for 15 min. After discarding the supernatants, the pellets were resuspended in 500 mL pre- chilled ddH_2O, and centrifuged as above. This step was repeated. Then, the cells were resuspended in 35 mL of pre- chilled and sterile 10% glycerol, and centrifuged at 4,000xg and 4°C for 15 min. Supernatants were discarded and the pellets finally resuspended in 1 mL of cold, sterile 10% glycerol. 80 µL aliquotes of electrocompetent cells in Eppendorf tubes were either stored on ice for instant use or frozen in liquid N_2 for storage.

ELECTROTRANSFORMATION

For electrotransformation, 80 µL of electrocompetent cells were mixed with plasmid DNA (2-4 µL) in pre- chilled electroporation cuvettes and pulsed with the EC2 program for 5-6 ms at 2.5 kV. Immediately after transformation, 920 mL SOC-media were added and the whole broth was incubated on a thermomixer at 650 rpm and 37°C for 1 hour. Defined amounts of the cells were plated on LB-Amp plates and incubated at 37°C overnight.

DNA RESTRICTION

For preparative restriction cuts, the full volume of a single plasmid purification event was mixed with 3 µL of restriction enzyme(s) and appropriate buffer at the recommended concentration. The mixtures were incubated for at least 3 h at 37°C.

For control restriction cuts, 3 µL of the purified plasmids were mixed with 0.5 µL of restriction enzyme, 1 µL of recommended buffer (10x) and 5.5 µL of ddH_2O. The reaction was incubated for 2 h at 37°C.

DNA LIGATION

Vector backbone and insert were used at 1:3 molar ratio. 1 µL T4-ligase [10 U/µL], 2 µL ligase buffer (10x) and the volume adjusted to 20 µL with ddH_2O. A useful tool for calculation of size-dependant insert amounts was the online ligation calculator offered by http://www.insilico.uni-duesseldorf.de/Lig_Input.html.

Reactions were performed at 19°C overnight or at room temperature for two h. Afterwards, mixtures were heated to 65°C for 10 min to inactivate the enzyme, followed by desalting for 30 min on a 0.025 µm Millipore filter floating on ddH_2O. Solutions were transferred to sterile Eppendorf tubes and immediately used for electrotransformation or stored at -22°C.

RECOMBINANT EXPRESSION OF HPO AND CPR

GENERATING OF RECOMBINANT VECTORS

The following genes had been cloned into a pMS470 vector backbone by T. Wriessnegger.

TABLE 9: VECTORS PROVIDED BY T. WRIESSNEGGER

Optimization done for	Vector with genes	Short name
P. pastoris	pMS470_CPR4_HPO1	CPR4-HPO1
	pMS470_dsbC_CPR4_HPO1	CPR4-HPO1 DsbC
	pMS470_CPR5_HPO2	CPR5-HPO2
	pMS470_dsbC_CPR5_HPO2	CPR5-HPO2 DsbC
	pMS470_CPR6_HPO3	CPR6-HPO3
	pMS470_dsbC_CPR6_HPO3	CPR6-HPO3 DsbC
E. coli	pMS470_CPR10_HPO7	CPR10-HPO7
	pMS470_dsbC_CPR10_HPO7	CPR10-HPO7 DsbC
	pMS470_CPR11_HPO8	CPR11-HPO8
	pMS470_dsbC_CPR11_HPO8	CPR11-HPO8 DsbC
	pMS470_CPR12_HPO9	CPR12-HPO9
	pMS470_dsbC_CPR12_HPO9	CPR12-HPO9 DsbC

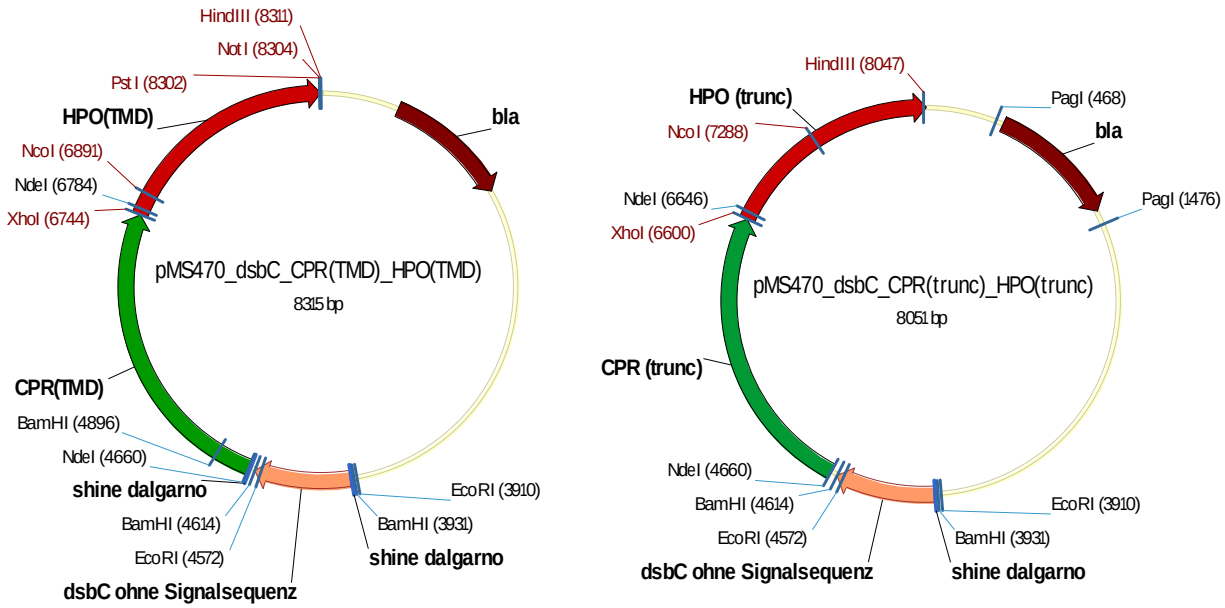


FIGURE 2: DIFFERENT VECTOR TYPES OPTIMIZED FOR *P. PASTORIS* (WITH TRANSMEMBRANE DOMAIN, TMD), OR OPTIMIZED FOR *E. COLI* (TRUNCATED, TRUNC)

All vectors had also been generated without *dsbC*, which was cut out by its *BamH*I restriction sites.

Genes optimized for *P. pastoris* code for an N-terminally linked transmembrane domain, indicated by the attachment "TMD". The truncation of this domain in case of genes optimized for *E. coli* is labeled with "trunc".

FERMENTATION

For overnight cultures, 15 mL of LB or TB medium in a 50 mL Greiner tube containing 100 µg/mL of ampicillin were inoculated with colonies freshly grown on LB-Amp plates. After an overnight incubation of 24 h at 20°C and 160 rpm, two to five mL there of were used to inoculate 100 mL of main culture in 300 mL shake flasks. The main cultures were incubated at 20°C and 160 rpm until an OD_{600} of 0.5 to 0.7 was reached, then induced by 0.1 mM IPTG and grown overnight. Simultaneously with induction, δ-ALA (5-aminolevulinic acid) and FeSO4 heptahydrate were added to final concentrations of 0.5 mM and 2 µM, respectively.

CELL HARVEST

For cell harvest, each culture was harvested in a 50 mL Greiner tube at 3,500xg for 15 min at 10°C. The supernatants were discarded and the cell pellets were resuspended in 5 mL of 50 mM KP_i buffer, 1 mM $MgCl_2$, pH 8.0. The tubes were spun again at 3,500xg for 15 min at 10°C. Finally, the pellets were weighed and resuspended in 20 mL of 50 mM KP_i buffer, 1 mM $MgCl_2$, pH 8.0.

CELL DISRUPTION

The cell suspensions were transferred to ice- water pre- cooled pulping bottles and sonicated for 5 min with 80% duty cycle and output control level 8. Whole cell lysates were immediately put on ice and if required centrifuged in 50 mL centrifuge tubes at 75,000 g and 4°C for 1 h. (For in vitro assays, cell homogenates were directly used, without centrifugation.) Cell free extracts were sterile filtered (0.2 µM filters) and stored at 4°C.

RESUSPENSION OF PELLETS

Insoluble cell fractions were resuspended in 1 mL of 50 mM KP_i buffer (1 mM $MgCl_2$, pH 8.0), by the use of Dounce homogenizers and finally frozen at -22°C.

OD_{600} VERSUS CELL DRY WEIGHT

To determine cell dry weights indirectly by OD_{600} measurement, a calibration curve was generated. Therefore, a cell pellet of *E. coli* BL21 carrying pMS470_CPR4_HPO1 was taken from a freshly grown LB-Amp plate to inoculate 20 mL of LB-Amp medium. After overnight incubation at 25°C, 10 mL were taken to inoculate 110 mL of main culture. The next day, OD_{600} was measured and the cell pellet resuspended in 6.26 mL buffer. Again, OD_{600} was measured and certain numbers of OD_{600} units were pipetted into preweighed cuvettes (in dublicate). These cuvettes were dehydrated for two days at 65°C and dry cells were weighed.

QUANTIFICATION AND ANALYSIS OF PROTEINS

BIORAD PROTEIN ASSAY

Protein concentrations were determined based on the method of BioRad according to a 96 well plate protocol established by A. El-Heliebi. Therefore 200 µL of 1:5 diluted BioRad reagent were transferred into each well of a standard microtitre plate. Cell lysates or resuspended insoluble cell fractions were diluted between 1:5 and 1:20 with ddH_2O and 10 µL added to each well. The plate was mixed thoroughly for 10 min on a shaker and absorptions were recorded at 595 nm in a Microplate reader. Bovine serum albumin (BSA) was used for calibration between 0 and 20 mg protein/mL.

SDS-PAGE

SDS-PAGE was performed according to the manual of NuPage®, Invitrogen. Before loading onto the ready-to-use-gels, cell homogenates, supernatants or resuspended cell pellets containing 5 µg protein were mixed with 2.5 µL LDS sample buffer (4x), 1 µL reducing agent (10 x) and 6.5 µL of ddH_2O. The mixture was denatured at 70°C for 10 minutes. As standard, 10 µL of protein ladders were used. Gel was run at 110 mA for 50 min. SDS-PAGE gels were placed in a well sized plastic box, covered with Coomassie blue staining solution and tightly closed with a lid. Staining was carried out overnight under moderate shaking at room temperature. Gels were destained with 10% acetic acid, which was repeatedly exchanged until protein bands were clearly seen.

CLONING OF FLAG-TAGGED AND *MYC*-TAGGED HPO AND CPR GENES

TAGGING OF GENES BY PCR

Templates for tagging genes by PCR were isolated from *E. coli* Top10F' strains (Table 10).

TABLE 10: TEMPLATES, PRIMERS AND CONDITIONS FOR TAGGING HPO AND CPR WITH RELATED PAIR OF PRIMERS, SPECIFIC ANNEALING TEMPERATURE AND NAME OF PRODUCT

N°	template	fw/rev primer	anneal.-temp. [°C]	product
1	pMS470_CPR10_HPO7	P7/ P62	55	HPO7-FLAG
2	pMS470_CPR11_HPO8	P19/ P63	61	HPO8-FLAG
3	pMS470_CPR12_HPO9	P27/ P64	55	HPO9-FLAG
4	pMS470_CPR4_HPO1	HPO1 fw/ HPO1 rv	57	HPO1-FLAG
5	pMS470_CPR5_HPO2	HPO2 fw/ HPO2 rv	57	HPO2-FLAG
6	pMS470_CPR6_HPO3	HPO3 fw/ HPO3 rv	57	HPO3-FLAG
7	pMS470_CPR4_HPO1	CPR4 fw/ CPR4 rv	51	CPR4-myc
8	pMS470_CPR5_HPO2	CPR5 fw/ CPR5 rv	51	CPR5-myc
9	pMS470_CPR6_HPO3	CPR6 fw/ CPR6 rv	55	CPR6-myc

For all reactions, 1 µL of template was mixed with 2 µL of forward as well as reverse primers, 5 µL dNTPmix [2 mM each], 5 µL Dream Taq Buffer [10x], 0.5 µL Dream Taq Polymerase (Fermentas) [5 U/µL] and 34.5 µL ddH_2O.

Cycling program: 95°C/5' – (95°C/30'' – anneal. temp. [°C]/30'' – 72°C/2') x 30 – 4°C/∞

PCR products: size and restriction sites

- **HPO7-FLAG, HPO8-FLAG, HPO9-FLAG: 1479 bp**

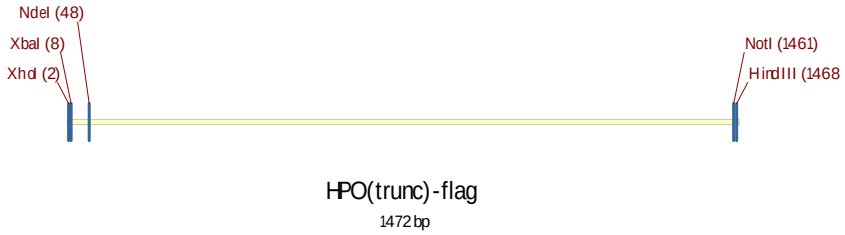


FIGURE 3: TRUNCATED FLAG-TAGGED HPO, PCR PRODUCT

- **HPO1-FLAG, HPO2-FLAG, HPO3-FLAG: 1592 bp**

FIGURE 4: FLAG-TAGGED HPO WITH TMD, PCR PRODUCT

- **CPR4-myc, CPR5-myc, CPR6-myc: 2197 bp**

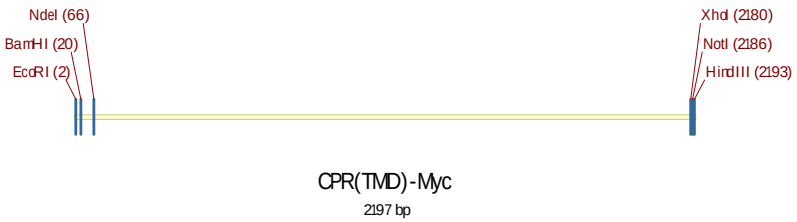


FIGURE 5: *MYC*-TAGGED CPR WITH TMD, PCR PRODUCT

Subsequently, PCR products were purified via 1% agarose gel at 90 V. DNA fragments were excised and cleaned up with the Wizard® SV Gel and PCR Clean Up System before quantifying DNA oncentrations by 1% agarose gels.

CLONING OF TAGGED GENES OPTIMIZED FOR *P. PASTORIS*

For vector backbone preparation, pMS470_CPR4_HPO1 was isolated from the respective *E. coli* Top10F' cells. Subsequently, CPR4 was replaced by CPR4-myc, CPR5-myc and CPR6-myc, respectively. In the second step, HPO1 was displaced by the tagged HPO versions in vectors already harboring the *myc*-tagged CPR genes.

Thirty µL of pMS470_CPR4_HPO1 vector were cut with 1.5 µL *Xho*I and 1.5 µL *EcoR*I in Buffer R at 37°C for 3 h. The PCR products CPR4-myc, CPR5-myc and CPR6-myc were prepared the same way. Then, all restriction reactions were cleaned up via preparative gel, gel bands of the correct sizes were excised and eluted with the Wizard® SV Gel and PCR Clean Up System.

Based on DNA concentrations determined by gel electrophoresis, ligation reactions were set up using 1 µL T4 DNA ligase in the appropriate buffer. Inactivated and desalted ligation preparations were transformed into *E. coli* Top10F' by electroporation. Regenerated cells were plated onto LB-Amp media. Plates were incubated overnight at 37°C, a few colonies streaked onto fresh LB-Amp plates and, vectors were isolated with the Gene Jet™ Plasmid Miniprep Kit to be tested for positive insertions via restriction analysis. Only constructs showing the expected restriction patterns were selected for further cloning steps.

The constructs pMS470_CPR4-myc_HPO1, pMS470_CPR5-myc_HPO1 and pMS470_CPR6-myc_HPO1 served as backbones for replacement of HPO1 with restriction enzymes. Vectors were isolated from *E. coli* Top10F' strains and 30 µL of plasmid preparations were cut with 1.5 µL *Xho*I and 1.5 µL *Hind*III in 3 µL Buffer Tango for 3 h at 37°C. PCR products HPO1-FLAG, HPO2-FLAG and HPO3-FLAG were cut with the same enzymes under the same conditions. A standard preparative gel was run after restriction procedures and fragments of the correct sizes were excised. Purification and elution were performed with the Wizard® SV Gel and PCR Clean Up System. FLAG-tagged HPO genes were ligated into vectors containing *myc*-tagged CPR genes to yield co- expression constructs harboring two genes optimized by the same principles. Both genes were either synthetic but native (pMS470_CPR4-myc_HPO1-FLAG), codon optimized by T. Wriessnegger (pMS470_CPR5-myc_HPO2-FLAG) or by a DSM proprietary algorithm (pMS470_CPR6-myc_HPO3-FLAG). Vectors were tested for positive inserts by restriction cut and gel electrophoresis.

CLONING OF TAGGED GENES OPTIMIZED FOR *E. COLI*

From previous work of T. Wriessnegger vectors containing *myc*-tagged genes optimized for *E. coli* already existed. CPR10-myc, CPR11-myc and CPR12-myc had been cloned into a pMS470-dsbC vector backbone. Vectors with these inserts had been cut with *Xho*I and *Hind*III and the linear fragments purified by preparative gel a few months ago. Finally, the frozen preparations were thawed for concentration

estimations and directly used for cloning. To prepare the inserts for ligation, the PCR products HPO7-FLAG, HPO8-FLAG and HPO9-FLAG were also cut with 1.5 µL *Xho*I and 1.5 µL *Hind*III in 3 µL Buffer Tango for 3 h at 37°C. After the standard procedure of preparative gel purification and estimation of DNA concentration, the PCR products were ligated into the vector backbones. Inactivation and desalting of the preparations was done as described above and 80 µL of pre- chilled electrocompetent *E. coli* Top10F' were mixed with 3 µL of ligation reaction. After electroporation, regeneration and isolation of constructs, restriction control indicated positive clones. Final products were named

- pMS470_CPR10-myc_HPO7-FLAG,
- pMS470_CPR11-myc_HPO8-FLAG and
- pMS470_CPR12-myc_HPO9-FLAG.

Finally, all vectors containing *myc*-tagged CPR and FLAG-tagged HPO were isolated from *E. coli* Top10F' strains by Gene Jet™ Plasmid Miniprep Kit and transformed into *E. coli* BL21 Gold™ (DE3) for protein expression and subsequent Western Blot Analysis.

WESTERN BLOT

SDS-PAGE was performed as described above using ready-to-use-gels according to the manual of NuPAGE®. Blotting pads, filter paper and nitrocellulose membrane were soaked in 700 mL 1x transfer buffer to remove air bubbles. The blotting sandwich was built as described in figure 6 using soaked pads, gel(s) and filters. Air bubble formation was thoroughly avoided.

FIGURE 6: LEFT SCHEME: BLOTTING SANDWICH USED FOR BLOTTING ONE GEL, RIGHT SCHEME: SANDWICH FOR BLOTTING TWO GELS, SOURCE: MANUAL NUPAGE®

The blotting sandwich was then fixed with a Gel Tension Wedge in the XCell II unit and blotting was carried out according to the manual, exposing the sandwich to 250 mA, maximum voltage and maximum power for one h.

Membranes were stained with Ponceau S to detect transfer efficiency of proteins and an image thereof was taken. After washing away excessive Ponceau S with ddH_2O, the membranes were blocked with 100 mL TBS-milk at 4°C overnight. Thereupon membranes were rinsed once with TBST and incubated with the primary antibody under moderate shaking at room temperature for one h. This step was followed by washing five times with TBST for 5 min each. Then, the membranes were treated with the secondary antibody for one h at room temperature and moderate shaking, followed by five washing steps with TBST, each for five min. Detection was carried out by covering the membranes with 4 mL of SuperSignal West Pico Chemoluminescent Substrate mixture and chemoluminescence detection in a G:Box after an incubation of two to three min.

HPO ACTIVITY ASSAYS

In nature, HPO catalyzes the synthesis of the phytoalexin solavetivone. Within this project, the sesquiterpene valencene should be oxidized to intermediately form the two isostereomers cis- and trans-nootkatol, which can further be converted to the finally appreciated product nootkatone by a second oxidation event at the C-2 atom.

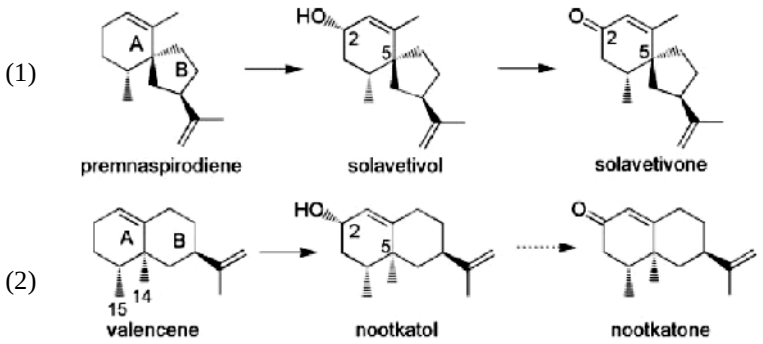


FIGURE 7: (1) NATURAL SUBSTRATE AND REACTION CATALYZED BY HPO VERSUS (2) INDUSTRIALLY DESIRED TRANSFORMATION[1]

IN VIVO ASSAY: WHOLE CELL BIOCATALYSTS

For in vivo assays, a standard protein expression culture was performed in 300 mL baffeled flasks. Main cultures were grown overnight in 50 mL LB-Amp and TB-Amp media, respectively. After induction and addition of δ-ALA and $FeSO_4$ heptahydrate, gene expression proceeded for one h. Then, 1 mL of 100 mM valencene in DMSO was added to yield in a final substrate concentration of 2 mM. In same experiments, 10 mL dodecane were added.[3]

Biotransformations were carried out at 20°C and 140 rpm for 20-26 h. Finally, OD_{600} was determined and the reaction cultures were extracted with 10 mL of ethylacetate (EtOAc) in extraction funnels. Organic layers were transferred to Pyrex tubes and centrifuged for 15 min at 2,500xg. Within the first assays, extracts were either concentrated in a rotary evaporator (100 mbar, 30°C water bath) or under N_2 stream before being redissolved in 1 mL ethylacetate. Later, extracts were directly used for GC measurements.

About 50 µL of the (concentrated) extracts were filled into glass crimp neck GC vials with inserts, and GC-MS or GC-FID measurement was performed.

IN VIVO ASSAY: RESTING CELLS

For in vivo assays with resting cells, cultures were performed in 50 mL LB-Amp supplemented with δ-ALA and $FeSO_4$ heptahydrate during induction with IPTG. After 24 h of main cultures at 20°C and 160 rpm. OD_{600} was determined. Cell pellets were harvested in 50 mL Greiner tubes for 15 minutes at 4,000xg, weighed and carefully resuspended in 2 mL of 50 mM KP_i buffer (pH8.0) containing 1 mM $MgCl_2$ and 2% glycerol. For bioconversions, 40 µL of 0,1 M valencene in DMSO were added. For selected conversions, 400 µL of dodecane were added. The reactions were carried out at 20°C and 160 rpm in Pyrex tubes for 20.[12] Then, 5 mL of EtOAc were added and the tubes were vortexed vigorously. After centrifugation for 15 min at 2,500xg, the organic layers were concentrated via N_2 stream, dissolved in 1 mL of EtOAc and used for GC-MS or GC-FID measurement.

IN VITRO ASSAY

Usually, 5 mL of cell free extract (20 to 40 mg of total protein) were used for in vitro assays. First, the mixture defined in Table 11 was generated in a Pyrex tube on ice without addition of NADPH in the first instance. Then, the tube was incubated for 10 min at 30°C and 160 rpm in the shaker. After addition of 500 µL of 2 mM NADPH in 50 mM KP_i buffer, pH 8.0, containing 1 mM $MgCl_2$, reactions were carried out for 16-20 h. Conversion were performed with and without implementation of the GDH based cofactor regeneration system. Therefore,, 35 U GDH and 5.4 mg glucose (final concentration in assay: 4 mM) were combined in 1.60 mL buffer and immediately added to each reaction. In assays without cofactor regeneration, 1.60 mL of buffer were added.[16]

TABLE 11: COMPOSITION OF IN VITRO ASSAY

substance	concentration	amount
KP_i buffer + GDH system	50 mM, pH 8,0	1.60 mL
DMSO + valencene (100 mM)	2% (v/v)	150 µL
cell free extract		5 mL
PMSF	1 M	7,5 µL
NADPH	2 mM	750 µL

FIGURE 8: PYREX TUBES WITH REACTION MIXTURE

Pyrex tubes were fixed as sloped as possible to generate highest turbulence for keeping samples well-mixed. The final substrate concentration used for the assay was 2 mM, but the concentration of cofactor in contrast was only 0.2 mM. Taking into account the reaction mechanism (Figure 9), NADPH regeneration is therefore essential for optimal conversion conditions for HPO. Thus, in assays without GDH system, ten times more NADPH was used for supplying in vitro assays with amounts equimolar to valencene.

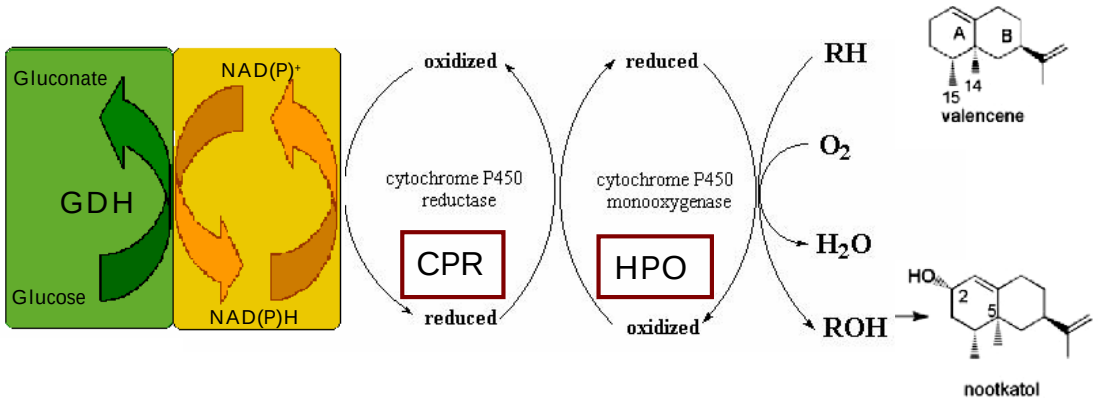


FIGURE 9: COFACTOR RECYCLING

After 16-20 h of conversion at 160 rpm and 30°C, reaction solutions were worked up for analysis. In case of concentrating via rotary evaporator (rotavap) or N_2 stream, the reaction solutions were extracted with 10 mL EtOAc in an extraction funnel. The organic layers were centrifuged for 15 min at 2,500xg, cell-debris free fractions were concentrated and redissolved in 0.5 mL or 1 mL of EtOAc. For direct measurement of the extracts, each tube was provided with 3 mL of EtOAc, vortexed vigorously and then centrifuged for 15 minutes at 2,500xrpm. Extracts were analyzed by GC-MS or GC-FID.

GC-MS AND GC-FID

GC measurements were performed in cooperation with Professor Erich Leitner and the Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology. The methods were established for the efficient separation and analysis of terpenes of interest by Erich Leitner.

- For GC-MS, the following method was used:

Injection volume 1.0 µL, inlet temperature: 220°C, detector: 280°C
Oven program: **70°C (1min) – 10°C/min – 200°C – 30°C/min – 290°C (2 min)**
Run time: 19.00 minutes, constant flow: on, constant flow pressure: 28 kPa, constant flow temperature: 70°C
Column length: 28.80 m, column diameter: 0.250 mm, film thickness 0.25 µm
Acquisition Mode: scan, solvent delay: 11.00 min, low mass: 40, high mass: 250 amu

- Method information for GC-FID

Injection volume 1.00 µL, inlet temp: 250 °C (On), detector: 320 °C (On)
Oven program: **100°C (1 min) – 20°C/min – 190°C – 45°C/min – 280°C (0.5 min)**
Run time: 8.00 min, Mode: Split
Column length: 10.0 m, column diameter: 0.10 mm, film thickness 0.10 µm
Mode: constant flow, initial flow: 0.4 mL/min, nominal init pressure: 293.5 kPa, average velocity: 36 cm/sec

Computer program:

Instrument #1 Data Analysis, MSD Chemstation D.03.00552, Agilent Technologies

Agilent ChemStation B.0302, Agilent Technologies

RESULTS

FERMENTATIONS

CALIBRATION OF BIORAD PROTEIN ASSAY

To determine protein concentrations of cell homogenates, a calibration of the protein assay was done. Therefore, standard solutions with defined concentrations of Bovine Serum Albumine (BSA) in ddH_2O were prepared. Calibration was performed following the instructions of a 96- well plate BioRad assay developed by A. El-Heliebi.

TABLE 12: ABSORBANCES MEASURED FOR DIFFERENT CONCENTRATIONS OF BSA

BSA [µg/µL]	A595-1 [-]	A595-2 [-]	A595-3 [-]
0,625	0,080	0,064	0,081
1,25	0,208	0,154	0,169
2,5	0,337	0,310	0,320
5	0,613	0,580	0,634
10	0,968	0,972	0,990
20	1,301	1,295	1,303

Absorbance values showed saturation effects at BSA concentrations higher than 10 µg/µL. Therefore, the last value was not used for linear regression, which still guaranteed a useful calibration interval for our cell homogenates. To generate the calibration curve, an average of the three measured absorbances was calculated and plotted against BSA concentrations (Figure 10).

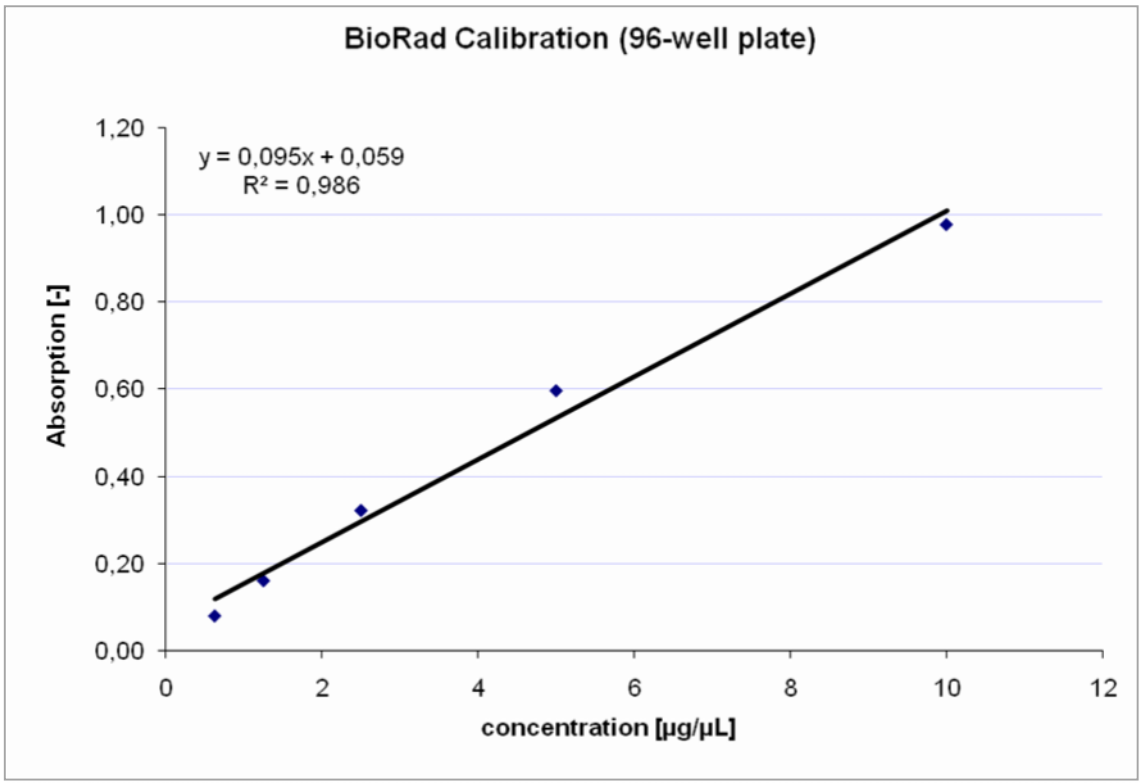


FIGURE 10: BIORAD PROTEIN ASSAY CALIBRATION

FERMENTATION RESULTS

Fermentations were done multiple times to generate cell free extracts for in vitro assays. All constructs harboring genes optimized for *P. pastoris* were expressed in *E. coli* BL21 Star™(DE3) and *E. coli* Origami B (DE3). Data for expression of genes optimized for *E. coli* are not shown since they turned out to be irrelevant for the progress of ACIB project 4.2. In the following tables either selected results or average values are presented.

CELLS GROWN IN LB-AMP MEDIA

TABLE 13: FERMENTATION RESULTS FOR HPO AND CPR EXPRESSED IN *E. COLI* BL21(DE), AVERAGE VALUES OF THREE FERMENTATIONS

N°	variants	OD_{600} at induction [-]	OD_{600} at harvest [-]	Cell wet weight [g]	Protein concentration [µg/µL]
1	pMS470_dsbC_CPR4_HPO1	0.613 ± 0.152	4.56 ± 0.26	0.92 ± 0.32	2.17 ± 1.18
2	pMS470_dsbC_CPR5_HPO2	0.488 ± 0.141	4.04 ± 0.32	1.09 ± 0.02	2.57 ± 1.00
3	pMS470_dsbC_CPR6_HPO3	0.472 ± 0.165	4.10 ± 0.32	0.70 ± 0.17	2.26 ± 0.98
4	pMS470_CPR4_HPO1	0.481 ± 0.072	3.55 ± 0.43	0.66 ± 0.16	2.06 ± 0.29
5	pMS470_CPR5_HPO2	0.630 ± 0.014	4.22 ± 0.19	1.14 ± 0.09	2.77 ± 0.42
6	pMS470_CPR6_HPO3	0.479 ± 0.276	3.93 ± 0.30	0.83 ± 0.20	2.81 ± 0.73
7	pMS470 linker	0.478 ± 0.170	2.17 ± 0.99	0.62 ± 0.27	0.88 ± 0.02

TABLE 14: FERMENTATION RESULTS FOR HPO AND CPR EXPRESSED IN *E. COLI* ORIGAMI B (DE3), SINGLE FERMENTATION

N°	variants	OD_{600} at induction [-]	OD_{600} at harvest [-]	Cell wet weight [g]	Protein concentration [µg/µL]
1	pMS470_dsbC_CPR4_HPO1	0.514	1.61	0.56	1.69 ± 0.09
2	pMS470_dsbC_CPR5_HPO2	0.342	2.33	0.57	2.32 ± 0.06
3	pMS470_dsbC_CPR6_HPO3	0.122	0.65	0.47	0.15 ± 0.06
4	pMS470_CPR4_HPO1	0.600	2.33	0.73	2.44 ± 0.02
5	pMS470_CPR5_HPO2	0.690	4.51	0.85	2.50 ± 0.15
6	pMS470_CPR6_HPO3	0.366	1.31	0.47	1.06 ± 0.15
7	pMS470 linker	0.660	3.31	0.84	3.26 ± 0.06

Unfortunately, *E. coli* Origami B (DE3) showed instable and extremely slow growth at 20°C. In case of BL21(DE) strain backgrounds, cells reached inducible OD_{600} values within 5 h after inoculation of main cultures, whereas Origami B had to be grown for at least 8 to 9 h for the same cell densities. Mostly, cells

did not grow at all or protein concentrations after sonication were too low for reasonable in vitro assays. In combination with bad results of in vivo conversions, the use of *E. coli* Origami B (DE3) as expression host was not continued.

CELLS GROWN IN TB-AMP MEDIA

TABLE 15: TABLE: FERMENTATION RESULTS FOR HPO AND CPR EXPRESSED IN *E. COLI* BL21(DE), AVERAGE VALUES OF FOUR FERMENTATIONS

N°	variants	OD_{600} at induction [-]	OD_{600} at harvest [-]	Cell wet weight [g]	Protein concentration [µg/µL]
1	pMS470_dsbC_CPR4_HPO1	0.488 ± 0.16	8.37 ± 2.93	1.92 ± 0.50	9.06 ± 3.60
2	pMS470_dsbC_CPR5_HPO2	0.445 ± 0.19	8.36 ± 3.67	2.02 ± 0.71	8.91 ± 4.09
3	pMS470_dsbC_CPR6_HPO3	0.379 ± 0.16	7.17 ± 4.16	1.74 ± 0.47	7.30 ± 4.92
4	pMS470_CPR4_HPO1	0.580 ± 0.12	8.16 ± 2.86	1.97 ± 0.87	8.72 ± 3.33
5	pMS470_CPR5_HPO2	0.486 ± 0.08	8.30 ± 3.58	2.18 ± 0.72	9.03 ± 4.02
6	pMS470_CPR6_HPO3	0.323 ± 0.06	7.77 ± 3.78	1.96 ± 0.76	8.48 ± 4.14
7	pMS470 linker	0.364 ± 0.14	2.07 ± 0.17	0.55 ± 0.06	2.26 ± 1.46

Cells grew to very high cell densities in TB media, even when exposed to low temperatures. Interestingly, cultures showed inconstant fitness at different fermentations, which is represented in the relatively big standard deviations of different OD_{600} values and protein concentrations.

CORRELATING OD_{600} OF CULTURES TO CELL DRY WEIGHTS (CDW)

After dehydration of a defined number of OD_{600} units, dry cells were weighed and values of experiments in duplicate were plotted against optical densities. The resulting calibration served as regression line for evaluation of in vivo assays. To normalize results, OD_{600} was determined after bioconversion, and the values were extrapolated for cell dry weight.

TABLE 16: DOUBLE DETERMINATION OF CDW

OD_{600}	cdw (1) [mg]	cdw (2) [mg]
67.0	11.6	11.5
44.7	8.8	7.6
33.5	6.3	5.5
22.3	3.9	4.1
11.2	1.6	1.9
0.0	0.0	0.0

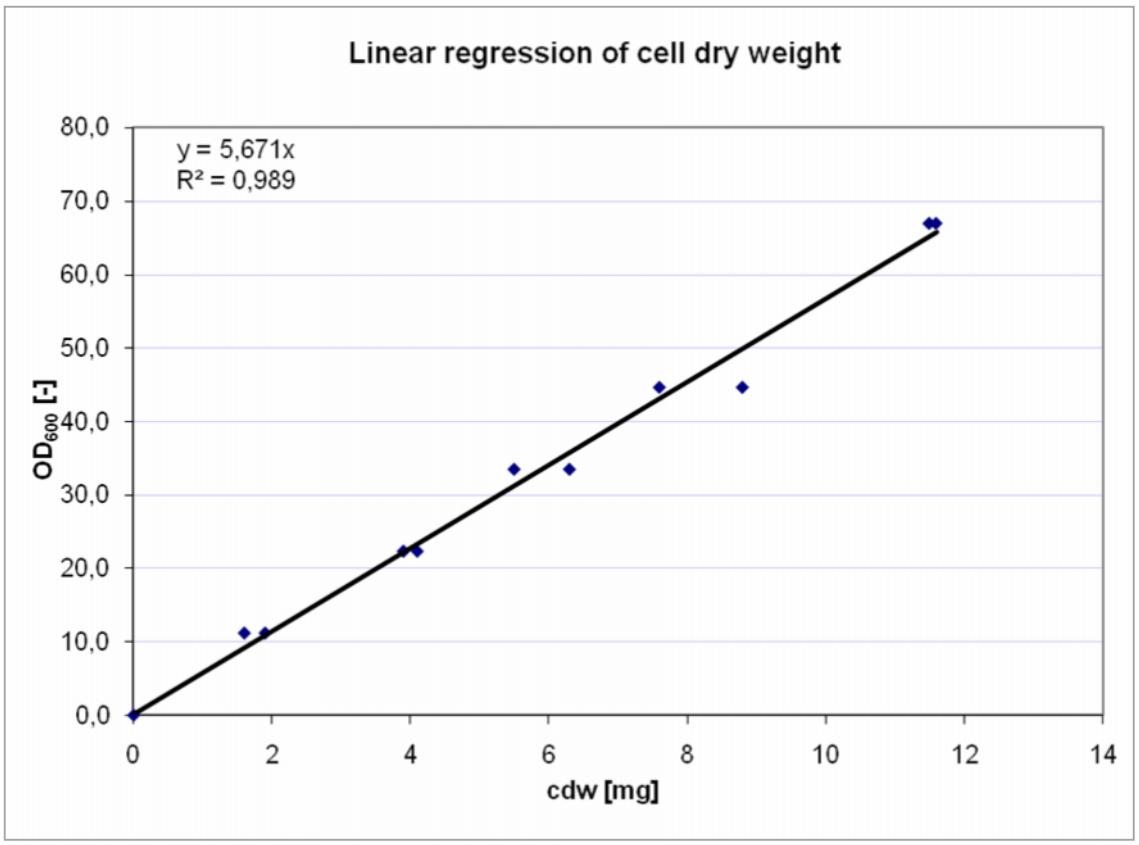
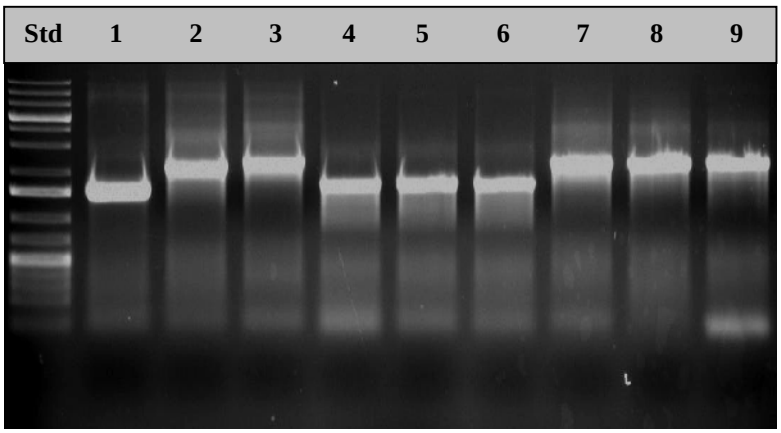


FIGURE 11: REGRESSION LINE FOR CELL DRY WEIGHT AND OD_{600} CORRELATION

TAGGING BY PCR

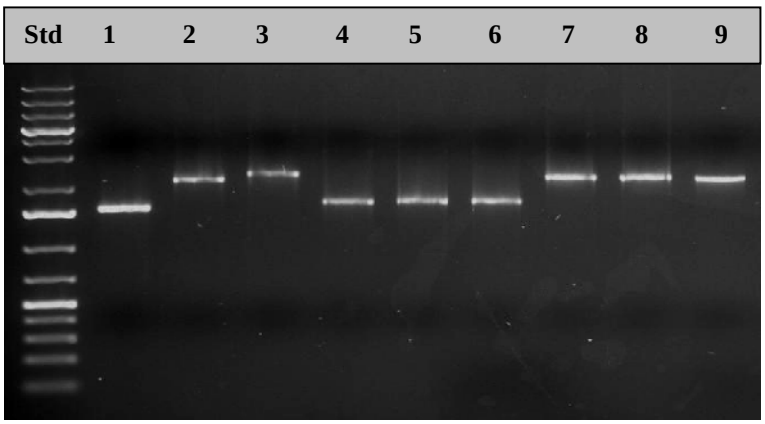
PCRs were performed to tag HPO genes with FLAG-tags and CPR genes with *myc*-tags. Afterwards, a standard preparative gel was run for fragment purification. In every case, satisfying amounts of constructs with the correct sizes were amplified.



ID	PCR product	~ size [bp]
1	HPO7-FLAG	1500
2	HPO8-FLAG	2100
3	HPO9-FLAG	2300
4	HPO1-FLAG	1600
5	HPO2-FLAG	1600
6	HPO3-FLAG	1600
7	CPR4-myc	2200
8	CPR5-myc	2200
9	CPR6-myc	2200

FIGURE 12: PREPARATIVE GEL OF CPR-*MYC* AND HPO FLAG FRAGMENTS

After isolation and cleanup, concentrations of the DNA fragments were determined by comparing band intensities with those of the standard.



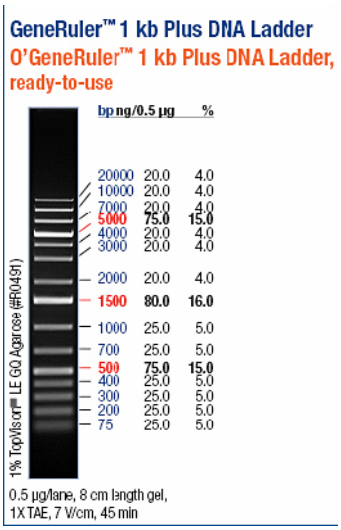


FIGURE 13: CONCENTRATION ESTIMATION BASED ON COMPARING BAND INTENSITIES WITH THE STANDARD GENERULER™ 1 KB PLUS DNA LADDER

Based on the use of 2 µL DNA preparation for concentration estimation, the following values were determined.

TABLE 17: ESTIMATED DNA CONCENTRATIONS

ID	PCR product	~ size [bp]	c [ng/µL]
1	HPO7-FLAG	1500	40
2	HPO8-FLAG	2100	30
3	HPO9-FLAG	2300	20
4	HPO1-FLAG	1600	25
5	HPO2-FLAG	1600	25
6	HPO3-FLAG	1600	25
7	CPR4-myc	2200	30
8	CPR5-myc	2200	30
9	CPR6-myc	2200	30

The strongly varying sizes of the PCR products HPO7-FLAG, HPO8-FLAG and HPO9-FLAG stem from using sequencing primers as forward primers, which did not bind at the equivalent position of each different template.

VECTOR PREPARATION WITH GENES OPTIMIZED FOR *P. PASTORIS* AND *E. COLI*

In case of genes optimized for *P. pastoris*, pMS470_CPR4_HPO1 and the constructs CPR4-myc, CPR5-myc and CPR6-myc were cut with *Xho*I and *Eco*RI.

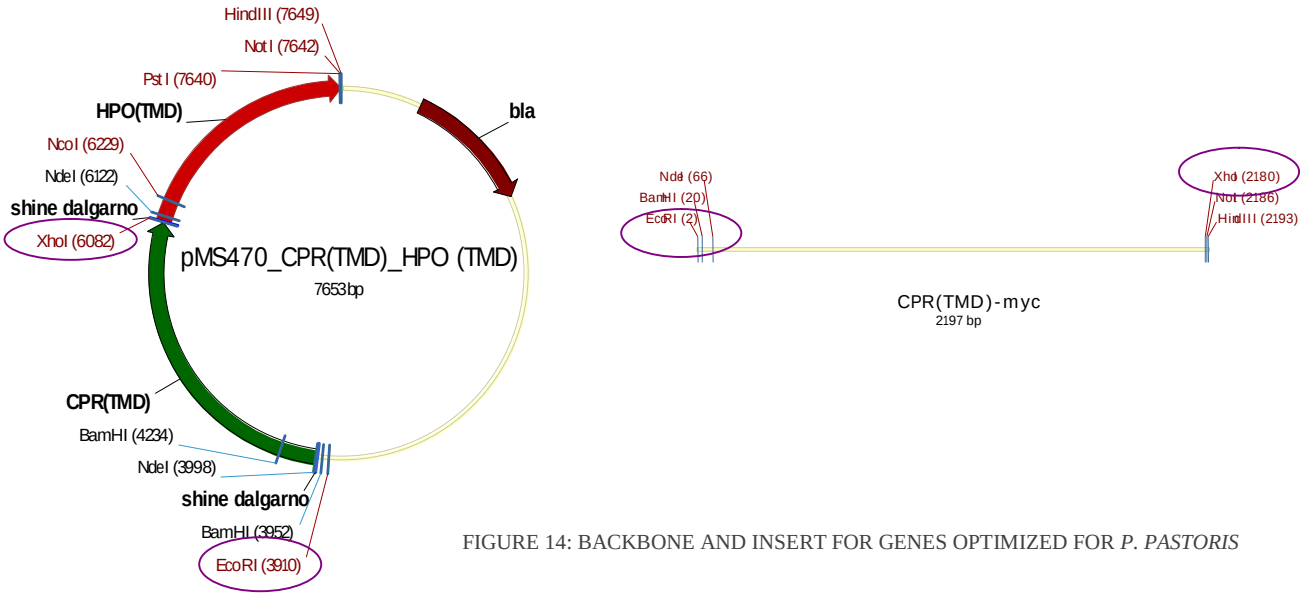


FIGURE 14: BACKBONE AND INSERT FOR GENES OPTIMIZED FOR *P. PASTORIS*

In case of genes optimized for *E. coli*, CPR10-myc, CPR11-myc and CPR12-myc had already been cloned into a pMS470 vector backbone by T. Wriessnegger. Plasmids had been isolated and linearized with *Xho*I and *Hind*III. The backbone fragments were thawed for concentration estimation and directly used for cloning. To prepare the inserts for ligation, the PCR products HPO7-FLAG, HPO8-FLAG and HPO9-FLAG were also cut with *Xho*I and *Hind*III.

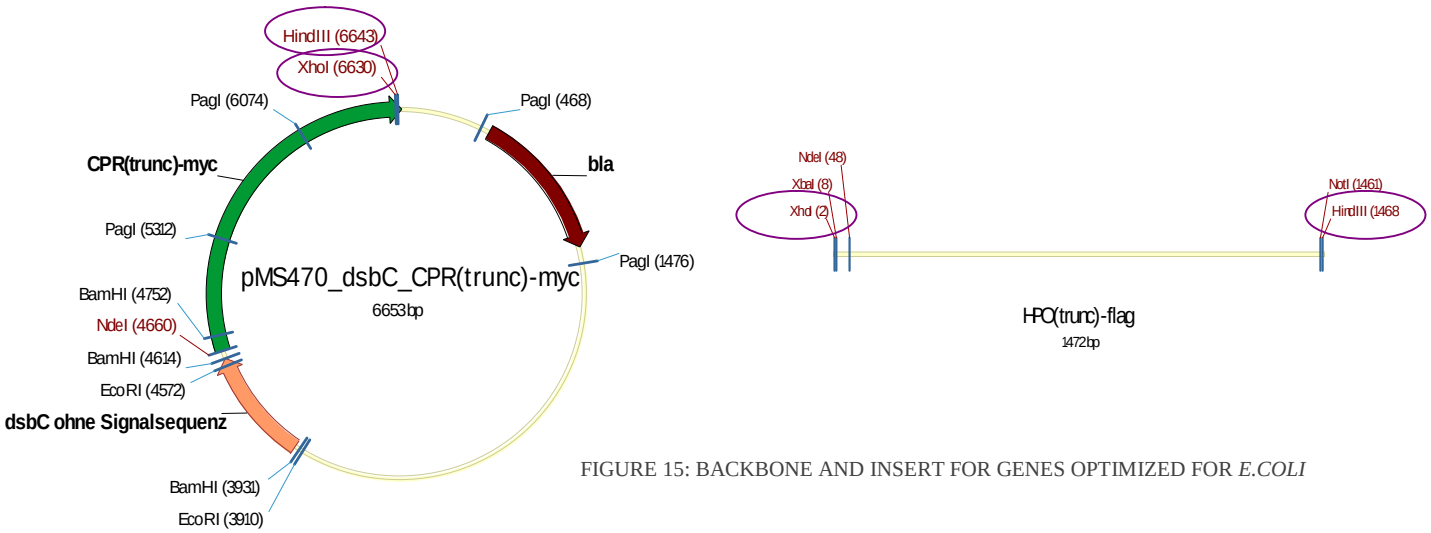


FIGURE 15: BACKBONE AND INSERT FOR GENES OPTIMIZED FOR *E.COLI*

All processed DNA fragments were purified via preparative gel, and after elution from miniprep columns, the concentrations were estimated using standard agarose gels. The preparative gel is only shown in case of the pMS470_CPR4_HPO1 vector to give an idea of the size of the excised vector backbone (yellow). Concentration estimation by gel electrophoresis is shown for all constructs included.

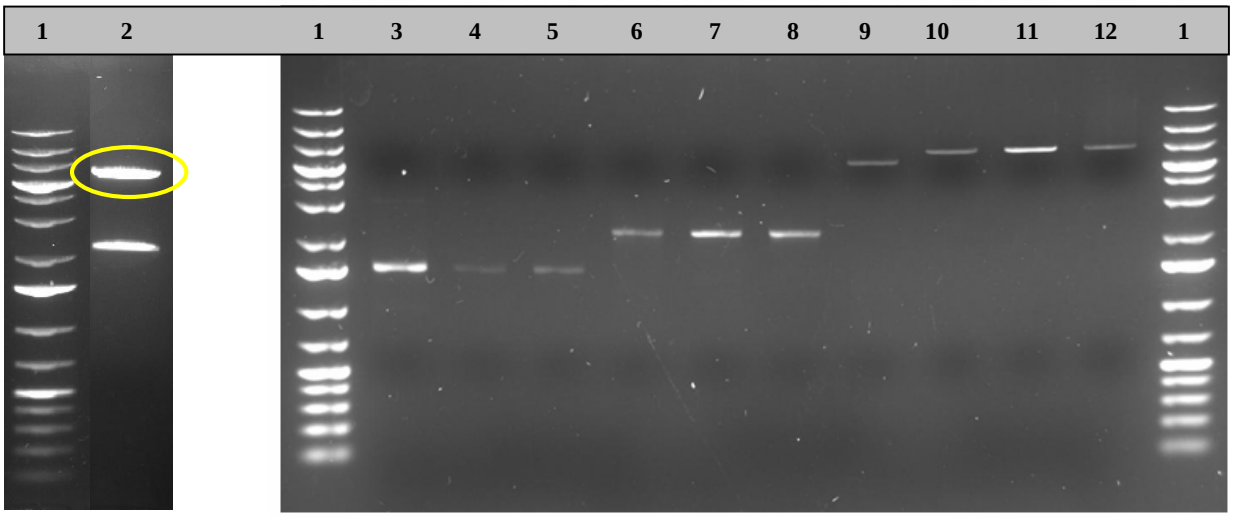
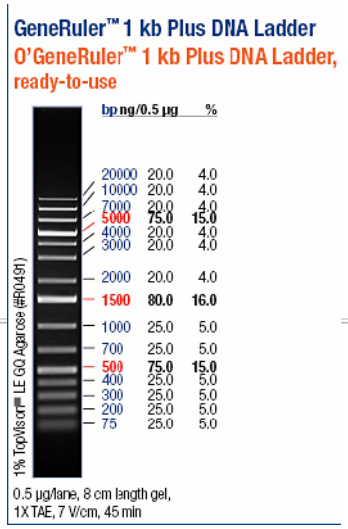


FIGURE 16: (1) PREPARATIVE GEL OF THE CUT PMS470_CPR4_HPO1 AND (2) CONCENTRATION ESTIMATIONS OF DIFFERENT FRAGMENTS.

N°	construct	size [bp]	c [ng/µL]
1	GeneRuler™ 1 kb Plus DNA Ladder	-	-
2	pMS470_CPR4_HPO1	5511 bp, 2142 bp	-
3	HPO7-FLAG	1479 bp	30
4	HPO8-FLAG	1479 bp	3
5	HPO9-FLAG	1479 bp	5
6	CPR4-myc	2197 bp	10
7	CPR5-myc	2197 bp	15
8	CPR6-myc	2197 bp	15
9	pMS470_HPO1	5511 bp	5
10	pMS470_dsbC_CPR10-myc	6653 bp	5
11	pMS470_dsbC_CPR11-myc	6653 bp	10
12	pMS470_dsbC_CPR12-myc	6653 bp	5



Ligation was performed to create the final vectors containing HPO-FLAG and CPR-myc in case of genes optimized for *E. coli* and to finish the first insertion step for constructs containing genes optimized for *P.*

pastoris. After electrotransformation into *E. coli* Top10F', plasmids were isolated and control cut by specially chosen restriction enzymes listed in the Table underneath.

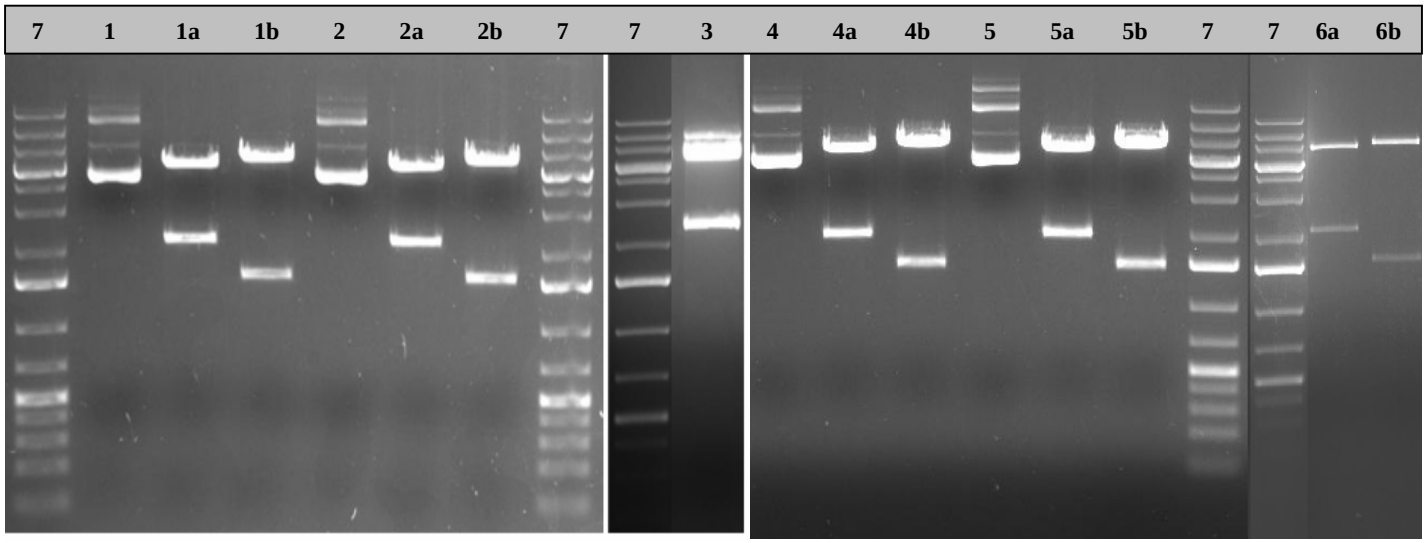


FIGURE 17: CONTROL CUT OF CLONED VECTORS (*P. PASTORIS* AND *E. COLI* OPTIMIZED GENES)

N°	construct	restriction enzymes	~ size [bp]
1	pMS470_CPR5-myc_HPO1	-	-
1a	pMS470_CPR5-myc_HPO1	*Eco*RI, *Xho*I	5560, 2200
1b	pMS470_CPR5-myc_HPO1	*Hind*III, *Xho*I	6200, 1560
2	pMS470_CPR6-myc_HPO1	-	-
2a	pMS470_CPR6-myc_HPO1	*Eco*RI, *Xho*I	5560, 2200
2b	pMS470_CPR6-myc_HPO1	*Hind*III, *Xho*I	6200, 1560
3	pMS470_CPR4-myc_HPO1	*Eco*RI, *Xho*I	5560, 2200
4	pMS470_dsbC_CPR11-myc_HPO8-FLAG	-	-
4a	pMS470_dsbC_CPR11-myc_HPO8-FLAG	*Nde*I, *Xho*I	6150, 1970
4b	pMS470_dsbC_CPR11-myc_HPO8-FLAG	*Hind*III, *Xho*I	6650, 1470
5	pMS470_dsbC_CPR12-myc_HPO9-FLAG	-	-
5a	pMS470_dsbC_CPR12-myc_HPO9-FLAG	*Nde*I, *Xho*I	6150, 1970
5b	pMS470_dsbC_CPR12-myc_HPO9-FLAG	*Hind*III, *Xho*I	6650, 1470
6a	pMS470_dsbC_CPR10-myc_HPO7-FLAG	*Nde*I, *Xho*I	6150, 1970
6b	pMS470_dsbC_CPR10-myc_HPO7-FLAG	*Hind*III, *Xho*I	6650, 1470
7	Gene Ruler™ 1 kb Plus DNA Ladder	-	-

Positive constructs were identified by the expected restriction patterns. In case of pMS470_CPR4-myc_HPO1 (N° 3), the vector was not entirely cut, because a band can be seen at 7700 bp, which means that one restriction enzyme did not work properly or the reaction time was too short.

For genes optimized for *E. coli*, the cloning was finished after this step. The three different vector combinations

pMS470_dsbC_CPR10-myc_HPO7-FLAG,

pMS470_dsbC_CPR11-myc_HPO8-FLAG and

pMS470_dsbC_CPR12-myc_HPO9-FLAG were generated.

Regarding the completion of the vectors composed of genes optimized for *P. pastoris*, the FLAG-tagged HPOs still had to be inserted. For this reason, plasmids harboring CPR-myc genes were isolated from positive clones to serve as vector backbones. Untagged HPO1 was cut out of the vectors by *Xho*I and *Hind*III. PCR products HPO1-FLAG, HPO2-FLAG and HPO3-FLAG were subjected to the same procedure. Finally, vector backbones and inserts were ligated, electroporated into *E. coli* Top10F' and the isolated vectors control cut by specially chosen restriction enzymes.

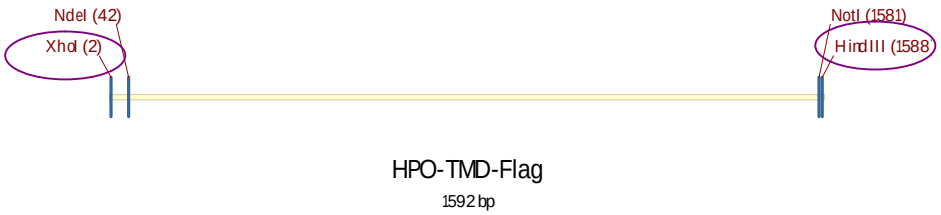


FIGURE 18: FLAG-TAGGED PCR PRODUCTS WITH TRANSMEMRANE DOMAIN

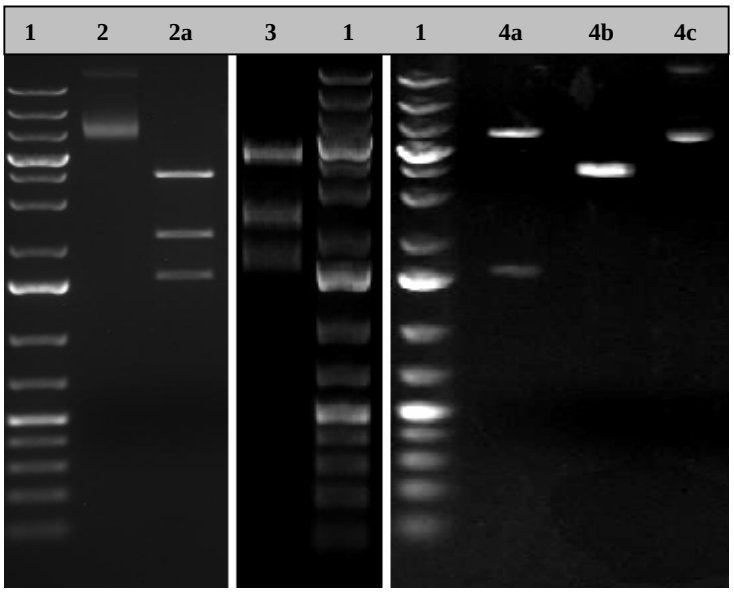


FIGURE 19: RESTRICTION PATTERN OF POSITIVE CONSTRUCTS PMS470_CPR4-MYC_HPO1-FLAG, PMS470_CPR5-MYC_HPO2-FLAG AND PMS470_CPR6-MYC_HPO3-FLAG

N°	Construct	restriction enzymes	~ size [bp]
1	Gene Ruler™ 1 kb Plus DNA Ladder		
2	pMS470_CPR4-myc_HPO1-FLAG	-	-
2a	pMS470_CPR4-myc_HPO1-FLAG	*Hind*III, *Eco*RI, *Xho*I	4000, 2200, 1600
3	pMS470_CPR5-myc_HPO2-FLAG	*Hind*III, *Eco*RI, *Xho*I	4000, 2200, 1600
4a	pMS470_CPR6-myc_HPO3-FLAG	*Xho*I, *Hind*III	6200, 1600
4b	pMS470_CPR6-myc_HPO3-FLAG	*Eco*RI, *Hind*III	3950, 3850
4c	pMS470_CPR6-myc_HPO3-FLAG	-	-

Altogether, tagged genes were successfully cloned into a pMS470 vector backbone and transformed into *E. coli* BL21 Gold™ (DE3).

SDS-PAGE AND WESTERN BLOT ANALYSIS

EXPRESSION OF TAGGED GENES

For Western blot analysis, tagged genes were expressed in *E. coli* BL21 Gold™ (DE3) as described above.

TABLE 18: FERMENTATION RESULTS FOR HPO AND CPR EXPRESSED IN *E. COLI* BL21 GOLD™ (DE3)

N°	variant	OD_{600} at induction [-]	OD_{600} at harvest [-]	cell wet weight [g]
1	pMS470_CPR4-myc_HPO1-FLAG	0.544	6.36	6.36
2	pMS470_CPR5-myc_HPO2-FLAG	0.540	4.99	4.99
3	pMS470_CPR6-myc_HPO3-FLAG	0.414	5.46	5.46
4	pMS470_dsbC_CPR10-myc_HPO7-FLAG	0.432	7.64	7.64
5	pMS470_dsbC_CPR11-myc_HPO8-FLAG	0.474	9.52	9.52
6	pMS470_dsbC_CPR12-myc_HPO9-FLAG	0.538	6.04	6.04
7	pMS470 linker	0.069	2.35	2.35

PROTEIN CONCENTRATIONS

BioRad Assays were performed with three different types of cell lysates. On the one hand, cell homogenates were directly used after sonication, on the other hand, cleared cell extracts and insoluble fractions were determined for protein concentration separately. Protein content was determined in triplicate.

TABLE 19: PROTEIN CONCENTRATIONS OF DIFFERENT FRACTIONS

N°	Strain: Plasmid	A595-1	A595-2	A595-3	dilution	Average	c [µg/µL]
Cell homogenates							
1	pMS470_CPR4-myc_HPO1-FLAG	0.8210	0.8610	0.8077	10	0.8299	8,11 ± 0.29
2	pMS470_CPR5-myc_HPO2-FLAG	0.8146	0.8057	0.8426	10	0.8210	8,01 ± 0.20
3	pMS470_CPR6-myc_HPO3-FLAG	0.8133	0.8127	0.8564	10	0.8275	8,08 ± 0.26
4	pMS470_dsbC_CPR10-myc_HPO7-FLAG	0.8875	0.8641	0.9702	10	0.9073	8,92 ± 0.17
5	pMS470_dsbC_CPR11-myc_HPO8-FLAG	0.9407	0.9763	1.0079	10	0.9750	9,63 ± 0.24
6	pMS470_dsbC_CPR12-myc_HPO9-FLAG	0.9223	0.9590	0.9654	10	0.9489	9,36 ± 0.24
7	pMS470 linker	0.7745	0.7695	0.7828	5	0.7756	3,77 ± 0.04
Supernatants							
1	pMS470_CPR4-myc_HPO1-FLAG	1.4973	1.4577	1.5458	2	1.4999	2,89 ± 0.07
2	pMS470_CPR5-myc_HPO2-FLAG	1.1944	1.1820	*	4	1.1882	4,75
3	pMS470_CPR6-myc_HPO3-FLAG	1.2621	1.2425	*	4	1.2523	5,02
4	pMS470_dsbC_CPR10-myc_HPO7-FLAG	1.2844	1.2957	1.0808	4	1.2203	4,89 ± 0.03
5	pMS470_dsbC_CPR11-myc_HPO8-FLAG	1.3580	1.3418	1.1656	4	1.2885	5,17 ± 0.05
6	pMS470_dsbC_CPR12-myc_HPO9-FLAG	1.4068	1.3721	*	4	1.3895	5,60
7	pMS470 linker	0.8195	0.7902	*	4	0.8049	3,14
Insoluble cell fractions							
1	pMS470_CPR4-myc_HPO1-FLAG	0.8575	0.8802	0.8810	20	0.8729	17,12 ± 0.28
2	pMS470_CPR5-myc_HPO2-FLAG	0.6913	0.7266	0.7196	20	0.7125	13,75 ± 0.10
3	pMS470_CPR6-myc_HPO3-FLAG	0.9260	0.9258	0.9095	20	0.9204	18,12 ± 0.20
4	pMS470_dsbC_CPR10-myc_HPO7-FLAG	1.0808	1.0632	1.0834	20	1.0758	21,39 ± 0.23
5	pMS470_dsbC_CPR11-myc_HPO8-FLAG	1.1656	1.2016	1.1836	20	1.1836	23,66 ± 0.27
6	pMS470_dsbC_CPR12-myc_HPO9-FLAG	0.6408	0.6758	0.6899	20	0.6688	12,83 ± 0.21
7	pMS470 linker	0.6092	0.6127	0.6216	10	0.6145	5,84 ± 0.07

*…outliers

SDS-PAGE

For SDS-Page, approximately 10-15 µg of total protein was used for loading the gel. Exact values are listed in the table. Truncated enzymes were separated on a different gel than those still having the transmembrane domain. (Std…protein standard)

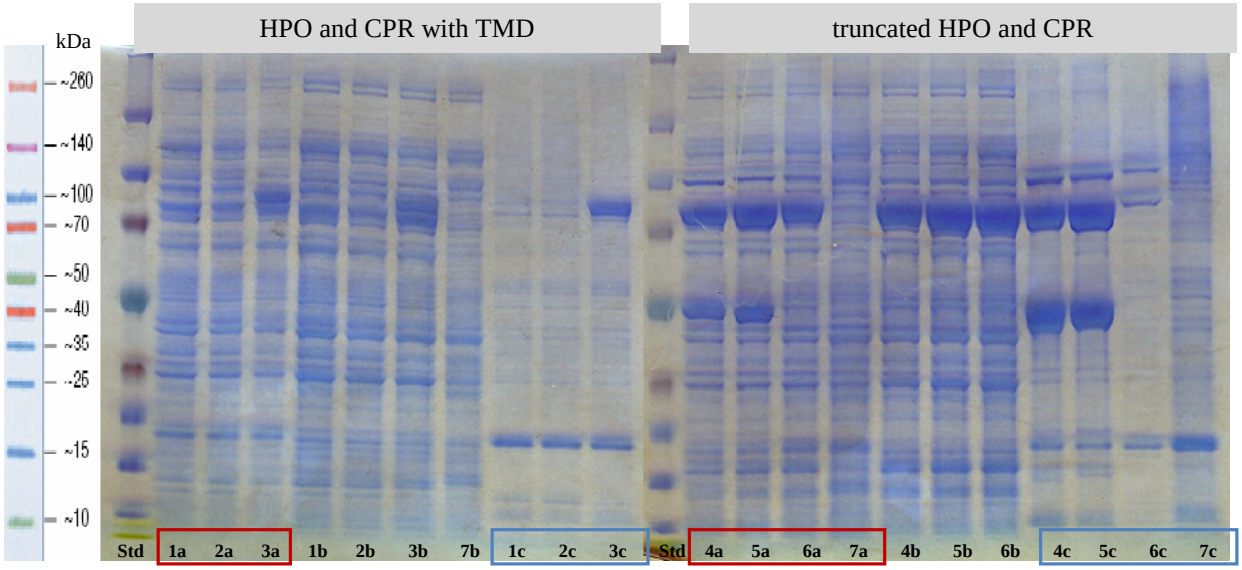


FIGURE 20: SDS-PAGE OF TAGGED ENZYMES

variant	total cell homogenate	cell free extract	insoluble cell fraction	amounts loaded [µg]
pMS470_CPR4-myc_HPO1-FLAG	1a			12,2
		1b		8,7
			1c	6,8
pMS470_CPR5-myc_HPO2-FLAG	2a			12,0
		2b		14,3
			2c	5,5
pMS470_CPR6-myc_HPO3-FLAG	3a			12,1
		3b		15,1
			3c	7,2
pMS470_dsbC_CPR10-myc_HPO7-FLAG	4a			13,4
		4b		14,7
			4c	8,6
pMS470_dsbC_CPR11-myc_HPO8-FLAG	5a			14,5
		5b		15,5
			5c	9,5
pMS470_dsbC_CPR12-myc_HPO9-FLAG	6a			14,0
		6b		16,8
			6c	5,1
pMS470 *dsbC* linker	7a			18,8
		7b		15,7
			7c	14,6

HPO and CPR including their transmembrane domain and carrying tags have approximately the following molecular weights:

- HPO with TMD: **73 kDa**
- CPR with TMD: **54 kDa**

Truncated HPO and CPR carrying tags are:

- HPO truncated: **70 kDa**
- CPR truncated: **51 kDa**

On SDS gels it can clearly be seen, that truncated genes optimized for *E. coli* were expressed to much higher levels. The enzymes with their transmembrane domains can hardly be allocated on the gel. Therefore, Western blot analysis was performed.

Western Blot Analysis

Each Western blot is demonstrated in two versions. Ponceau S stained gels for whole protein detection and the corresponding chemoluminescence pictures after specific immunodecoration.

A Western blot performed previously made it reasonable to apply variable amounts of total protein to achieve detectable signals.

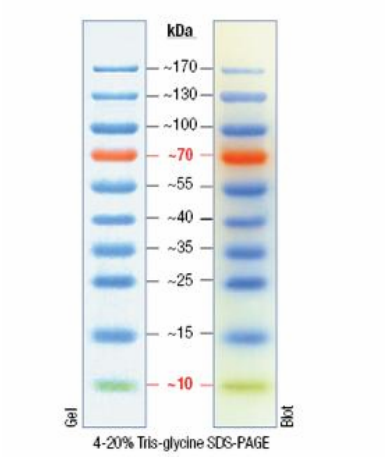


FIGURE 21: PROTEIN LADDER USED, PAGERULER™ PRESTAINED PROTEIN LADDER, FERMENTAS

First, results for enzymes with transmembrane domains are presented.

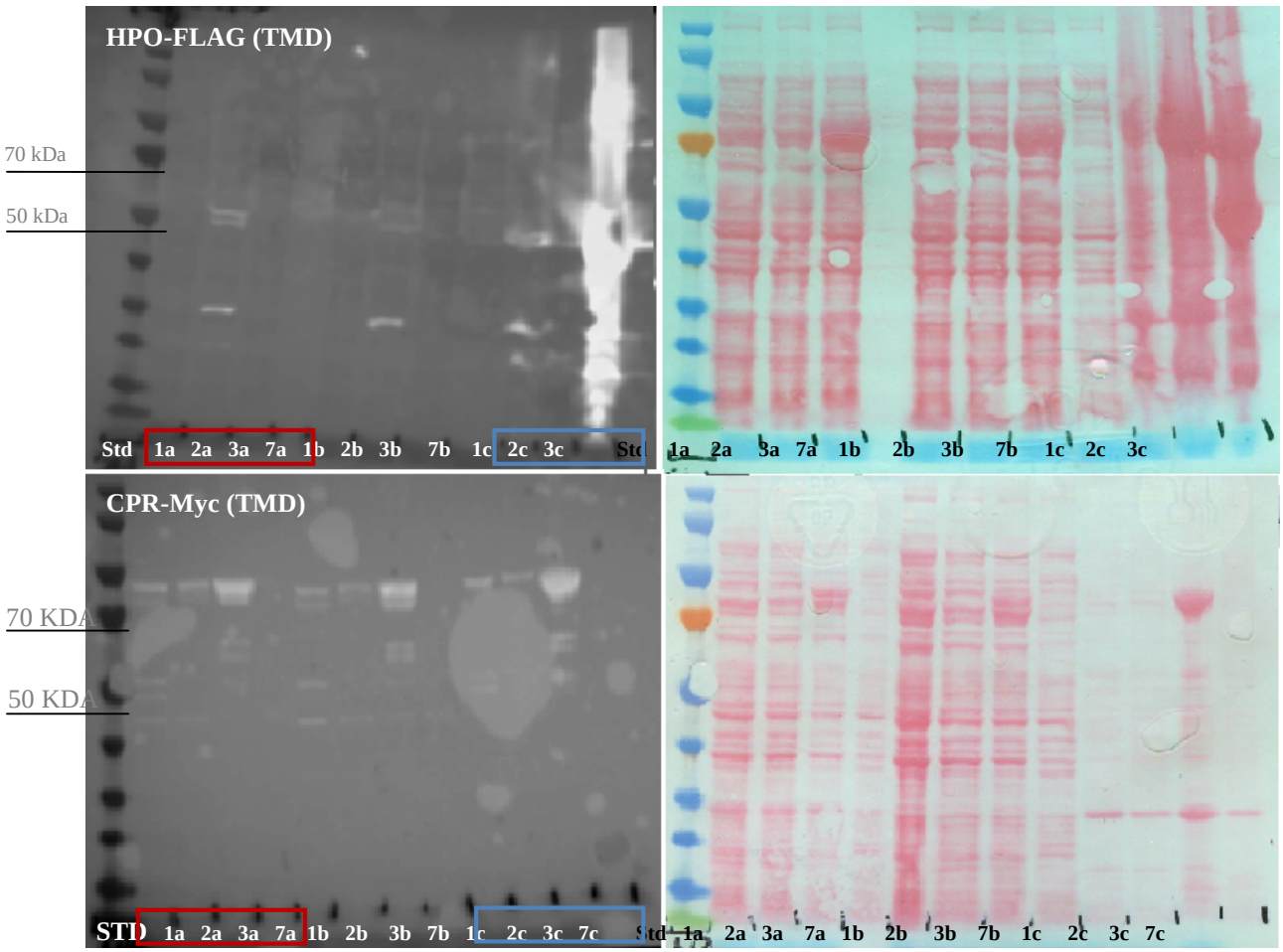


FIGURE 22: WESTERN BLOTS OF ENZYMES WITH TRANSMEMBRANE DOMAIN

variant	total cell homogenate	cell free extract	insoluble cell fraction	protein amounts (FLAG) [µg]	protein amounts (*myc*) [µg]
pMS470_CPR4-myc_HPO1-FLAG	1a			65	24
		1b		23	20
			1c	72	7
pMS470_CPR5-myc_HPO2-FLAG	2a			64	24
		2b		38	19
			2c	*	5
pMS470_CPR6-myc_HPO3-FLAG	3a			65	16
		3b		40	15
			3c	*	7
pMS470 linker	7a			30	8
		7b		25	16
			7c	47	12

*data not available

Results of Western blot analysis for truncated HPO and CPR.

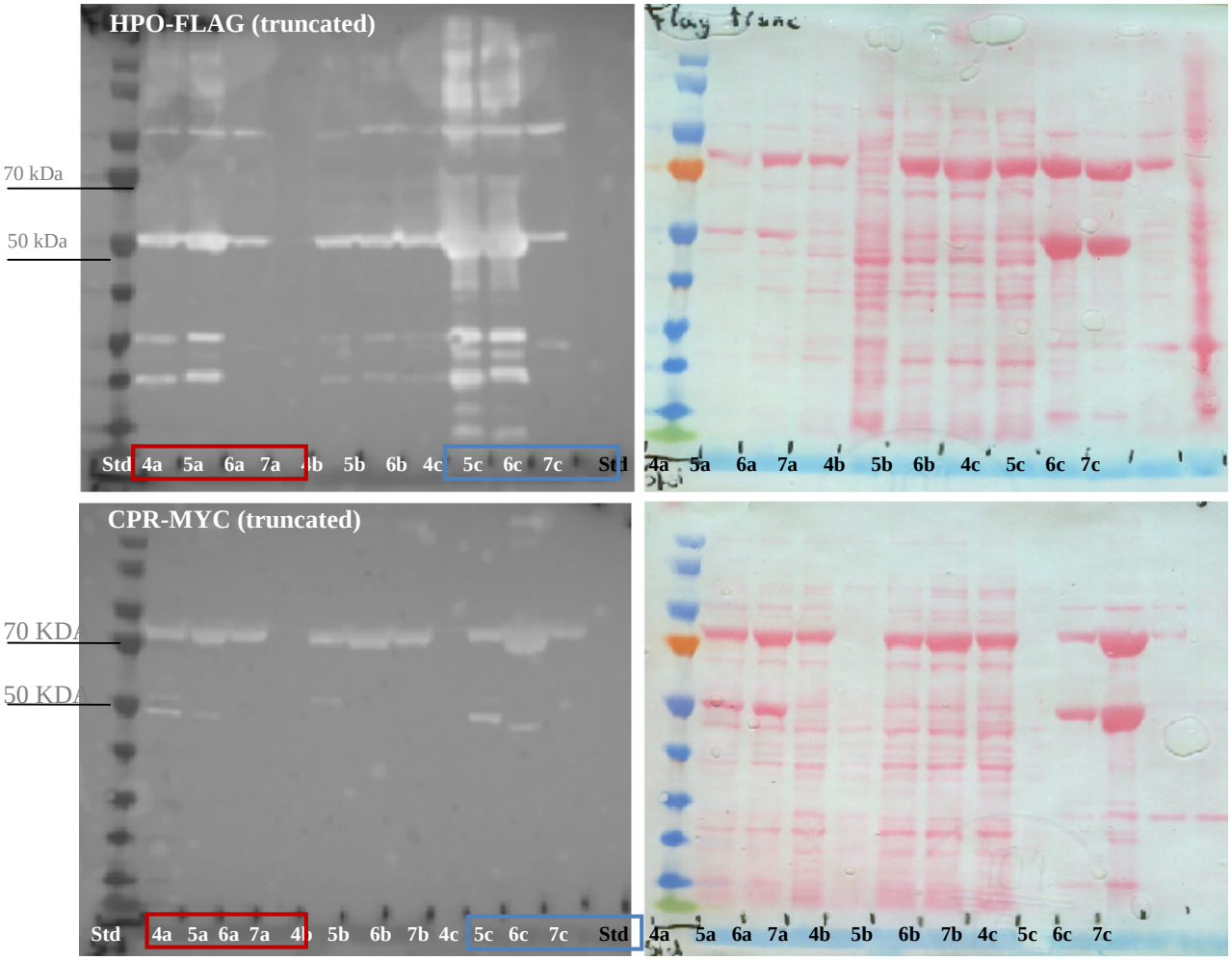


FIGURE 23: WESTERN BLOTS OF TRUNCATED HPO AND CPR PROTEINS

variant	total cell homogenate	cell free extract	insoluble cell fraction	protein amounts (FLAG) [µg]	protein amounts (*myc*) [µg]
pMS470_dsbC_CPR10-myc_HPO7-FLAG	4a			9	19
		4b		15	15
			4c	9	9
pMS470_dsbC_CPR11-myc_HPO8-FLAG	5a			10	19
		5b		16	16
			5c	9	9
pMS470_dsbC_CPR12-myc_HPO9-FLAG	6a			9	8
		6b		17	17
			6c	6	5
pMS470 linker	7a			30	6
		7b		25	16
			7c	47	12

A mistake was made probably during dilution of insoluble cell fractions for FLAG-tag immunodetection (Figure 23). On Ponceau S stained gels, excessive amounts of proteins were located that finally resulted in highly unspecific binding of antibodies.

Additionally, protein concentrations were sometimes not easy to determine, especially in case of insoluble cell fractions. Membrane particles and parts of disrupted cells showed tendency to form aggregates. In BioRad assays this caused troubles during absorption measurement due to irreproducible results.

Enzyme Activity Assays

Quantification of terpenes

For quantification of the substrate valencene and the products cis-, trans-nootkatol and nootkatone dilutions of terpene standards supplied by DSM were prepared. Standards with defined concentrations were analyzed by GC-MS or GC-FID and peak areas were integrated for quantification. Calibration curves were created by correlating peak areas with concentrations. Based on such a calibration curve, a special method for evaluating GC-FID data was programmed by Erich Leitner, enabling automatic peak recognition and integration followed by direct quantification of sought-after substances.

Purities of standard compounds, valencene: 70%, trans-nootkatol: 86%, cis-nootkatol: 98%, nootkatone: 75%, were considered in the calculations.

In case of GC-MS quantification, standards of different concentrations were generated and analysed (Figure 24).

standard	retention time [min]
valencene	12,559
cis-nootkatol	14,867
trans-nootkatol	15,003
nootkatone	15,742

FIGURE 24: CHROMATOGRAM OF STANDARDS ANALYSED BY GC-MS

For calibration, peaks were integrated and peak areas plotted against concentrations. Linear regressions revealed the following correlations of concentrations and integral peak areas.

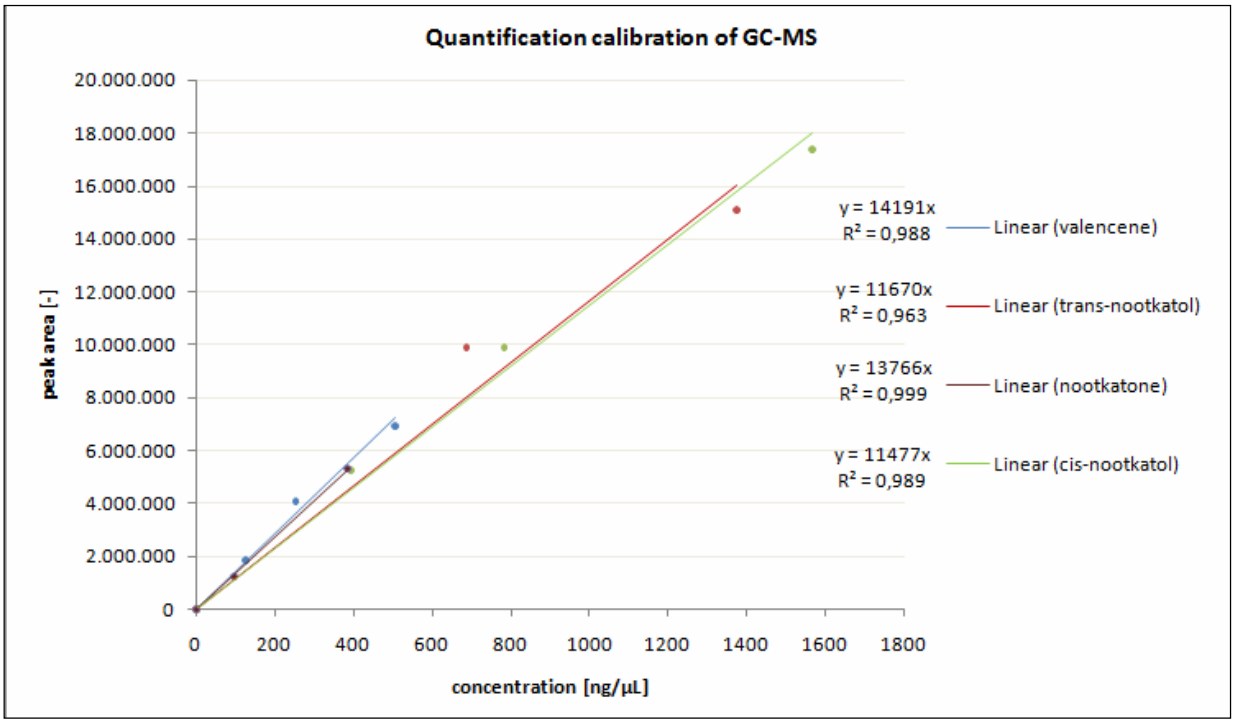


FIGURE 25: LINEAR REGRESSION FOR GC-MS QUANTIFICATION OF TERPENES

TABLE 20: INTEGRATION OF PEAK AREAS

valencene		trans-nootkatol		cis-nootkatol		nootkatone	
c [ng/µL]	peak area [-]	c [ng/µL]	peak area [-]	c [ng/µL]	peak area [-]	c [ng/µL]	peak area [-]
0	0	0	0	0	0	0	0
127	1,887,220	688	9,894,599	392	5,241,558	96	1,214,432
254	4,082,340	1,376	15,125,347	784	9,894,599	384	5,313,058
508	6,954,936	-	-	1,568	17,.361,103	-	-

.

Analysis via GC-MS offers the possibility to support compound identification by comparing mass spectra with those of the standards.

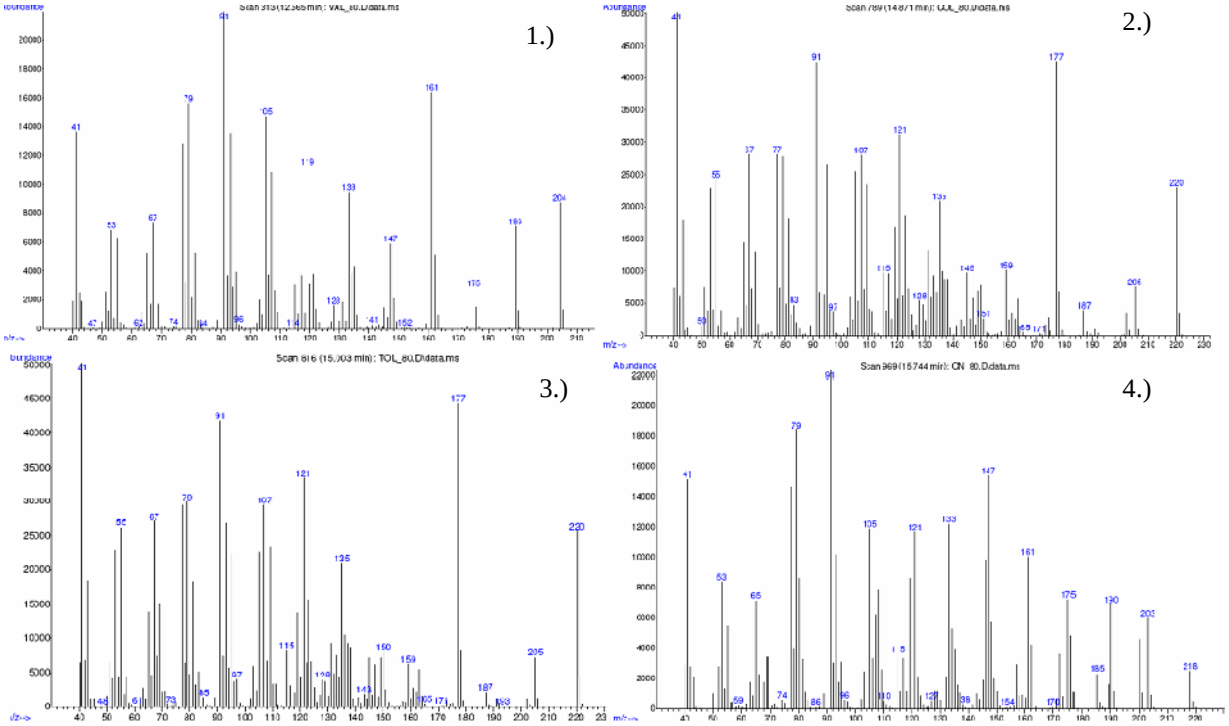


FIGURE 26: MASS SPECTRA OF 1.) VALENCENE, 2.) CIS-NOOTKATOL, 3.) TRANS-NOOTKATOL AND 4.)NOOTKATONE

Configuration and molecular masses of terpenes of interest are shown in Figure 27.

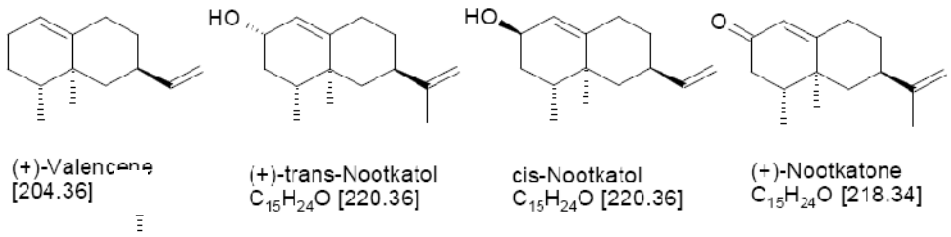
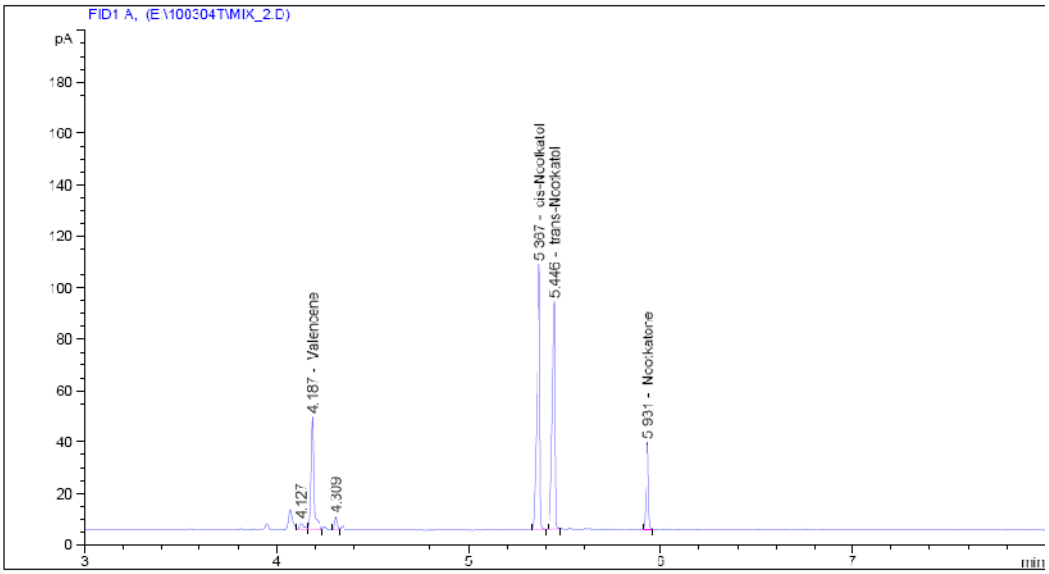


FIGURE 27: TERPENES WITH MOLECULAR MASS

Quantification and allocation of terpenes by GC-FID was automatically done by a method programmed for this task. A representative output file can be seen in Figure 28.




```
=====================================================================
                        External Standard Report
---------------------------------------------------------------------

Sorted By             :      Signal
Calib. Data Modified  :      05.03.2010 09:24:42
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A,

RetTime  Type     Area      Amt/Area   Amount    Grp   Name
 [min]           [pA*s]                [ng/ul]
-------|------ ---------- ----------|---------- --|------------------
  4.187 BV       43.67273    1.61163   70.38439    Valencene
  5.367 BB      115.51733    1.70664  197.14593    cis-Nootkatol
  5.446 BB       86.65300    1.91818  166.21641    trans-Nootkatol
  5.931 BB       30.53579    1.11982   34.19470    Nootkatone

Totals :                               467.94143
```

FIGURE 28: CHROMATOGRAM OF STANDARDS MEASURED BY GC-FID COUPLED TO DIRECT QUANTIFICATION

IN VIVO ASSAYS

Three different in vivo assays representing varying reaction conditions are shown. In every diagram, the initial amount of valencene is shown to give an idea of substrate loss.

TABLE 21: OVERVIEW OF DIFFERENT IN VIVO ASSAYS PRESENTED

	reaction volume [mL]	medium during conversion	valencene used [mg]	extraction method	measurement
In vivo assay 1	50	LB	14.25	concentrated via N_2 stream	GC-FID
In vivo assay 2	2	KP_i buffer	0.57	concentrated via N_2 stream	GC-MS
In vivo assay 3	50	LB, TB	14.25	directly measured	GC-FID

For **in vivo assay 1** cells were grown in LB media and δ-ALA was added. Organic phases were concentrated via N_2 stream, dissolved in 1 mL EtOAc and analyzed by GC-FID.

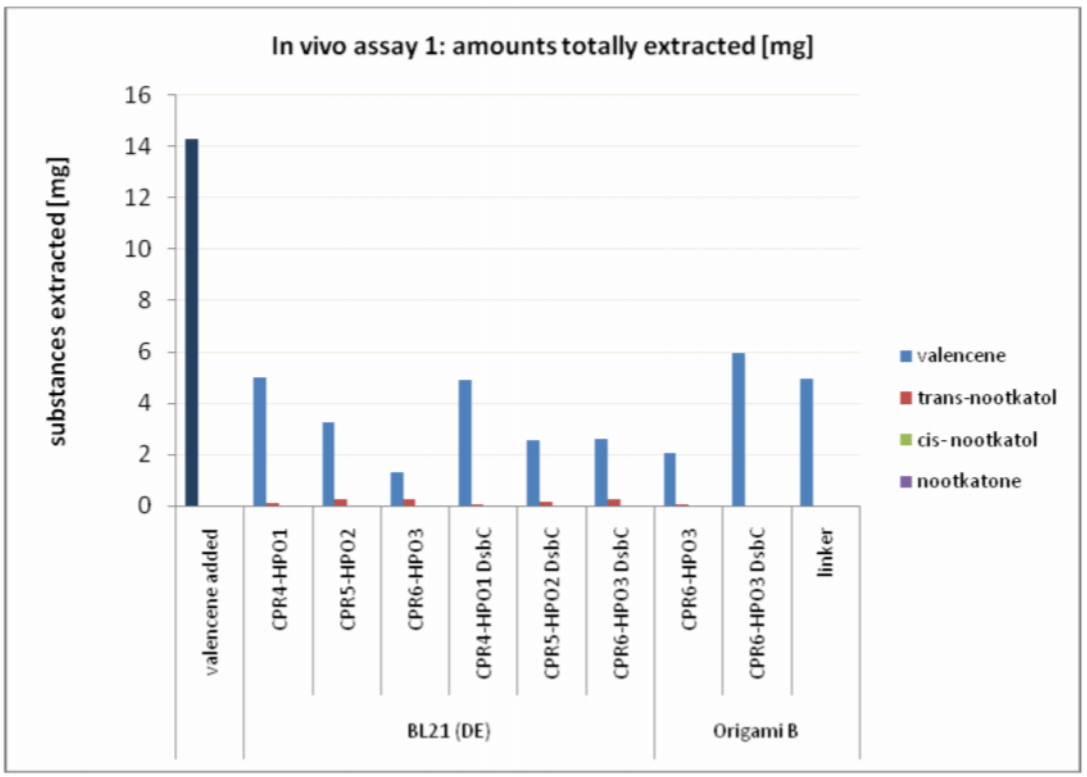


FIGURE 29: TOTALLY EXTRACTED TERPENES (IN VIVO ASSAY 1)

In vivo assay 2 was performed with resting cells. Therefore, the total amount of valencene used was smaller. Every sample was prepared and analyzed twice. Once with the addition of 20% dodecane and once without. After conversion, extracts were concentrated via N_2 stream, dissolved in 1 mL EtOAc and analyzed by GC-MS.

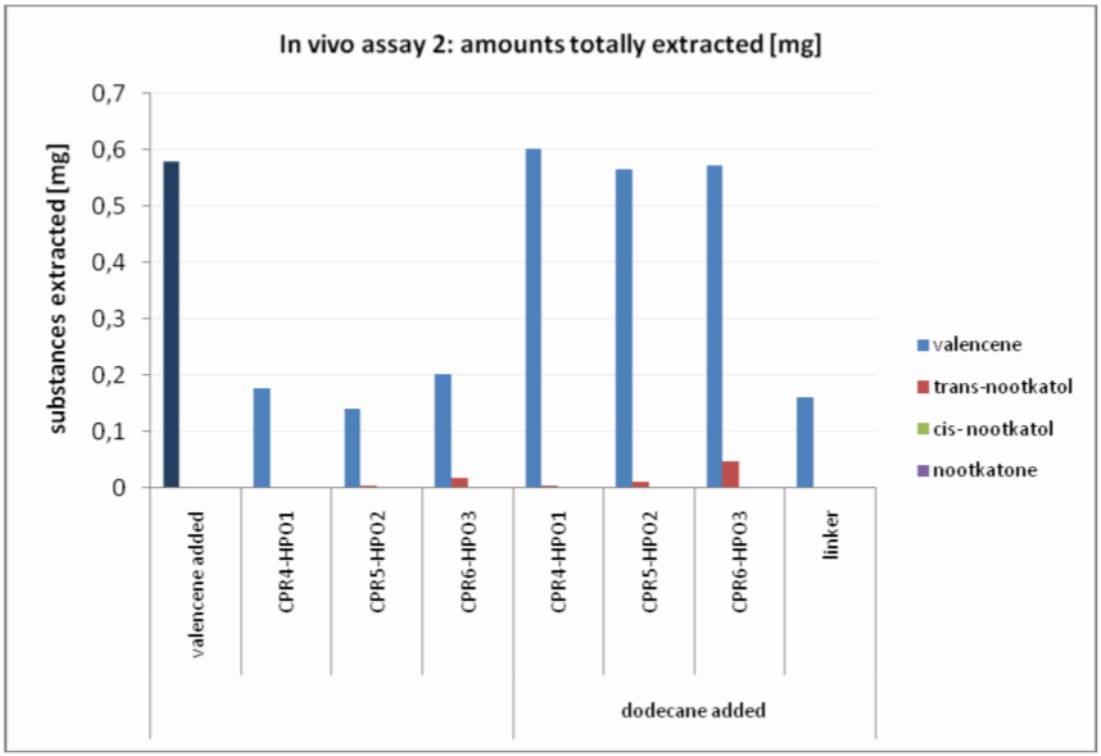


FIGURE 30: TOTALLY EXTRACTED TERPENES (IN VIVO ASSAY 2)

In vivo assay 3 was exactly performed as **in vivo assay 1** with the only difference, that organic layers were not concentrated but directly analyzed by GC-FID. The assay was performed in two different media, TB and LB. Additionally, strains harboring tagged genes were tested.

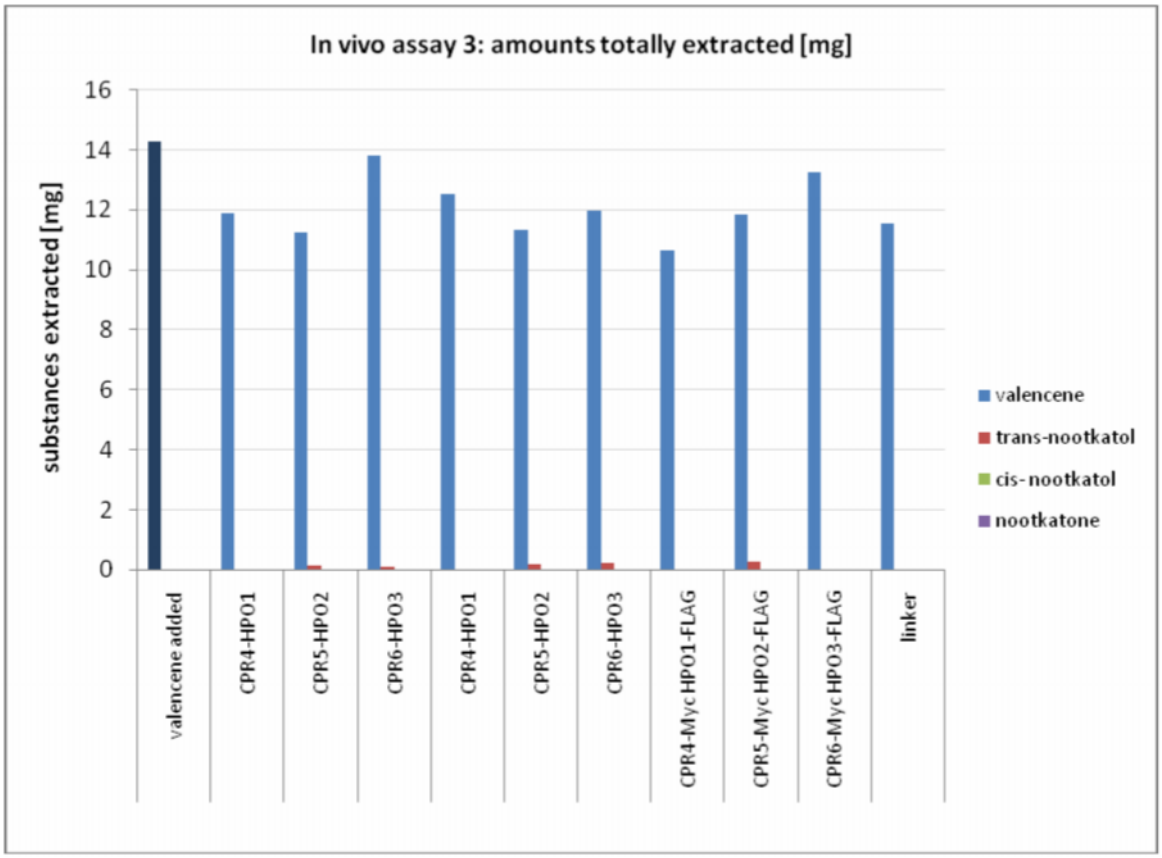


FIGURE 31: TOTALLY EXTRACTED TERPENES (IN VIVO ASSAY 3)

OD_{600} of cell cultures after bioconversion were determined to extrapolate for cell dry weight. Total amount of products found in extracts was normalized for cell dry weights and conversion times.

TABLE 22: CELL DRY WEIGHTS DETERMINED FOR IN VIVO ASSAYS

	Conversion time [h]	*E.coli* phenotype, media	Genes expressed	end OD_{600} [-]	cdw [mg]
In vivo assay 1	22	BL21 Gold (DE3), LB medium	CPR4-HPO1	4.14	36.5
			CPR5-HPO2	4.87	42.9
			CPR6-HPO3	3.78	33.3
			CPR4-HPO1 DsbC	4.74	41.8
			CPR5-HPO2 DsbC	4.78	42.1
			CPR6-HPO3 DsbC	4.42	39.0
		Origami B (DE3), LB medium	CPR6-HPO3	0.62	5.5
			CPR6-HPO3 DsbC	2.04	18.0
			linker	2.38	21.0
In vivo Assay 2	20	BL21 (DE), KPi buffer	CPR4-HPO1	9.26	83.2
			CPR5-HPO2	8.76	78.7
			CPR6-HPO3	7.83	70.3
		BL21 (DE), KPi buffer, dodecane	CPR4-HPO1	8.72	78.3
			CPR5-HPO2	8.29	74.5
			CPR6-HPO3	7.10	63.8
			linker	2.19	19.7
In vivo Assay 3	20	BL21 (DE), LB medium	CPR4-HPO1	3.34	29.4
			CPR5-HPO2	4.85	42.8
			CPR6-HPO3	3.48	30.7
		BL21 Gold™(DE3), LB medium	CPR4-myc HPO1-FLAG	1.29	11.4
			CPR5-myc HPO2-FLAG	2.14	18.9
			CPR6-myc HPO3 FLAG	0.97	8.6
		BL21 (DE), TB medium	CPR4-HPO1	7.99	70.4
			CPR5-HPO2	8.77	77.3
			CPR6-HPO3	9.45	83.3
			linker	3.52	31.0

Linker was always expressed in *E. coli* BL21(DE) and served as vector control.

Results of all three in vivo assays were compared to each other regarding product recovered in µg per h and mg cell dry weight.

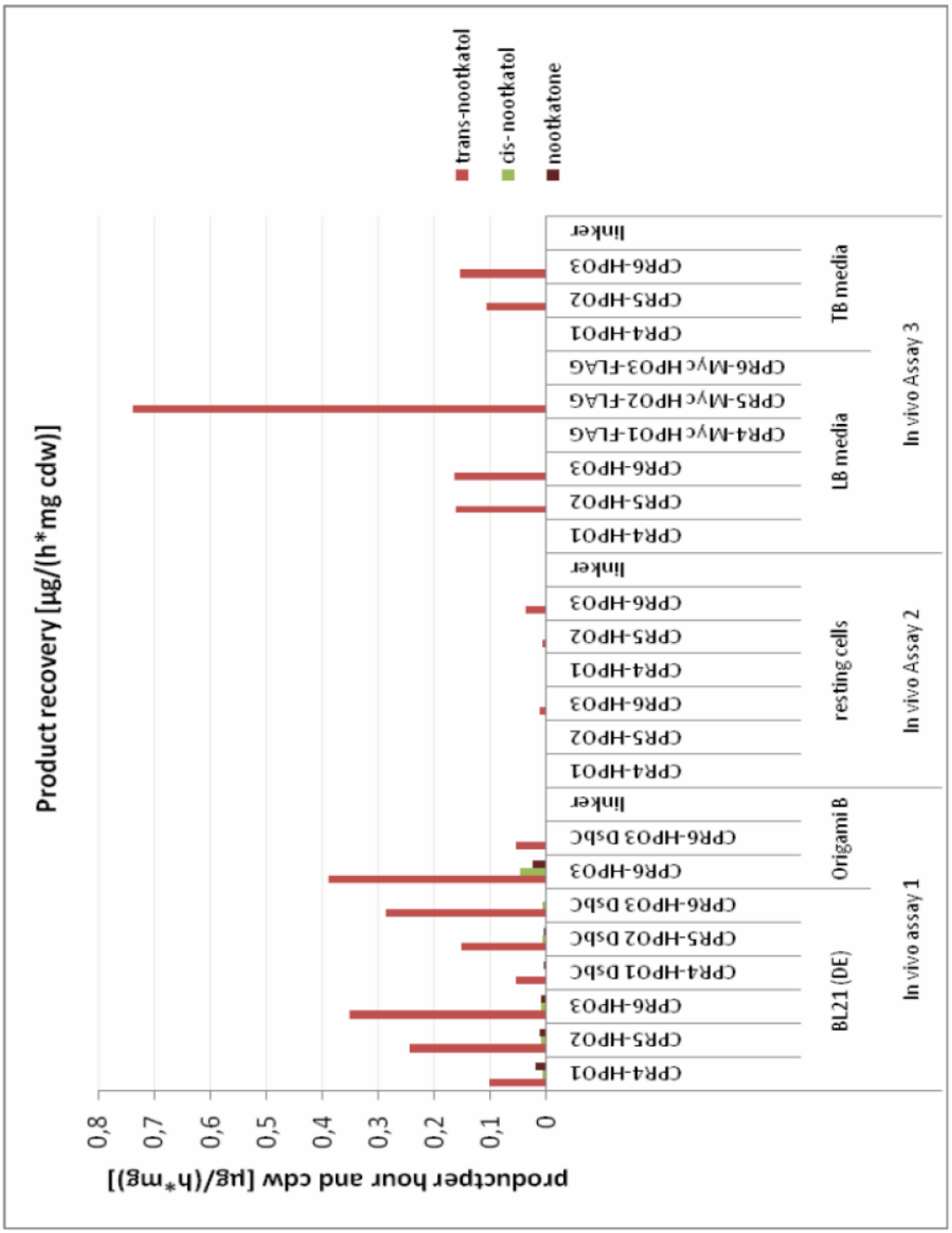


FIGURE 32: RESULTS OF IN VIVO ASSAY 1, 2 AND 3

IN VITRO ASSAYS

Results of three assays performed under different conditions were chosen to highlight differences and the effects of different variables.

TABLE 23: ASSAY CONDITIONS FOR IN VITRO ASSAYS PRESENTED

	media conditions	GDH system	assay volume [mL]	work up	measurement	duration [h]
In vitro assay 1	LB media, no δ-ALA or $FeSO_4$	no	15 mL	extracts concentrated via rotavap	GC-MS	16
In vitro assay 2	TB media, δ-ALA and $FeSO_4$	yes	7.5 mL	extracts concentrated via N_2 stream	GC-MS	26
In vitro assay 3	TB media, δ-ALA and $FeSO_4$	yes	7.5 mL	extracts not concentrated	GC-FID	16

In vitro assay 1 was performed in a total volume of 15 mL. Therefore, the double amounts of all substances were used in the assay. Cells were grown in LB medium without addition of δ-ALA or $FeSO_4$ during protein expression. There was no cofactor regeneration system. Reaction mixtures were concentrated via rotavapor and analyzed by GC-MS. The initially used amount of valencene is shown in dark blue.

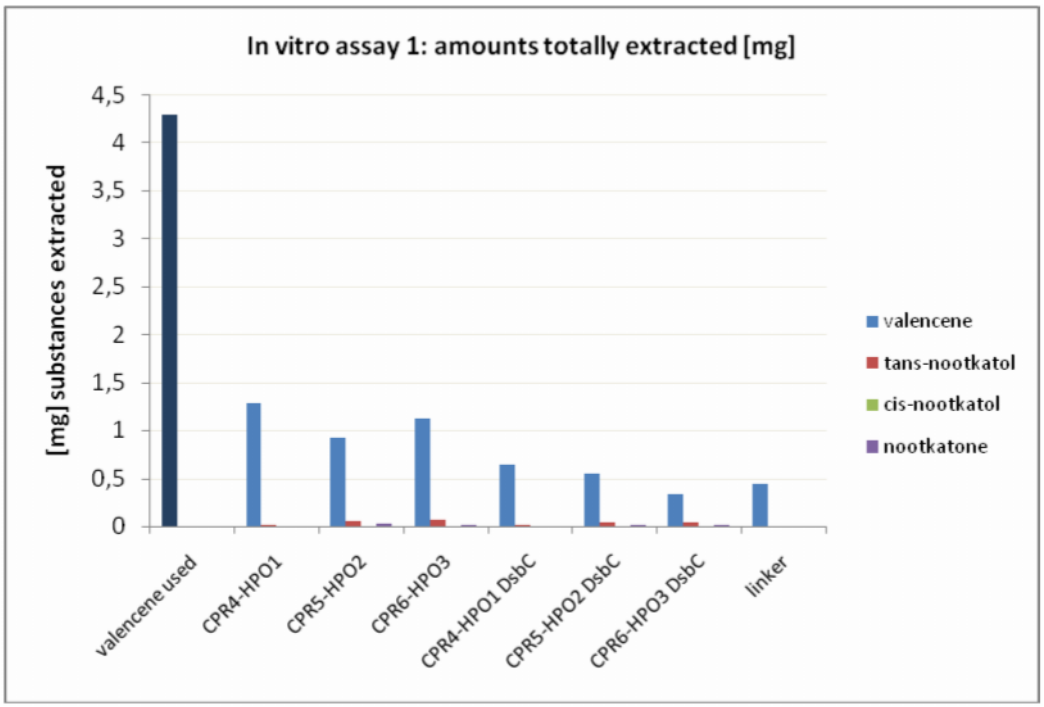


FIGURE 33: SUBSTANCES TOTALLY EXTRACTED (IN VITRO ASSAY 1)

In vitro assay 2 was exactly performed as described in Materials and Methods (page 32) and during fermentation, δ-ALA and $FeSO_4$ were added. Glucose dehydrogenase was provided for cofactor recycling. The initially used amount of valencene was 2.15 mg. Organic extracts were concentrated via N_2 stream, dissolved in 0.5 mL EtOAc and analyzed by GC-MS.

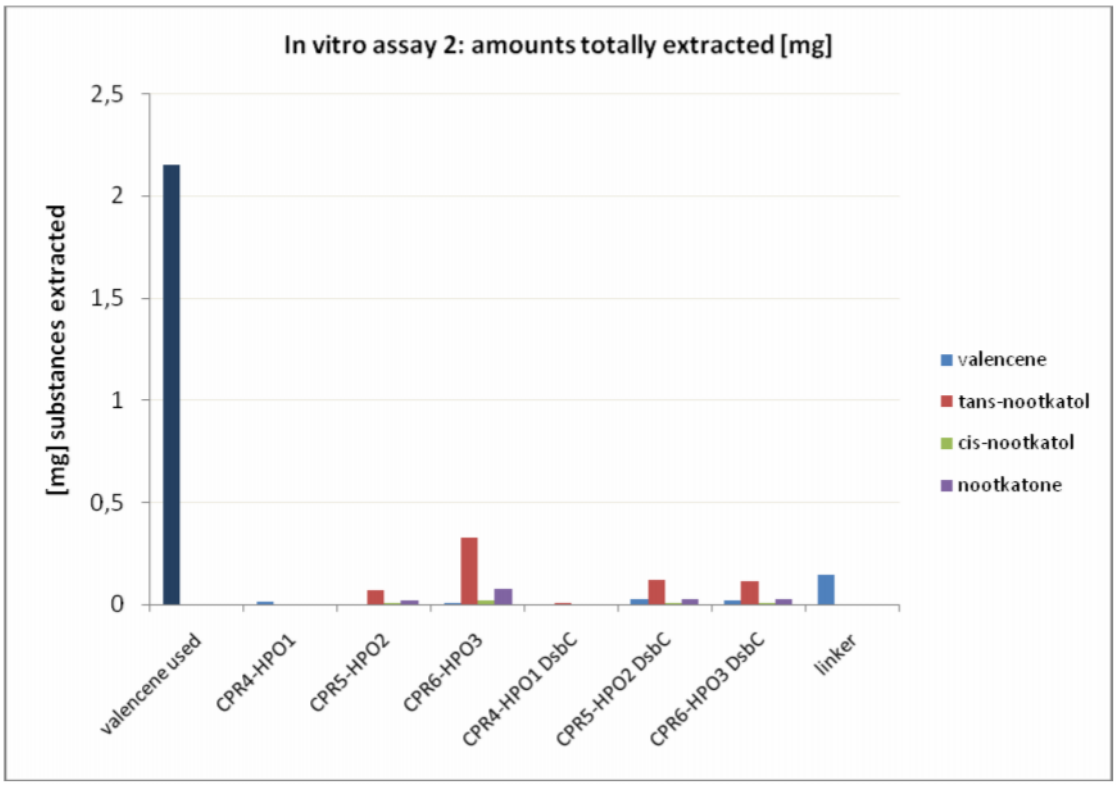


FIGURE 34: SUBSTANCES TOTALLY EXTRACTED (IN VITRO ASSAY 2)

In vitro assay 3 resembled **in vitro assay 2**, with the exception that organic extracts were directly transferred to GC-FID analysis. Amount of valencene initially used was 2.15 mg.

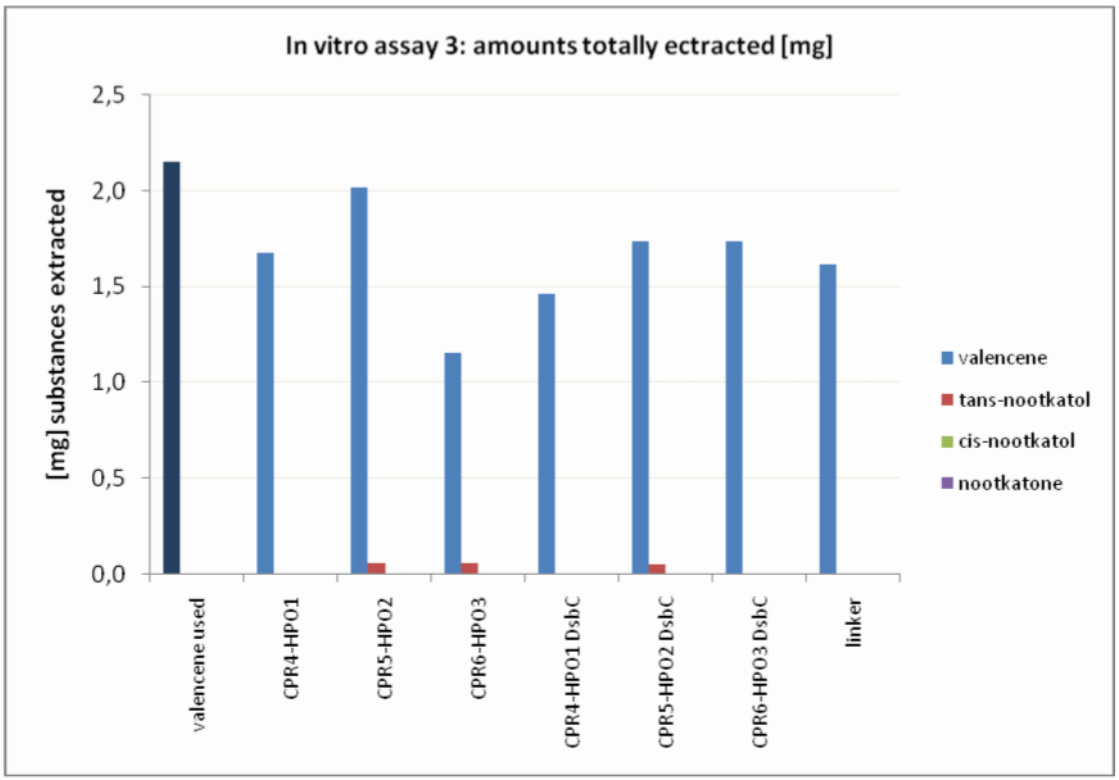


FIGURE 35: SUBSTANCES TOTALLY EXTRACTED (IN VITRO ASSAY 3)

For comparing all three assays, totally extracted amounts were normalized considering conversion time and mg of total protein used.

TABLE 24: PROTEIN AMOUNTS USED FOR IN VITRO ASSAYS

	in vitro assay 1 [mg]	**in vitro assay 2** [mg]	**in vitro assay 3** [mg]
CPR4-HPO1	19	44	22
CPR5-HPO2	25	44	26
CPR6-HPO3	20	40	24
CPR4-HPO1 DsbC	19	45	25
CPR5-HPO2 DsbC	19	43	25
CPR6-HPO3 DsbC	22	27	24
linker	9	12	12

The three different in vitro assays were compared with regard to product per time and total amount of protein.

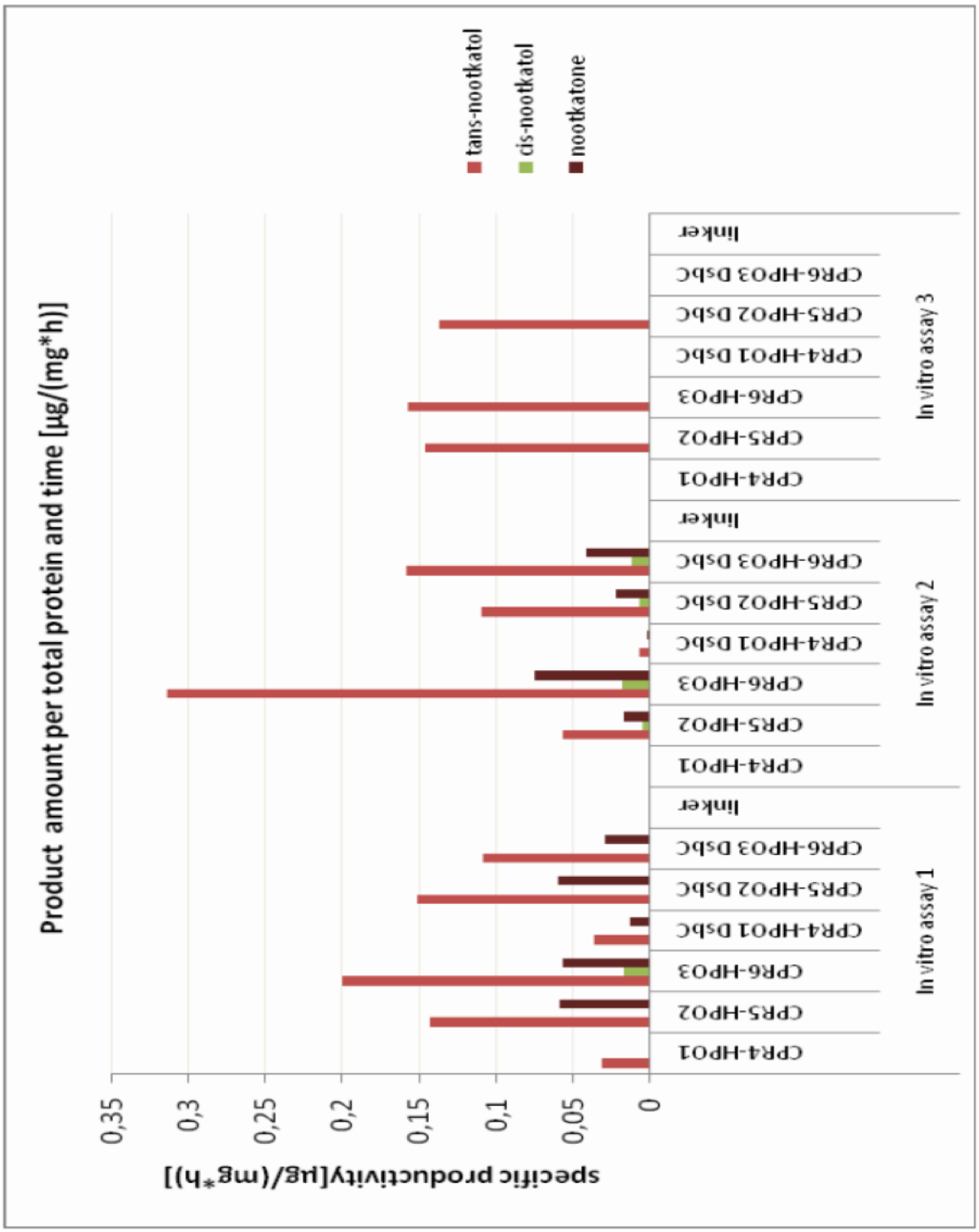


FIGURE 36: RESULTS OF IN VITRO ASSAY 1, 2 AND 3

DISCUSSION

Native HPO is N-terminally fused to a transmembrane domain, which was truncated in case of genes optimized for *E. coli*. According to literature, this should result in high-level expression and activity, due to better solubility of the enzyme.[17] At the beginning of the diploma thesis, all vectors were constructed using truncated genes, which became more and more irrelevant as the project progressed for strategy reasons. As we observed extremely low activity despite high- level expression of HPO, which was proven by SDS-PAGE and Western blot analysis, we hypothesized that truncated HPO is mainly stored in inclusion bodies. For this reason, we switched to the expression of genes optimized for *P. pastoris* with transmembrane domains in *E. coli*. Only the Shine-Dalgarno sequence had to be added before cloning into the pMS470 expression vector.

All cloning steps were performed in *E. coli* Top10F', to generate sufficient amounts of plasmid DNA for the individual molecular operations. For fermentation and assay setup, *E. coli* BL21 (DE) modified for gene expression was used. This expression strain is lacking the two proteases Lon and OmpT, leading to increased stability of recombinant proteins, and is definitely beneficial if crude whole cell lysates are directly used for in vitro assays

To correlate enzyme activities to expression rates, genes were tagged using PCR methods. A FLAG-tag[18] was C-terminally fused to all HPO genes, a *myc*-tag was chosen for CPR genes. Both tags are unlikely to denature or inactivate proteins to which they are appended, due to their small size and optimization for compatibility. Expression of tagged proteins was successfully detected by Western blot analysis, using antibodies for recognition of the tag-epitopes and secondary antibodies linked to horseradish peroxidase. Peroxidase activity yielded a chemoluminescence signal when incubated with commercially available peroxide substrates.

FERMENTATION

Fermentation conditions were improved gradually. Therefore, growth and expression temperature were lowered to 20°C and the concentration of the inducing agent IPTG reduced to slow down intercellular protein synthesis and favour correct protein folding.

These conditions evidently influenced the fitness and growth characteristics of cells, which was accepted due to initially successful expression of active enzymes. Unfortunately, *E. coli* Origami B (DE3) had severe problems to grow at 20°C. Because of non- reproducible growth and extremely low overall enzyme concentrations, expression with this host was terminated. Also, the cloning strain *E. coli* Top10F' was tested as expression host, but both, living cells and lysates showed no activity. Finally, *E. coli* BL21 (DE) has proven to be the best strain for expression. However, the highest levels of P450 expression were achieved by addition of δ-aminolevulinic acid (δ-ALA) and $FeSO_4$ to the medium culture, presumably permitting heme biosynthesis to keep up with P450 polypeptide synthesis[3,13]. Altogether, activity found was by far too low for industrial applications.

WESTERN BLOT ANALYSIS

HPO and CPR expression was proven by Western blot analysis for FLAG- and *myc*-tags that had been fused to the proteins expressed in *E. col.i* To get a subtle hint about enzyme localization within the cell and solubility, cell homogenates, cell free extracts and insoluble cell fractions were investigated.

As SDS-PAGE had already proven, genes optimized for *E. coli* were much higher expressed than those optimized for *P. pastoris*. On the one hand this could be explained by the adapted codon usage and, therefore, faster translational processes, but also the truncation of transmembrane domains might have played a significant role. Considering the hydrophobic character of these transmembrane domains originating from plants, it appears highly probable that only a small fraction can be functionally embedded into the cytoplasmic membrane of *E. coli*. Compared to the vast space of the cytosol, membranes only offer very limited space for proteins to be attached, which in some cases even can seriously harm the plasmid-bearing host by reducing viability and stability.[19] If expressions of native sequences (with and without TMD) are compared, enzymes with transmembrane domains were much lower expressed.

Antibodies for FLAG-tag detection recognized HPO- bands of the correct size but labelled also smaller bands probably representing degradation products. Interestingly, a larger band about twice the size of HPO-FLAG was stained specifically. The lysates of the expressed CPR6-myc_HPO3-FLAG gave no signal for expression of HPO, although positive cloning was proven by restriction control. Remarkably, the same strain showed no activity during **in vivo assay 3**. The reason for the lacking protein synthesis is still unclear. Maybe the Shine-Dalgarno sequence was destroyed or mutated during cloning, which could easily be found out by sequencing, which was however not done so far.

Comparing HPO and CPR expression of genes still having their transmembrane domains, all constructs were expressed to about the same level, no matter if codon usage was optimized by DSM or by T. Wriessnegger. This is not surprising, due to the fact, that genes were optimized for expression in *P. pastoris* and therefore an effect in *E. coli* is not necessarily expected. What can clearly be stated is that insoluble cell fractions contained three to four times more of tagged proteins than the cell free extract of the same cell homogenate.

Similar observations were made for expression of *E. coli* optimized genes. Just a very slightly improved expression rate could be seen for optimized genes compared to the native sequences. Interestingly, again approximately the three- fold amounts of tagged proteins was detected in the insoluble cell fraction compared to whole cell lysates, although sequence optimization included truncation of transmembrane domains to reach better enzyme solubility. Obviously, HPO and CPR show hydrophobic tendencies based

on tertiary protein structures, even if truncated. This is definitely feasible, considering the fact that the enzymes naturally act in direct proximity of cell membranes in plant, which is evidently an absolutely different environment.

ACTIVITY ASSAYS

In general, genes optimized for *E. coli* showed no activity, despite being highly expressed. This fact was proven by Western blot analysis finally substantiating the assumption of inclusion body formation. By request of our industrial partner we soon focused on activity assays performed with genes attached to transmembrane domains. Although or maybe even because optimized for *P. pastoris*, active enzymes could be obtained. A possible explanation might be the slower translation of codons usage foreign to *E. coli* and, therefore, higher chances for correct protein folding.

Due to constantly varying conditions for improving bioconversion, assays of one setup were often only performed once. Reproduction of results also turned out to be difficult, because of low overall activities. Therefore, only trends can be pointed out and no exact data or conversion rates.

IN VIVO ASSAYS

- Loss of valencene

The diagram showing totally extracted amounts of **in vivo assay 1** indicates that a huge loss of the substrate valencene occurs without it being converted to one of the desired products. According to literature, some CYPs may tend to form overoxidized side products[13]. Although this possibility was not supported by unidentified peaks in gas chromatograms, a strategy to overcome this unwanted effect was the addition of dodecane. Dodecane is a rather non-toxic organic solvent for *E. coli* and confers better substrate and product solubility than aqueous phase.[13] **In vivo assay 2** (Figure 30) shows, that substrate loss was almost totally abrogated by addition of dodecane. This could either be caused by avoiding side product formation or preventing substrate evaporation during bioconversion or workup. Therefore, organic extracts were directly measured, without concentrating steps during the final stage of this work. After extraction during **in vivo assay 3** almost all of the instantly used substrate was retrieved, which disproves the possibility of side product formation. Concentrating via N_2 stream or rotavapor caused irreproducible and therefore untrustworthy results based on uncontrollable loss of substances.

- DsbC coexpression

As presented in Figure 29 and 32, coexpression of DsbC did not yield higher HPO activity as compared to assays without DsbC. In most cases, activities were slightly decreased, but differences were hardly significant considering overall conversion fluctuations.

- Codon optimization

Although codon usage was optimized for *P. pastoris*, conversion rates were improved three to seven times compared to native sequences. All assays performed revealed the highest activities for CPR and HPO optimized by T. Wriessnegger. In case of **in vivo assay 3** no results were obtained for CPR6-myc HPO3-FLAG. That is the reason for the apparently higher conversion by CPR5-myc and HPO2-FLAG, for which the genes were optimized by DSM.

- Resting cells

Looking at the diagram showing totally extracted amounts of **in vivo assay 3**, conversions reached with resting cells seem to be the most promising of all in vivo assays presented. This conclusion is deceiving, as indicated by comparing results of all assays normalized for cell dry weights and conversion times (Figure 32). Because of the number of cells used for this particular in vivo assays, only small amounts of valencene were converted under normalized conditions.

- Addition of dodecane

Addition of dodecane prevents terpenes from being evaporated due to high affinity for the compounds. Basically, this is beneficial for assay setup and quantification, but a huge drawback considering conversion aspects. Dodecane, consequently also prevents valencene to get in contact with the cell surface of biotransformants, which enforces substrate limitation.

Furthermore, another point to be considered is substrate uptake into the cell. As long as valencene and also nootkatole are toxic for *E. coli*, like most hydrophobic terpenes are, the cell always will try to get rid of the substances by activating intracellular processes. In most cases, membrane associated transport systems such as efflux pumps are involved. Knocking out these systems has been shown to increase substrate uptake.[20]

- TB and LB media

Another interesting effect will be discussed with the help of **in vivo assay 3**. TB media enable cells to grow to higher densities that understandably show higher conversion performance per unit of time and conversion volume. But if conversions per h and cell dry weight are compared, no difference between cells grown in TB or LB media can be determined. In conclusion, single cells harbour a defined amount of active enzymes that is not influenced by type of media.

Tagged genes were only tested once to compare activities with expressed protein amounts determined by Western blot analysis. Positive results in both cases could be obtained for CPR5-myc and HPO2-FLAG. Nevertheless, activities in general seem to be very low compared to expressed protein amounts, which again might be a hint for inclusion body formation. Western blotting will detect proteins by recognizing *myc*- or FLAG-tags without discriminating if enzymes are active or not.

Addition of tags also sometimes increases enzymatic activities based on different aspects like protein stability or enhanced translation due to better mRNA stability[21,22].

- *E. coli* Origami B (DE3)

Normalizing for conversions per hour and cell dry weights, *E. coli* Origami B (DE3) seems to be a reasonable host for HPO/CPR expression and bioconversions. But on overall extremely low optical densities and huge ups- and downs in cell growth forced us to quit using this strain.

IN VITRO ASSAYS

In vitro assays worked out generally better than in vivo assays, although almost all of the observations to be discussed count for both types of assays. This might support the argument of troubles with substrate uptake and abidance in living cells. Relatively more intermediate product nootkatol was converted to the final product nootkatone in vitro. Once, overall conversion rates were very low and miner improvements were achieved, but did not get close to industrially relevant results. Cell homogenates for conversion always had to be freshly prepared, because freezing and thawing destroys sensitive CYP enzymes[23]. This made the assays very time consuming and optimization implementation laborious.

- Loss of valencene

Concentrating extracts by N_2 stream or rotavapor leads also to a remarkably loss of substances in this case, which can be seen in the diagrams showing total extracts of **in vitro assay 1** and **in vitro assay 2** (Figures 33 and 34). This could be overcome by directly analyzing organic phases after extraction, as represented by **in vitro assay 3** (Figure 35). Renouncing of concentrating samples made results reproducible and more reliable. The absence of nootkatone in the crude extracts can be explained by its very low concentration, although GC-MS and GC-FID are both highly sensitive quantification methods.

- DsbC coexpression

Coexpression of disulfide bond isomerase C had no real effect on HPO activity. At the most, a slight decrease could be seen as already discussed for in vivo assays.

- Codon optimization

Compared to native sequences, codon optimization leads in all assays to improved enzyme activity, whereas constructs optimized by T. Wriessnegger again achieved the best results.

- GDH system

For in vitro assays, implementation of cofactor regeneration system was definitely an improvement. Conversion was enhanced, which can clearly be seen for **in vitro assay 2** (Figure 34). Based on long conversion time, product amount per h of conversion and mg of protein used is most likely lowered due to the possibility that HPO activity is not stable for 26 h. Other assays performed with and without glucose dehydrogenase addition (data not shown) showed lower or no activity if GDH was omitted. Theoretically, equimolar amounts of NADPH are needed for Valencene conversion. In assay 1 (in vivo), ten times more Valencene than NADPH was used. Considering this fact, even without addition of cofactor regeneration system, there should have been enough cofactor to support the very low conversion rates. However, an explanation for the improved rates by regeneration of NADPH (Figure 36) might be better availability of

NADPH if present at higher concentrations, but also the regeneration of spontaneously hydrolyzed cofactor due to photo- and thermosensitivity.

Based on the fact, that gluconic acid is the most abundant by-product resulting from cofactor regeneration, pH values of reaction mixtures were measured after in vitro assays to make sure that enzymes do not get inactivated by acidic environment. pH values remained stable, which is a sign of either very low depletion of NADPH or low activity of glucose dehydrogenase.

An interesting approach was tested for overcoming the apparent substrate limitation of enzymatic reactions. Therefore, both in vitro and in vivo the detergent Triton X-100 was added to support enzyme-substrate-complex formation by facilitating phase transfer of substrate, which is considered to be the rate limiting step. The creation of emulsions on the one hand facilitates substrate solubility, but also stabilizes hydrophobic membrane proteins in cell homogenates.[24] In case of conversion with living cells, low concentrations of detergent support pemeabilization of cell walls and therefore could be beneficial for substrate uptake into the cell. Although the strategy appeared to work out well within the first assays, experiments could not be continued. Unfortunately, Triton X-100 is coextracted by organic solvents during workup due to its amphiphilic character and consequently severely damaged the GC column.

An easy and comparatively fast way to quantify cytochrome P450 enzymes are CO difference spectra. If mature CYPs are treated with carbon monoxide, which shows high affinity for the reduced ferrous active side, enzymes are inactivated and absorptions shifts from 420 nm to 450 nm[25]. Peak integration gives an idea of enzyme concentration. This method was also tested during this master thesis, but revealed no significant results due to obviously low amounts of (active) HPO.

Head space analysis of reaction mixtures is still in progress for directly analyzing the products after bioconversion but was not subject of this diploma thesis. This one hand saves time and on the other hand reduces falsification of results based on extraction with organic solvents, which has proven to be responsible for irreproducibility.

Indeed, most of the implemented optimization and adaptation steps for in vitro and in vivo conversions seemed to have positive effects on HPO activity. Nevertheless, the interaction of all limitations like uneasy substrate handling, sensitive and complex enzymes and long-time assays made this project very challenging.

Altogether, *E. coli* does not seem to be the most qualified expression host for this type of enzyme. Nevertheless, considering the undeniable advantages offered by heterologous expression in *E. coli*, it was definitely worth a trial. At least, the low activities were quantified and different types of assays were established for future work.

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