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MSc Protein Biotechnology
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MSc Thesis

Functional expression and characterization of cytochrome P450s, associated with pesticide toxicity

by Stavroula Kaili

Committee
Prof. John Vontas
Prof. George Chalepakis
Assist. Prof. Ioannis Pavlidis

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Abstract

Insecticide selectivity and resistance is an ongoing challenge concerning both crop pest control and insect-born diseases control, and it has been largely associated with cytochrome P450 monooxyganases' metabolism. Functional characterization of P450s is of great importance, in order to validate their involvement in insecticide resistance and to develop selective and safe insecticides (e.g. for bee pollinators) and insecticide synergists. In the present study, two distinct cases of P450s that have been associated with resistance to insecticides were heterologously expressed in *E. coli* for their functional characterization. Firstly, *Ha*CYP6B7, which was previously found to be associated with fenvalerate metabolism in *Helicoverpa armigera*, was co-expressed in *E. coli* together with *Musca domestica* CPR (*Md*CPR). *Ha*CYP6B7 was expressed in its native and truncated form in the membrane or in the cytoplasm of bacteria, respectively. The recombinant enzyme was assayed for its catalytic activity against model substrates. In the second part of this study, the aim was to heterologously express in *E. coli Bo*CYP6G2 and *Bo*CYP6A23 that have been related with resistance to α-cypermethrin in *Bactrocera oleae.* The two P450s were successfully cloned into expressing vectors and *Bo*CYP6G2 proceeded to membrane targeting experiments together with *Bactrocera oleae* CPR (*Bo*CPR).

Περίληψη

Η ανθεκτικότητα των εντόμων στα εντομοκτόνα καθώς και η εκλεκτικότητα των εντομοκτόνων αποτελούν μεγάλες προκλήσεις όσον αφορά την αντιμετώπιση των εντόμων-γεωργικών εχθρών αλλά και αυτών που είναι φορείς ασθενειών και απειλούν τη δημόσια υγεία. Τα ένζυμα κυτοχρωμικές μονοξυγενάσες P450s διαδραματίζουν πολύ σημαντικό ρόλο όσον αφορά ανάπτυξη ανθεκτικότητας στα εντομοκτόνα καθώς και στην εκλεκτικότητα των εντομοκτόνων. Η λειτουργική έκφραση των ενζύμων αυτών, με σκοπό την κατανόηση της εμπλοκής στους μηχανισμούς ανθεκτικότητας αλλά και του σχεδιασμού νέων, ασφαλών και εκλεκτικών εντομοκτόνων είναι ύψιστης σημασίας. Στην παρούσα εργασία, δυο διακριτές περιπτώσεις ενζύμων P450s, που έχουν συσχετιστεί με ανθεκτικότητα σε συγκεκριμένα εντομοκτόνα εκφράστηκαν ετερόλογα σε βακτηριακά κύτταρα *E. coli* με στόχο το λειτουργικό χαρακτηρισμό τους. Αρχικά, το ένζυμο *Ha*CYP6B7 το οποίο έχει βρεθεί ότι συσχετίζεται με τον μεταβολισμό του fenvalerate στο έντομο *Helicoverpa armigera* εκφράστηκε σε *E. coli* μαζί με το CPR από *Musca domestica* (*Md*CPR). Το *Ha*CYP6B7 εκφράστηκες στη φυσική και στη διαλυτή του μορφή, στη μεμβράνη και στο κυτταρόπλασμα των βακτηρίων, αντίστοιχα. Το ανασυνδυασμένο ένζυμο δοκιμάστηκε για την καταλυτική του δράση σε ορισμένα μοντέλα-υποστρώματα. Στο δεύτερο κεφάλαιο της παρούσας εργασίας, σκοπός ήταν η ετερόλογη έκφραση σε *E. coli* των *Bo*CYP6G2 και *Bo*CYP6A23 που έχουν συσχετιστεί με την ανθεκτικότητα που εμφανίζει ο δάκος (*Bactrocera oleae*) στο πυρεθροειδές εντομοκτόνο α-cypermethrin. Τα δύο κυτοχρώματα P450 κλωνοποιήθηκαν επιτυχώς σε κατάλληλους πλασμιδιακούς φορείς έκφρασης και με την *Bo*CYP6G2 προχωρήσαμε σε πειράματα έκφρασης στις βακτηριακές μεμβράνες του *E. coli* και σε περεταίρω βιοχημικό χαρακτηρισμό

Contents

1. Introduction

Development of resistance to insecticides is a major problem concerning both agricultural economy and public health. Scientists have focused in the discovery and development of new active and selective insecticides to control pest populations without killing beneficial arthropods such as bee pollinators. Various mechanisms are responsible for insecticide resistance development. Increased metabolic detoxification of insecticides, decreased sensitivity of target site proteins on which an insecticide acts, increased efflux of insecticides (Li et al., 2007) or cuticle alterations which reduce the rate of insecticide penetration (Balabanidou et al., 2019). Focusing on metabolic resistance, it involves three major metabolic gene families esterases, glutathione-S-transferases (GSTs) cytochrome and P450 monooxygenases (P450s). The latter have been also shown to determine insecticide selectivity.

1.1 P450 enzymes

1.1.1 General characteristics of P450s

Cytochrome P450s comprise one of the largest known protein families, with representatives in every kingdom of life. Each P450 protein is the product of a distinct CYP gene. P450s contain an iron–porphyrin group incorporated in their core and are therefore classified as hemoproteins. Their name results from a characteristic absorption peak near 450 nm, which they exhibit when Fe II is complexed with carbon monoxide (Omura & Sato, 1964). In procaryotes, P450s are soluble proteins. In eykaryotes, they are usually bound in the endoplasmatic reticulum or inner mitochondrial membranes. P450 enzymes catalyze a variety of metabolic pathways, such as carbon source assimilation, synthesis of hormones and secondary metabolites, and degradation of xenobiotics. In insects, P450 enzymes, except for their implication to the metabolism of xenobiotics (naturally occurring plant allelochemicals or synthetic insecticides), they are found in the biosynthetic pathways of ecdysteroids and juvenile hormones, which are major regulators of insect growth, development, and reproduction (Feyereisen, 1999). P450s are best known for their monooxygenase role, catalyzing the insertion of one atom of molecular oxygen to a substrate reducing the other to water. The stoichiometry of this reaction is:

$(S) + (NADPH + H^+) + O_2 \rightarrow S(O) + NADP^+ + H_2O$ *S: substrate

For microsomal P450s, the electrons required for this reaction are transferred from nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase and/or cytochrome b5.

P450 genes are classified on the basis of amino-acid identity, phylogenetic criteria and gene organization (Nelson et al., 1996). The root symbol CYP is followed by number for families (proteins that share >40% amino-acid sequence identity), a letter for subfamilies (greater that 50% identity) and a number for the gene. With the increasing number of sequences, and therefore CYP families, a higher level of nomenclature, the CYP clan, was introduced. Until recently insect CYPomes were distributed in four clans the CYP2, CYP3, CYP4 and mitochondrial clans, but recently, according to a recent survey that widened to arthropods, two clans, the CYP16 and CYP20 clans were added (Dermauw et al., 2020).

1.1.2 Redox partners of P450s

Most animal P450s require a redox partner in order to receive electrons from NADPH. NADPH cytochrome P450 reductase (CPR) and cytochrome *b5* are the electron donors in P450s found in microsomes and ferredoxin and ferredoxin reductase in mitochondria. Some bacterial and fungal P450s are fused with their redox partners, and for this reason they are called self-sufficient, as they require no external electron donor. CPR belongs to a family of flavoproteins utilizing both FAD and FMN as cofactors. This protein accepts two electrons from NADPH and transfers them, one at a time, to P450 enzymes (Feyereisen, 2012). The additional potential electron donor, cytochrome b5 is a heme protein whose presence may be obligatory or without effect in the reaction catalyzed from P450 (Porter, 2002)

1.1.3 Structural features

P450s sequences show extremely low identity and this is because there are very few conserved amino acids. These conserved amino acids, are found in the five conserved motifs of the protein: **W**xxx**R**, GxE/D**T**T/S , **E**xL**R** , PxxFx**P**E/D**R**F, and PFxxGxRx**C**xG/A. The last one includes the absolutely conserved Cys that serves as fifth ligand to the heme iron. Despite this sequence variability there is high conservation on a structural level. The highest structural conservation is found in the core of the protein, around heme. The conserved core is formed of a 4-helix bundle (D, E, L and I), helices J and K, two β-sheets and a coil called “the meander” (**Figure 2**). The term P420 is given to P450 proteins in which heme is not liganded, Cys is reduced and the enzyme is thus inactivated (often irreversibly). The most variable regions are those associated with either

N-terminal anchoring or targeting of membrane bound proteins or substrate recognition sites (SRS). Concerning N-terminal sequence, P450s targeted to the endoplasmic reticulum have an extended part of about 20 hydrophobic amino acids (the membrane anchor), followed by a cluster of basic residues and a cluster of prolines and glycines that acts as a “hinge” that slaps the globular domain of the protein onto the surface of the membrane while the N-terminus is anchored through it. A hydrophobic region between helices F and G is thought to penetrate the lipid bilayer, thus increasing the contact of the P450 with the hydrophobic environment from which many substrates can enter the active site. The substrate enter channel is usually assumed to be located in close contact with the membrane (Feyereisen, 1999; Werck-Reichhart & Feyereisen, 2000).

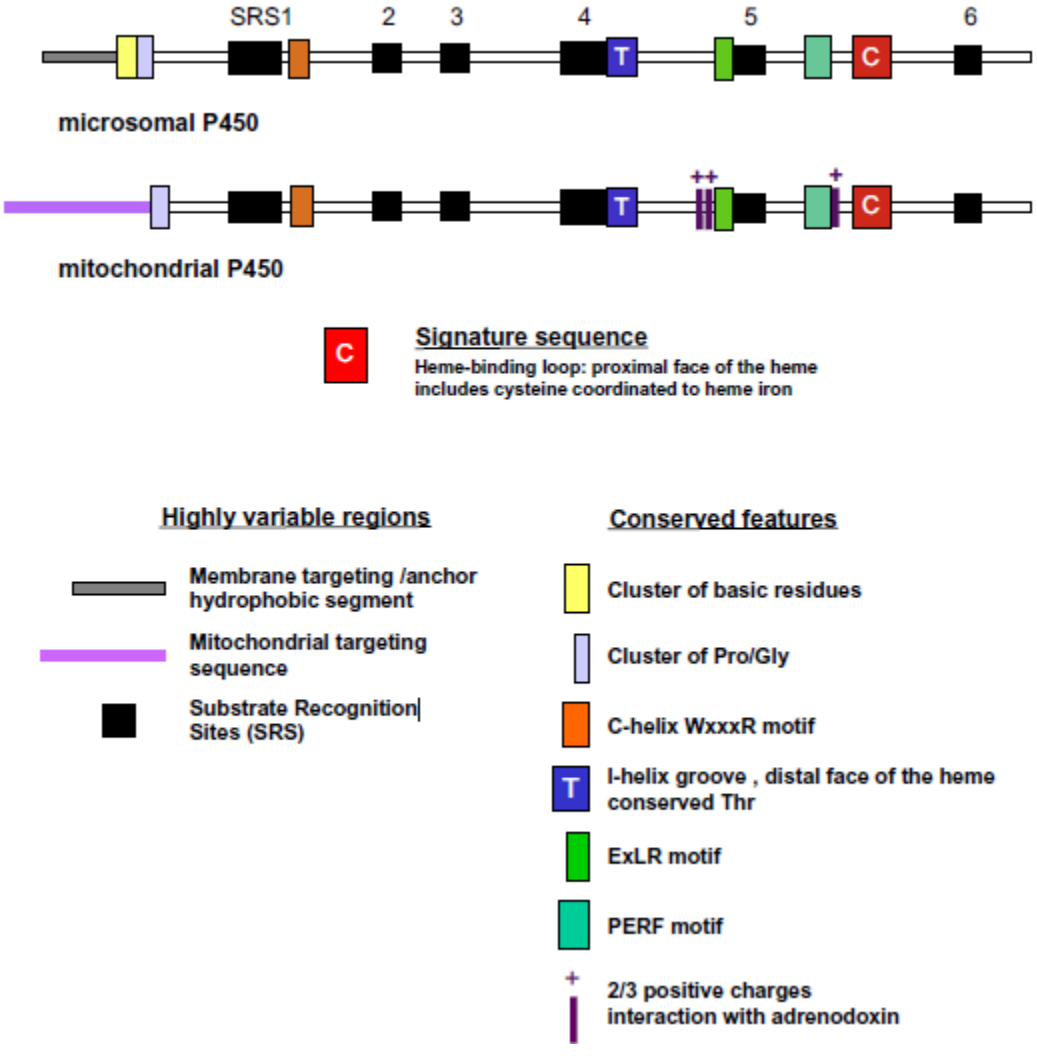


Figure 1. Primary structures of mitochondrial and microsomal P450 proteins. (Feyereisen, 2012)

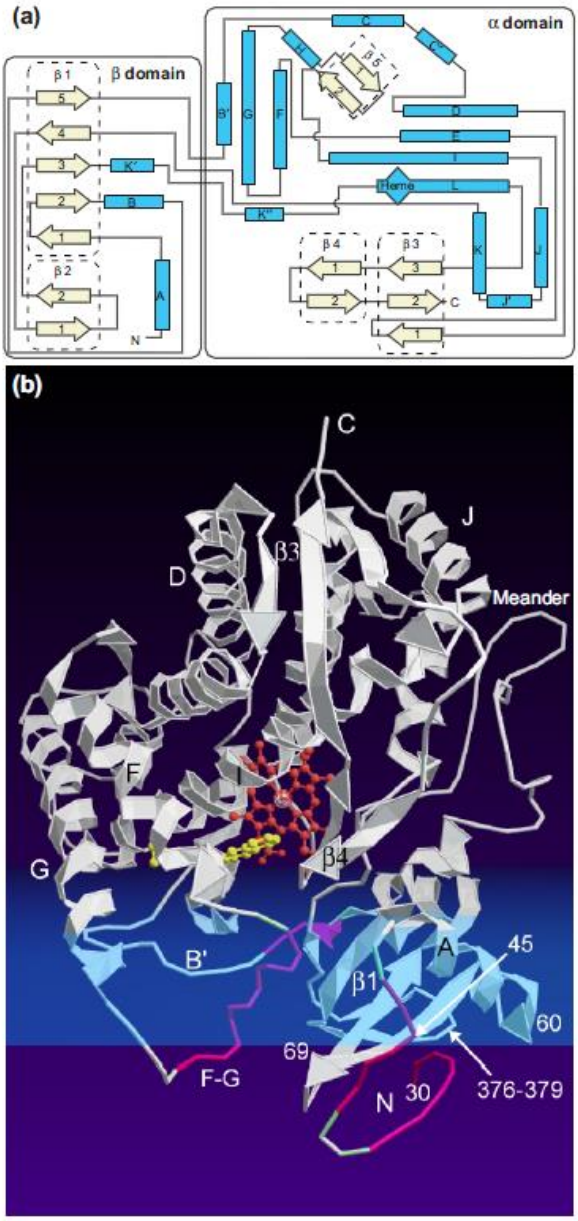


Figure 2. Secondary and tertiary structures of P450 proteins. (a) Topology diagram showing the arrangement of the secondary structural elements of a typical P450 protein (CYP102). Blue boxes, α helices; groups of cream arrows outlined with dotted lines, β sheets; lines, coils and loops. The first domain is often associated with substrate recognition and the access channel, the second with the catalytic center. (b) A cartoon representation of the tertiary structure of CYP2C5 protein showing its putative association with the ER membrane (purple). Heme is in orange, substrate in yellow. The transmembrane N-terminal segment, removed for crystallization, and an additional 11 residues that are disordered in the crystal structure, are not shown (Werck-Reichhart & Feyereisen, 2000)

1.1.4 Catalytic mechanism

Resting P450 is in the ferric form and partially six-coordinated with a molecule of solvent. The first step of the catalytic reaction is the displacement of the solvent from the substrate, leading to a shift to high spin and a decrease in the redox potential of P450. The P450-substrate complex receives a first electron from the redox partner and ferrous P450 (FeII) binds molecular oxygen. Then a second reduction step follows, leading to an activated oxygen species. The exact nature of the oxidizing species that performs the substrate attack has long remained unclear. Recently, a key intermediate named compound I, was characterized. This intermediate is an iron (IV) oxo species with a delocalized oxidizing equivalent. Hydroxylation of an inactivated C–H bond therefore follows a "rebound" mechanism, where hydrogen is abstracted from the substrate by compound I, forming an iron (IV) hydroxide that then recombines quickly with the substrate radical (**Figure 3**) (Feyereisen, 2012).

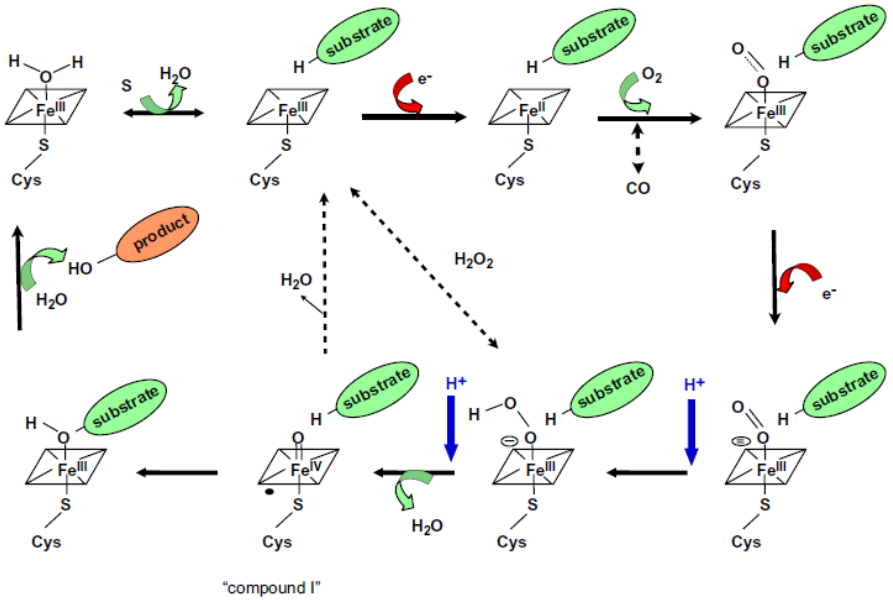


Figure 3. Mechanism of monooxygeneration reaction catalyzed by P450 enzymes (Feyereisen, 2012).

1.1.5 P450 gene expression associated with insecticide resistance

P450-mediated insecticide resistance may result from qualitive and quantitive changes in the expression of protein and occur singly or simultaneously (Zimmer et al., 2018). Quantitative changes may be constitutive or induced. Constitutive changes are the most common mechanism and results from cis acting (Le Goff & Hilliou, 2017) and/or trans acting regulatory factors (Amezian et al., 2021) or gene duplication and amplification (Bass et al., 2013; Schmidt et al.,

2010). Induced quantitative changes occur as a response to the exposure to a xenobiotic (plant allelochemicals or pesticides) (Liu et al., 2015). More rarely, downregulation of a P450 involved in pro-pesticide activation can also mediate resistance (Vlogiannitis et al., 2021). Less commonly reported, qualitative changes in coding sequence of a P450 can alter its metabolizing capacity. These changes may enhance activity of an already active P450 or may be gain-of function mutations (Ibrahim et al., 2015). Concerning the expression of P450s in different tissues, the enzymes are not only expressed in “first line of defense" tissues that are involved in xenobiotic detoxification (e.g. midgut, Malpighian tubules, and fat body) (Chung et al., 2007), but also at sites of insecticide action, such as brain (Zhu et al., 2010). Temporal changes in expression of P450s seems to follow age-specific expression of resistance (S.-F. Xu et al., 2019).

1.1.6 Functional characterization of P450s

Several tools have been employed for the functional characterization P450s in order to validate their involvement in insecticide resistance and on the applied side, to develop selective and safe insecticides (e.g for bee pollinators) and insecticide synergists. These strategies include in silico methods, in vivo approaches achieved by RNAi-based suppression, transgenic overexpression or P450 knock-outs and in vitro recombinant protein expression.

1.1.6.1 Heterologous expression of insect P450s

Multiple expression systems have been employed for functional recombinant expression of insect P450s **(Figure 4)**. Challenges are faced due to the requirement of successful incorporation of heme group, need of appropriate coupling with the redox partner, as well as the fact that many P450s are membrane bound proteins. Baculovirus expression system in insect cells has been used extensively because it offers a cellular environment closer to the native one of the insect proteins. It enables post-translation modification but it has the disadvantage of background P450 activity. Stable insect cell lines have also been used as a heterologous expression system but they suffer from low protein yield and the fact that growth and selection of stable cells is laborious. Yeast, as an expression system offers the necessary inner organelles for membrane bound proteins, enables some post-translational modifications, but it also expresses endogenous P450s (Nauen et al., 2020).

1.1.6.1.1 Heterologous expression in *E.coli*

E. coli is a well-studied and the most popular expression system for production of recombinant proteins. It offers a great variety of engineered strains, a big number of expression plasmids and many cultivation strategies. All of this, lead to high level production of recombinant proteins. But when it comes for expression of eukaryotic proteins some difficulties are faced (Rosano & Ceccarelli, 2014). Thus, expression of insect P450s in *E. coli* is complicated. Firstly, *E. coli* lacks the inner membrane structures in which P450s are anchored and as a result insoluble P450s agglomerate to inclusion bodies. Also, difficulties in translation due to the difference in codon usage observed in bacteria may be faced. Furthermore, *E. coli* does not perform post translational modification which may be important for the catalytic activity of the enzyme. On the other hand, *E. coli* offers the advantage that lacks endogenous P450 activity. The difficulties mentioned are mostly faced through modifications performed in the N-terminus region, responsible for membrane anchoring. These modifications may be sequence alteration that direct the P450 to the outer membrane of *E. coli,* or truncation to produce soluble P450 in the cytoplasm. For membrane targeted P450s, the sequence that is most commonly used is MALLLAVF(L) that comes from the bovine P450 and enhances P450 integration in the membrane (Barnes et al., 1991). For P450s that are intended to be soluble in the cytoplasm, complete or partial truncation of the N-terminus has proven effective. Also, another strategy for expression of soluble P450s is the N-terminus fusion with sequences of bacterial proteins that increase solubility. Successful expression of P450s can be further enhanced by co-expression of chaperones. Possible difficulties in protein translation and folding due to rare codons in bacteria, can be eliminated with codon optimization. Another important aspect in P450 expression is the optimization of cultivating parameters, including oxygen, temperature, pH, concentration of inducer and heme-precursor. Strategies for functional coupling of the P450 with CPR include expression of two proteins from two independent plasmids, expression of both proteins from a single plasmid and fusion of the two proteins. Pairing P450s with CPR from the same species, instead of using a general CPR (e.g. *Musca domestica* CPR) has been also proven effective in some cases (Hausjell et al., 2018; Nauen et al., 2020).

1.2 *Ha*CYP6B7 of *Helicoverpa armigera*

Helicoverpa armigera, known as cotton bollworm, is a major insect pest of a wide variety of agricultural and commercial crops, all over the world. Thus, it is characterized as polyphagous and cosmopolitan insect pest. Constant use of insecticides for its control lead to insecticide

resistance to almost all kind of insecticides (Downes et al., 2017). Subsequently, there is a big interest for the elucidation of the mechanisms of this insecticide resistance.

*Ha*CYP6B7 was reported by to be responsible for fenvalerate metabolism in *Helicoverpa armigera* and its relationship with fenvalerate resistance was confirmed later via Northern blot, real-time quantitative PCR, and RNAi (Ranasinghe & Hobbs,1998; Ranasinghe & Hobbs, 1999; Tang et al., 2012; L. Xu et al., 2016; Zhang et al., 2010). Later, *Ha*CYP6B7 was co-expressed together with *Ha*CPR in *Pichia pastoris* and its activity against 7-ethoxycoumarin, *p*-nitroanisole, fenvalerate and chlorpyrifos was confirmed (Zhao et al., 2018).More recently, CYP6B7 was heterologously expressed in SF9 insect cells together with *Ha*CPR and showed activity against esfenvalerate (Shi et al., 2021).

Concerning CYP6B subfamily in general, is reported to be associated with the metabolism of host plant allelochemicals and insecticides. CYP6B8 and CYP6B1 proteins were expressed in baculovirus system and CYP6B8 showed metabolic activity against six biosynthetically diverse plant allelochemicals (xanthotoxin, quercetin, flavone, chlorogenic acid, indole-3-carbinol, and rutin) and three insecticides (diazinon, cypermethrin, and aldrin), whereas CYP6B1 found active against two allelochemicals (xanthotoxin and flavone) and diazinon (Li et al., 2004). CYP6B6 from *H. armigera* expressed *in E. coli* together with *Ha*CPR showed ability to hydroxylate esfenvalerate. Additionally, CYP6B6 and CYP6B2 when heterologously expressed together with *Ha*CPR in SF9 cells exhibited activity against esfenvalerate (Shi et al., 2021).

1.3. *Bo*CYP6G2 and *Bo*CYP6A23 of *Bactrocera olea*

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is the most important pest of olive orchards worldwide, with major distribution in Mediterranean basin. It is a monophagous species feeding solely on olives. Females lay their eggs in ripening fruit, newly hatched larvae feed upon the pulp and either pupate in the olive or exit to pupate on the ground. Economic damage results from reduction of the production of table olives and olive oil, modification of the chemical properties, and deterioration of quality of these products. The olive fruit fly has been estimated to damage 15% of total olive production, resulting in economic losses of approximately 800 million USD per year (Daane & Johnson, 2010).

Insecticides have been the basis for the control of *B. oleae*. The intense use of insecticides has led to the development of field evolved resistance in *B. oleae*. Margaritopoulos et al., (2008) firstly reported resistance to α-cypermethrin. Resistance to α-cypermethrin have been increased over the last years (Kampouraki et al., 2018). The molecular mechanism of pyrethroid resistance was studied (Pavlidi et al., 2018). Sequencing analysis in a large number of resistant flies indicated

that resistance is not associated with target site mutations. Microarray analysis of the transcriptomic differences between resistant populations versus susceptible field and lab populations, revealed the up-regulation of several transcripts encoding detoxification enzymes. The up-regulation of several transcripts encoding detoxification enzymes was qPCR validated. Of note, the expression levels of contig00436 and contig02103, encoding CYP6 P450s, were significantly higher in all resistant populations. These findings were confirmed from RNA-seq that performed to more recent and more resistant populations. Based on the annotation of these contigs, contig02103 and contig00436 share homology with CYP6G2 and CYP6A23 of *Drosophilla melanogaster* respectively. From here on, P450s that are encoded from contig02103 and contig0046 will be referred as *Bo*CYP6G2 and *Bo*CYP6A23. Furthermore, treatment with PBO (a P450 inhibitor) caused a significant synergistic effect in α-cypermethrin toxicity. Moreover, RNAi silencing experiments of this two P450s conducted and silencing of *Bo*CYP6A23 resulted in increase of susceptibility in α-cypermethrin. Silencing of *Bo*CYP6G2 is in progress. These results suggest that the overexpression of these CYP6 P450s might be an important mechanism of pyrethroid resistance in *B. oleae*.

1.4 Aim of the study

Aim of this study is the heterologous expression in *E. coli* and functional characterization of 3 P450s that are related with metabolic resistance to insecticides. The first part includes the heterologous expression of CYP6B7 from *Helicoverpa armigera* that has found be related with fenvalerate metabolism in this insect. The protein will be expressed in native and truncated form and subsequently targeted in bacterial membrane or in the cytoplasm, respectively. As a redox partner, *Musca domestica* CPR (*Md*CPR) will be co-expressed. Membrane preparations and purified P450 will be assayed against model substrates and insecticides. The second part includes the heterologous expression of *Bo*CYP6G2 and *Bo*CYP6A23 of *Bactrocera oleae* that have found to be related with resistance to α-cypermethrin in *Bactrocera oleae*. The two P450s will be targeted in bacterial membranes and two different redox partners *Bactrocera oleae* CPR (*Bo*CPR) and *Musca domestica* CPR (*Md*CPR) will be tested. Membrane preparations of *Bo*CYP6G2 and *Bo*CYP6A23 will be checked for their catalytic activity against model substrates and insecticides. Successful expression of these P450s as recombinant enzymes will give answers to the association of these enzymes to insecticide resistance but also in the applied aspect, understanding of function may point to candidates for selective inhibition will be applied in development of new safe and selective insecticides and synergists. In order to exploit this technological potential of P450s for industrial applications sufficient and consistent yields are needed and *E. coli* as an expression system, is appropriate for this purpose.

2. Materials and methods

Chapter I

Heterologous expression in *E. coli* of *Ha*CYP6B7

2.1. Plasmid constructs for P450 and CPR expression

In order to functionally express *Ha*CYP6B7 together with *Md*CPR, multiple approaches were implemented. *Ha*CYP6B7 is targeted either on bacterial membranes or in the cytoplasm of bacteria. *Ha*CYP6B7 targeted in the bacterial membranes, was expressed in native form or together with the OmpA leader sequence fused at its N-terminus. The role of this sequence is to direct P450s to the bacterial membranes. *Ha*CYP6B7 that was targeted in the cytoplasm (soluble P450s) was N-terminally truncated (*ΔN*-P450). More specifically the transmembrane domain which was predicted using ΔG prediction server (http://dgpred.cbr.su.semembrane) was deleted. In addition, *Ha*CYP6B7 was C-terminally tagged with FLAG tag (DYKDDDD) and six histidine residues (His-tag) in order to track its expression via anti-Flag and anti-His antibody respectively during western blot, or to enable their purification. Regarding the co-expression of the *Md*CPR, two different approaches were followed: expression of both proteins from the same plasmid vector, each gene being under the control of T7 promoter (in this work this approach is referred as single plasmid experiment) and expression of two proteins from two different plasmid vectors (in this work this approach is referred as two plasmids experiment).

Gene encoding *Ha*CYP6B7 (codon optimized or not) was introduced into pCWOri or pCWOmpA which includes β-lactamase gene as a selection marker and IPTG-inducible tac-tac promoter upstream of the cloning site of P450 gene. *Md*CPR was introduced into pCDFDuet-1 vector which contains streptomycin resistance gene and IPTG-inducible T7 promoter upstream the cloning site of CPR gene. For single plasmid experiments *Ha*CYP6B7 gene was subcloned to the multi cloning site I of to pCDFDuet vector which bears MdCPR gene in the multi-cloning site II. In this construct both genes are under the control of separate T7 promoters.

Based on the strategies described above the following constructs were used for the heterologous expression of P450 in bacteria **Table 1**.

Table 1. Plasmid constructs of *Ha*CYP6B7 and characteristics.

Plasmid constructs	Subcellular location targeted for P450	Antibodies for western blot detection
a. pCW- *Ha*CYP6B7-Flag-His	Membranes	anti-His anti-Flag
b. pCW- *Ha*CYP6B7-Flag-His + pCDF Duet-1-*Md*CPR **(two plasmids)**	Membranes	anti-His anti-Flag anti CPR
c. pCDF Duet-1 - *Ha*CYP6B7-Flag-His/*Md*CPR **(single plasmid)**	Membranes	anti-His anti-Flag anti CPR
d. pCW-OmpA *Ha*CYP6B7	Membranes	-
e. pCW-OmpA *Ha*CYP6B7 + pCDF Duet-1-*Md*CPR **(two plasmids)**	Membranes	anti CPR
f. pCDF Duet-1 -OmpA *Ha*CYP6B7/*Md*CPR **(single plasmid)**	Membranes	anti CPR
g. pCW-*Δ*N *Ha*CYP6B7-Flag-His	Cytoplasm	anti-His anti-Flag

Cloning of P450 and CPR into expressing vectors that mentioned above **(Section 2.1)** was conducted during previous work in our laboratory.

2.2. Bacterial stains and antibiotics

E. coli strains used for the P450 and CPR expression are listed in **Table 2**. All of them are available as chemically competent, suitable for high efficiency transformation and are appropriate for

expressing proteins. Furthermore, Lemo21(DE3) andC43 cells are effective in overexpressing toxic membrane proteins.

Table 2. List of bacterial strain tested for P450 heterologous expression and their characteristics.(https://openwetware.org/wiki/E._coli_genotypes 07/2021; Rosano & Ceccarelli, 2014)

***E. coli* strain**	**Characteristics**
DH5α	High yield and quality of DNA due to endA mutation High insert stability due to recA1 mutation
BL21 Star (DE3)	Extremely high activity of T7 RNA polymerase Increased stability of mRNAs
JM109 (DE3)	Improved insert stability and quality of recombinant plasmids
Lemo21 (DE3)	Ability of tunable expression, Appropriate for membrane proteins, toxic proteins and proteins prone to insoluble expression.
C43 (DE3)	Effective in expressing toxic and membrane proteins from all classes of organisms

For selection of transformed bacterial cells with pCWOri and pCDF-Duet-1 plasmid vector, media were supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, respectively. Also concerning Lemo21 (DE3) strain, Lemo system requires 50 μg/mL chloramphenicol for its maintenance.

2.3. Small scale expression test of P450 in *E. coli* strains

Small scale expression tests of constructs including P450s bearing Flag-His (pCW-*Ha*CYP6B7-Flag-His, pCDF Duet-1-*Ha*CYP6B7-Flag-His/*Md*CPR, pCW-*ΔN Ha*CYP6B7-Flag-His) were performed in five bacterial expression strains (BL21 (DE3), JM109 (DE3), DH5α, Lemo21 and C43 (DE3)) under standard expression conditions. These conditions have been selected from previous experiments of P450s heterologous expression in our laboratory. A bacterial strain will be selected for downstream experiments based on two criteria of I) Successful expression (high expression of P450) and II) Protein stability.

In details: *100 µL of competent E. coli* cells were transformed with 50 ng of plasmid DNA. Transformation was conducted via heat-shock (1 min at 42°C). Transformed cells were plated on LB agar supplemented with the appropriate antibiotics and incubated at 37°C overnight. Two single colonies from the transformation plate were used for the inoculation of 3 mL LB each, supplemented with appropriate antibiotics. The pre-culture was incubated for 16 h at 37°C, while shaking at 150 -200 rpm. Pre-culture (100 µL) were used to inoculate 10 mL of Terrific Broth (1/100 dilution) also supplemented with appropriate antibiotics**.** Cell culture was grown at 37°C (150 rpm shaking) until the optical density at 595 nm is 0.8-0.9 cm^{-1}. Then, growth was continued at 25°C (150 rpm shaking) for 30 min. At this time point, a cell culture sample (–isopropyl-1-thio-b-D-galactopyranoside (IPTG) sample, 1 mL) was collected. Induction was initiated with the addition of IPTG to final concentration of 0.5 mM. Also, the heme precursor, δ-aminolevulinic acid (ALA) was added to final concentration of 1 mM. Culture was grown for 24 h at 25°C, 150 rpm shaking. Finally, a cell culture sample (+ IPTG sample, 1 mL) was collected. All the –/+ IPTG samples were normalized according to their OD value at 595 nm and the corresponding proteins were extracted via homogenization in 5x Laemmli Sample buffer.
Bacterial lysates were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blots.

2.4. SDS-PAGE electrophoresis

Principle

SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) is a method that is used for the separation of proteins by their molecular weight and it is based on the property of charged molecules to move in response to the electric field applied. Polyacrylamide gels are formed by the reaction of acrylamide and bis-acrylamide (*N,N*'-methylenebisacrylamide) that

results in highly cross-linked gel matrix. The gel acts as a sieve through which proteins move in response to the electric field. SDS acts as a surfactant, masking the proteins' intrinsic charge and conferring them very similar charge-to-mass ratios. By denaturing the proteins and giving them a uniform negative charge it is possible to separate them based on their size as they migrate towards the anode (Cleveland et al., 1977).

Protocol

Protein samples were loaded on 10% acrylamide gel.

2.5. Western blot

Principle

Western blot is a method used to visualize proteins that have been separated by gel electrophoresis. A nitrocellulose or PVDF (polyvinylidene fluoride) membrane it attached to the polyacrylamide gel and an electrical current causes the proteins to migrate from gel to the membrane. The membrane can then be probed by antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents (Renart et al., 1979). In the present study, Horseradish peroxidase (HRP) conjugated secondary antibodies were used. HRP – secondary antibodies are in conjunction with specific chemiluminescent substrates to generate visible signal.

Protocol

The polypeptides were analyzed by using anti-His antibody (penta-His, Qiagen) (1:2000 dilution in 1X Tris Buffered Saline, pH 7.5, with Tween 20, supplemented with 3 % w/v Bovine Serum Albumin) followed by anti-mouse-HRP (1:5000 dilution in 1X Tris Buffered Saline pH 7.5, with Tween 20, supplemented with 3 % w/v milk). As a positive control 1 µg of purified His-MDH was loaded. When necessary anti-CPR was used (1:5000 dilution in 1X Tris Buffered Saline pH 7.5 with Tween 20, supplemented with 3 % w/v milk) followed by anti-rabbit-HRP (1:10000 dilution in 1X Tris Buffered Saline pH 7.5 with Tween 20, supplemented with 3 % w/v milk). As a positive control 6 µg of semi-purified *Md*CPR was loaded on gel.

2.6. Large scale cultures for P450 expression

2.6.1. Membrane targeting experiments

2.6.1.a Expression

Selected bacterial strains from expression tests were used for large scale expression P450s targeted in bacterial membranes, in 200 mL TB liquid cultures. One hundred µL of competent *E. coli* cells were transformed with 50 ng of plasmid DNA. Transformation was conducted via heat-shock (1 min at 42$^{\circ C}$). Transformed cells were plated on LB agar supplemented with the appropriate antibiotics and incubated at 37$^{\circ C}$ overnight. One single colony from the transformation plate was used for the inoculation of 3 mL LB supplemented with appropriate antibiotics. The pre-culture was incubated for 16 h at 37$^{\circ C}$, while shaking at 150 -200 rpm. Two mL of the pre-culture were used to inoculate 200 mL of Terrific Broth (1/100 dilution) also supplemented with appropriate antibiotics. Cell culture was grown at 37°C (150 rpm shaking) until the optical density at 595 nm reached 0.8-0.9 cm^{-1}. Then, growth was continued at 25°C (150 rpm shaking) for 30 m. At this time point, a cell culture sample (–IPTG sample,1 mL) was collected. Induction was initiated with the addition of isopropyl-1-thio-b-D-galactopyranoside (IPTG) to final concentration of 0.5 mM. Also, the heme precursor, δ-aminolevulinic acid (ALA) was added to final concentration of 1 mM. Culture was grown for 24 hours at 25$^{\circ C}$, 150 rpm shaking. Finally, a cell culture sample (+ IPTG sample, 1 mL) was collected. All the –/ + IPTG samples were normalized according to their OD value at 595 nm and the corresponding proteins were extracted via homogenization in 5x Laemmli sample buffer. Bacterial lysates were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blots.

2.6.1.b Membrane preparation

Cultures were centrifuged at 6000 rpm for 25 min at 4°C. The cell pellets were resuspended in 20 mL of 1x TSE buffer (0.1 M Tris acetate, pH 7.6, 0,5M sucrose, 0.5 mM EDTA) and lysozyme was added in the solution for final concentration 0.25 mg/mL. The solution was incubated for 60 min at 4°C with gentle shaking. The solution was centrifuged at 2800 *g* for 25 min at 4°C. The spheroplast pellet was resuspended in spheroplast resuspension buffer (0.1 M potassium phosphate buffer, pH 7.6, 6 mM magnesium acetate, 20 % v/v glycerol) containing 0.1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mg/mL aprotinin and

1 mg/mL leupeptin. The suspension was sonicated (40% amplitude, 30 s, on 30 s off, 5 times) and the membrane fraction was pelleted by ultracentrifugation at 64000 rpm for 1 h, at 4°C. Membrane pellets were diluted in TSE buffer and homogenized using Dounce homogenizer. Membrane preparations were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blots. Membrane preparations were stored in aliquots at -80°C.

2.6.2 Soluble P450s

2.6.2.a Expression

Selected bacterial strain from expression tests was used for large scale expression of soluble *Δ*N *Ha*CYP6B7-Flag-His in 5x200 mL TB liquid cultures. One hundred µL of competent *E. coli* cells were transformed with 50 ng of plasmid DNA. Transformation was conducted via heat-shock (1 min at 42$^{\circ C}$). Transformed cells were plated on LB agar supplemented with the appropriate antibiotics and incubated at 37$^{\circ C}$ overnight. Five 200 mL TB liquid cultures (supplemented with 100 µg/mL ampicillin) were inoculated with 5 mL of preculture (1:50 inoculation). Cultures were incubated at 30°C and monitored for their OD at 595 nm. When OD reached 0.5 cm^{-1}, IPTG and ALA were added for final concentration of 0.5 mM and 1 mM, respectively. After induction, incubation was continued at 30°C for 24 h. One mL sample (-IPTG, +IPTG) was collected in order to check expression, -IPTG sample was collected before the induction and +IPTG sample was collected 24 h after induction. Cultures were centrifuged at 6000 rpm for 25 min at 4°C. Cell pellet was weighed and stored at –20°C.

2.6.2.b Cell lysis

Cell pellets stored at -20°C were thawed out, using 50 mL ice cold lysis buffer (50 mM Tris-HCl, pH 8.8, 10 % v/v glycerol, 0.1 % Triton v/v), mixing by pipetting and gentle vortexing. After the samples were completely homogenized, 2 mM β-mercaptoethanol, 0.5 mM EDTA, 2 mM PMSF and 1 mg/mL lysozyme were added. The samples were then incubated at 25°C shaking at 250 rpm for 1 h. After the lysozyme treatment, the lysates were left on ice for 30 min to cool down, followed by gentle sonication (40% amplitude, 20 s on, 20 s off, 3 times). After sonication, 500 mM NaCl were added to the samples. Following centrifugation at 18000 rpm, 4^{o} C for 30 minutes, the pellet was kept at -20 °C and the supernatant was kept on ice.

2.6.2.c Purification of P450s

2.6.2.c.i. Ni-NTA affinity chromatography

Principle

Metal affinity protein purification is based on the remarkable selectivity of Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins which contain an affinity tag of six or more histidine residues (consecutive or alternating) — the His tag. NTA occupies four of six ligand binding sites of the nickel ion, leaving two sites free for interaction with the His-tag. Histidine residues on the tag, connected to the C- or N-terminus of the protein, bind to the Ni-ions. The protein can be eluted by competitive displacement with imidazole (Bornhorst & Falke, 2000).

Protocol

Ni-NTA affinity chromatography was carried out at 4°C. P450 lysates (from previous cell lysis step), supplemented also with 5 mM imidazole, were loaded on 500 µL of pre-equilibrated, with 10 column volumes (c.v.) of equilibration buffer, Ni-NTA resin (Qiagen) with a slow flow rate (0.5 mL/min) and Flow Through (FT) was collected either directly or the column was left overnight at 4°C and FT was collected the next day. Then, the column was washed with 10 c.v. of equilibration buffer and the respective fraction (W1) was collected, followed by wash with 5 c.v. of wash 2 buffer and fraction (W2) was collected. Then, the bound proteins were eluted with 5 c.v. of elution buffer and 10 elution fractions were collected, 0.5 c.v. each one. Collected samples were loaded on 10% SDS-PAGE for Coomassie staining.

Buffers

Equilibration buffer: 50 mM Tris-HCl pH 8.8, 500 mM NaCl, 10% v/v glycerol, 0.1% Triton v/v, 5 mM imidazole

Wash 2 buffer: 50 mM Tris-HCl pH 8.8, 50 mM NaCl, 10% v/v glycerol, 0.1% v/v Triton, 5 mM imidazole

Elution buffer: 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% v/v glycerol, 0.1% v/v Triton, 200 mM imidazole

2.6.2.c.ii Ion exchange chromatography

Principle

Ion-exchange chromatography uses a resin to separate proteins according to their surface charges. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatography resin via coulombic interactions.

The net surface charge of proteins varies according to the surrounding pH. The pH at which a protein has no net charge is called isoelectric point (pI). Above its isoelectric point (pI), a protein will bind to a positively charged anion exchanger. Below its pI, a protein will bind to a negatively charged cation exchanger. High salt concentration buffer is applied to the column so it can compete with the ion exchanger for the bound proteins, and in this way the bound proteins can then be eluted. (Small, Stevens, & Bauman, 1975)

SP Sepharose platform

SP Sepharose (Cytiva) is composed of crosslinked 6% agarose beads with sulphopropyl (SP) strong cation exchange groups. Protein samples diluted in buffer (100mM Tris/HCl, pH 7.5, 10 mM NaCl, 10 % v/v glycerol) were loaded on 2 mL of pre-equilibrated (with 10 c.v. equilibration buffer) SP-sepharose (Cytiva) and FT was collected. Then the column was washed with 10 c.v. of equilibration buffer and the respective fraction (W) was collected. Bound proteins were eluted with 10 c.v. of elution buffer. Collected samples were loaded on 10% SDS-PAGE for Coomassie staining.

Buffers

Equilibration buffer: 100 mM Tris/HCl, pH 7.5, 10 mM NaCl, 10 % v/v glycerol
Elution buffer: 50 Mm Tris/HCl, pH 7.5, 1 M NaCl, 10 % v/v glycerol

Q Sepharose platform

Q Sepharose (Cytiva) is composed of crosslinked 6% agarose beads, with quaternary ammonium (Q) strong anion exchange groups. Protein samples diluted in buffer (50 mM Tris/HCl, pH 7.5, 0.1 % v/v Triton, 10 % v/v glycerol, 50 mM NaCl) were loaded on 1 mL of pre-equilibrated (with 10 c.v. of equilibration buffer) Q-sepharose (Cytiva) and flow through was collected. Then, the column was washed with 10 c.v. of equilibration buffer and the respective fraction (W) was collected. Bound proteins were eluted with salt gradient 50 - 500 mM NaCl (10 c.v. of each elution buffer were loaded). Collected samples were loaded on 10% SDS-PAGE for Coomassie staining.

Buffers

Equilibration buffer: 50 mM Tris/HCL pH 7.5, 0.1% v/v Triton, 10 % v/v glycerol, 50 mM NaCl
Elution buffer: 50mM Tris/HCL pH 7.5, 0.1% v/v Triton, 10 % v/v glycerol, 50 mM-500 mM NaCl

2.7. Bradford assay for P450s

Principle

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that Coomassie Brilliant Blue G-250 dye under acidic conditions has an absorption peak at 465 nm in the absence of protein in the solution and this peak shifts to 595 nm when dye binds to proteins. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex (Bradford, 1976).

Protocol

Different volumes of diluted protein samples were added on the 1 mL of Bradford reagent (Biorad) solution (dilution 1:5 in H_2O). Three hundred µL of the solution was loaded in the microplate and incubated for 5 min, RT. Absorbance at 595 nm was measured in a microplate reader. The total protein concentration was calculated according to Bovine Serum Albumin (BSA) standard curve.

2.8. P450 spectral assay

Principle

The principle of the P450 spectral assay is that the reduced (ferrous) form of the P450 reacts with carbon monoxide (CO) to form a complex that specifically produces a spectrum with a wavelength maximum at 450 nm, owing to the signature cysteine thiolate axial ligand to the heme iron in these proteins. Only the reduced (ferrous) form of P450 binds CO (Omura & Sato, 1964) Thus, the principle is that the reference sample contains only ferrous P450 reduced artificially with the reducing salt, sodium hydrosulfate, and the sample contains the same ferrous P450 bound to CO. A useful aspect of the assay, is that the loss of activity of P450 enzymes is associated with a loss of the 450-nm peak and conversion to a form with a wavelength maximum at 420 nm. Thus, the assays serve as a check on the integrity (correct folding) of the enzyme (Guengerich et al., 2009). The assay is also used to characterize recombinant or purified P450 preparations in the context of the stoichiometry of active P450 per unit protein.

Protocol

In plastic cuvette containing 1 mL of spectrum buffer (0.1 M Tris –HCl, pH 7.4, 20 % v/v glycerol, 1 mM EDTA), 10-75 µL of sample was added, followed by the addition of some grains of sodium hydrosulfate. After gentle inversion of the cuvette, the absorbance of the sample was measured

at 400-500 nm per 2 nm. This measurement was set as reference. In the same cuvette CO was added slowly for 45 s. The absorbance of the sample was measured at 400-500 nm per 2 nm. The concentration of P450 (μM) was calculated using the following formula:

$$\frac{A_{450} - A_{470}}{0.091} * dilution\ of\ sample$$

The value 0.091 is extinction coefficient calculated from (Omura & Sato, 1964).

2.9. CPR activity assay

Principle

In this assay, in order to measure NADPH-P450 reductase activity we measure the reduction of cytochrome c instead of P450, because this electron acceptor does not reoxidize in air as P450. In this assay, the reductase accepts electrons from the NADPH and transfers these (one at a time) to cytochrome c. The rate of reduction of cytochrome c is measured by recording kinetically the absorbance at 550 nm (Strobel & Dignam, 1978).

Protocol

In a cuvette containing 1 mL of cytochrome c solution 50 μM (diluted in 0.3 M potassium phosphate buffer, pH 7.7) 10 μL of membranes were added. This sample was considered as the reference sample. The absorbance of the reference sample was measured kinetically at 550 nm for 1:32 min per 2 s. Then, in order to start the reaction, 10 μL of NADPH solution 5 mM (diluted in water) were added. The rate of reduction of cytochrome c was measured by recording kinetically the absorbance at 550 nm for 1:32 min per 2 s.

Catalytic activity of CPR (nmol reduced cytochrome c min-1 mg^{-1} total protein) was calculated from the following formula:

$$\frac{\text{Vmax (mOD } min^{-1}\text{)}/21.4}{total\ protein\ concentration\ (mg\ ml^{-1})}$$

*value 21.4 is a constant that is related to the diameter of the cuvette

2.10. Catalytic activity of P450 against model substrates

7-Ethoxycoumarin

Principle

7-Ethoxycoumarin is widely used as a model substrate to monitor P450 catalytic activity. The enzymatic product of O-deethylation (ECOD) activity, 7-hydroxycoumarin, is measured at an excitation wavelength of 390 nm and an emission wavelength of 465 nm. Cytochrome b5 can also be included in the reaction (Aitio, 1978).

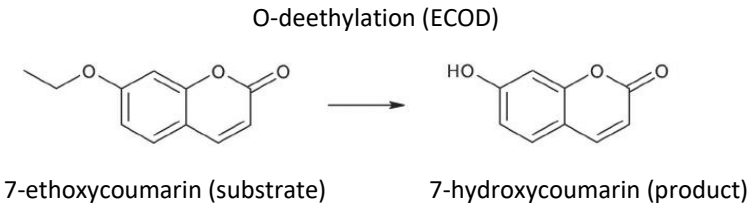


Figure 5. Reaction catalyzed from P450 enzymes

Protocol

Enzyme activity measurements were performed at 100 mM sodium phosphate buffer, pH 7.2. Different quantities (2.5-100 μg) of membrane preparation were added in the reaction (total volume 120 μL) in presence or absence of cytochrome b5 (1.2 μM final concentration). Membranes were incubated with 7-ethoxycoumarin (1.2 mM final concentration) for 30 min, in the dark at 30°C. For each sample two reactions are conducted, one in presence of NADPH (1.25 mM final concentration) and a second one with water instead of NADPH. After the incubation of 30 min the fluorescence of NADPH blocked with the addition of 20 μL of mix buffer (0.5 μL glutathione reductase, 10 μL oxidized glutathione 30 mM, 95 μL dH_2O). After 10 min incubation the reaction stopped with the addition of 140 μL of stop reaction buffer (70 mM Tris-HCl, pH 8.5, Acetonitrile 1:1). Fluorescence was measured at an excitation wavelength of 390 nm and an emission wavelength of 465 nm.

p-nitroanisole

Principle

p-nitroanisole is a model substrate that is used to monitor O-demethylation activity of P450s, the product of the reaction is p-nitrophenol and is measured by its absorbance at 405 nm.

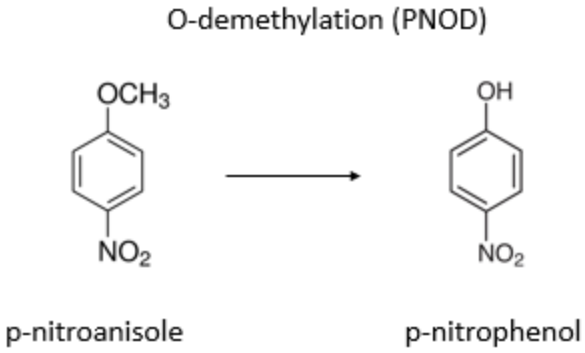


Figure 6. Reaction catalyzed from P450 enzymes

Protocol

For the p-nitroanisole O-dealkylation assay, enzyme reactions of total volume 1 mL were carried out in 0.1 M Tris-HCl buffer (pH 7.5). Two hundred µg of membrane preparations and 10 µL of 50 mM of p-nitroanisole (final concentration of 0.5 mM) were added in the reaction. Reactions were initiated by adding 50 µL of 10 mM NADPH to each microtube, yielding final concentrations of 0.5 mM NADPH. H_2O was added to the reactions as a control. After 60 min of incubation with 500 rpm shaking at 30°C, the assay was centrifuged at 14,000 *g* for 10 min at 4°C. The amount of the O-dealkylated product, p-nitrophenol, in the supernatant by measuring the absorbance at 405 nm. The above protocol has been described from (Hamada et al., 2020).

Chapter II

Heterologous expression in *E. coli* of *BoCYP6G2 and BoCYP6A23*

2.11 Plasmid constructs for P450 and CPR expression

Constructs of *Bo*CYP6G2 and *Bo*CYP6A23 were designed with the aim to target P450s in bacterial membranes. *Bo*CYP6G2 and *Bo*CYP6A23 will be expressed in native form or together with the OmpA leader sequence, fused N-terminally. In order to track their expression via anti-His antibody during western blot, P450s were fused C-terminally with His-tag. The redox partner will be co-expressed with the P450s either from the same plasmid or from two different plasmids. *Bactrocera oleae* (*Bo*CPR) will be used mainly as a redox partner but *Musca domestica* (*Md*CPR) has also been included in one construct. It is also planned to express P450s without His-tag in the case that affects folding and catalytic activity, but this approach it is not presented in this study. Genes encoding for *Bo*CYP6G2 and *Bo*CYP6A23 were introduced into pCWOmpA expressing vector and *Md*CPR and *Bo*CPR were introduced into pCDFDuet-1 expressing vector. For single plasmid experiments P450s genes were subcloned to the multi cloning site I of to pCDFDuet vector which bears CPR gene in the multi-cloning site II. For more information about vector characteristics (e.g. promoters, selection markers) see **Section 2.1**. Based on the above, the constructs that will be used for the heterologous expression of *Bo*CYP6G2 and *Bo*CYP6A23 in bacteria are presented in **Table 3**.

Table 3. Plasmid constructs and characteristics. All constructs target membranes.

Constructs	Codon optimization of P450/CPR	Antibodies for western blot
***Bo*CYP6G2**		
pCW-OmpA *Bo*CYP6G2-His	No	anti-His
pCW- OmpA *Bo*CYP6G2-His + **pCDF Duet-1-*Bo*CPR** (two plasmids)	No/Yes	anti-His, anti CPR
pCDF Duet-1- *Bo*CYP6G2-His/*Bo*CPR (single plasmid)	Yes/Yes	anti-His, anti CPR
***Bo*CYP6A23**		
pCDF Duet-1- *Bo*CYP6A23-His/*Md*CPR (single plasmid)	Yes/Yes	anti-His, anti CPR
pCDF Duet-1- *Bo*CYP6A23-His/*Bo*CPR (single plasmid)	Yes/Yes	anti-His, anti CPR

2.11.1 Cloning of P450s and CPR to expressing vectors

P450s and *Bo*CPR genes were double-digested with the appropriate pair of restriction enzymes and cloned to pCWOmpA, and pCDFDuet-1 expressing vectors (**Table 4)**.

Table 4. Primers and restriction enzymes used for cloning to expression vectors

Gene	Expressing vector	Restriction enzymes	Restriction site (5'-3')		Primer sequence (5'-3')
***Bo*CYP6G2-His**	pCDFDuet-1 MCSI	NdeI Not I	CATATG GCGGCCGC	F external	CATATGTTTATTAGCAGCGTTCTG
				R external	GATTATGCGGCCGTGTACA
				F internal	TTTTTTTCATCCCGATCATG
				R internal	CTTTACCTGGCTGCGTAG
*Bo*CYP6G2-His	pCWOmpA	NgOMIV Sac-I	GCCGGC GAGCTC	F external	GCCGGCATGTTTATATCAAGTGTGTTA TCC
				R external	GAGCTCTCAATGATGATGATGATGAAACAATGG ATCACGCA
				F internal	ACAGTCTCAACGATCC
				R internal	AACGGTTTCTTACCCTC
***Bo*CYP6A23-His**	pCDFDuet-1 MCSI	NdeI NotI	CATATG GCGGCCGC	F external	CATATGAGCCTGTTTCTG AAT
				R external	GATTATGCGGCCGTGTACA
				F internal	AATATCGTGAACGTACCG
				R internal	CACAATACCCATATAGAAATC
*Bo*CYP6A23-His	pCWOmpA	SacI XbaI	GAGCTC TCTAGA	F external	GAGCTCATGAGCTTGTTCTTGAACTTGTT
				R external	TCTAGATTAATGATGATGATGATGAT GCAAGCTCTCCACTCGCAG
				F internal	ATCGTGCGTGAAACTAT
				R internal	TTGGTTTTTAATTCCATTAAGA
***Bo*CPR**	pCDFDuet-1 MCSII	MfeI KpnI	CAATTG GGTACC	F external	TTGTACACGGCCGCATAAT
				R external	AGTTATTGCTCAGCGGTGG
				F internal	AGGTAAAGAAAAATATCAGAG
				R internal	TATTAATCAGGCTAAAAACG

* Genes with bold letters are codon optimized

**Underlying sequence denotes the introduction of restriction sites to facilitate cloning

***All restriction enzymes were bought from NEB except for XbaI that was bought from MinoTECH

Digestion reaction was conducted according to the restriction enzyme's manufacturer's instructions. Digested genes and expression vectors were extracted from agarose gel using DNA-RNA-protein purification-NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and then ligated using T4 DNA ligase (Promega) according to the manufacturer's instructions. Stellar *E. coli*

competent cells were transformed with the ligation reaction and plated to LB plates supplemented with the appropriate antibiotic. For selection of transformed bacterial cells with pCWOmpA and pCDF-Duet-1 plasmid vector, media were supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol correspondingly. Positive colonies were verified by colony PCR (25 μL reaction), exact reaction and conditions are shown in **Table 5, 6**. Positive colonies were cultured in liquid cultures and followed by plasmid DNA isolation with DNA-RNA-protein purification-NucleoSpin Plasmid kit of Macherey-Nagel, according to the manufacturer's protocol. Insertion of P450s and *Bo*CPR genes to the expressing vectors was verified with digestion with appropriate restriction enzymes **Table 4** and sequencing.

Table 5. Components of PCR reaction (25 μL) with Taq polymerase for amplification of *Bo*CYP6G2 and *Bo*CYP6A23.

Components	**Volume (μL)**
10 μM forward primer	1
10 μM reverse primer	1
10 mM dNTPs	1
10x Buffer A	2.5
KAPA Taq 5 U/μL	0.3
H_2O	19.2

Table 6. Conditions of PCR reaction with KAPA Taq polymerase for amplification of *Bo*CYP6G2 and *Bo*CYP6A23

Initial denaturation		**95°C, 5 min**
Denaturation	**35 cycles**	95°C, 30 s
Annealing		56°C, 30 s
Extension		72°C, 2 min
Final extension		72°C, 10 min
Hold		4°C

2.11.2 Membrane targeting experiments and biochemical characterization of *Bo*CYP6G2 and *Bo*CYP6A23

Small scale expression tests of constructs pCW-OmpA-*Bo*CYP6G2-His, pCDF Duet-1-*Bo*CYP6G2-His/*Bo*CPR were performed in five bacterial expression strains (BL21 DE3, JM109 DE3, DH5α, Lemo21 and C43 DE3) under standard expression conditions, following the procedure analyzed

in **Section 2.3** for *Ha*CYP6B7. Expression was tracked via western blot before and after the induction **(Section 2.4 -2.5).**

Co-expression of chaperones

In some cases, co-expression of chaperones was incorporated with the intention to facilitate folding and to enhance stability of the heterologous proteins. Specifically, plasmid pG-Tf2 (plasmid 4) (Takara Bio) encoding for groES-groEL-tig chaperones was used. This plasmid carries an origin of replication derived from pACYC and a chloramphenicol-resistance gene. The chaperone genes are situated downstream of the araBorPzt-1(tet) promoters, so addition of tetracycline is needed for induction of chaperone expression. In detail, the procedure followed for the co-expression of chaperones was: One hundred μL of competent *E. coli* cells containing plasmid 4 were transformed with 50 ng of plasmid DNA carrying P450s or/and CPR. Transformed cells were plated on LB agar supplemented with the appropriate antibiotics, including antibiotics for P450s and CPR plasmids but also with chloramphenicol (20 μg/mL) for selection of plasmid 4. Plates were incubated at 37$^{\circ C}$ overnight. Colonies from the transformation plate were used for the inoculation of 3 mL LB supplemented with appropriate antibiotics. The pre-culture was incubated for 16 h at 37$^{\circ C}$, while shaking at 150-200 rpm. Terrific Broth supplemented with appropriate antibiotics and with tetracycline (5 ng/μL), was inoculated with the preculture **(**1/50 inoculation). Cell culture was grown at 37°C (150 rpm shaking) until the optical density at 595 nm 0.5-0.6 cm^{-1}. Then, growth was continued at 30°C (150 rpm shaking) for 30 min. Induction was initiated with the addition of IPTG (final concentration of 0.5 mM), ALA (final concentration of 1 mM) and tetracycline (final concentration of 10 ng/mL). Culture was grown for 24 h at 30$^{\circ C}$, 150 rpm shaking.

Constructs 1) OmpA*Bo*CYP6G2-His together with groES-groEL-tig chaperones 2) OmpA*Bo*CYP6G2-His/*Bo*CPR (two plasmids) together with groES-groEL-tig chaperones and 3) pCDF Duet-1-*Bo*CYP6G2-His_*Bo*CPR with or without co-expression of groES-groEL were proceeded for large scale membrane targeting experiments in the selected strains indicated from small scale expression tests. Large scale expression and membrane's preparation procedure was the same analyzed for *Ha*CYP6B7 in **Section 3.2**. Expression was tested via western blot in bacterial cell extracts before and after the induction and in membrane preparations. Total protein concentration was calculated with Bradford assay (**Section 2.7**). Membrane preparations were tested for appropriate folding of P450 with CO assay and CPR activity against cytochrome c (**Section 2.8-2.9**).

3. Results

Chapter I

Heterologous expression of *Ha*CYP6B7 in *E. coli*

3.1. Small scale expression test of P450 in *E. coli* strains

Small scale expression tests of P450s bearing Flag-His (*HaCYP6B7*-Flag-His, *Ha*CYP6B7-Flag-His/*Md*CPR, *Δ*N*Ha*CYP6B7-Flag-His) were performed in five *E. coli* strains (BL21 DE3, JM109 DE3, DH5α, Lemo21 DE3 and C43 DE3) under standard expression conditions. According to the western blot analysis shown in **Figures 7-8-9,** *Ha*CYP6B7-Flag-His was expressed successfully in Lemo21 DE3 and only at lower levels in strain DH5α and JM109 DE3 (western blot with higher exposure time), while there was no expression in C43 DE3 and BL21Star DE3. Ha*Δ*NCYP6B7-Flag-His was expressed in all five bacterial strains tested, showing higher expression in DH5α, Lemo21 and BL21star DE3 cells. A slight proteolytic cleavage was noticed in all strains except for C43 DE3 and JM109 DE3. *Ha*CYP6B7-Flag-His and *Md*CPR were co-expressed (in single plasmid experiment) successfully in Lemo21 DE3 and BL21star DE3 cells. Evaluating the above results with the two criteria of i) successful expression (high expression of P450) and ii) protein stability, Lemo21 DE3 was selected for the expression of *Ha*CYP6B7-Flag-His alone or with the reductase partner *Md*CPR and BL21 DE3 for the expression of *HaΔN*CYP6B7-Flag-His. The summary of the expression experiment results are presented in **Table 7**.

Table 7. Results of small scale expression of P450s bearing Flag-His, performed in five *E.coli* strains under standard expression conditions. (√ √ : high expression, √: moderate expression, X: no expression)

Constructs	***E.coli* strains**				
	DH5α	JM109 DE3	BL21 STAR DE3	C43 DE3	Lemo21 DE3
***Ha*CYP6B7-Flag-His**	√ √	√	X	X	√ √
***Δ*N*Ha*CYP6B7-Flag-His**	√ √	√ √	√ √	√	√ √
***Ha*CYP6B7-Flag-His/*Md*CPR**	X	X	√ √	X	√ √

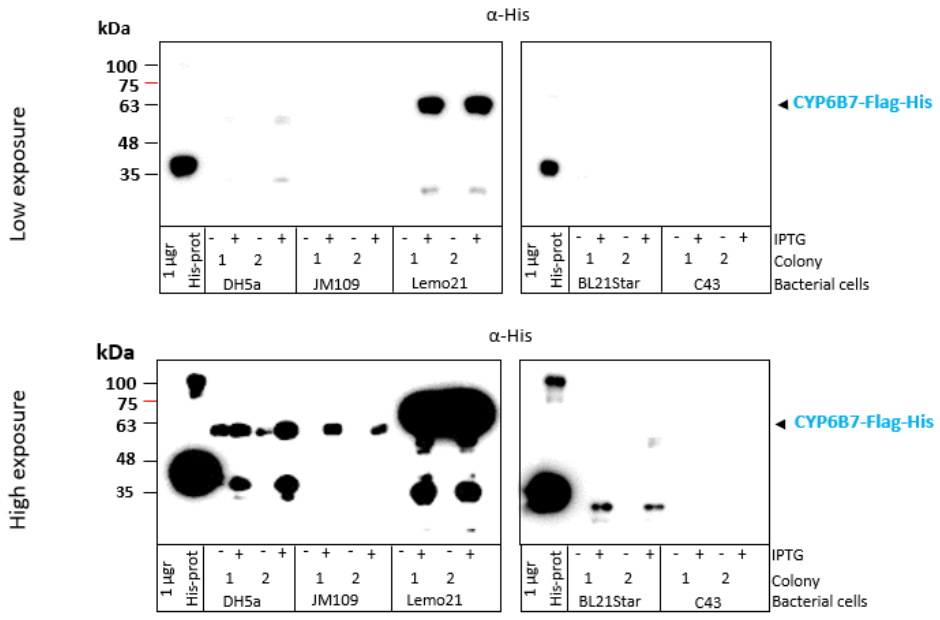


Figure 7. Western blot analysis of small-scale expression of *Ha*CYP6B7-Flag-His (predicted MW 60 kDa), performed in five *E. coli* strains under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 was tracked using anti-His antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As a positive control, 1 µg of purified His-MDH loaded.

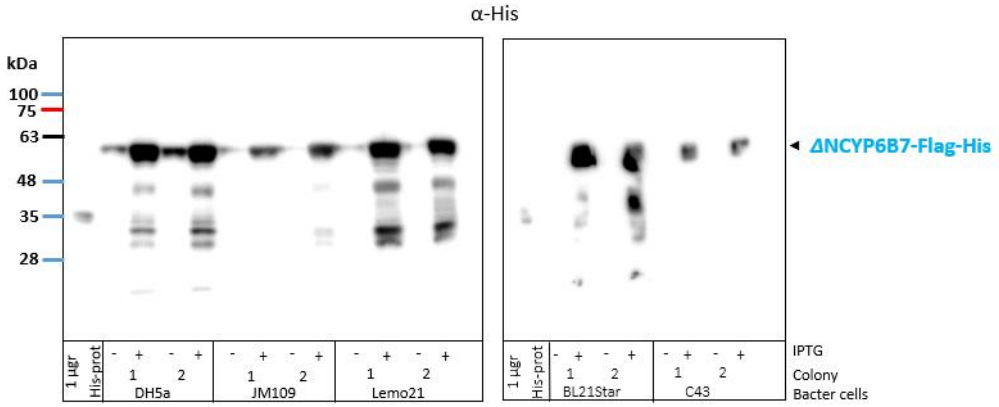


Figure 8. Western blot analysis of small-scale expression of *Δ*N*Ha*CYP6B7-Flag-His, performed in five *E. coli* strains under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR using anti-CPR antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As a positive control, 1 µg of purified His-MDH loaded.

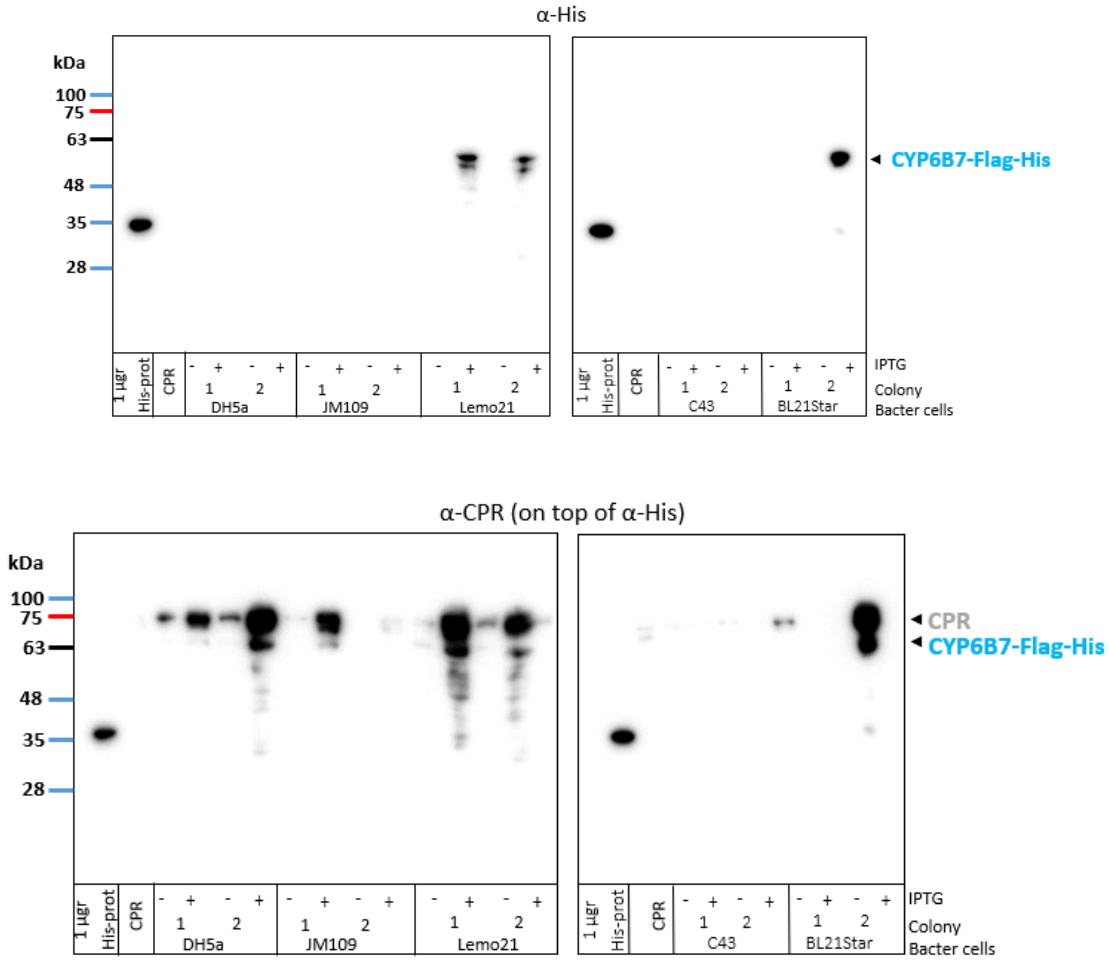


Figure 9. Western blot analysis of small scale expression of HaCYP6B7-Flag-His/MdCPR (predicted MW P450: 60 kDa , CPR: 76,4 kDa), performed in five *E. coli* strains under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR using anti-CPR antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As positive controls, 1 µg of purified His-MDH and 6 µg of purified CPR was loaded.

3.2. Membrane targeting experiments

Large scale expression (200 mL culture) of membrane targeted *Ha*CYP6B7 alone or together with *Md*CPR was conducted in Lemo21 DE3 *E.coli* cells, under standard expression conditions. Bacterial cells (-/+IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed via western blot. Two

independent biological replicates were conducted for each construct. Then, membrane preparations were checked for correct folding of P450 with CO spectrum assay, for the activity of CPR against cytochrome c and for catalytic activity of P450 against model substrate 7-ethoxycoumarin.

3.2.1. Western blot analysis of expression

As shown in **Figure 10**, expression of Native *Ha*CYP6B7-Flag-His was successful only in the second biological replicate. The protein was detected around 60 kDa, as expected, in both bacterial cells after induction and membranes. A slight proteolytic cleavage was noticed. According to **Figure 11** native HaCYP6B7-Flag-His together with *Md*CPR were expressed successfully from a single plasmid in both biological replicates. P450 was detected around 60 kDa in both bacterial lysate (+IPTG) and membranes. Alike, *Md*CPR was detected in +IPTG sample and membranes around 76,4 kDa, as expected. Concerning expression of Native HaCYP6B7-Flag-His together with *Md*CPR from two different plasmids, western blot analysis (**Figure 12**) revealed the successful expression of both proteins in membranes and +IPTG sample, on both biological replicates. P450 was detected around 60 kDa and *Md*CPR 76.4 kDa. A slight proteolytic cleavage of P450 was noticed.

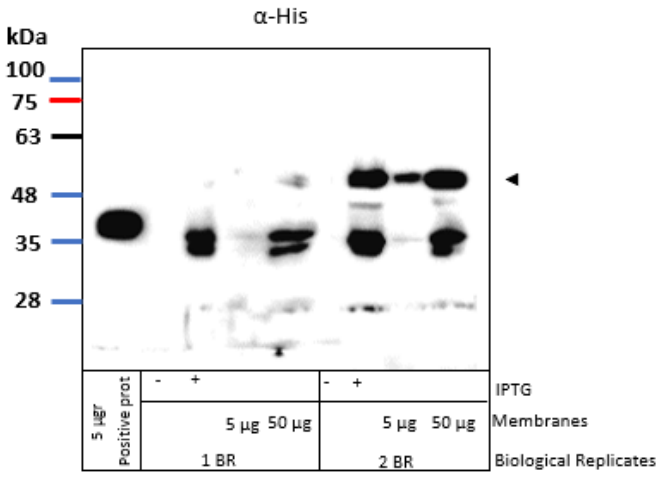


Figure 10. Western blot analysis of expression of native *Ha*CYP6B7-Flag-His (predicted MW: 60 kDa). Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 μg, 50 μg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 was tracked using anti-His antibody. As positive control, 5 μg of purified His-MDH and was loaded.

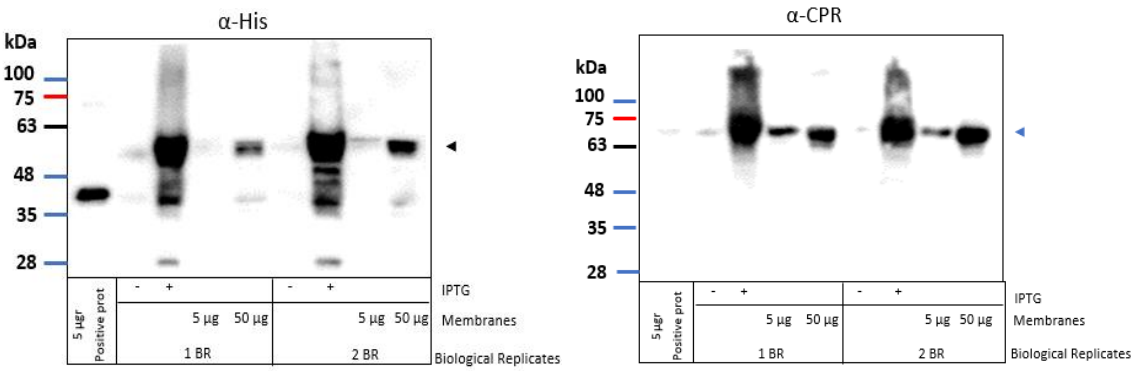


Figure 11. Western blot analysis of native *Ha*CYP6B7-Flag-His/*Md*CPR (single plasmid experiment). Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR was tracked via anti-CPR. As positive controls, 5 µg of purified His-MDH and 6 µg of purified CPR was loaded. *Ha*CYP6B7 is indicated with the black arrow (predicted MW 60 kDa) and *Md*CPR with the blue arrow (predicted MW 76.4 kDa).

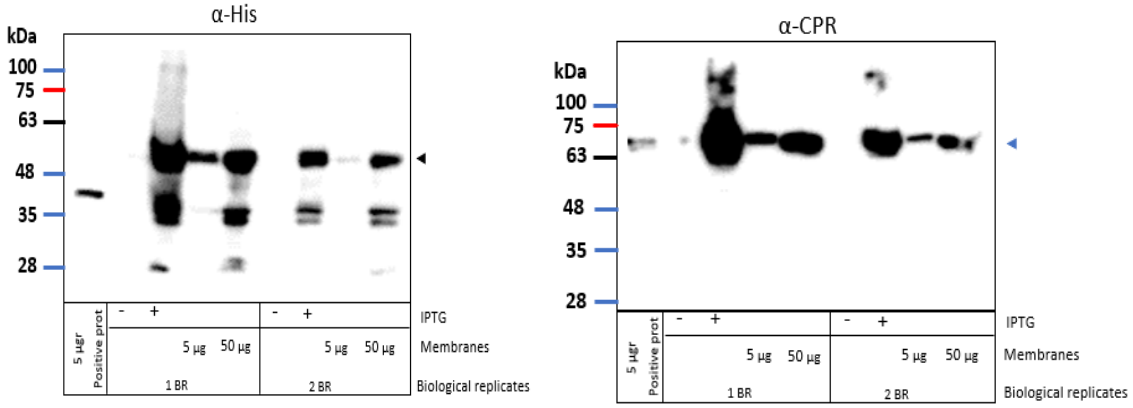


Figure 12. Western blot analysis of expression of native *Ha*CYP6B7-Flag-His/*Md*CPR (two plasmids experiment). Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR was tracked via anti-CPR. As positive controls, 5 µg of purified His-MDH and 6 µg of purified CPR was loaded. *Ha*CYP6B7 is indicated with the black arrow (predicted MW 60 kDa) and *Md*CPR with the red arrow (predicted MW 76.4 kDa).

*Ha*CYP6B7 that is merged with OmpA leader sequence does not bear Flag-His. Subsequently monitoring the expression of this P450 via western blot, was impossible. Albeit, in membranes containing OmpA*Ha*CYP6B7 and *Md*CPR, detection of CPR via western blot, was possible. As

shown in **Figure 13** and **Figure 14**, *Md*CPR was expressed successfully in membranes and +IPTG sample either in single plasmid experiment or in two plasmids experiment.

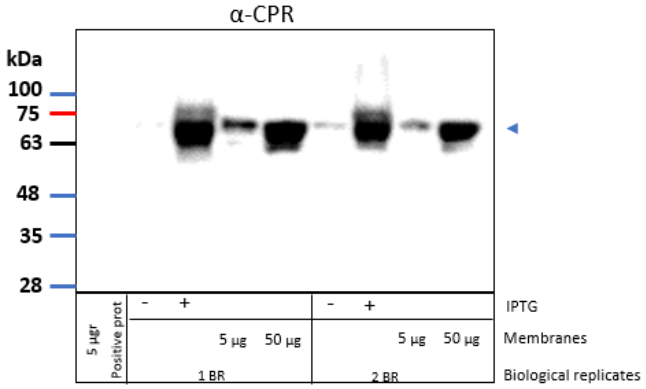


Figure 13. Western blot analysis of expression of *Md*CPR co-expressed with OmpA*Ha*CYP6B7-Flag-His in single plasmid experiment. Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of of CPR was tracked via anti-CPR. As positive control, 6 µg of purified CPR was loaded. *Md*CPR with the blue arrow (predicted MW 76.4 kDa).

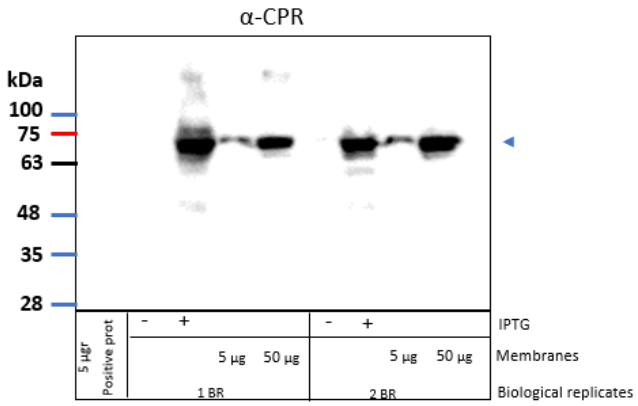


Figure 14. Western blot analysis of expression of *Md*CPR co-expressed with OmpA*Ha*CYP6B7-Flag-His in two plasmids experiment. Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of CPR was tracked via anti-CPR. As positive control, 6 µg of purified CPR was loaded. *Md*CPR with the blue arrow (predicted MW 76.4 kDa).

3.2.2. Bradford assay

Total protein concentration of membrane preparations was calculated based on the BSA standard curve y=0.0454x, with R^2=0.989. The calculated total protein concentration is presented in **Table 8**.

Table 8. Bradford total protein concentration of membrane preparations.

Construct	Biological replicate	Total protein mg/mL
***Ha*CYP6B7-Flag-His**	1	36.6
	2	33.5
***Ha*CYP6B7-Flag-His/*Md*CPR** (single plasmid)	1	35.5
	2	29.3
***Ha*CYP6B7-Flag-His and *Md*CPR** (two plasmids)	1	19.4
	2	26.1
OmpA*Ha*CYP6B7	1	43.6
	2	38.1
OmpA*Ha*CYP6B7/*Md*CPR (single plasmid)	1	38.2
	2	28.7
OmpA*Ha*CYP6B7 and *Md*CPR (two plasmids)	1	40.8
	2	25.6

3.2.3. Spectral assay

In order to check the integrity of P450s, P450 spectral assay was conducted. According to carbon monooxide (CO)-difference spectra in **Figure 15**, *Ha*CYP6B7-Flag-His showed a not well-formed

peak around 450 nm, shifted (about 3-5 nm) and a significant peak at 420 nm in both biological replicates. In **Figure 16** spectral characteristics of *Ha*CYP6B7-Flag-his when co-expressed with *Md*CPR from a single plasmid, are shown. Both biological replicates showed a well-formed peak around 450 nm. More specifically, the first biological replicate showed a predominant peak at 450 nm. These data indicate the presence of P450 which maintains its integrity. Concerning the spectral characteristics of *Ha*CYP6B7-Flag-His when co-expressed with *Md*CPR from two different plasmids, both biological replicates showed a well-formed but shifted (about 4-10 nm) peak around 450 nm and a significant peak around 420 nm (**Figure 17**). Regarding OmpA*Ha*CYP6B7 membrane preparation (**Figure 18**), both biological replicates showed a not well formed, shifted (about 5 nm) peak. Also, in the second biological replicate a peak at 420 nm was noticed. OmpA*Ha*CYP6B7 when co-expressed with *Md*CPR from a single plasmid showed promising spectral characteristics **(Figure 19).** Both biological replicates showed a well-formed, predominant peak at 450 nm, indicating correct folding. Albeit, a small at 420 nm was noticed. Subsequently these spectral characteristics, indicate the presence of P450 which mainly maintains its integrity. Finally, OmpA*Ha*CYP6B7-Flag-His, co-expressed with *Md*CPR from two plasmids, showed a not well formed, sifted (about 5-6 nm) peak in both biological replicates (**Figure 20**). The P450 content of membrane preparations was calculated according to the formula described in **Section 2.8** and is shown in the **Table 9**.

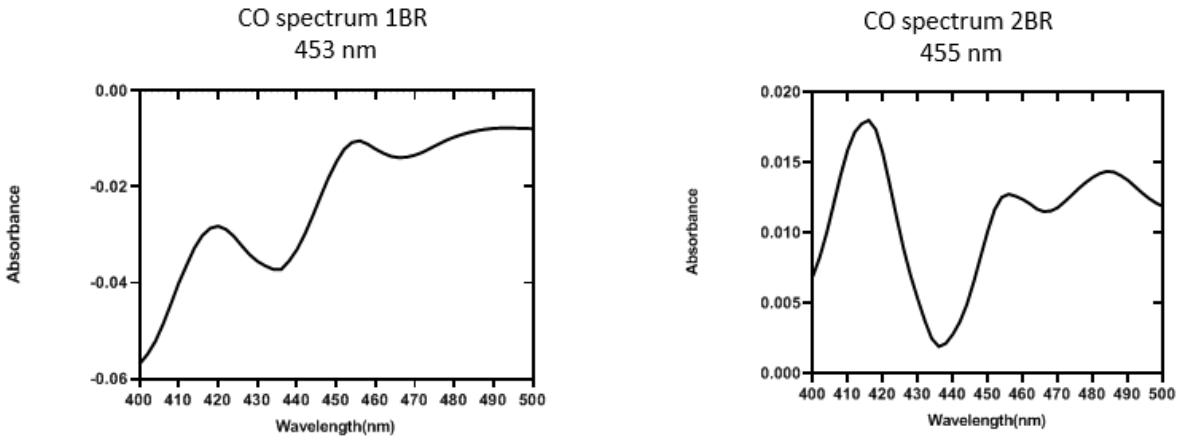


Figure 15. Carbon monoxide (CO)-difference spectra of native *Ha*CYP6B7-Flag-His. The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

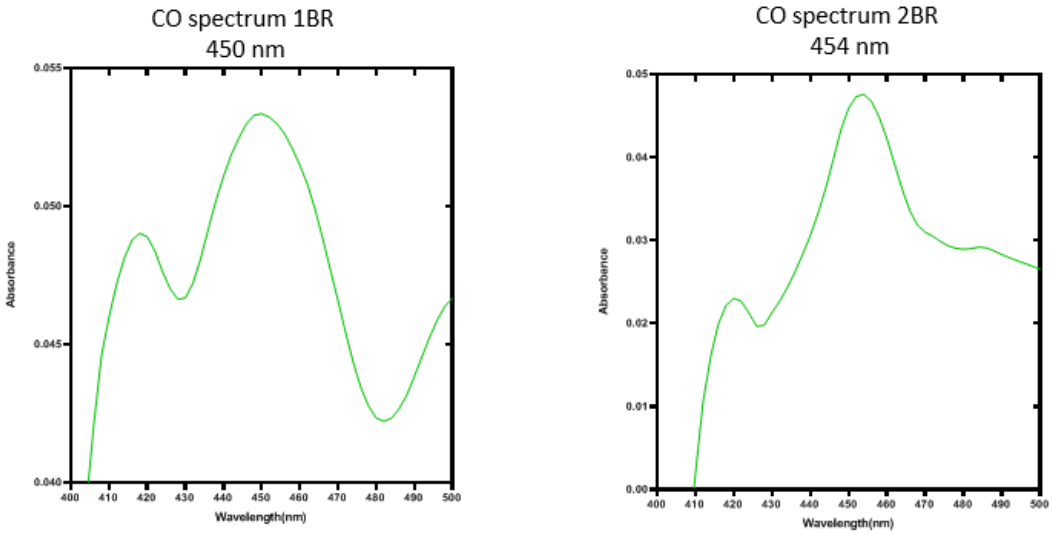


Figure 16. Carbon monooxide (CO)-difference spectra of native *Ha*CYP6B7-Flag-His/*Md*CPR (single plasmid experiment). The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

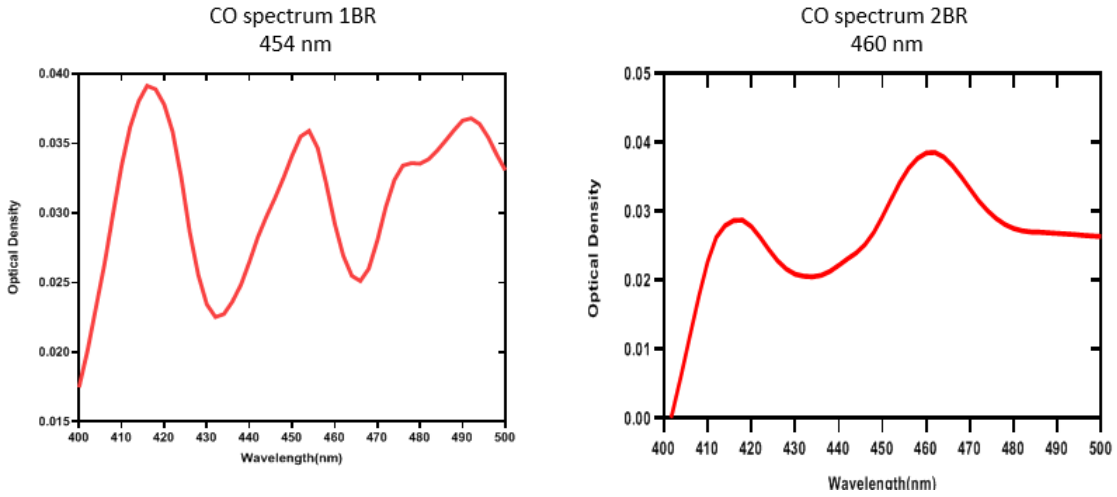


Figure 17. Carbon monooxide (CO)-difference spectra of native *Ha*CYP6B7-Flag-His/*Md*CPR (two plasmids experiment). The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

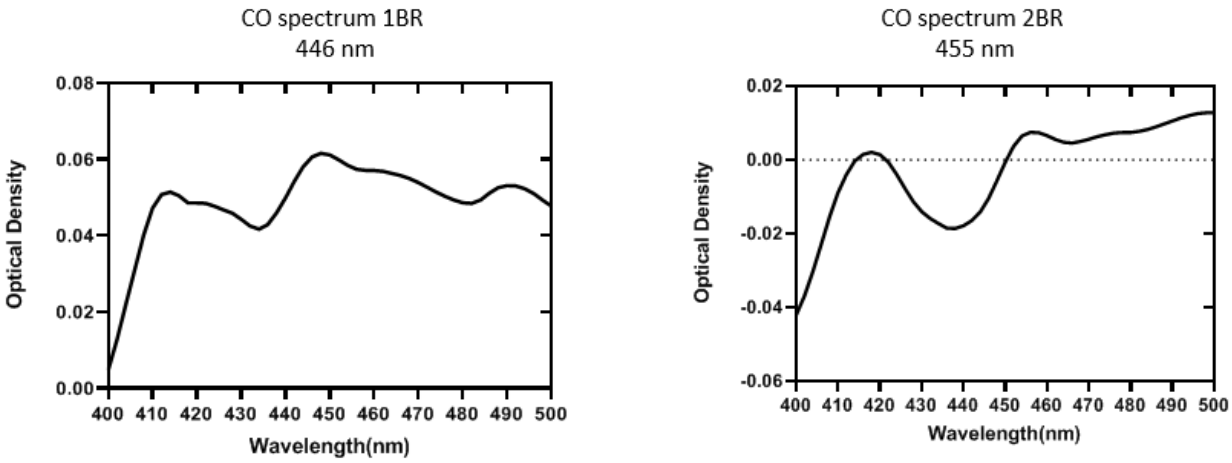


Figure 18. Carbon monooxide (CO)-difference spectra of OmpA*Ha*CYP6B7-Flag-His. The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

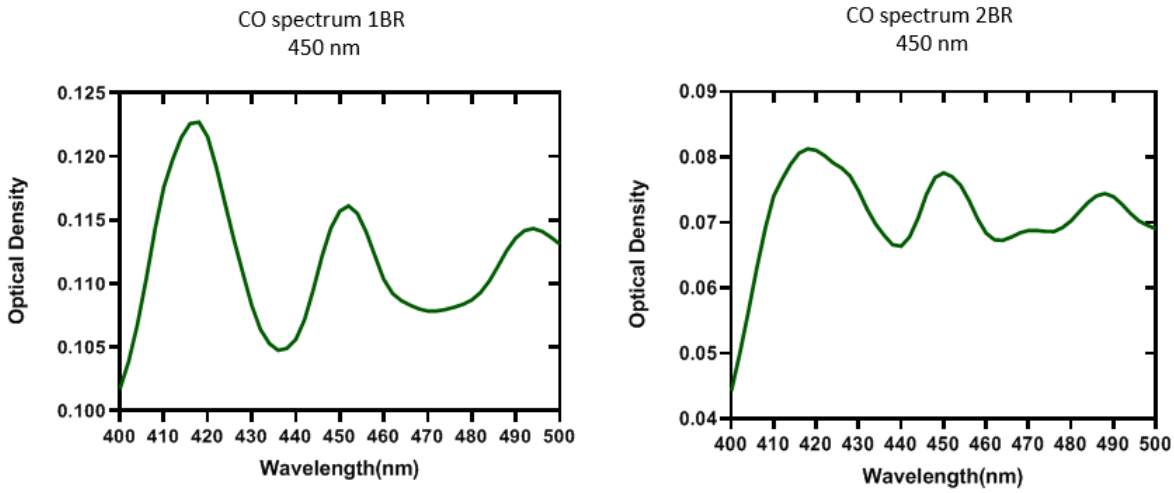


Figure 19. Carbon monooxide (CO)-difference spectra of OmpA*Ha*CYP6B7-Flag-His/*Md*CPR (single plasmid experiment). The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

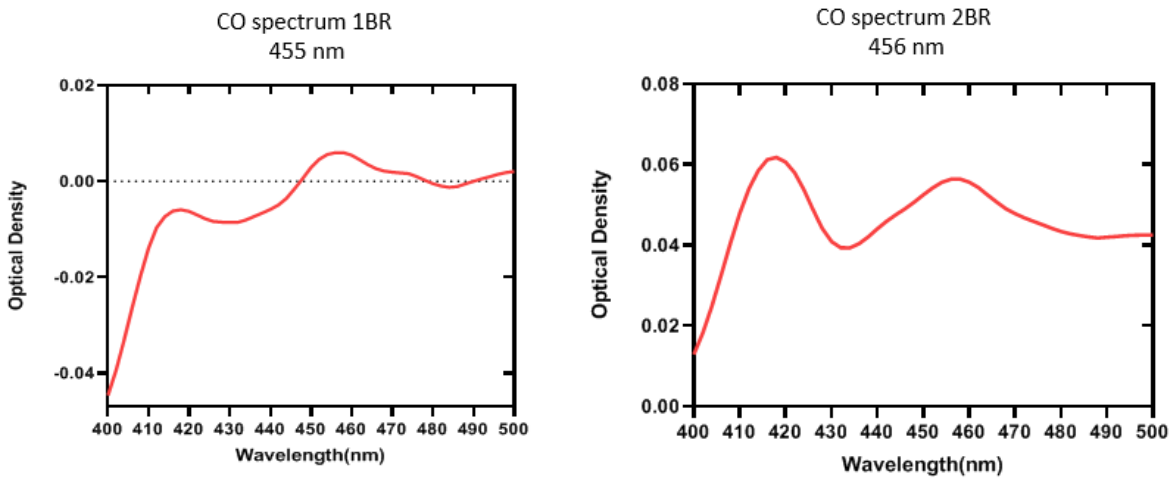


Figure 20. Carbon monooxide (CO)-difference spectra of OmpA*Ha*CYP6B7-Flag-His/*Md*CPR (two plasmids experiment). The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

Table 9. Spectral characteristics and P450 content of membrane preparations.

Construct	Biological replicate	P420 nm	P450 nm	nmol P450/mg total protein	Total Yield nmol/L
***Ha*CYP6B7-Flag-His**	1	416	453	0.05	7.03
	2	415	455	0.02	2.37
***Ha*CYP6B7-Flag-His/*Md*CPR** (single plasmid)	1	422	450	0.71	100.22
	2	417	454	0.85	74.51
***Ha*CYP6B7-Flag-His and *Md*CPR** (two plasmids)	1	420	454	0.25	19.34
	2	417	460	0.52	47.69
OmpA*Ha*CYP6B7	1	-	446	0.18	38.46
	2	420	455	0.03	4.55
OmpA*Ha*CYP6B7/*Md*CPR (single plasmid)	1	416	450	0.12	32.62
	2	420	450	0.21	20.77
OmpA*Ha*CYP6B7 and *Md*CPR (two plasmids)	1	415	455	0.22	31.54
	2	418	456	0.11	10.30

3.2.4. CPR activity assay

Membrane preparation containing *Md*CPR, were tested for CPR activity against cytochrome c (electron acceptor) in presence of NADPH (electron donor). The rate of reduction of cytochrome c was measured by recording kinetically the absorbance at 550 nm. Finally, catalytic activity of CPR (nmol reduced cytochrome c min-1 mg-1 total protein) was calculated according to the formula mentioned in **Section 2.9**. Measurements of CPR activity are shown in **Table 10**. High activity of CPR against cytochrome c was noticed in all membrane preparation.

Table 10. Measurements of CPR activity against cytochrome c in presence of NADPH.

Construct	Biological replicate	nmol of product min^{-1} mg^{-1}
***Ha*CYP6B7-Flag-His**	1	-
	2	-
***Ha*CYP6B7-Flag His/*Md*CPR** (single plasmid)	1	6151.18
	2	6803.10
***Ha*CYP6B7-Flag-His and *Md*CPR** (two plasmids)	1	3110.60
	2	1368.87
OmpA*Ha*CYP6B7	1	-
	2	-
OmpA*Ha*CYP6B7/*Md*CPR (single plasmid)	1	6380.45
	2	2647.96
OmpA*Ha*CYP6B7 and *Md*CPR (two plasmids)	1	1431.50
	2	1491.69

3.2.5. Catalytic activity assay against model substrates

All membrane preparations were tested for their catalytic activity against model substrates 7-ethoxy coumarin and p-nitroanisole and no catalytic activity was detected.

3.3 Soluble P450

3.3.1 Expression and purification

*Δ*N*Ha*CYP6B7-Flag-His, namely *Ha*CYP6B7 without the hydrophobic anchoring part was expressed in the cytoplasm of BL21Star DE3 cells, in 5x200 mL TB liquid cultures. P450 purification conducted via Ni-NTA affinity chromatography and ion exchange chromatography. Fractions collected during different phases of chromatography were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed via Coomassie Brilliant Blue (R-250) staining and western blot.

In **Figure 21**, analysis of Ni-NTA purification of *Δ*N*Ha*CYP6B7-Flag-His is presented. *Δ*NHaCYP6B7-Flag-His band is detected around 63 kDa close to the predicted molecular weight (57 kDa). Focusing on the +IPTG sample, the band of the protein is very thick, which means that expression was not satisfying. *Δ*N*Ha*CYP6B7-Flag-His band is detected mainly in elution fractions, where unfortunately is attended by other proteins. The protein band is also detected in flow through fraction and wash 1 fraction, indicating that part of the protein didn't bind in the resin. Thus, the purification was not satisfying. CO assay performed in elution fractions (**Figure 22**) and showed a not well-formed peak at 447 nm and a significant peak around 420 nm, revealing that P450 is unfolded. Afterwards, cation exchange chromatography (SP-sepharose) was conducted. ΔN*Ha*CYP6B7 was diluted in Tris/HCl buffer, pH 7.5, where according to the isoelectric point (pI 8.8) the protein has a positive surface charge and is expected to bind to the negatively charged SP-Sepharose. In **Figure 23**, analysis of cation exchange chromatography is presented. *Δ*N*Ha*CYP6B7-Flag-His band is detected mainly in flow through and wash fraction, while a very thick band is also detected in elution fraction. These results indicate that P450 didn't bind in the SP-sepharose. Another attempt of purification via Ni-NTA affinity chromatography was conducted. This time, bacterial lysate was incubated with the resin overnight, for more efficient binding. The results of this attempt are presented in (**Figure 24**). *Δ*N*Ha*CYP6B7-Flag-His band is detected mainly in elution fractions together with some contaminants, while a very thick band is also detected in flow through and wash fractions. As these results suggest, *Δ*N*Ha*CYP6B7-Flag-His is semi-purified. Finally, anion exchange chromatography (Q-sepharose) was conducted. P450 was not expected to bind to the positively charged Q Sepharose because it was positively charged (diluted in Tris/HCl buffer, pH 7.5). Negatively charged Q Sepharose was used with the intense to bind the protein contaminants and not *Δ*N*Ha*CYP6B7-Flag-His, which will be eluted in the flow through. According to **Figure 25,** *Δ*N*Ha*CYP6B7-Flag-His was detected in both flow through and elution fractions. Focusing on the flow through fraction, we can see that the major contaminants

are not detected and P450 is purified to a great extent. Flow through was concentrated using Amicon filter (Merck, Milipore) with molecular weight cut-off 10 kDa and the Bradford concentration of the semi-purified protein was calculated 1 mg/mL based on the BSA standard curve. CO assay performed in flow through, showed a not well-formed peak at 446 nm, revealing a P450 with a doubtful quality (**Figure 26)**.

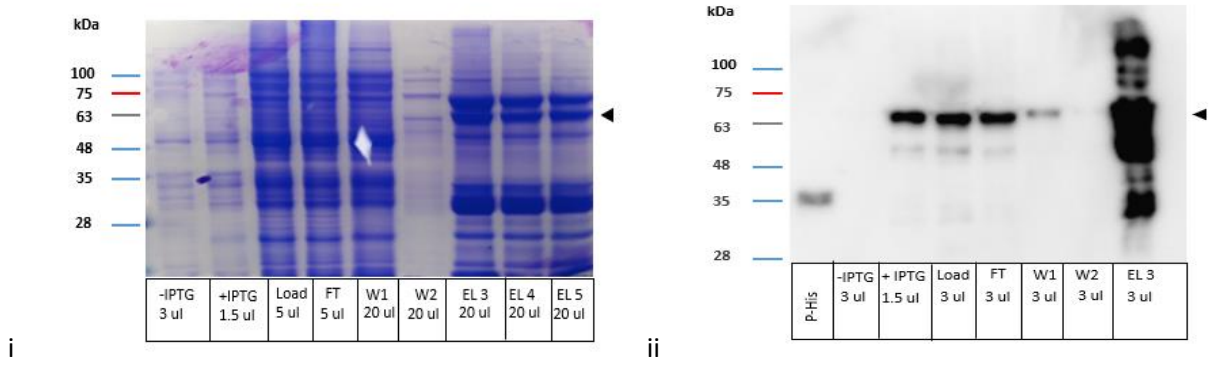


Figure 21. Analysis of Ni-NTA purification of *Δ*NHaCYP6B7. Elution conducted with 200 mM imidazole. Fractions collected during different phases of chromatography and bacterial cells before and after the induction (-/+IPTG samples) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed via i. Coomassie Brilliant Blue (R-250) staining and ii. Western blot with anti-His. Load: bacterial lysate that was loaded in the Ni-NTA resin, FT: flow through, W1: Wash 1, W2: Wash 2, EL 3,4,5: elution fractions. The volume of each fraction that was loaded in the gel is noted under each lane. As positive control for western blot, 1 μg of purified His-MDH was loaded. *Δ*N*Ha*CYP6B7-Flag-His is indicated with the black arrow (predicted MW 57 kDa).

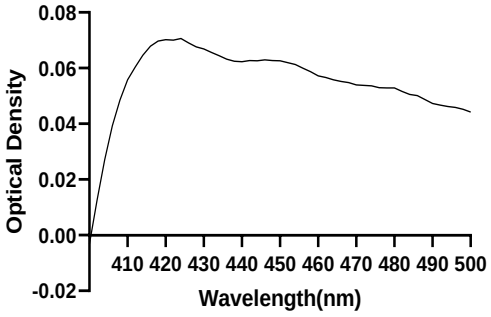


Figure 22. Carbon monoxide (CO)-difference spectrum of elution fractions from Ni-NTA affinity chromatography.

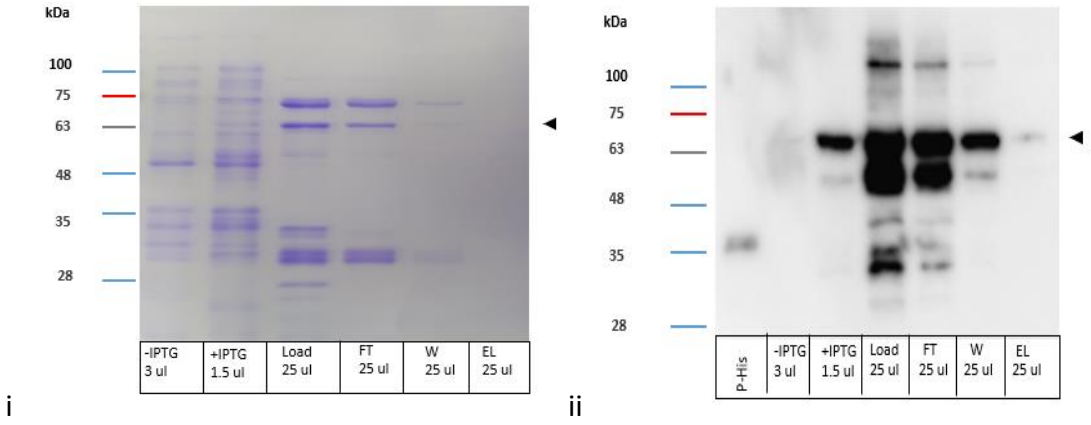


Figure 23. Analysis of cation exchange chromatography-SP Sepharose. Elution conducted with 1 M NaCl. Fractions collected during different phases of chromatography and bacterial cells before and after the induction (-/+IPTG samples) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed via i. Coomassie Brilliant Blue (R-250) staining and ii. Western blot with anti-His. Load: Pooled elution fractions from Ni NTA chromatography, FT: flow through, W: Wash, EL: elution fraction. The volume of each fraction that was loaded in the gel is noted under each lane. As positive control for western blot, 1 μg of purified His-MDH was loaded. *Δ*N*Ha*CYP6B7-Flag-His is indicated with the black arrow (predicted MW 57 kDa).

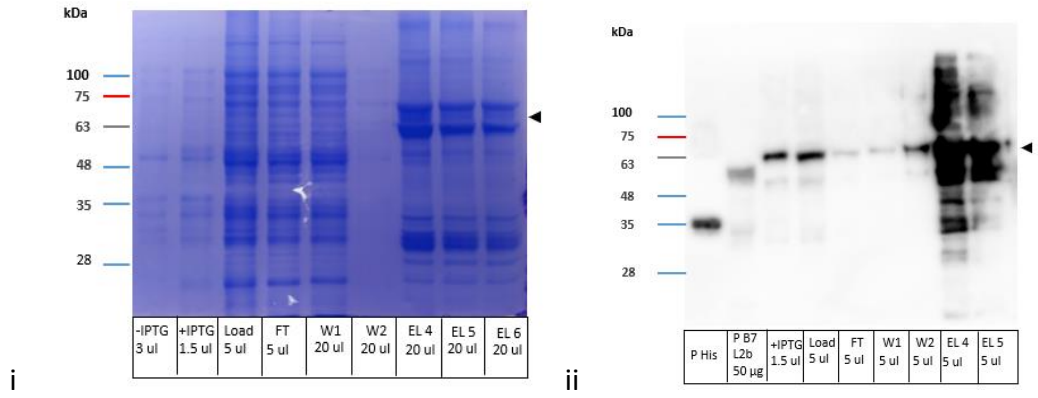


Figure 24. Analysis of Ni-NTA purification of ΔNHaCYP6B7. Elution conducted with 200 mM imidazole. Fractions collected during different phases of chromatography and bacterial cells before and after the induction (-/+IPTG samples) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed via i. Coomassie Brilliant Blue (R-250) staining and ii. Western blot with anti-His. Load: bacterial lysate that was loaded in the Ni-NTA resin, FT: flow through, W1: Wash 1, W2: Wash 2, EL 4,5,6: elution fractions. The volume of each fraction that was loaded in the gel is noted under each lane. As positive controls for western blot, 1 μg of purified His-MDH and 50 μg of membrane preparation of native *Ha*CYP6B7/*Md*CPR were loaded. *Δ*N*Ha*CYP6B7-Flag-His is indicated with the black arrow (predicted MW 57 kDa).

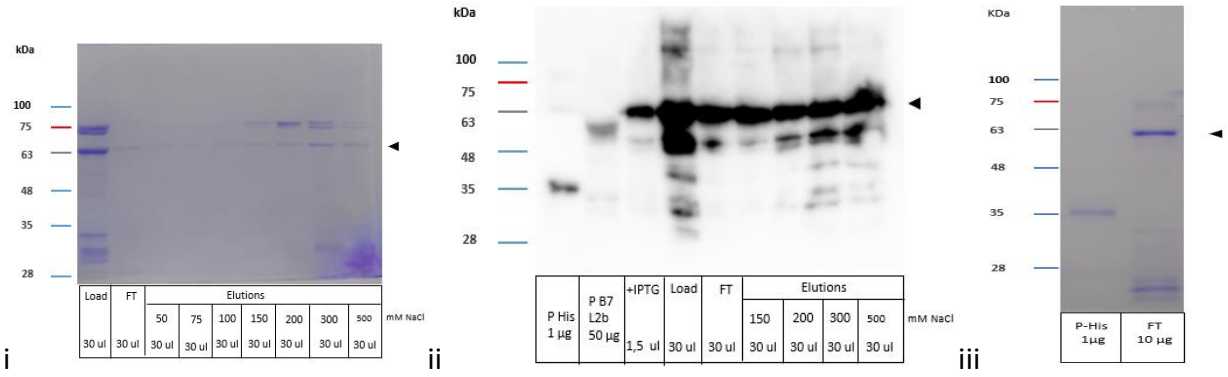


Figure 25. Analysis of anion exchange chromatography-Q Sepharose. Elution conducted with salt gradient (50-500 mM NaCl). Fractions collected during different phases of chromatography and bacterial cells before and after the induction (-/+IPTG samples) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analysed via i. Coomassie Brilliant Blue (R-250) staining and ii. Western blot with anti-His. Load: Pooled elution fractions from Ni NTA chromatography, FT: flow through, W: Wash. Under each lane the volume of fraction that was loaded in the gel is noted. As positive controls for western blot, 1 µg of purified His-MDH and 50 µg of membrane preparation of native *Ha*CYP6B7/*Md*CPR single plasmid were loaded. iii: concentrated FT analysed via Coomassie Brilliant Blue (R-250) staining.

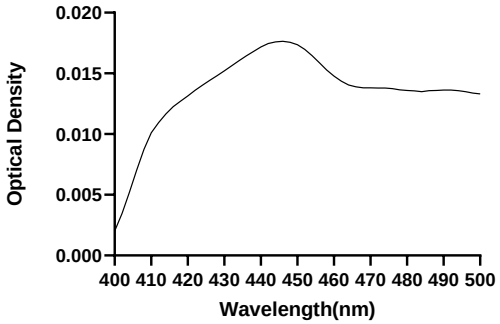


Figure 26. Carbon monoxide (CO)-difference spectrum of elution fractions from Ni-NTA affinity chromatography.

Moreover, it is worth mentioning that *Δ*NCYP6B7-Flag-His and native *Ha*CYP6B7-Flag-His showed an anomalous migration behavior on SDS gel (**Figure 24.ii**). Comparing the mobility of the two proteins, native P450 had higher mobility than the truncated P450. This fact is unexpected because *Ha*CYP6B7-Flag-His has higher molecular weight than *Δ*NCYP6B7-Flag-His. This anomalous mobility of the two proteins can be explained based on the hydrophobic characteristics of *Ha*CYP6B7-Flag-His. According to (Shirai et al., 2008) gel mobility is influenced

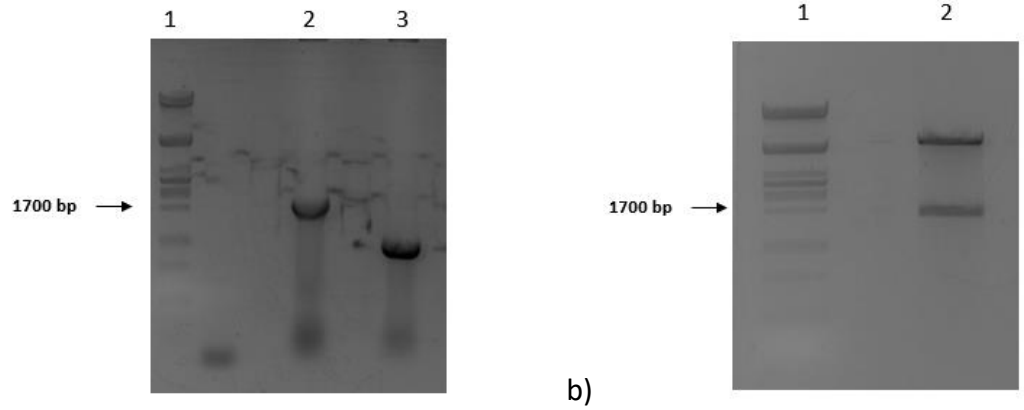
by various properties of proteins. Among them, the hydrophobicity has the greatest influence on gel mobility. In general, SDS binds to a protein at a ratio of approximately one molecule/two amino acid residues. However, because this binding is mediated via the hydrophobic interaction, SDS preferentially binds to the hydrophobic region of proteins. As a result, it is likely that hydrophobic proteins bind higher amounts of SDS than hydrophilic ones and therefore migrate faster in SDS-PAGE. *Ha*CYP6B7 has the hydrophobic transmembrane anchoring part and subsequently shows faster mobility than the truncated that lacks this hydrophobic part.

Chapter II

Heterologous expression of *BoCYP6G2 and BoCYP6A23* in *E. coli*

3.4. Cloning of P450s and CPR to expressing vectors

Gene sequence (not codon optimized) encoding for *Bo*CYP6G2 was double digested with NgoMIV/SacI and ligated to the pCWOmpA expressing vector. Successful insertion of *Bo*CYP6G2-His to pCWOmpA was verified by amplification with primers specific for the insert, digestion with NgoMIV/SacI and sequencing **Figure 27**. In addition, gene sequence encoding for *Bo*CPR (codon optimized) was double digested with restriction enzymes MfeI/KpnI and ligated to the MCSII of pCDF-Duet-1 expressing vector. Cloning *Bo*CPR to pCDF-Duet-1 was verified by amplification with primers specific for the MCSII of pCDF-Duet-1, digestion with MfeI/KpnI and sequencing **Figure28**. Moreover, sequences of *Bo*CYP6G2-His and *Bo*CYP6A23-His (codon optimized) were double digested with NdeI/NotI restriction enzymes and ligated to the MCSI of pCDF-Duet-1 which had incorporated *Bo*CPR, in the MCSII. Successful cloning was confirmed by amplification with primers specific for *Bo*CYP6A23 and *Bo*CYP6G2, digestion with NdeI/NotI restriction enzymes and sequencing (**Figure 29-30**). Finally, *Bo*CYP6A23 gene sequence (codon optimized) was double digested with NdeI/NotI restriction enzymes and ligated to the MCSI of pCDF-Duet-1 which had incorporated *Md*CPR, in the MCSII. Successful cloning was confirmed by amplification with primers specific for *Bo*CYP6A23, digestion with NdeI/NotI restriction enzymes and sequencing **(Figure 31)**.



a) b)

Figure 27. Electrophoresis of a) PCR products amplified with specific primers b) NgoMIV/SacI digestion products for confirming the integration of *Bo*CYP6G2-His (not codon optimized) to pCWOmpA expressing vector. Lane a1, b1: DNA ladder λPstI, lane a2: PCR product amplified with external primers specific for *Bo*CYP6G2 (not codon optimized), lane a3: PCR product amplified with internal primers specific for *Bo*CYP6G2 (not codon optimized) lane b2: product after digestion of pCWOmpA-*Bo*CYP6G2-His with NgoMIV/SacI. Size of *Bo*CYP6G2 gene is 1637 bp.

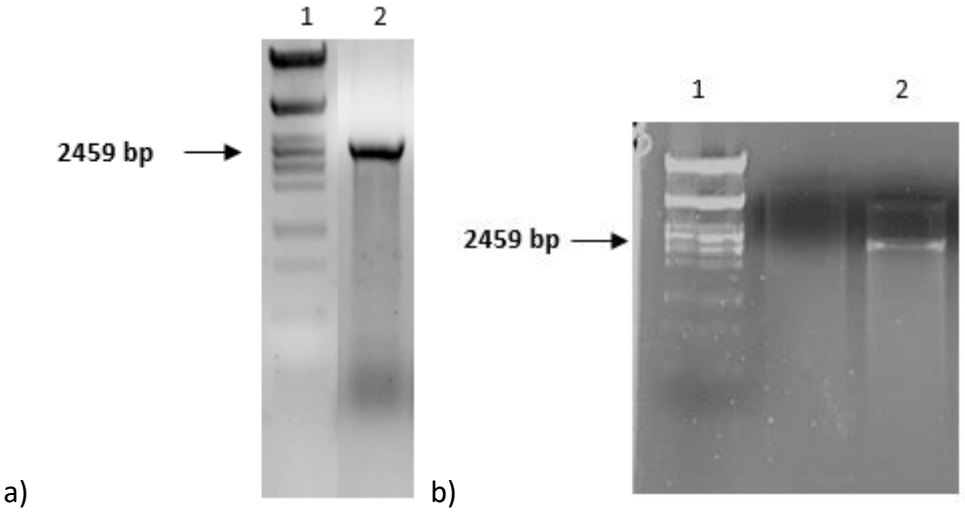


Figure 28. Electrophoresis of a) PCR product amplified with specific primers b) MFeI/ KpnI digestion products for confirming the integration of *Bo*CPR to MCSII of pCDFDuet-1 expressing vector. Lane a1, b1: DNA ladder λPstI, lane a2: PCR product amplified with primers specific for MCSII of pCDFDuet-1 expressing vector, lane b2: product after digestion of pCDFDuet_*Bo*CPR with MFeI/ KpnI. Size of *Bo*CPR gene is 2094 bp.

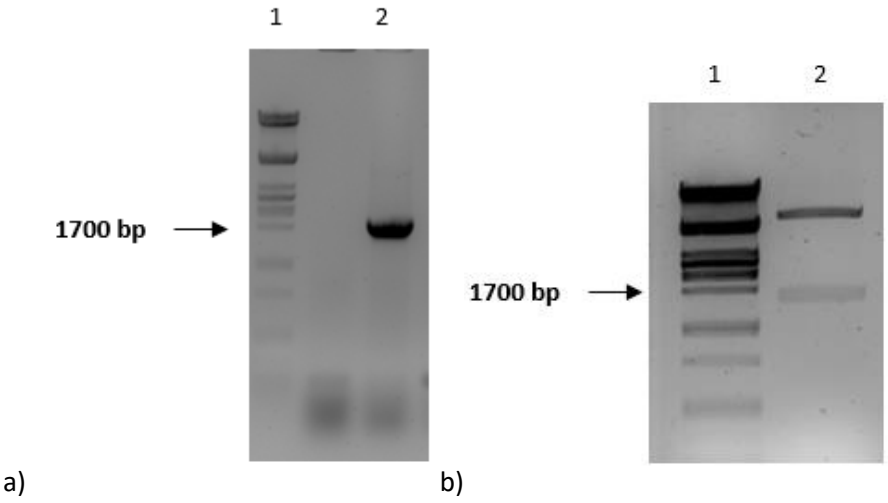


Figure 29. Electrophoresis of a) PCR product amplified with specific primers b) NdeI/NotI digestion products for confirming the integration of *Bo*CYP6G2 (codon optimized) to MCSI of pCDFDuet_*Bo*CPR expressing vector. Lane a1, b1: DNA ladder λPstI, lane a2: PCR product amplified with primers specific for *Bo*CYP6G2 (codon optimized), lane b2: product after digestion of pCDFDuet_*Bo*CYP6G2-His_*Bo*CPR with NdeI/NotI. Size of *Bo*CYP6G2 gene is 1637 bp.

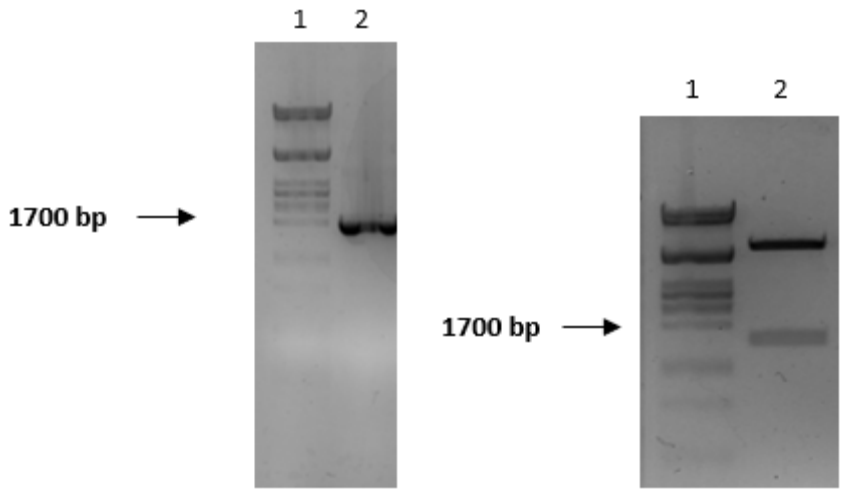


Figure 30. Electrophoresis of a) PCR product amplified with specific primers b) NdeI/NotI digestion products for confirming the integration of *Bo*CYP6A23 (codon optimized) to MCSI of pCDFDuet_*Bo*CPR expressing vector. Lane a1, b1: DNA ladder λPstI, lane a2: PCR product amplified with primers specific for *Bo*CYP6A23 (codon optimized), lane b2: product after digestion of pCDFDuet_*Bo*CYP6A23-His_*Bo*CPR with NdeI/NotI. Size of *Bo*CYP6A23gene is 1526 bp.

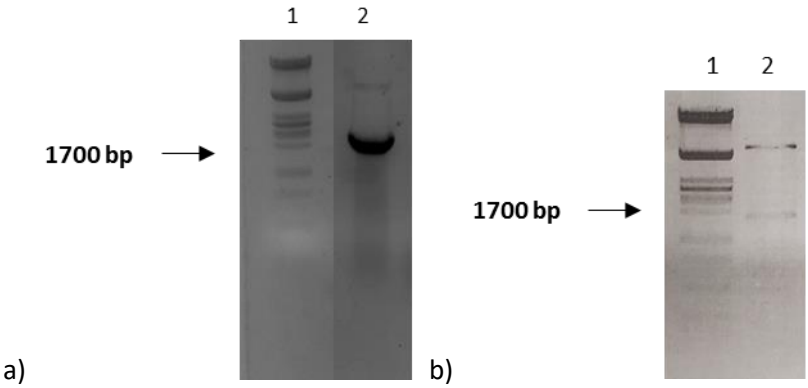


Figure 31. Electrophoresis of a) PCR product amplified with specific primers b) NdeI/NotI digestion products for confirming the integration of *Bo*CYP6A23 (codon optimized) to MCSI of pCDFDuet_*Md*CPR expressing vector. Lane a1, b1: DNA ladder λPstI, lane a2: PCR product amplified with primers specific for *Bo*CYP6A23 (codon optimized), lane b2: product after digestion of pCDFDuet_*Bo*CYP6A23-His_*Md*CPR with NdeI/NotI. Size of *Bo*CYP6A23 gene is 1526 bp.

3.5 Small scale expression tests of *Bo*CYP6G2 and *Bo*CYP6A23

Small scale expression tests of constructs pCW-OmpA-*Bo*CYP6G2-his and pCDF Duet-1-*Bo*CYP6G2-His/*Bo*CPR were performed in five bacterial expression strains (BL21 DE3, JM109 DE3, DH5α, Lemo21 and C43 DE3) under standard expression conditions. According to the western blot analysis shown in **Figure 32,** *OmpA BoCYP6G2-His* was expressed successfully in all bacterial strains, but with a more detailed look two protein bands are detected around the predicted molecular weight of the protein (62.7 kDa), indicating proteolytic cleavage. For this reason, small scale expression was conducted including co-expression of chaperons groES-groEL. As shown in **Figure 33** expression of *OmpA BoCYP6G2-His* in BL21Star DE3 and C43 *E. coli* cells that express chaperones groES-groEL-ti was successful but proteolytic cleavage didn't improve. *Bo*CYP6G2-His and *Bo*CPR were co-expressed (in single plasmid experiment) successfully in JM109 DE3, Lemo 21 DE3 and BL21Star DE3 cells. Higher expression for both P450 and CPR was shown in BL21Star cells (**Figure 34)**. Evaluating the above results with the two criteria of i) successful expression (high expression of P450) and ii) protein stability, C43 DE3 containing plasmid 4 was the selected strain for the expression of OmpA*BoCYP6G2*-His and BL21Star DE3 cells for the co-expression of *Bo*CYP6G2-His and *Bo*CPR in single plasmid experiment.

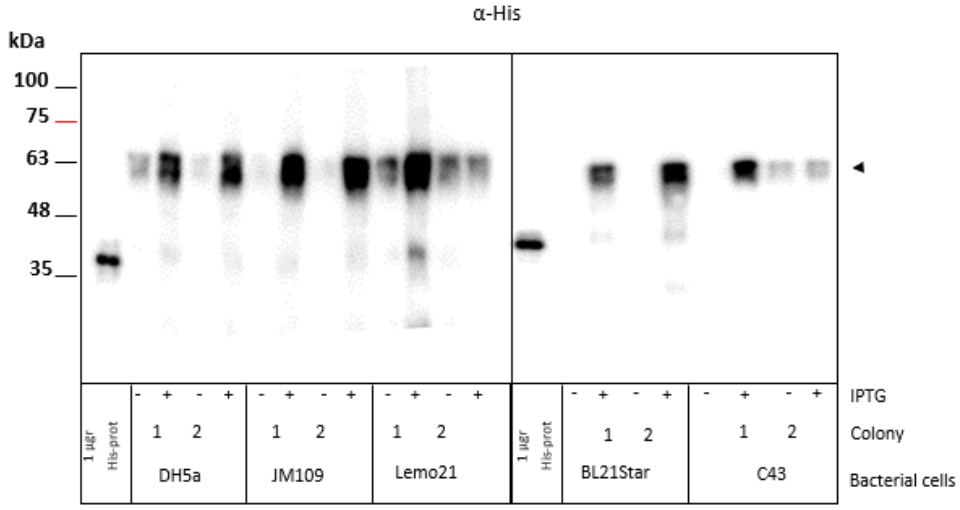


Figure 32. Western blot analysis of small scale expression of OmpA*Bo*CYP6G2-His (predicted MW 62.7 kDa), performed in five *E.coli* strains under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 was tracked using anti-His antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As a positive control, 1 μg of purified His-MDH was loaded. OmpA*Bo*CYP6G2-His is indicated with the black arrow.

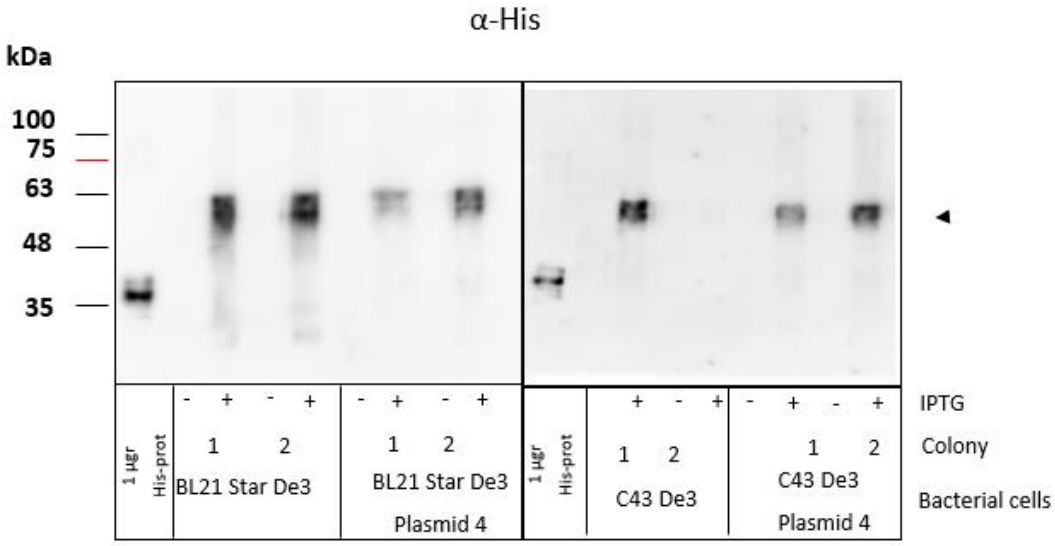


Figure 33. Western blot analysis of small scale expression of OmpA*Bo*CYP6G2-His (predicted MW 62.7 kDa), performed in *E.coli* strains: BL21 Star DE3, BL21 Star DE3 expressing chaperones, C43 DE3 and C43 DE3 expressing chaperones, under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 was tracked using anti-His antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As a positive control, 1 µg of purified His-MDH was loaded. OmpA*Bo*CYP6G2-His is indicated with the black arrow.

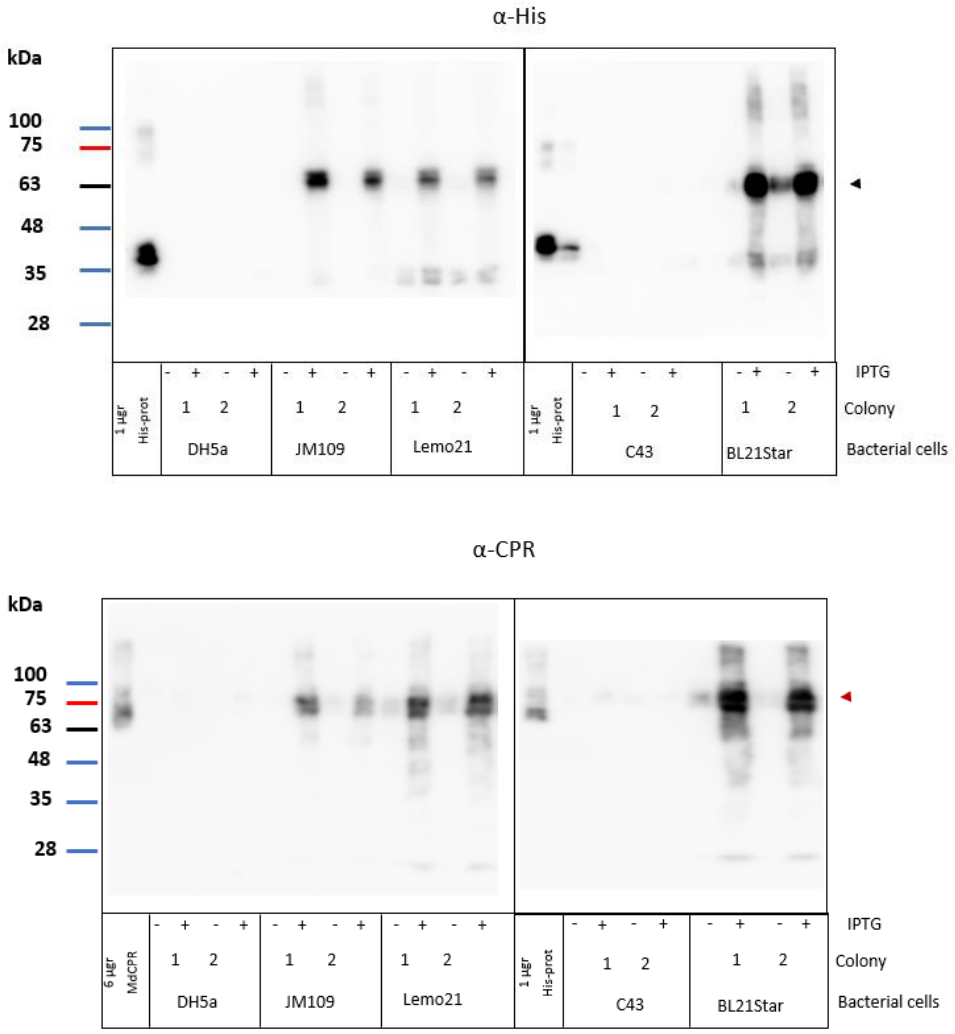


Figure 34. Western blot analysis of small scale expression of *Bo*CYP6G2-His/*Bo*CPR (predicted MW P450: 62.7 kDa , CPR: 78,8 kDa), performed in five *E. coli* strains under standard expression conditions. Bacterial cells were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by Western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR using anti-CPR antibody. For each strain expression was checked before (-IPTG) and after the addition of IPTG (+IPTG) for two different colonies. As positive controls, 1 µg of purified His-MDH and 6 µg of purified CPR was loaded. OmpA*Bo*CYP6G2-His is indicated with the black arrow and *Bo*CPR with the red one.

3.6. Membrane targeting experiments

3.6.1. Western blot analysis of expression

As shown in **Figure 35**, expression of OmpA-*Bo*CYP6G2-His was successful in both biological replicates. The protein was detected around 62.7 kDa, as expected, in both bacterial cells (+IPTG sample) and membranes. In the case of co-expression of OmpA-BoCYP6G2-His together with *Bo*CPR from two different plasmids, P450 was expressed successfully in both bacterial lysate (+IPTG) and membranes for both biological replicates. On the other hand, expression of *Bo*CPR was detected high in bacterial cells (+IPTG sample) but very low in membranes **(Figure 36)**. Expression of *Bo*CYP6G2 together with *Bo*CPR in a single plasmid experiment was conducted with and without the co-expression of chaperones. Concerning the experiment in absence of chaperones expression of *Bo*CYP6G2 was successful in both bacterial cells (+IPTG sample) and membranes and expression of *Bo*CPR was detected high in bacterial cells (+IPTG sample) but very low in membranes **(Figure 37).** In the co-expression of chaperones experiment, expression of both P450 and CPR was not successful (**Figure 37).** More biological replicates are needed to clarify if co-expression of chaperones hampers expression of P450s for this construct.

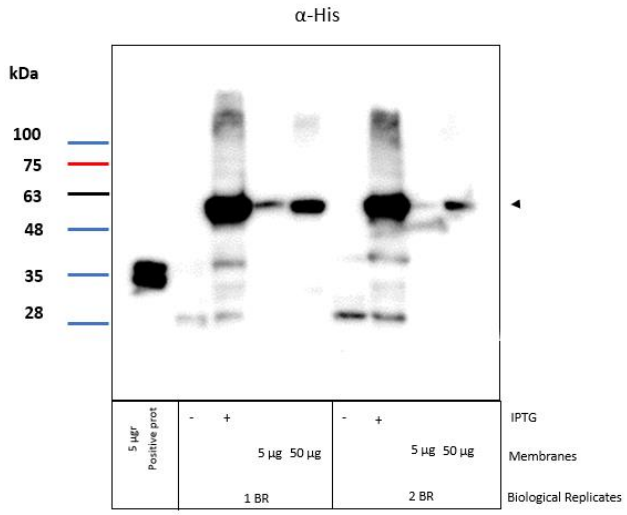


Figure 35. Western blot analysis of expression of OmpA*Bo*CYP6G2-His. Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 mg, 50 μg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 was tracked using anti-His antibody. As positive control, 5 μg of purified His-MDH was loaded. OmpA*Bo*CYP6G2 is indicated with the black arrow (predicted MW 62.7 kDa)

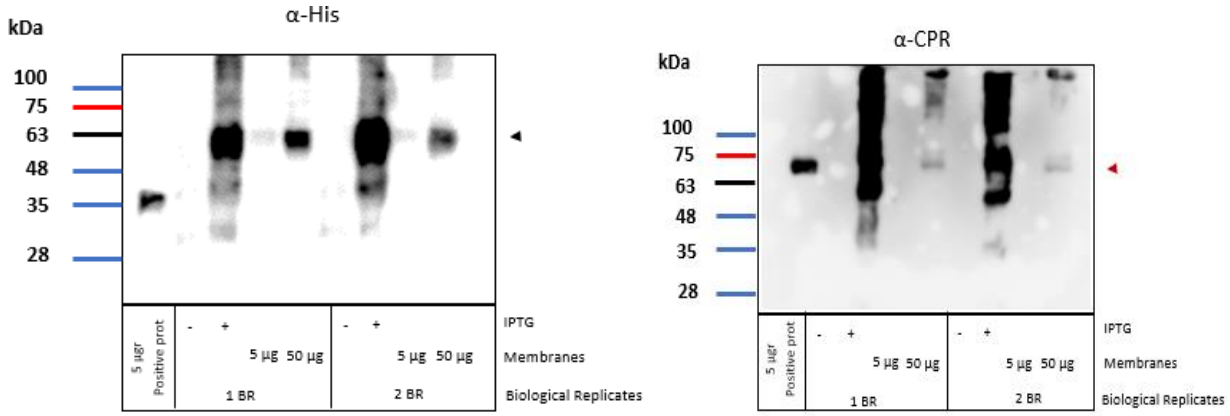


Figure 36. Western blot analysis of co-expression of OmpA*Bo*CYP6G2His/*Bo*CPR (two plasmids) and groES-groEL-tig chaperones. Two independent biological replicates were conducted for each experiment. Bacterial cells (-/+IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR was tracked via anti-CPR. As positive controls, 5 µg of purified His-MDH and 6 µg of purified CPR were loaded. OmpA*Bo*CYP6G2 is indicated with the black arrow (predicted MW 62.7 kDa) and *Bo*CPR with the red arrow (predicted MW 78.8 kDa).

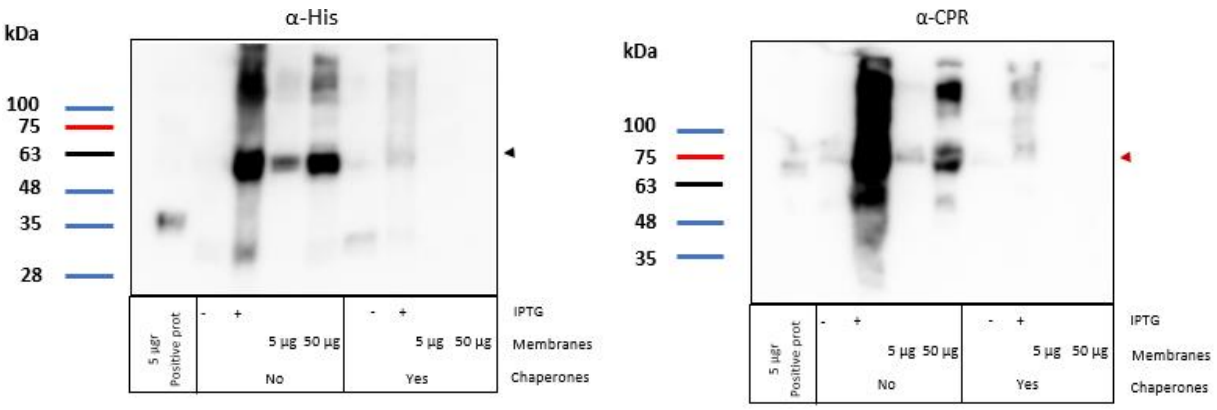


Figure 37. Western blot analysis of expression of OmpA*Bo*CYP6G2His/*Bo*CPR (two plasmids) with or without co-expression of groES-groEL-tig chaperones. One biological replicate was conducted for each experiment. Bacterial cells (-/+ IPTG samples) and membrane preparation (5 µg, 50 µg) were loaded on 10% acrylamide gel and upon separation, the polypeptides were analyzed by western blot. Expression of P450 bearing His-Flag was tracked using anti-His and expression of CPR was tracked via anti-CPR. As positive controls, 5 µg of purified His-MDH and 6 µg of purified CPR were loaded. OmpA*Bo*CYP6G2 is indicated with the black arrow (predicted MW 62.7 kDa) and *Bo*CPR with the red arrow (predicted MW 78.8 kDa).

3.6.2 Bradford assay

Total protein concentration of membrane preparations was calculated based on the BSA standard curve y=0.0454x R^2=0.989. Calculated total protein concentration is presented in the **Table 11** below.

Table 11. Bradford total protein concentration of membrane preparations.

Construct	Biological replicate	Total protein mg/mL
pCWOmpA_*Bo*CYP6G2-his + plasmid 4	1	49.7
	2	42.9
pCWOmpA_*Bo*CYP6G2-his)+pCDF_*Bo*CPR (two plasmids) **+plasmid 4**	1	18.9
	2	16.9
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid)	1	35.20
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid) **+plasmid 4**	1	4.00

3.6.3. Spectral assay

In order to check the integrity of P450s, P450 spectral assay was conducted. According to carbon monooxide (CO)-difference spectra in **Figure 38**, OmpA *Bo*CYP6G2-His showed a peak at 450 nm in the first biological replicate and a shifted (about 8 nm) peak in the second biological replicate. Also, in the first biological replicate a significant peak at 420 nm was noticed, indicating the presence of unfolded P450. In **Figure 39** spectral characteristics of OmpA*Bo*CYP6G2-His when co-expressed with *Bo*CPR from two different plasmids, are shown. Both biological replicates showed a peak around 450 nm (sifted about 2-4 nm) indicating the presence of P450 which possibly maintains its integrity. Concerning the spectral characteristics of *Bo*CYP6G2-His when co-expressed *with Bo*CPR in single plasmid experiment, P450 gave a peak at 452 nm revealing correct folding of the protein (**Figure 40**). Regarding the spectral characteristics of *Bo*CYP6G2-His when co-expressed with *Bo*CPR in single plasmid experiment, together with chaperones, a not well-formed peak at 450 nm was detected (**Figure 40**). The P450 content of membrane preparations was calculated according to the formula described in **Section 2.8** and is shown in the **Table 12.**

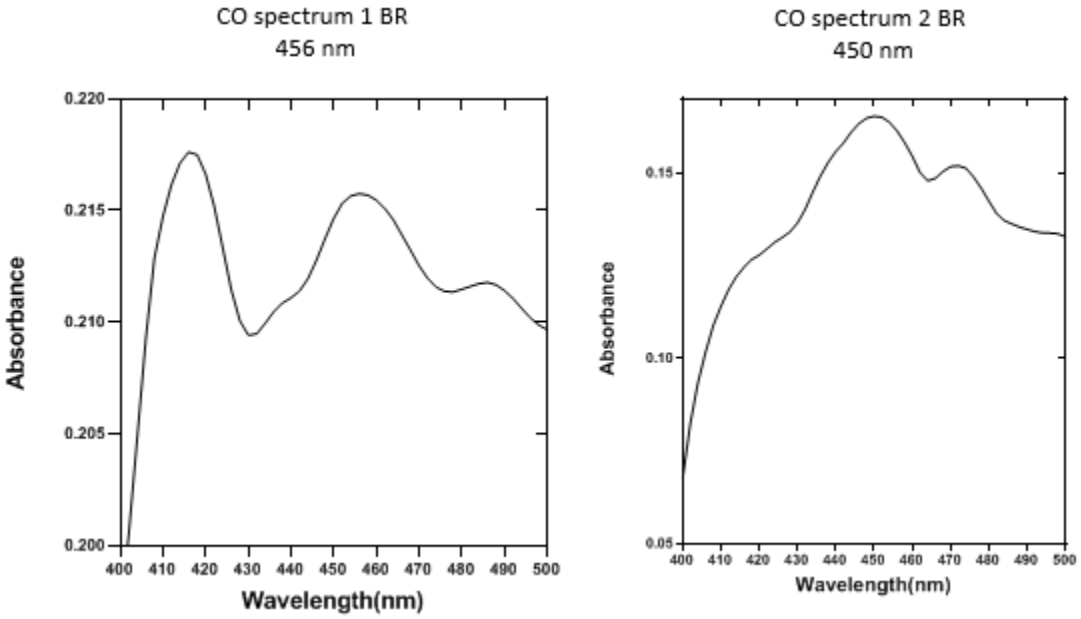


Figure 38. Carbon monooxide (CO)-difference spectra of OmpA-02013-His. The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

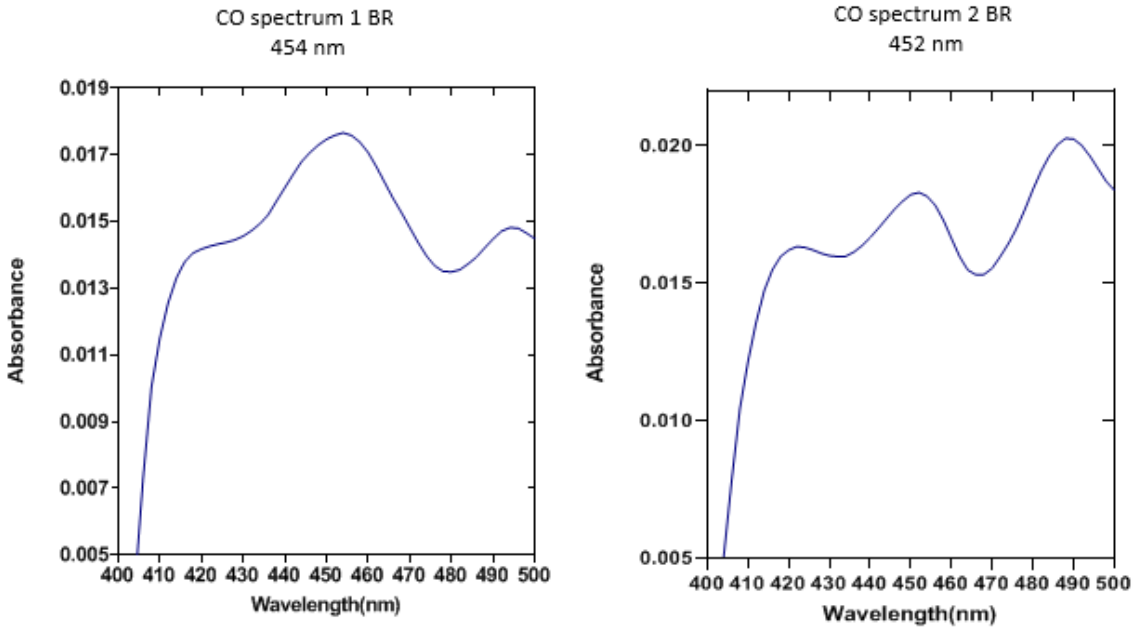


Figure 39. Carbon monooxide (CO)-difference spectra of OmpA-02013-His in co-expression with *Bo*CPR from two different plasmids. The construct was expressed in two independent biological replicates. 1BR: first biological replicate, 2BR: second biological replicate.

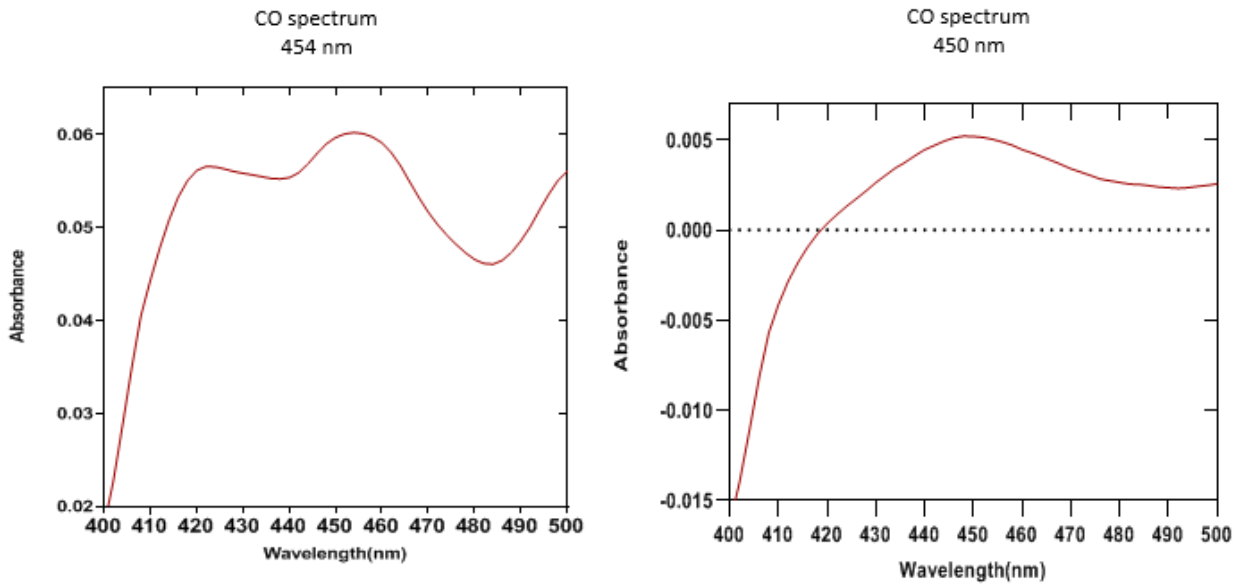


Figure 40. Carbon monooxide (CO)-difference spectra of 02013-His in co-expression with *Bo*CPR (from single plasmid experiment) in absence of groES-groEL-tig chaperones (left image) or in presence of groES-groEL-tig chaperones (right image)

Table 12. Spectral characteristics and P450 content of membrane preparations.

Construct	**Biological replicate**	**P450 nm**	**nmol P450/mg total protein**	**Total Yield nmol/L**
pCWOmpA_*Bo*CYP6G2-his + plasmid 4	1	456	0.19	36.26
	2	450	0.22	35.85
pCWOmpA_*Bo*CYP6G2-his)+ pCDF_*Bo*CPR (two plasmids) **+plasmid 4**	1	454	0.49	32.31
	2	452	0.40	23.85
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid)	1	454	0.44	76.92
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid) **+plasmid 4**	1	450	0.82	9.89

3.6.5. CPR activity assay

Membrane preparation containing *Bo*CPR, were tested for CPR activity against cytochrome c (electron acceptor) in presence of NADPH (electron donor). Catalytic activity of CPR (nmol reduced cytochrome c min-1 mg-1 total protein) was calculated according to the formula mentioned in **Section 2.9**. Measurements of CPR activity are shown in **Table 13**. Low activity of CPR against cytochrome c was noticed in all membrane preparation.

Table 13. Measurements of CPR activity against cytochrome c in presence of NADPH.

Construct	**Biological replicate**	**nmol of product min^{-1} mg^{-1}**
pCWOmpA_*Bo*CYP6G2-his + plasmid 4	1	nd
	2	nd
pCWOmpA_*Bo*CYP6G2-his)+pCDF*Bo*CPR (two plasmids) **+plasmid 4**	1	140,57
	2	87,34
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid)	1	106,93
pCDF_*Bo*CYP6G2-his_*Bo*CPR (single plasmid) **+plasmid 4**	1	nd

4. Discussion

Insect P450s play a crucial role in metabolism of insecticides. Heterologous expression of P450s and reconstitution of purified enzymes has been typically employed for the functional characterization of these enzymes in order to elucidate their involvement in insecticide resistance and selectivity. *E. coli* is commonly used and well-studied expression system that produces high yields of recombinant protein, but more specifically for expression of insect P450s includes some major challenges. The major experimental bottlenecks are that *E. coli* lacks the inner membrane structures in which P450s are anchored and does not perform post translational modification which may be important for the catalytic activity of the enzyme. In the present study, two distinct cases of P450s that have been associated with resistance to insecticides were heterologously expressed in *E. coli* with the aim of their functional characterization.

Concerning first chapter, this is the first time that CYP6B7 from *Helicoverpa armigera,* which has been reported to be associated with fenvalerate metabolism (Ranasinghe & Hobbs, 1998), is expressed successfully in *E. coli* expression system. *Ha*CYP6B7 has also been heterologously expressed together with *Ha*CPR (Zhao et al., 2018) in *Pichia pastoris* and found active against 7-ethoxycoumarin, *p*-nitroanisole, fenvalerate and chlorpyrifos. Furthermore, recently *Ha*CYP6B7 was co-expressed together with *Ha*CPR in SF9 insect cells (Shi et al., 2021) and found active against esfenvalerate. Other members of CYP6B subfamily, have been functionally characterized by heterologous expression and reported to metabolize host plant allelochemicals and insecticides (**Section 1.2**). In this study, in order to achieve functional expression of *Ha*CYP6B7, N-terminal modifications of *Ha*CYP6B7 and different co-expression strategies of the redox partner *Md*CPR were explored. *Ha*CYP6B7 was targeted either on bacterial membranes (in native form or together with OmpA leader sequence) or in the cytoplasm of bacteria (in truncated form). Regarding co-expression of the redox partner *Md*CPR, the two proteins were co-expressed either from the same vector or from two different vectors. Expression of the multiple constructs was tested in small scale cultures in five different *E. coli* strains and then proceeded to large scale with the selected strain.

Small scale expression tests in five *E. coli* strains showed that Lemo21 DE3 was the selected strain for the expression of *Ha*CYP6B7-Flag-His alone or with the reductase partner *Md*CPR and BL21 DE3 for the expression of *HaΔN*CYP6B7-Flag-His. Lemo21 DE3, an *E. coli* strain suitable for expression of membrane and toxic proteins, showed successful and stable expression of all constructs, indicating that is possibly a preferable choice for expression of P450s in general.

Regarding large scale membrane targeting experiments, *Ha*CYP6B7 and *Md*CPR expressed successfully as western blot analysis revealed, in all constructs. Spectral characteristics of P450s

when complexed with CO varied for different constructs. In general, all constructs showed a characteristic peak around 450 nm but some constructs also showed peak at 420 nm revealing existence of unfolded protein. The most satisfying spectral characteristics arose from *Ha*CYP6B7-Flag-His when co-expressed with *Md*CPR in a single plasmid experiment, where a predominant peak at 450 nm was noticed. *Md*CPR showed high activity against cytochrome c in all approaches. Highest activity was noticed from *Md*CPR when co-expressed with HaCYP6B7-Flag-His in single plasmid experiment. Also, *Md*CPR when co-expressed with OmpACYP6B7 in co-transformation experiment with two plasmids showed very high activity. All constructs when assayed against 7-ethoxy coumarin and *p*-nitroanisole, didn't show any catalytic activity. Further screening against model substrates and insecticides is needed in order to reach conclusions about catalytic activity. Priority must be given to those insecticides in which *Ha*CYP6B7 has been found active such as chlorpyrifos and fenvalerate (Zhao et al., 2018) and esfenvalerate (Shi et al). In the case that P450 is catalytically inactive, this may be attributed to unsuccessful coupling with the redox partner *Md*CPR. Coupling of *Ha*CYP6B7 with *Ha*CPR may lead to a more active complex. In conclusion, despite the lack of data about catalytic activity, based on yield of expression, spectral characteristics and CPR activity, *Ha*CYP6B7-Flag-His when expressed from a single plasmid together with *Md*CPR was the most successful and promising approach for the membrane targeting experiments of *Ha*CYP6B7 and will be proceeded to further screening with model substrates and insecticides.

Concerning expression of soluble *Ha*CYP6B7 (*HaΔN*CYP6B7), employment of Ni-NTA affinity chromatography and anion exchange chromatography lead to reconstitution of semi-purified P450 with low yield (1 mg/mL). CO assay showed a not well-formed peak at 446 nm, revealing a P450 with a doubtful quality. Because of the very low yield and semi-purification of the P450, the enzyme was not further characterized for its metabolic activity. A major problem during Ni-NTA purification was the weak binding of P450 in the resin. A possible explanation is that His-tag that is C-terminally fused, may be hidden and thus not accessible for binding with nickel ions of the resin. So, design of a new construct that will bear the His tag N-terminally and in some distance from the protein may be a solution for more effective binding.

Regarding second chapter, heterologous expression of BoCYP6G2 and BoCYP6A23 that have found to be related with resistance to α-cypermethrin in Bactrocera oleae is still in progress and multiple experiments have to be done in order to reach conclusions for their functional characterization. So far, cloning of the two P450s and BoCPR to expression vectors was completed successfully but only BoCYP6G2 proceeded in membrane targeting experiments in the present study. Small scale experiments indicated the need of co-expression of chaperones because moderate proteolytic cleavage was noticed. So, membrane targeting experiments of OmpABoCYP6G2-His/BoCPR (single plasmid) BoCYP6G2-His/BoCPR (two plasmids) were

conducted in presence of chaperones groES-groEL-tig. BoCYP6G2 was expressed successfully in all constructs as western blot analysis revealed and maintained its integrity as CO assay indicated. On the other hand, the redox partner BoCPR, showed very low expression and activity in all constructs. Because of the inactive redox partner (BoCPR) metabolic activity assays for BoCYP6G2-His were not conducted. MdCPR might be used as redox partner in future experiments in order to achieve an active complex that will be proceeded for further functional characterization.

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Supplementary

Nucleotide sequences

>*Ha*CYP6B7 Native codon optimized

ATGTGGGTTCTGTATCTGCCTGCAGTTCTGAGCGTTCTGATTGTTACCCTGTATCTGTATTTTACCCGCACCTTCAACTATTGGAAAAAACGTAA
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>*Ha*CYP6B7 Truncated codon optimized

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>*Md*CPR codon optimized

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>*Bo*CYP6G2 no codon optimized

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>*Bo*CPR codon optimized

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