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Executive Summary

ACTPAC consortium aims to develop a complete path for re/up-cycling of polyethylene plastic waste. The proposed process contains multiple chemical and biological/enzymatic steps ranging from degrading polyethylene into defined chain length alkanes to functionalizing them to repolymerize into biodegradable new plastics. One key step in this complete transformation is the enzymatic functionalization of short/medium chain alkanes into terminal diols and diacids.

CYP153 monooxygenase enzymes have been chosen as the ideal biocatalyst group for this task since they were shown to exclusively perform terminal oxidation of alkanes and fatty acids. In the first 6 months of the project, we have carried out an extensive literature search and *in silico* gene mining to identify CYP153 orthologs that are ideal for our purpose concerning following aspects; 1) α, ω-diol production from C6-C12 alkanes by successive hydroxylations, 2) further (over) oxidation activity to produce α, ω-diacids, 3) proper chain length selectivity, 4) avoiding product mixtures, 5) efficiency as whole cell/crude lysate catalysts (in *E. coli*) under process-like conditions (space-time yields, substrate conc.). Overall, we identified six CYP153 monooxygenase enzymes, designed their codon-optimized genes inside expression vectors with desired tags, and got them synthesized from gene synthesis companies according to our specifications. Two reductase partner proteins (putidaredoxin and putidaredoxin reductase), commonly used for CYP153s, were also obtained in a single plasmid for concomitant expression. After obtaining the synthesized plasmid vectors, we started expression optimization of the enzymes followed by activity screening. Moreover, we established a detailed analytical methodology for the identification and quantification of the substrates and possible products that can be obtained (diols, diacids, omega-hydroxy acids, aldehydes). Furthermore, various extraction methods (solvent extraction) have been tested and compared to determine the best method for product recovery after the biocatalytic reactions.

We now have detailed data on the expression conditions of the enzymes. Although the enzyme activity screenings have not yet revealed conclusive results, optimization of conditions is ongoing to get robust and consistent conversions. The results from our protein expression analysis, coupled with literature info on the six CYP153 monooxygenases enabled us to choose a subset of the genes to move forward in Task 3.2.

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Acronyms & Abbreviations

Term	Description
DX.X	Deliverable X.X
WP	Work Package
PE	Polyethylene
CYP	Cytochrome P450 Enzyme
TB	Terrific Broth
aa	Amino Acid
TON	Turnover Number
TTN	Total Turnover Number
STY	Space-time Yield
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectroscopy
OD	Optical Cell Density at 600 nm
Pdr	Putidaredoxin Reductase
Pdx	Putidaredoxin

1 Introduction

1.1 Overview

The main objective of WP3 is to optimize and engineer CYP153 monooxygenase enzymes as multi-enzyme/whole cell biocatalysts for efficient α,ω-dihydroxylation/oxidation of short to medium chain length alkanes (C6-C12 alkanes that will be supplied by catalytic metathesis from WP1 & WP2) into α, ω-diols/diacids as monomers for PE-like polyester (for polymerization in WP6-WP9). Cytochrome P450 enzyme sub-family of CYP153, originating from bacteria, has been shown to perform highly selective ω-hydroxylation of fatty acids as well as terminal oxidation of alkanes (almost 100 % terminal oxidation selectivity)[1-4]. This is remarkable given that almost all other CYP families, except the fungal CYP52 family, prefer oxidation of sub-terminal (in-chain) positions[3]. In the recent decade, many orthologs of the CYP153 family have been characterized and engineered with different efficiency, substrate selectivity, and oxidation patterns[1, 4-12]. Thus, the most suitable CYP153 orthologs for WP3 should be selected among the reported enzymes. Once suitable CYP153 orthologs are identified in Task 3.1, the enzymes will be engineered to obtain variants that are highly efficient and specific towards defined chain lengths, for sequential double hydroxylation (α and ω) and for further oxidation of diol functionality to diacid. Whole cell *E. coli* catalysts or cell-free extracts will be used for efficient and feasible large-scale bioconversions with optimized process conditions in the further tasks and WPs.

Within WP3, the main scope of this first deliverable was to identify and obtain (through gene synthesis) 6-8 different CYP153 monoxygenase orthologs (enzymes from different organisms) as well as common reductase partner proteins based on extensive literature analysis. Moreover, this deliverable aimed to carry out initial screening of enzyme activity to confirm and complete literature data and further reveal 1) α, ω-diol production from C6-C12 alkanes by successive hydroxylations, 2) further (over) oxidation activity to produce α, ω-diacids, 3) chain length selectivity, 4) avoiding product mixtures, 5) efficiency as whole cell/crude lysate catalysts (in *E. coli*) under process-like conditions (space-time yields, substrate conc.). Based on this initial evaluation, we aimed to choose 2-3 orthologs that have the best potential to either alone or in cascade produce α, ω-diols and diacids from C6-C12 alkanes efficiently (i.e. high expression levels, high TTN, high STY, high stability). The data in Task 3.1 will be guiding the selection of suitable orthologs for protein engineering efforts in Task 3.2.

1.2 Relation to other tasks and deliverables

Since this is the first deliverable, D3.1 receives no input from other tasks or deliverables. D3.1 provides output to the following other ACTPAC tasks and deliverables (Table 2):

Provides outputs to:

Table 1. D3.1 Output for other tasks and deliverables

Deliverable	Due Date	Output from D3.1
D3.2	M20	CYP153 monooxygenases for further engineering in Task 3.2 identified
D3.3	M32	Some of the protein expression and reaction condition data from D3.1 will be useful for D3.3

1.3 Structure of the deliverable

In this deliverable report, our results are described in section 2, divided into 4 sub-sections; ***1) selection of the genes; 2) expression optimization; 3) setting-up of analytical methods; 4) activity screening***; ***5) problems and troubleshooting***. Our conclusion from the results is summarized in section 3.

2 Results on characterization of CYP153 orthologs for their capacity of C6-C12 alkane to α, ω-diol and -diacid transformation

2.1 Selection of CYP153 genes and associated reductase partners

2.1.1 Selection of the CYP153 encoding genes for synthesis and plasmid design

As we indicated in our WP3 description, our main aim is to use CYP153 monooxygenases for terminal (α, ω) oxidation reactions. Thus, we searched the literature thoroughly to evaluate different CYP153 orthologs that have been characterized to date. During our evaluation, we aimed to choose the enzymes that showed 1) broad diversity in oxidation patterns that match our purpose (diterminal oxidation of C6-C12 alkanes), 2) exhibited good turnover numbers, 3) good amount of literature data, including engineering data, being available, 4) potential for novel reactivity patterns. Based on these criteria, we selected six CYP153 genes as outlined below (Table 2). We designed the genes in suitable pET expression vectors with N-terminal (6x)His-tag attached to enable purification (protease cleavage sites were included in case His-tag needs to be removed)[13]. The gene sequences were codon optimized for expression in *E. coli*. Once the plasmid designs for the different genes were finalized, we placed orders from gene synthesis companies (Genescript and Twist Bioscience) and the vectors containing our inserts were received as pure plasmids that are ready to transform and express in *E. coli* cells.

Table 2. CYP153 encoding genes selected for gene synthesis and further study

Gene Name	Expression Vector & cloning sites & tags	Important Characteristics	Literature References
CYP153A16 ***(Mycobacterium marinum)***	pET-28a (+) & *Nde*I and *Hind*III & N-terminal his-tag	463 aa; can perform efficient terminal diol formation from C5-C12 alkanes	Scheps et al., 2011[14]
CYP153A7 ***(Sphingopyxis macrogoltabida)***	pET-28a (+) & *Nde*I and *Hind*III & N-terminal his-tag	416 aa; broad substrate spectrum from C6-C10 alkanes, engineering examples in literature	Van Beilen et al., 2006[15], Funhoff et al., 2007[16], Dong et al., 2022[5]
CYP153A71 **(*Alloalcanivorax dieselolei*)**	pET-28a (+) & *Nde*I and XhoI & N-terminal His-tag	470 aa; selective towards medium-chain alkanes, can perform	Jacobs et al,. 2022[8]

		overoxidations to diols and acids	
CYP153A6 (*Mycobacterium sp. HXN-1500*)	pET-28a (+) & *Nde*I and XhoI & N-terminal His-tag	420 aa; effective towards C6-C11 alkanes, with TON over 1 s^{-1} towards octane, overoxidations to diols and acids shown	Funhoff et al., 2006[7], Jacobs et al,. 2022[8]
CYP153A33 *(Marinobacter aquaeolei, Also known as Marinobacter nauticus VT8*)	pET-28a (+) & *Nde*I and XhoI & N-terminal His-tag	470 aa; one of the most studied CYP153, extensive literature data, engineering studies, plasticity in substrate spectrum	Fiorentini et al,. 2018[17], Park et al., 2022[10], Park et al., 2020[2], Honda et al., 2012[1]
CYP-Nfa22290 (*Nocardia farcinica*)	pET-28a (+) & *Nde*I and XhoI & N-terminal His-tag	403 aa; newly discovered, efficient diol production for medium chains, potential for novel selectivity and oxidation patterns	Park et al,. 2020[2]

2.1.2 Selection of Reductase partners for gene synthesis

CYP enzymes require external electron donors to activate molecular oxygen, thus carrying out their respective reactions. Two electrons are required for each reaction cycle and these electrons need to be injected one by one to the active site at different stages of the reaction (electrons eventually end up in the water as the by-product)[3] (Figure 1). Thus, CYP enzymes require special reductase partner proteins for this timed electron supply. Indeed, CYP enzymes are divided into 5 groups depending on the nature and organization of their reductase partners that range from two separate components to one fused partner proteins[4]. CYP153s require a ferredoxin and ferredoxin reductase partner enzymes, which transfer electrons from NAD(P)H to heme center of CYP153. Ferredoxin is the enzyme that interacts with CYP153 and injects electrons, whereas ferredoxin reductase reduces ferredoxin using NAD(P)H.

According to our literature search putidaredoxin (CamB) and putidaredoxin reductase (CamA) (both from *Pseudomonas putida*) turned out to be the common and effective heterologous reductase partner proteins used for multiple CYP153 enzymes[2, 14, 18] (Figure 1). They have high amino acid sequence similarity (about 65%) with redox partners located in the vicinity of some CYP153A genes[14]. Moreover, CamA and CamB are well-characterized redox proteins that can be expressed in *E. coli*. Thus we designed the insertion of CamA and CamB encoding genes on a pETduet-1 expression vector (a common vector for simultaneous expression of two genes from one plasmid) and obtained the synthesized ready-to-express vector from a gene synthesis company (Table 3).

Table 3. Reductase partner genes selected for gene synthesis

Gene Name	Expression Vector & cloning sites & tags	General Charcateristics	Literature References
Putidaredoxin reductase (Pdr) + Putidaredoxin (PdX)	pETduet-1 & *NcoI and HindIII for Pdr and NdeI and XhoI for PdX* & no tag	Putidaredoxin – 107 aa Putidaredoxin red. – 422 aa	Scheps et al., 2011[14], Park et al., 2020[2]

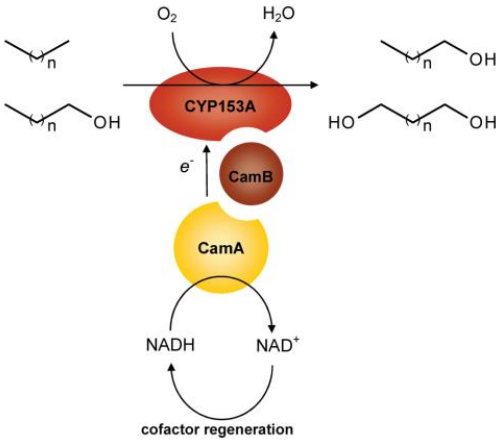

Figure 1. CYP153 monooxygenase reaction with reductase partners for alkane functionalization. Figure was taken from ref. [14]

2.2 Expression of CYP153 genes and reductase partners

2.2.1 Expression of CYP153 genes in *E. coli*

The genes obtained from gene synthesis companies were ready to express in the corresponding pET expression vectors (Table 2). Thus, we immediately started by transforming the plasmid vectors into *E. coli* BL21(DE3) cells, the most common host used for protein expression in bacteria, using chemical transformation protocols. All the plasmids were successfully transformed as indicated by cell growth as single round colonies on antibiotic selection agar plates (kanamycin for all CYP153 genes). Colonies from these plates were then grown in liquid cultures to start the expression procedure. Small volume overnight cultures were used to inoculate larger volume cultures for IPTG-induced expression (with TB-media). We also tested auto-induction media (ZYM & ZYP media) as well as various temperatures for the initial optimization of the expression. Below is a summary of the conditions tested for expression:

Table 4. Summary of the expression screening conditions of CYP153 encoding genes[1,2,3]

Media	IPTG [mM]	Temperature [°C]	Culture time [h]	Expression level	Soluble enzyme level
TB-media	0.25	25	18	High	Low
TB-media	0.25	18	18	High	Intermediate
ZY(M)-5052	0 (auto-ind.)	18	48	Intermediate	Intermediate
ZY(P)-5052	0 (auto-ind.)	18	48	Intermediate	Intermediate

[1] Temperature is given as either the post-induction temperature for the case of TB media, or the temperature during the entire expression for the case of ZYM + ZYP media (auto-induction media).

[2]All cultures were inoculated with starter cultures in LB, (1% of culture volume) and incubated in Non-baffled Erlenmeyer, media up to 20% of flask volume. Induced with IPTG at OD 1-2 and incubated at 200 RPM. Fe(II)SO4 and ALA was added 20 minutes prior to IPTG addition.

[3]Either single antibiotic: 100 µg/mL ampicillin or 50 µg/mL kanamycin, or double antibiotic: 80 µg/mL ampicillin or 40 µg/mL kanamycin.

2.2.2 Expression of reductase partner genes in *E.Coli*

The pETduet-1 plasmid vector containing both putidaredoxin and putidaredoxin reductase was obtained from gene synthesis company according to our design instructions. The plasmid vector was transformed into *E.c oli* BL21(DE3) cells and expression was carried out by IPTG induction.

SDS-PAGE analysis showed that both proteins are expressed in detectable amounts at expected molecular weights (Table 5, Figure 2).

Table 5. Best expression conditions for each CYP153 encoding gene.

Genes	Media	Conditions	Final OD	Expression	Soluble enzyme
CYP153A6	ZYM-5052	T = 20 °C, culture time = 44 h	11.6	High	High
CYP153A7	ZYM-5052	T = 20 °C, culture time = 40 h	7.8	High	Low
CYP153A16	ZYM-5052	T = 20 °C, culture time = 40 h	7	High	Intermediate
CYP153A33	ZYM-5052	T = 20 °C, culture time = 44 h	10.2	High	High
CYP153A71	ZYM-5052	T = 20 °C, culture time = 40 h	4.4	Intermediate	Low
Anfa22290	ZYM-5052	T = 20 °C, culture time = 40 h	3.2	Very low	N/A
Redox	TB-media	[IPTG] = 0.25 mM, T = 25 °C, culture time = 18 h	8.2	Intermediate	High

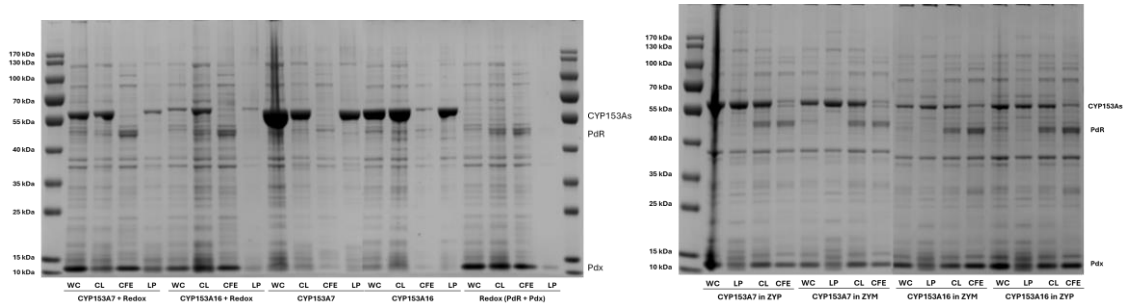

Figure 2. SDS-PAGE gels for the expression of CYP153A7 and CYP153A16 genes with and without redox partners. Left gel is the expression in TB-media and right gel is expression in ZYP and ZYM media. (WC, whole cell; CL, crude lysate; CFE, cell free extract; LP, pellet).

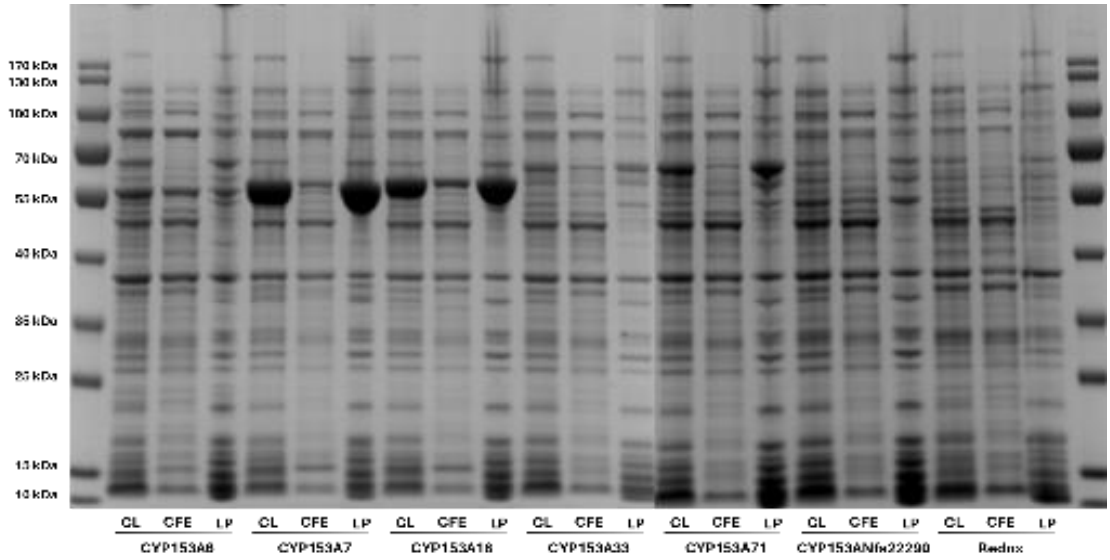

Figure 3. SDS-PAGE for the expression of CYP153A6, CYP153A7, CYP153A33, CYP153A71 and Nfa22290 genes. WC, whole cell; CL, crude lysate; CFE, cell free extract; LP, pellet.

2.3 Establishment of Analytical Methods for Indetification and Quantification of Substrates and Possible Products

Analysis of activity and product scope of enzymatic reactions is highly dependent on the establishment of proper, robust, and reliable analytical methods for the identification and quantification of the products. GC is one of the most convenient methods for the analysis of fatty acids, fatty alcohols, alkanes, and similar compounds. When coupled with suitable derivatization methods like trimethylsilylation or methylation, GC-FID enables precise quantification. Moreover, GC-MS can be used for identification in cases where suitable product standards are not available. Therefore, we decided to use GC-FID and GC-MS, which are both available in our labs, as the main analytical tools for the analysis of our reaction content. Below are the details of the analytical methods.

2.3.1 GC-FID Analysis of Substrate and Product Standards

Selected product standards were prepared and derivatized by using BSFA-TMCS to introduce trimethysilyl groups to the active hydrogens (hydroxy groups). The samples were analysed using a Bruker Scion 436 GC-FID hosting a SPB®-1 (30 m, 0.25 mm i.d., 0.25 µm film thickness), using the following conditions (The initial column temperature was 80 °C and held for 2 min, increased to 230 °C at 20 °C/min, and maintained for 3 min, finally increased to 280 °C at 40 °C/min and maintained for 7 minutes).

To evaluate GC-FID identification and quantification profile as well as optimizing extraction efficiency (see 2.3.2), nonane-derived products were chosen due to their wider standard availability. The standards used were: nonane, nonanol, nonanediol, nonanoic acid, 9-hydroxynonanoic acid, and nonanedioic acid (azelaic acid).

2.3.2 Evaluation of Various Extraction Methods for Product Recovery

5 different extraction methods were tested for better substrate and product recovery using standards. A comparison of extraction methods and their efficiencies is displayed in the GC-FID chromatogram below (Figure 4). Based on this evaluation, ethyl acetate extraction together with acidification was chosen as the most suitable extraction method for reaction extractions, since almost all the product standards can be obtained at high efficiency (see ratios of extracted product standards in Figure 5). Because our main product targets are diols and diacids, we believe that ethyl acetate extraction (with acidification), also given its simplicity, will be the most convenient method. However, we will keep in mind the better extraction efficiency of alcohols with Bligh Dyer, and apply it as needed during our analyses throughout the project.

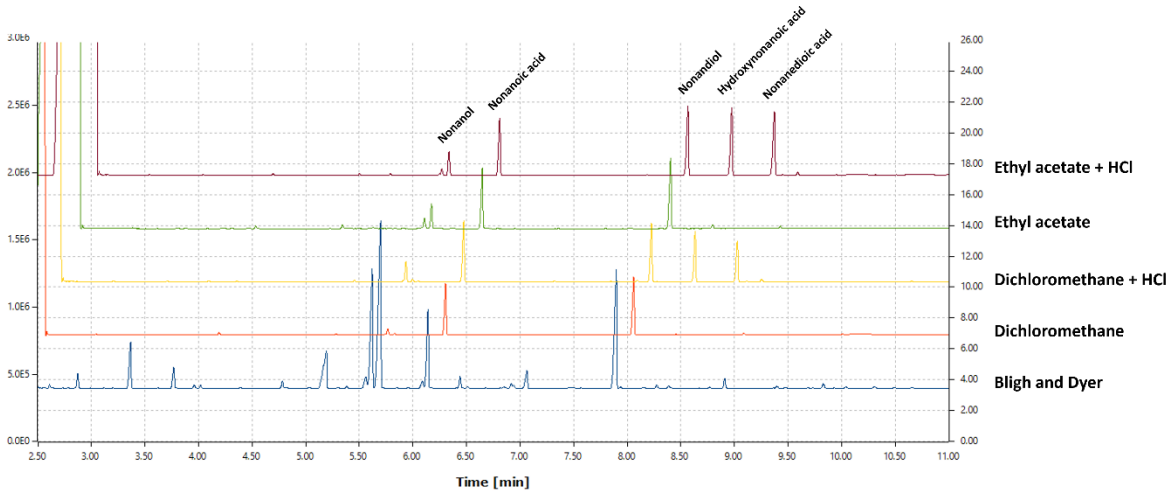

Figure 4. Comparison of extraction efficiencies of possible product standards (C9 derivatives) using GC-FID analysis after extraction.

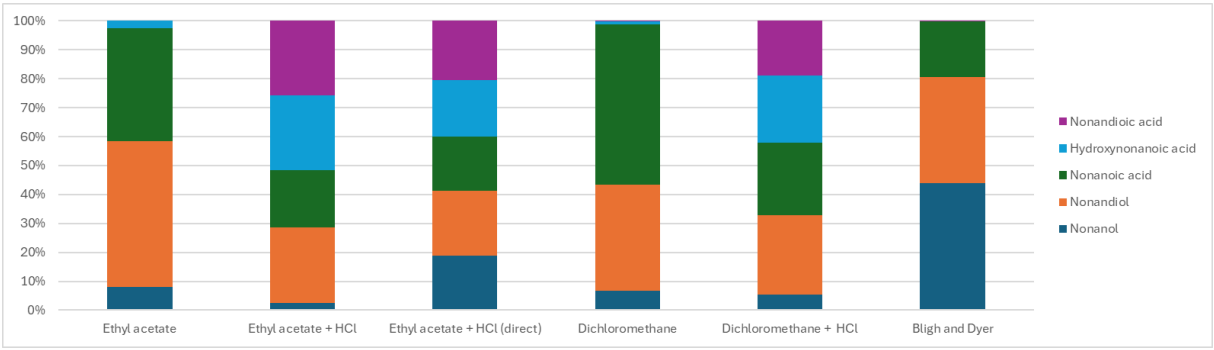

Figure 5. Comparison of extraction efficiencies of possible product standards (C9 derivatives). Reaction buffer containing 5 mM of standards were extracted with corresponding methods and derivatized by silylation before being analyzed by GC-FID. The ratios of extracted species are shown. An even extraction distribution can be seen with ethyl acetate + HCl.

2.3.3 GC-FID Analysis of Reaction Content

Once the most suitable extraction method has been determined as mentioned above, reactions were extracted accordingly and derivatized for trimethylsilylation (before extraction, internal standard is added to assess extraction efficiency as well as chromatography performance – internal standard was chosen as alkanoic acid with one more carbon, e.g. nonanoic acid for substrate octane). Afterwards, samples were loaded onto GC-FID for analysis. Internal standards and substrates were detected as expected according to elution pattern obtained with standards. Although some peaks were detected in the product region, no clear peak indicating the products yet been observed (see section 2.4).

2.3.4 GC-MS Analysis

As an alternative to GC-FID analysis, especially to identify unexpected peaks that might appear during our analyses in the project, we also tested GC-MS for our C9 product standards. All the peaks were able to be identified by their mass spectra (Figure 6). Conditions of GC-MS analysis using Thermo Scientific Trace GC Ultra with DSQ II MS detector were similar to previously described GC-FID. An identical SPB®-1 column was employed, and the method was altered slightly to better align with working GC-MS methods employed by the group (initial column temperature was 80 °C and held for 2 min, increased to 220 °C at 20 °C/min, and maintained for 38 min).

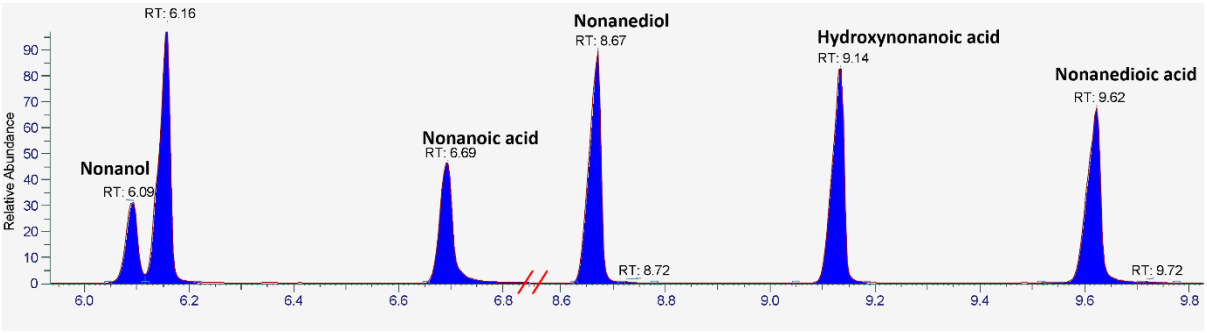

Figure 6. A representative GC-MS chromatogram showing the elution of C9 product standards. Same capillary column as GC-FID has been used.

2.4 Activity Assay Screening with CYP153s

After the successful expression of 5 out of 6 of the CYP153 monooxygenase genes and the reductase partner proteins, and establishing robust analytical methods, our aim was to screen activity of the enzymes towards C6-C12 alkanes as well as fatty acids within the same chain-length range. Below is the summary and results of various assays we have carried out for this purpose.

2.4.1 Crude lysate activity screening with reductase partners

In initial trials both the CYP153 genes and reductase partners were expressed in the same cell (both plasmids transformed into the same cell) and the resulting whole cells were lysed and used as catalysts. Crude lysate was chosen over whole-cell biotransformations to circumvent problems in relations to mass transfer limitations of alkanes across the cell membrane. (Several literature articles have indicated that using lysed cells or clear lysate results in significantly higher product yields when

compared to whole-cell biotransformation when no transporter gene is co-expressed[19]). In these trials, octane, octanol, and octanoic acid were used as substrates, as all have been proven in literature.

Table 6. Activity screening with CYP153A7 and CYP153A16 enzymes as crude lysates.

Enzyme	Preparation	Substrate	Conditions	Activity
(A7+Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH* = 0.1 U	No
(A16+Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH = 0.1 U	No
(A7+Redox)	Crude lysate	Octane	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A7+Redox)	Crude lysate	Octanol	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A7+Redox)	Crude lysate	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A16+Redox)	Crude lysate	Octane	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A16+Redox)	Crude lysate	Octanol	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A16+Redox)	Crude lysate	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No

**Glucose dehydrogenase was used for cofactor (NADH) regeneration.*

No product peaks were observed using either GC-FID or GC-MS from reactions containing double transformed cell crude lysate, towards octane, octanol, or octanoic acid (Table 6). Reaction procedures were derived from the literature showing effective hydroxylation using either CYP153A7 or CYP153A16. Due to the lack of activity, it was decided to explore various methods for achieving activity.

2.4.2 Whole-cell activity screening with reductase partners

To rule out enzyme deactivation during lysis as the cause of the lack of activity observed with crude lysate, double-transformed whole cells were used as biocatalysts (Table 7). We chose octanoic acid as the substrate because it can cross the cell membrane and has been proven effective in other studies. Furthermore, using whole cells removes the requirement of NADH addition and co-factor regeneration systems (to a certain extent), and will keep the CYPs and reductase partners in closer proximity compared to lysed cells[19].

Table 7. Activity screening with CYP153A7 and CYP153A16 whole cells.

Enzyme	Preparation	Substrate	Conditions	Activity
(A7+Redox)	Whole cells	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A16+Redox)	Whole cells	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No

With this method, however, still no product was observed.

2.4.3 Activity screening with reconstituted reductase partners

Due to the lack of activity using double expressing (CYP153 monooxygenase and reductase partners) biocatalyst, it was decided to attempt individual expression of CYPs and reductase partners, followed by reconstitution (Table 8). This approach allowed for precise control over the amount of reductase added to each reaction. Additionally, expressing CYPs individually could lead to higher soluble yields, as co-expression with other proteins often increases inclusion body formation, particularly for difficult-to-express genes like CYPs. Octanoic acid was used as the substrate, since it would be more soluble compared to octane and alkanoic acids as has been shown in literature to achieve higher conversions, compared to the alkane counterparts.

Table 8. Activity screening of all the six CYP153 enzymes in clear lysate form with separate addition of reductase partners.

Enzyme	Preparation	Substrate	Conditions	Activity
(A7)+(Redox)	Clear lysate	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A16)+(Redox)	Clear lysate	Octanoic acid	OD = 20, NADH = 1 mM, GluDH = 0.1 U	No
(A6)+(Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No
(A7)+(Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No
(A16)+(Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No
(A33)+(Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No
(A71)+(Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No
(Anfa22290)+ (Redox)	Clear lysate	Octanoic acid	OD = 50, NADH = 2 mM, GluDH = 0.1 U	No

Still, under these conditions, we have not observed any product formation from octanoic acid.

2.4.4 Investigation of NADH and regeneration system for troubleshooting lack of activity

The decoupling of the electron transfer system from product formation often results in the generation of harmful hydrogen peroxide and other unstable oxygen species due to the reaction of NADH or Pdx with oxygen. Furthermore, regeneration of NADH using glucose dehydrogenase will produce gluconic acid, eventually acidifying the reaction media. In the case this issue was preventing activity, different concentrations of NADH and the inclusion of a regeneration system were investigated (Table 9).

Table 9. Screening concentration of NADH and glucose dehydrogenase with CYP153A6 and CYP153A33.

Enzyme	Preparation	Substrate	Conditions	Activity
(A6)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 1 mM, GluDH = 0 U	No
(A6)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH = 0 U	No
(A6)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH = 0.1 U	No
(A33)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 1 mM, GluDH = 0 U	No
(A33)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH = 0 U	No
(A33)+(Redox)	Crude lysate	Octane	OD = 20, NADH = 0.5 mM, GluDH = 0.1 U	No

2.4.5 Activity screening with reconstituted commercially available ferredoxin reductase and ferredoxin from *Spinacia oleracea*

Despite employing various methods and conditions, no enzymatic activity was detected. This lack of activity led us to hypothesize that faulty reductase partners might be the underlying cause. To test this, we used commercially available ferredoxin and ferredoxin reductase from *Spinacia oleracea* with clear lysates of CYP153A6 and CYP153A33 (Table 10). This approach aimed to evaluate the impact of these alternative reductase partners on enzyme activity.

Table 10. Screening activity using commercially available ferredoxin and ferredoxin reductase from ***Spinacia oleracea***

Enzyme	Preparation	Substrate	Conditions	Activity
(A6)+(FdR+Fdx)	Clear lysate	Octane	OD = 20, NADH = 2 mM, GluDH = 0 U	No
(A33)+(FdR+Fdx)	Clear lysate	Octane	OD = 20, NADH = 2 mM, GluDH = 0 U	No

Even with the use of commercial reductase partners, no activity was observed. However, these are the very first initial results and we will continue to test the other CYP153 genes with this ferredoxin and ferredoxin reductase system.

2.5. Possible reasons for lack of activity with tested CYP153 enzymes and measures to solve the problems

2.5.1. Possible reasons leading to lack of activity

We followed almost the exact literature protocols (scientific articles published on CYP153 enzymes) for all the five CYP153 monooxygenases that we have tested for activity. Although it is not uncommon that some literature studies on enzymes might be challenging to repeat, especially with enzymes like CYP family (due to their redox requirements), we suspect below possible reasons for lack of activity:

1) **Problem with redox system:** Although we used the Pdr-Pdx (CamA-CamB) system similar to literature [2, 14] and get the gene synthesized in a common pET-duet1 vector, we think that there might be an issue with the gene that leads to low levels of (possibly) inactive expression of (at least) one of the enzymes. Since this is the common system that we used for all the five enzymes that we tested, this is likely to be the problem.
2) **Improper folding of the CYP153 enzymes:** Although we can see soluble expression on the SDS-PAGE gel for all the five CYP153 enzymes we tested, there might be lack of proper folding of the enzymes, which can also affect the proper incorporation of the heme cofactor.

2.5.2. Measures to be taken to solve the problems

For the two most likely causes mentioned above, we are performing/will perform the following troubleshooting workflow

1) <u>Measures for redox system:</u>

a) Testing Pdr-Pdx (CamA-CamB) system as separate genes in separate plasmids with histidine-tags, which we can purify and test separately at desired ratios. The genes were already received and we started experiments as of last week of June.
b) We are also testing now heterologous ferredoxin/ferredoxin reductase systems (commercially available systems under different conditions).
c) We started testing H_2O_2 as redox partner. It is well known that CYP enzymes can use H_2O_2 directly bypassing the O_2 and electron requirement[3]. This reaction, also known as "peroxide shunt", may not be very efficient, but may give us sufficient activity profile.
d) Moreover, we plan to test the native reductase partners for each enzyme, at least for some of the CYP153 enzymes.
e) We will test other reductase partners from other groups of CYP enzymes, like the single domain CPR as fused or as separate proteins.

2) <u>Improper folding:</u>

a) In order to evaluate heme incorporation and confirm amount of active (correctly folded with heme incorporated), we will soon start carbon monoxide (CO) binding assays with our CYP153 enzymes (this method is a common and well established method to evaluate

correct construction of the heme binding site for catalysis)[7]. Currently, we are working on the regulatory aspects of setting-up the system due to health hazard of CO.

b) If the CO binding analysis show improper folding, we will screen different expression conditions in order to obtain correctly folded enzyme that has maximum amount of heme protein incorporated. These will include different growth media, temperatures, shaking speed, IPTG conc. vs auto.induction, inclusion of chaperone proteins in the cell (with external plasmids), screening different *E.Coli* cell lines, concentrations of externally added iron and aminolevulinic acid.

We believe that taking these measures will give us active enzymes that we can go forward with for Task 3.2 in the shortest time.

3 Conclusions

In this starting task of our WP3 of the ACTPAC project, we first identified six genes encoding CYP153 monooxygenase enzymes and two reductase partner enzymes based on literature investigation. We received all the encoding genes designed inside suitable expression vectors and synthesized by gene synthesis companies (Genscript and Twist Bioscience). Afterward, we started detailed work on the expression analyses of the enzymes. Our results showed that 5 out of the 6 CYP153 genes expressed, 4 of them showing both good expression and a good amount of soluble protein (Table 5). We analyzed various expression conditions including; IPTG-based induction versus auto-induction and different expression temperatures to optimize protein expression. Overall, we now have detailed information and methodology on the expression of all the genes we have, including reductase partner enzymes.

Moreover, during this task, we established methods necessary for the identification and quantification of substrates and products. Convenient GC-FID and GC-MS methods have been confirmed by analyzing substrate and product standards. Moreover, various extraction methods have been tested, and the best extraction efficiency has been obtained with acidified ethyl acetate, which we will be using to extract substrates and products from our reaction mixtures.

We started activity assays using medium-chain alkanes/fatty acids to test all 5 enzymes that exhibited expression. So far we have not been able to observe any noticeable activity. Although we followed established protocols, it has not been yet possible to reproduce literature results. Our earlier experience as well as discussions with colleagues working with CYP enzymes suggest that establishing the activity with CYP enzymes can be initially tedious. *As mentioned in section 2.5, we strongly think that a problem with the reducing system might be preventing activity, possibly due to a problem with the CamA/CamB encoding genes in the pET-duet1 vector. Thus, we have now started testing new reductase systems (heterologous ferredoxin/ferredoxin reductase systems as well as H_2O_2) as well as ordered the Pdr-Pdx (CamA-CamB) system as genes in separate plasmids with His-tags, which we can purify and test separately at desired ratios. Moreover, we plan to test the native reductase partners. We will also soon start carbon monoxide binding assays with our CYP enzymes to evaluate the correct folding and successful incorporation of heme group inside the protein. If we observe lack of proper CO binding, we will start measures to increase correct folding and heme incorporation, including screening expression conditions, different cell lines and chaperone proteins, as described in section 2.5.2.*

Although we have not yet seen activity, our expression evaluation, together with literature observations has guided us to choose the genes to move forward with for Task 3.2. Among the 5 genes that were successfully expressed, the following four genes encoding for; CYP153A6, CYP153A7, CYP153A16, CYP153A33 demonstrated consistent and significant expression levels. Moreover, their oxidation profile according to the literature fits our aims for Task 3.2. We will continue with these four genes for the next stages. Once we establish the activity patterns, we will further narrow down our selection to 2 or 3 of them for extensive engineering efforts.

Overall, although we were not able to establish all the results we aimed for Task 3.2 yet, current results are sufficient to choose a subset of the CYP153 enzymes to move forward. We have now multiple CYP153 encoding genes and reductase systems in our hands and established all the analytical methods for the quantification of substrates and products. Once we overcome the problem of lack of activity, we expect to quickly transition to Task 3.2 without any delay, by M8 of the project.

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Appendix

Genes FASTA format

>CAH04396.1 cytochrome P450 alkane hydroxylase CYP153A6 [*Mycobacterium* sp. HXN-1500]

MTEMTVAASDATNAAYGMALEDIDVSNPVLFRDNTWHPYFKRLREEDPVHYCKSSMFGPYWSVTKYRDIM
AVETNPKVFSSEAKSGGITIMDDNAAASLPMFIAMDPPKHDVQRKTVSPIVAPENLATMESVIRQRTADL
LDGLPINEEFDWVHRVSIELTTKMLATLFDFPWDDRAKLTRWSDVTTALPGGGIIDSEEQRMAELMECAT
YFTELWNQRVNAEPKNDLISMMAHSESTRHMAPEEYLGNIVLLIVGGNDTTRNSMTGGVLALNEFPDEYR
KLSANPALISSMVSEIIRWQTPLSHMRRTALEDIEFGGKHIRQGDKVVMWYVSGNRDPEAIDNPDTFIID
RAKPRQHLSFGFGIHRCVGNRLAELQLNILWEEILKRWPDPLQIQVLQEPTRVLSPFVKGYESLPVRINA

>CAH61448.1 cytochrome P450 alkane hydroxylase 1 CYP153A7 [*Sphingopyxis macrogoltabida*]

MEHTGQSAAATMPLDSIDVSIPELFYNDSVGEYFKRLRKDDPVHYCADSAFGPYWSITKYNDIMHVDTNH
DIFSSDAGYGGIIIDDGIQKGGDGGLDLPNFIAMDRPRHDEQRKAVSPIVAPANLAALEGTIRERVSKTL
DGLPVGEEFDWVDRVSIEITTQMLATLFDFPFEERRKLTRWSDVTTAAPGGGVVESWDQRKTELLECAAY
FQVLWNERVNKDPGNDLISMLAHSPATRNMTPEEYLGNVLLLIVGGNDTTRNSMTGGVLALHKNPDQFAK
LKANPALVETMVPEIIRWQTPLAHMRRTAIADSELGGKTIRKGDKVVMWYYSGNRDDEVIDRPEEFIIDR
PRPRQHLSFGFGIHRCVGNRLAEMQLRILWEEILTRFSRIEVMAEPERVRSNFVRGYAKMMVRVHA

>ACC41588.1 cytochrome P450 153A16 Cyp153A16 [*Mycobacterium marinum* M]

MSNIREAVTAKAQATIPMDRIIQGAHLYDRTRRWVTGTNGEKIFIERPIPPADEVELTDIDLSNPFLYRQ
GRWKSYYERLRNEAPVHYQAHSAFGPFWSVTRHADIVAVDKNHEVFSSEPFIVIGSPPRFLDIAMFIAMD
PPKHDRQRQAVQGVVAPKNLREMEGLIRERVVDVLDALPLGEPFNWVQHVSIELTARMLATLLDFPFEQR
RKLVQWSDLATSMEQANGGPSDNDEIFRGMVDMARGLSAHWRDKAARTAAGELPGFDLITMLQSDESTKD
LIDRPMEFLGNLVLLIVGGNDTTRNSMSGGVLALNEFPDQFEKLKANPELIPNMVSEIIRWQTPLAHMRR
IAKADTVLNGQFIRKGDKVLMWYASGNRDERVFDRPDDLIIDRANARNHISFGFGVHRCMGNRLAEMQLR
ILWEELLPRFENIEVVGEPEYVQSNFVRGISKLMVRLTPKGGA

>ABM17701.1 cytochrome P450 CYP153A33 [*Marinobacter nauticus* VT8]

MPTLPRTFDDIQSRLINATSRVVPMQRQIQGLKFLMSAKRKTFGPRRPMPEFVETPIPDVNTLALEDIDV
SNPFLYRQGQWRAYFKRLRDEAPVHYQKNSPFGPFWSVTRFEDILFVDKSHDLFSAEPQIILGDPPEGLS
VEMFIAMDPPKHDVQRSSVQGVVAPKNLKEMEGLIRSRTGDVLDSLPTDKPFNWVPAVSKELTGRMLATL

LDFPYEERHKLVEWSDRMAGAASATGGEFADENAMFDDAADMARSFSRLWRDKEARRAAGEEPGFDLISL

LQSNKETKDLINRPMEFIGNLTLLIVGGNDTTRNSMSGGLVAMNEFPREFEKLKAKPELIPNMVSEIIRW

QTPLAYMRRIAKQDVELGGQTIKKGDRVVMWYASGNRDERKFDNPDQFIIDRKDARNHMSFGYGVHRCMG

NRLAELQLRILWEEILKRFDNIEVVEEPERVQSNFVRGYSRLMVKLTPNS

>ACQ99381.2 cytochrome P450 alkane hydroxylase CYP153A71 [*Alloalcanivorax dieselolei*]

MSTKSGTNDAMQTKMINATSRLVPMHLQIKALKSLMKAKKKALGSTRPQVKFLERPVPDVNTLALEDIDT

SNPFLYRQDQWGAYFKRLRDEAPVHFQKSSQFGPFWSVTRYEDILFVDKNHELFSSEPQIILGDPPEGLS

VEMFIAMDPPKHDVQRRAVQGVVAPQNLKEMEGLIRQRAAEVLDSLPLDKAFNWVPAVSKELTGRMLATL

LDFPYEQRHKLVDWSDRLSGASSATGGEFTDEDIMFDDAADMAWSFSRLWRDKEARRKAGEPPGFDLISM

LQSNKDTRDLINRPMEFIGNLALLIVGGNDTTRNSMSGGVLALNQFPEEFIKLKKNPELIPNMVSEIIRW

QTPLAHMRRVATQDVELRGQTIKKGDRVLMWYASGNRDERKFENPDQLIIDRKDARNHISFGYGIHRCMG

NRLAELQLRILWEELLKRFDNIEVVGEPERVQSNFVRGYSKLMVKLTAKN

>CRY76464.1 Biotin biosynthesis cytochrome P450 [*Nocardia farcinica*]

MSAATSWIEDITMEELERNPYPFYERLRREAPLAFIPILGTYAATTKELCRTIANSPDFEAIITPAGGRT

FGHPAVIGVNGEIHEDLRSMVDPALQPSEVDRWVDGLVRPIARRYLAEFENDGHADLVAQFCEPVSVRAL

GDLLGLRDVGSDKLREWFHKLSNSFTNAAMNEDGTFANPAGFDQGDEAKAEIRAIVDPLIDHWIAHPDDS

AISHWLHDGMPEGQVRDRDYIYPTLYVFLLGAMQEPGHAMASTVAGLFTRPDQLERVIDDPALIPRAVAE

SLRWTSPIWSATARTNTVDVTIDGVFLPKGSVVMMAYGSANHDENDYNAPSAYDMDRPPLPHLAFGAGDH

ACAGTYFANKVCQIGLEELFEAIPNIERDDRKPIDFWGWGFRGPTELHVTWEV

>sp|P16640|CAMA_PSEPU Putidaredoxin reductase CamA OS=*Pseudomonas putida* OX=303 GN=camA PE=1 SV=1

MNANDNVVIVGTGLAGVEVAFGLRASGWEGNIRLVGDATVIPHHLPPLSKAYLAGKATAE

SLYLRTPDAYAAQNIQLLGGTQVTAINRDRQQVILSDGRALDYDRLVLATGGRPRPLPVA

SGAVGKANNFRYLRTLEDAECIRRQLIADNRLVVIGGGYIGLEVAATAIKANMHVTLLDT

AARVLERVTAPPVSAFYEHLHREAGVDIRTGTQVCGFEMSTDQQKVTAVLCEDGTRLPAD

LVIAGIGLIPNCELASAAGLQVDNGIVINEHMQTSDPLIMAVGDCARFHSQLYDRWVRIE

SVPNALEQARKIAAILCGKVPRDEAAPWFWSDQYEIGLKMVGLSEGYDRIIVRGSLAQPD

FSVFYLQGDRVLAVDTVNRPVEFNQSKQIITDRLPVEPNLLGDESVPLKEIIAAAKAELS

SA

>sp|P00259|PUTX_PSEPU Putidaredoxin OS=*Pseudomonas putida* OX=303 GN=camB PE=1 SV=3

MSKVVYVSHDGTRRELDVADGVSLMQAAVSNGIYDIVGDCGGSASCATCHVYVNEAFTDK

VPAANEREIGMLECVTAELKPNSRLCCQIIMTPELDGIVVDVPDRQW