HETEROLOGOUS EXPRESSION OF CYTOCHROME P450 MONOOXYGENASES FROM *Aspergillus terreus* AND *Cryptococcus neoformans*

by

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Dedicated to my parents for faith during the humble beginnings and my brother for immeasurable sacrifices in the course of my quest for dreams.

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CHAPTER 1

Literature review: Fungal cytochrome P450 monooxygenases and their heterologous expression

1.1. Introduction

Fungal enzymes enable carbon and nitrogen utilization from unconventional environmental sources for adaptation and survival (Van den Brink *et al.*, 1998). One family of enzyme which has been identified to play prominent roles in diverse fungal metabolic processes are the cytochrome P450s (CYP450) (Van den Brink *et al.*, 1998). The discovery of a carbon monoxide (CO) binding pigment with a characteristic A_{450} peak in rat liver microsomes unlocked an enzyme family known as CYP450. Interest in this family of enzymes grew with the establishment of the role of CYP450s in C-21 hydroxylation of 17-hydroxyprogesterone, which confirmed that CYP450 enzymes are involved in hydroxylation reactions (Omura, 1999). Currently, over 18,500 CYP450s are known (Nelson, 2013), while over 2,700 CYP450s are reported to be present within the fungal kingdom (Hlavica, 2013). These enzymes are ubiquitous to the different kingdoms of life, although they are lacking in organisms such as the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (Guengerich & Isin, 2008).

The main reaction catalysed by CYP450s is the addition of a single oxygen atom to a non-activated carbon atom (Omura, 2010). However, they play a functional role in other reactions such as N-, O- and S-dealkylation, sulphoxidation, epoxidation, deamination, desulphuration, dehalogenation, peroxidation and N-oxide reduction. They mainly catalyse conversion of organic hydrophobic compounds which includes fatty acids, steroids, prostaglandins, organic solvents, pesticides and drugs (Bernhardt, 2006). Chemical oxidation of these substrates generally requires high temperatures and produces unspecific products, while CYP450 enzymes catalyse site specific oxidation of the substrates at physiological temperatures (Werck-Reichhart & Feyereisen, 2000).

The increase in number of named fungal CYP450s was facilitated by several genome sequencing projects conducted in the last few years. The diversity of CYP450s present within fungi implies that their catalytic abilities can be exploited for several biotechnological applications. Functional libraries of a few fungal CYPomes have been constructed, but there is still a very large number of uncharacterized fungal CYP450s (Ide *et al.*, 2012). In this review, the heterologous expressions, catalytic reactions and characterization of fungal CYP450s are discussed.

1.2. Cytochrome P450 monooxygenase

Cytochrome P450 monooxygenases comprise a large family of heme-thiolate enzymes present in all biological kingdoms (Ichinose, 2009). The name cytochrome P450 monooxygenases does not refer to the function of these enzymes as seen with other enzymes, but to an uncommon spectral characteristic. These enzymes display a maximum absorption peak at 450 nm from a reduced CO bound complex formed when the spectrum of the reduced enzyme is subtracted from reduced-CO complex. The absorption peak at 450 nm is used in estimating the concentration of properly folded catalytically active CYP450. This unusual feature is due to the cysteine thiolate group forming the fifth ligand of the heme iron and CO the sixth (Bernhardt, 2006; Hannemann *et al.*, 2007).

The variations observed within the different CYP450s indicate that they evolved from a common ancestral gene and are then divided as the different organisms evolved (Ichinose & Wariishi, 2012). The identification of a new CYP450 requires the presence of conserved domains involved in heme binding and proton transfer (Van Bogaert *et al.*, 2011). Additionally, the primary structure of the CYP450 enzyme must also differ by more than 3% from a similar CYP450 (Anzenbacher & Anzenbacherova, 2003). Amino acid identities between these enzymes are used in classification into different families. Enzymes which share an amino acid identity greater or equal to 40% are classified within the same family using an Arabic numeral e.g. CYP21. Similarly, subfamily classifications require a degree of sequence identity of 55% or more and are identified with an alphabetic letter e.g. CYP3A4, CYP3A7.

An active eukaryotic CYP450 monooxygenase system usually comprises of a CYP450 enzyme and an NAD(P)H-CYP450 reductase (Fig. 1.1) with FAD and FMN cofactors shuttling electrons from NAD(P)H (Van Bogaert *et al.*, 2011). However, there has been discoveries of self-sufficient CYP450s having the reductase component fused to the CYP450. Cytochrome P450s are found both in the endoplasmic reticulum (microsomes) and mitochondria of animal cells while they are present only in the endoplasmic reticulum in fungal and plant cells. Microsomal CYP450s and their reductase components are membrane bound while mitochondrial CYP450s are also membrane bound but their reductases are soluble (Omura, 2010).

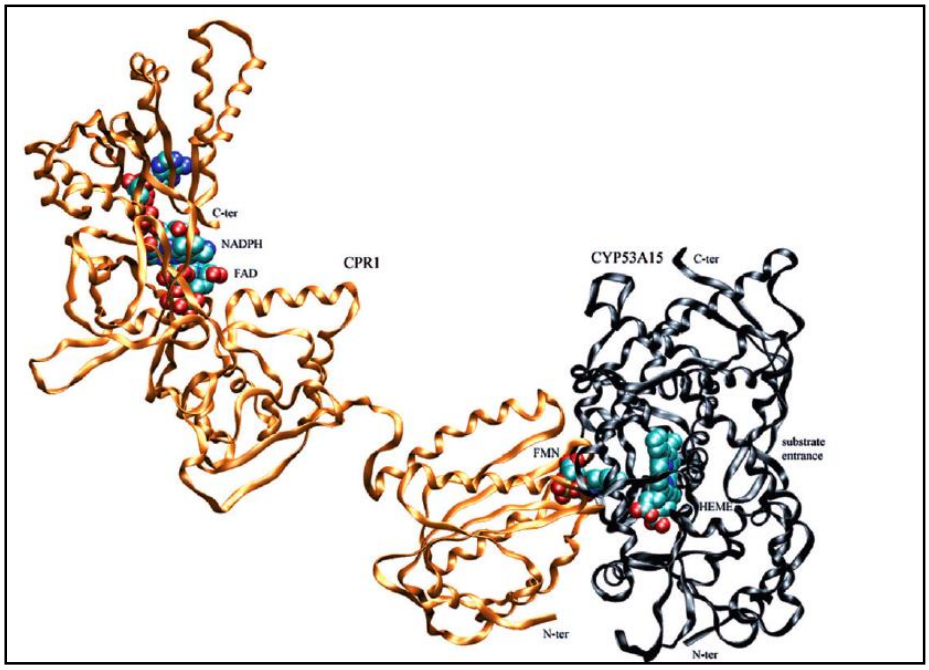


Figure 1.1. Model of a cytochrome P450 enzyme (CYP53A15; black) with heme interacting with its reductase, showing the FAD and FMN binding domains within the cytochrome P450 reductase (CPR; yellow) (Lah *et al.*, 2011).

1.3. Cytochrome P450 reaction mechanism

Oxidation of organic molecules with molecular oxygen at low temperatures does not happen spontaneously because of high energy barriers or a spin-forbidden state. Hence, living organisms require metal dependent oxygenases such as CYP450s capable of performing such reactions. The CYP450 enzyme catalyse the insertion of an oxygen atom into a substrate with the simultaneous reduction of the other oxygen atom to water as shown in Fig. 1.2 (Meunier *et al.*, 2004).

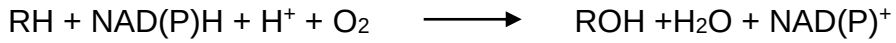


Figure 1.2. Scheme for a hydroxylation reaction most commonly catalysed by cytochrome P450s.

This reaction, involves a series of steps summarized in Fig. 1.3 before the final products are obtained. The initial stage of the reaction is a ferric state enzyme with a resting low-spin state and substrate binding around the distal region of the heme. Substrate binding at a resting state of low-spin may result in a change to a high-spin state. The binding often disturbs water, coordinated as the sixth ligand of the heme iron, to change the spin to a high-spin ferric substrate bound complex. However, some CYP450s are in a low-spin state when they are substrate free, while some have a high spin state (Guengerich, 2001; Denisov *et al.*, 2005). Generally, this step is considered to be fast in CYP450s with small binding sites such as CYP101A1 and CYP2A6 while those with large active sites are quite slow (Guengerich & Isin, 2008).

In eukaryotes, the electrons needed for reduction of ferric CYP450 is most often supplied by NADPH *via* NADPH-CYP450 reductase containing an FAD and FMN (Guengerich, 2001; Guengerich & Isin, 2008). The ferrous CYP450 produced from ferric reduction binds an oxygen molecule to form an unstable complex. This complex was first observed in CYP101A1 and can be broken down into a ferric iron and a superoxide anion. However, studying these complexes with mammalian proteins has been challenging due to slow interactions between the CYP450 and NADPH-CYP450 reductase (Guengerich & Isin, 2008).

Characterization of intermediates beyond this step has encountered lots of challenges. However, suggestions of possible steps indicate that a second electron is added *via* a NADPH-CYP450 reductase or a cytochrome b_5 to form a peroxo-ferric intermediate (Guengerich, 2001). This intermediate is protonated to produce a hydroperoxo-ferric intermediate. A second protonation occurs at the distal oxygen atom which results in the heterolysis of the O-O bond, release of water, formation of compound I and finally oxygenation of the substrate (Denisov *et al.*, 2005).

Alternatively, substrate monooxygenation occurs *via* the shunt pathway in some CYP450 cycles. This allows for direct substrate hydroxylation in the presence of peroxides such as hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide. This system serves as a by-pass to the necessary dependence on an

NADPH regeneration system and also allows cell-free dependent catalysis by CYP450s (Bernhardt, 2006).

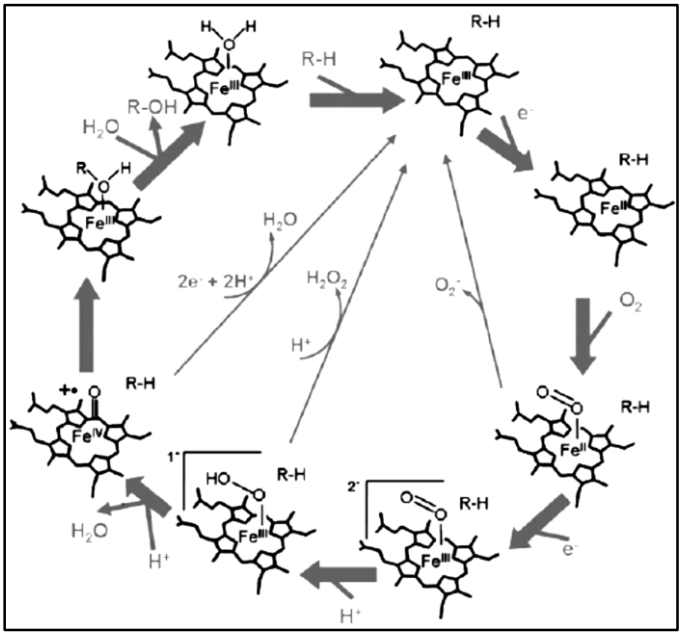


Figure 1.3. The cytochrome P450 monooxygenation catalytic cycle (Munro *et al.*, 2007).

1.4. Cytochrome P450 classifications by electron transfer

Cytochrome P450s were initially classified into two broad groups of either the bacterial/mitochondrial or microsomal types based on their electron transfer mechanism. The microsomal class which are membrane bound require a NADPH-CYP450 reductase for electron transfer. The bacterial/mitochondrial class of CYP450s are soluble and the active system comprises of three components which include a NADH(P)-CYP450 reductase containing an FAD, an iron-sulphur protein and the CYP450 (Degtyarenko, 1995; Bernhardt, 2006). Isolation of a self-sufficient CYP450, CYP102A1 (P450BM3), from *Bacillus megaterium*, which displayed an unusual electron transfer mechanism, lead to the identification of other unique electron transfer systems. Based on the mode of electron transfer, ten classes of CYP450s were identified. However, only three classes are found in fungi (Hannemann *et al.*, 2007).

1.4.1. Class II

CYP450s belonging to class II comprises a two component system and is mostly present within the endoplasmic reticulum of eukaryotes. These components include a CYP450 and an NADPH-CYP450 reductase containing FAD and FMN. The prosthetic groups are involved in the transfer of two electrons from NADPH to the CYP450 as shown in Fig. 1.4. The homology between the N-terminal region of the reductase and the FMN-containing bacterial flavodoxins demonstrates that the reductase evolved from a fusion of two ancestral proteins. More evidence can also be observed from the homology of the C-terminal region with FAD-containing ferredoxin $NADP^+$ reductases and NADH cytochrome *b5* reductase.

Besides the above mentioned components of the class II CYP450s, some schemes also consider cytochrome *b5* for transfer of electrons. The 17 kDa heme protein is associated with eukaryotic microsomal and mitochondrial cell fractions. The protein independently transfers both electrons or only the second electron from NADPH-CYP450 reductase or NADH-cytochrome *b5* reductase to the CYP450 (Fig. 1.5). In addition, it also supports allosteric stimulation of CYP450 catalysis without electron transfer.

Class II CYP450s catalyse diverse reactions which include the oxidative metabolism of fatty acids, steroids, prostaglandins, drugs and carcinogens in mammals. In plants they are involved in the synthesis of cutin, lignin and defence substances while in fungi they synthesise sterols, mycotoxins and detoxify phytoalexins (Hannemann *et al.*, 2007; Crešnar & Petrič, 2011).

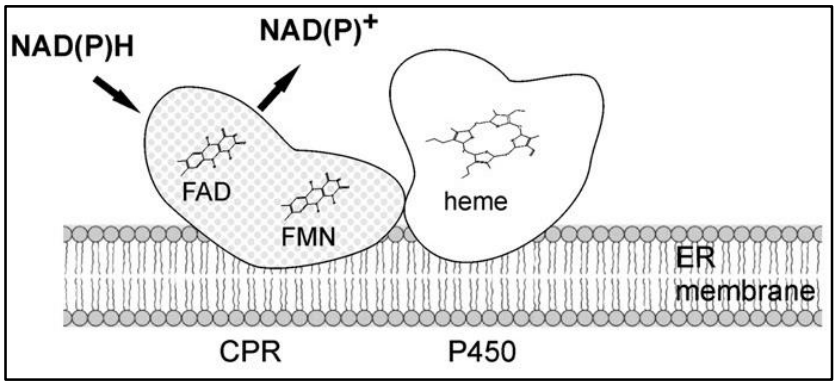


Figure 1.4. Class II electron transfer proteins with the cytochrome P450 and the flavoprotein containing reductase bound to the membranes of the endoplasmic reticulum (Crešnar & Petrič, 2011).

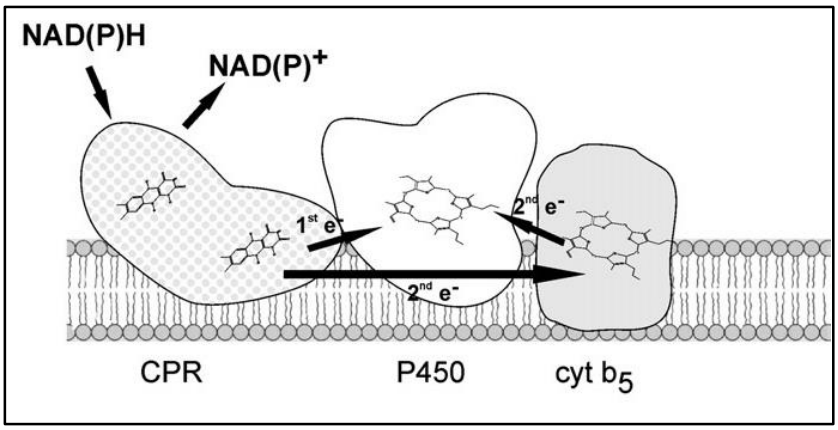


Figure 1.5. Alternate scheme of Class II electron transfer involving a cytochrome *b5* (Crešnar & Petrič, 2011).

1.4.2. Class VIII

CYP450s belonging to class VIII are fused to their diflavin reductase. Hence, they are catalytically self-sufficient as shown in Fig. 1.6. This class has its CYP450 hydroxylase N-terminal domain connected to the FAD/FMN containing CYP450 reductase C-terminal domain *via* a 20 to 30 amino acid linker region (Crešnar & Petrič, 2011). They have been identified in several prokaryotes and lower eukaryotes and include the extensively studied *Bacillus megaterium* CYP102A1 ($P450_{BM3}$) as well as CYP505A1 from *Fusarium oxysporum* ($P450_{foxy}$). They are known for their characteristic ω-1 to ω-3 sub-terminal hydroxylation of saturated and unsaturated fatty acids (Hannemann *et al.*, 2007; Crešnar & Petrič, 2011).

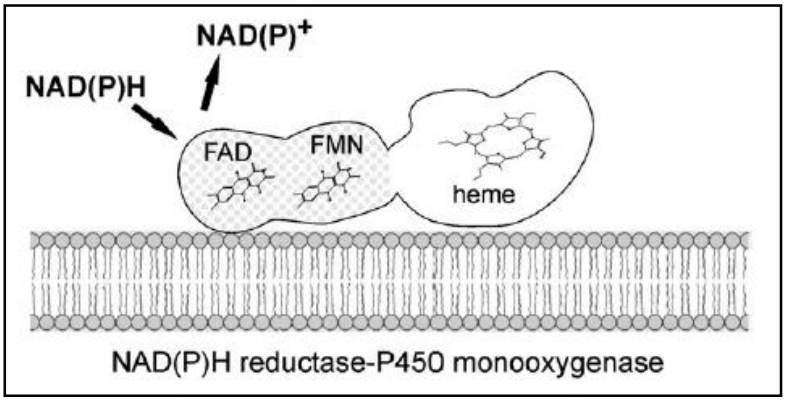


Figure 1.6. Class VIII electron transfer fusion systems of the cytochrome P450 and the reductase (Crešnar & Petrič, 2011).

1.4.3. Class IX

Members of class IX differ from other monooxygenases and are known as P450nor. The P450nor belongs to the CYP55A subfamily and are reported to be present only within eukaryotes (Zhang *et al.*, 2002). The P450nor is the only soluble eukaryotic CYP450 localized within the mitochondrial and cytosolic faction as shown in Fig. 1.7. They catalyse the reduction of two molecules of NO to gaseous N_2O in fungal denitrification. They obtain electrons directly from either NADH or NADPH without an additional redox partner. Their physiological role protects the fungal mitochondria from inhibition by NO during dioxygen limitation. These CYP450s have been isolated from several fungi including *F. oxysporum, Cylindrocapon tonkinense* and *Aspergillus oryzae* (Hannemann *et al.*, 2007; Crešnar & Petrič, 2011).

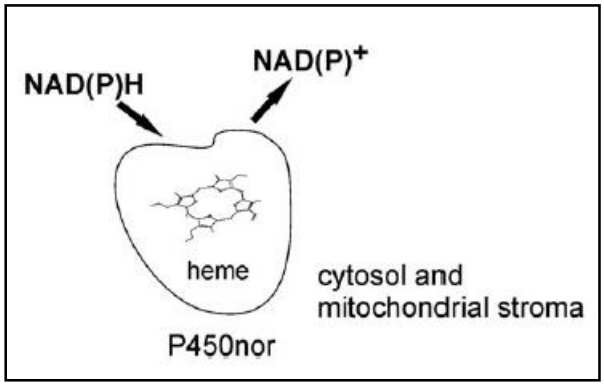


Figure 1.7. Class IX electron transfer soluble system (Crešnar & Petrič, 2011).

1.5 Fungal cytochrome P450s

Filamentous fungi and to a lesser extent yeast possess a flexible metabolism supported by numerous enzymes which enable their survival in various ecological niches (Van den Brink *et al.*, 1998). They produce various metabolites using specific pathways often catalysed by CYP450s during nutrient recycling and environmental detoxification (Kelly *et al.*, 2009). Some of the metabolic pathways involving fungal CYP450s are discussed below and are also shown in Table 1.1.

Ergosterol is an essential primary metabolite in the cell membranes of fungi. The synthesis of this compound requires the catalytic function of CYP51 and CYP61. CYP51 is common to all kingdoms of life and catalyses the 14 α-demethylation of

lanosterol or eburicol to produce $\Delta^{14,\ 15}$ desaturated intermediates (Fig. 1.8). CYP61 is only conserved within the fungal kingdom and functions as a Δ^{22}-desaturase during membrane ergosterol synthesis. The enzyme also carries out aromatic hydrocarbon detoxification in the production of 3-hydroxybenzo(*α*)pyrene from benzo(*α*)pyrene (Crešnar & Petrič, 2011).

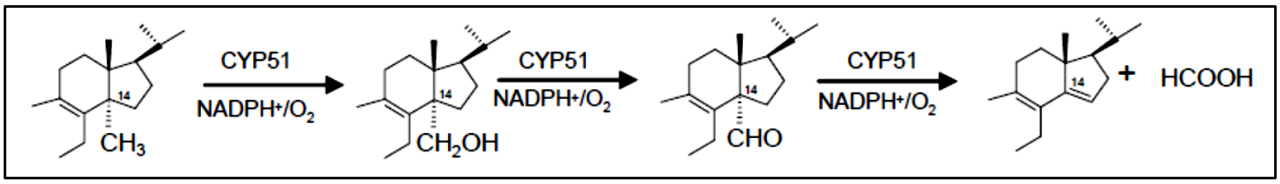


Figure 1.8. Lanosterol demethylation catalysed by CYP51 to produce $\Delta^{14,\ 15}$ desaturated intermediates for the ergosterol pathway (Waterman & Lepesheva, 2005).

N, N-bisformyl dityrosine is the most abundant amino acid of the yeast spore wall and protects the outer spore layer from severe environmental conditions (Briza *et al.*, 1996). The polymer is synthesised in a two-step reaction catalysed by enzymes from *DIT1* and *DIT2* genes (Gómez-Esquer *et al.*, 2004). *DIT2* gene was identified in *Candida albicans* and *Saccharomyces cerevisiae* to code for CYP56 enzyme (Melo *et al.*, 2008) which catalyses the conversion of two molecules of N-formyl tyrosine to N, N-bisformyl dityrosine. While N, N-bisformyl dityrosine formation is crucial to the outer spore wall of *S. cerevisiae*, in *C. albicans* it was identified to play a role in the vegetative cell wall (Crešnar & Petrič, 2011).

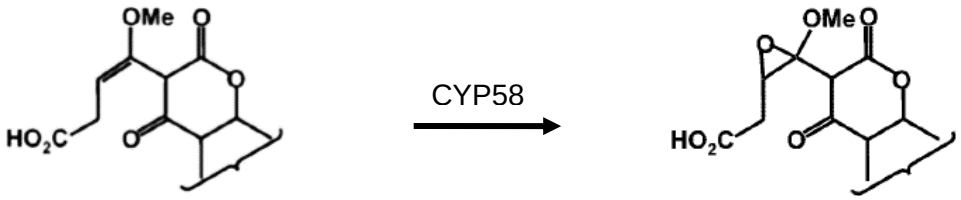
The CYP52 family in fungi regulates the initial and rate limiting steps for the hydroxylation of n-alkanes and fatty acids within the ω-oxidation pathway. These enzymes catalyse the production of ω-hydroxy fatty acid intermediates which are further converted into α, ω-diacids which are ultimately degraded *via* β-oxidation to provide energy and acetyl CoA building blocks. These diacids can be used for production of perfumes, polymers, adhesives and macrolide antibiotics (Craft *et al.*, 2003).

The white rot fungus *Phanerochaete chrysosporium* is the only known organism to completely biodegrade the heterogeneous wood polymer lignin. However, lignin mineralisation by *P. chrysosporium* involves several enzymes including the CYP450 enzyme family (Hirosue *et al.*, 2011). Syed and co-workers (2014) have identified multiple copies of CYP63, CYP512, CYP5035, CYP5037, CYP5136, CYP5141,

CYP5144, CYP5146, CYP5150, CYP5348 and CYP5359 families in the genomes of the wood degrading fungi *P. chrysosporium*, *P. carnosa*, *Agaricus bisporus*, *Postia placenta*, *Ganoderma* sp. and *Serpula lacrymans*. The CYP450 families from enriched wood degrading fungi support lignin degradation by oxidizing resins and coumarin which are plant defence chemicals. The detoxification of plant defence systems enable fungal wood colonization. Additionally, transcriptome studies have indicated the up-regulation of CYP450 genes in wood degrading fungi during fungal growth on wood constituent or colonization (MacDonald *et al.*, 2011).

Synthesis of fungal secondary metabolites such as mycotoxins entails complex pathways. The biosynthetic pathway of aflatoxin, a mycotoxin produced in *A. flavus* and *A. parasiticus* includes four CYP450s namely, CYP58, CYP59, CYP60 and CYP64 catalyse epoxidation, oxidation, hydroxylation and desaturation reactions in the pathway (Fig. 1.9). The pathway for the synthesis of trichothecenes, a class of sesquiterpene mycotoxins produced by *Fusarium* spp., also involve three CYP450 namely CYP58, CYP65 and CYP68. Fumonisins are also synthesised by *Fusarium* spp. and their synthesis involves two CYP450 enzymes. The modifications on fumonisins by CYP450 enzymes include C-14 and C-15 oxidation by members of the self-sufficient CYP505B family, while members of the CYP65 family introduce a hydroxyl group at C-10 (Crešnar & Petrič, 2011; Hlavica, 2013).

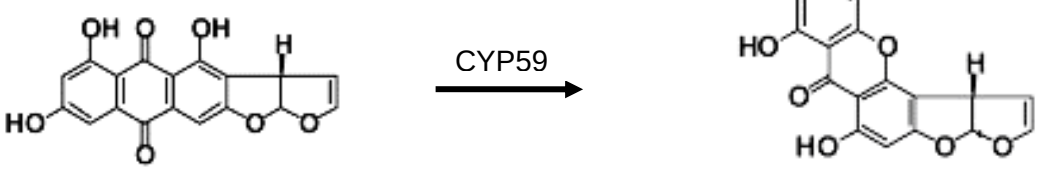
A



Aflatoxin B1 intermediate

Aflatoxin G1 intermediate

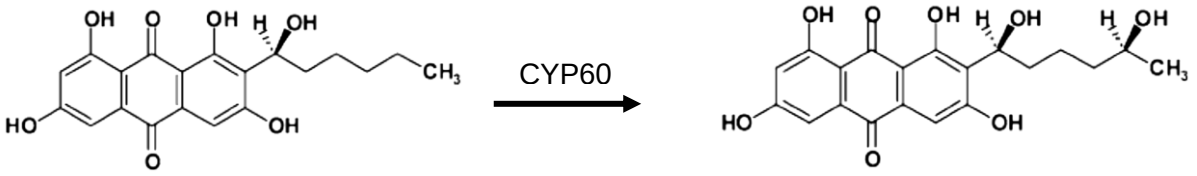
B



Versicolorin A

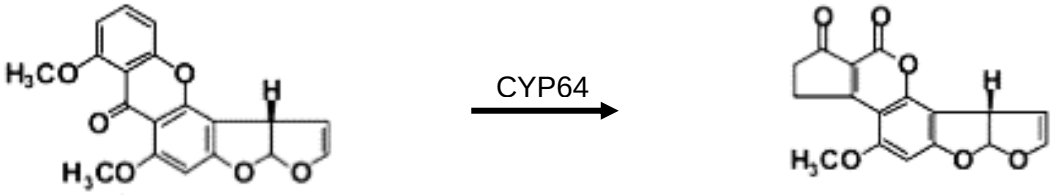
Demethylsterigmatocystin

C



Averantin 5-hydroxyaverantin

D



O-methylsterigmatocystin Aflatoxin B1

Figure 1.9. Reactions catalysed by CYP450s in the mycotoxin synthesis pathway of *A. flavus* and *A. parasiticus* (Ehrlich *et al.*, 2004; Yabe & Nakajima, 2004).

Hydroxylated C20 polyunsaturated fatty acids known as eicosanoids have been identified in several organisms including fungi. These important signaling molecules which include prostaglandins, leukotrienes and thromboxanes are produced from oxidative pathways catalysed by cyclooxygenases (COX), lipoxygenases (LOX) and CYP450s (Singh & Del Poeta, 2011). In fungi, these compounds are essential for pathogenesis, growth and survival (Noverr *et al.*, 2003). The fungus *Dipodascopsis uninucleata* was identified to produce 3-hydroxy-5, 8, 11, 14 eicosatetraenoic acid (3-HETE) when fed with arachidonic acid (AA). However, the presence of aspirin a known cyclooxygenase inhibitor, significantly reduced 3*R*-HETE production and also hampered ascospore release during the sexual life cycle (Kock *et al.*, 2003). Eicosanoid concentration is highest in the family *Dipodascaceae* during ascosporegenesis, which indicates the transition from a sexual to an asexual stage (Noverr *et al.*, 2003).

Fungal pathogenesis involving colonization and infection are supported by eicosanoids. Investigations carried out on the pathogenic fungus *C. albicans* shows that germ tube and biofilm formation were enhanced when immunomodulatory prostaglandin E_2 was exogenously added to cells (Noverr *et al.*, 2003). Yeast to

hypha transition is preceded by germ tube formation and has been associated with the progression of infection, while the colonization is characterized by formation of biofilm on host surfaces (Andreou *et al.*, 2009). Although mammalian CYP450s have been identified to catalyse the production of eicosanoids from arachidonic acid (Zeldin, 2001), the fungal CYP450s have not been associated with production of eicosanoid in the identified eicosanoid-producing fungi.

The fungal CYP505A1 found in *Fusarium oxysporum* catalyses the hydroxylation of saturated and unsaturated fatty acids at ω-1 to ω-3 positions. The enzyme which is self-sufficient shares close amino acid identity with the bacterial CYP102s (Kitazume *et al.*, 2002). CYP505 is membrane associated and shows optimum activity towards medium chain fatty acids. The function of ω-1 to ω-3 hydroxylated fatty acids produced in *Fusarium* spp. is still not clear, but it has been suggested to be responsible for pathogenicity and survival (Hlavica & Lehnerer, 2010).

Fungal CYP450s also degrade toxins produced as a means of defence by their host. Pisatin is produced by pea plants (*Pisum sativum*) as a fungitoxic substance in response to microbial attacks. However, pisatin induces pea pathogens such as *Nectria haematococca* to release CYP57A1 which detoxifies pisatin by demethylation (Crešnar & Petrič, 2011).

Xenobiotics such as polycyclic aromatic hydrocarbons and benzoate derivatives are environmental pollutants degraded by fungi. Fungal species such as *P. chrysosporium* and *A. niger* carry out these processes with CYP450s often catalysing the first step in the degradation of these compounds (Crešnar & Petrič, 2011). Benzoic acid, a fungal inhibitor, is converted by CYP450s of the CYP53 family into a para-hydroxylated product which is further degraded *via* the β-ketoadipate pathway (Podobnik *et al.*, 2008).

Table 1.1. Examples of catalytic functions of fungal cytochrome P450 (Crešnar & Petrič, 2011).

CYP450	ORGANISM	FUNCTION
CYP51, CYP61	*S. cerevisiae* *C. albicans*	Membrane ergosterol synthesis
CYP52	*Candida* spp.	n-Alkane & fatty acid degradation
CYP53	*A. niger* *A. nidulans*	Benzoate detoxification
CYP505	*F. oxysporum*	ω-1 to ω-3 Fatty acid hydroxylation, mycotoxin production
CYP56	*Candida* spp.	N,N′-bisformyl dityrosine production
CYP58, CYP69, CYP60, CYP64	*A. flavus,* *A. parasiticus*	Aflatoxin biosynthesis
CYP55	*F. oxysporum,* *Cylindrocapon tokinese,* *A. oryzae,* *Trichosporon cutaneum*	Denitrification process

1.6. Characterization of Fungal CYP450s

Numerous CYP450 encoding genes have been identified in fungi (Table 1.2), with over 150 CYP450 genes representing more than 1% of the entire genome identified in some fungal species (Lah *et al.*, 2011). Functional classification of these genes are essential to understand the numerous diverse metabolic pathways they are involved in (Hirosue *et al.*, 2011). Although, gene function may be predicted based on sequence similarity with known CYP450s (Kelly *et al.*, 2009), similarity between fungal CYP450s is generally low and involve only a small number of conserved residues.

Additionally, metabolic function of a CYP450 enzyme can be considerably influenced by the change of a single amino acid (Moktali *et al.*, 2012). Assigning function to large numbers of fungal CYP450s depends on the successful heterologous expression of individual genes (Ichinose & Wariishi, 2013). This is required to identify

the probable substrates of individual fungal CYP450s, because the natural substrates are unknown (Kelly *et al.*, 2009).

Table 1.2. CYP450 genes identified in fungal species (Floudas *et al.*, 2012; Park *et al.*, 2008).

FUNGAL SPECIES	NUMBER OF CYP450
Postia placenta	250
Auricularia delicata	249
Coniophora puteana	238
Stereum hirsutum	215
Wolfiporia cocos	206
Trametes versicolor	190
Fusarium oxysporum	170
Aspergillus flavus	159
Aspergillus niger	156
Phanerochaete chrysosporium	149
Heterobasidion annosum	144
Aspergillus terreus	125

1.7. Heterologous expression of fungal cytochromes P450

Purification of functional proteins from their natural source is challenging and produces very low concentration of the desired proteins. Hence, the heterologous expression of functional proteins is essential to produce large amounts for further investigations and applications (Sørensen & Mortensen, 2005). The most commonly used hosts for heterologous expression is *E. coli*. However, other hosts such as yeast, insect cells, mammalian cells and the slime mold *Dictyostelium discoideum* have been employed (Rai & Padh, 2001). The features of *E. coli*, which makes it an attractive host, include growth on cheap carbon sources, rapid biomass accumulation and high-cell density fermentation. However, it also has some drawbacks such as the lack of post-translational modification (Sahdev *et al.*, 2008), protein misfolding, codon bias and mRNA stability (Khow & Suntrarachun, 2012).

Heterologous expression of CYP450 enzymes has been carried out using *E. coli* and yeasts such as *S. cerevisiae*, *Yarrowia lipolytica* and *Pichia pastoris* (Bernhardt, 2006). The expression of CYP450 genes using their native sequences has been successful with insect and mammalian cell cultures as well as yeast hosts (Gillam, 2008). However, expression of eukaryotic CYP450s by *E. coli* often requires N-terminal nucleotide and amino acid modifications (Ichinose & Wariishi, 2013).

Modification of eukaryotic CYP450 genes include substitution of the second amino acid with alanine followed by sequence alteration at the N-terminus to obtain an improved AT content (Gillam, 2008). It has been proposed that these modifications improve codon preference and reduce secondary structure formation in *E. coli*. Alternatively, sequence truncation of the hydrophobic transmembrane domain (TMD) at the N-terminus, has been shown to enhance expression of eukaryotic CYP450 in *E. coli* (Fig. 1.10) (Ichinose & Wariishi, 2013). These TMDs have 20-30 amino acid sequences which anchor the CYP450s to the membrane, but these sequences are not required to produce functional eukaryotic CYP450s in *E. coli* (Shukla *et al.*, 2009). Although truncation of the N-terminal region improves expression of eukaryotic CYP450s in *E. coli*, disruption of the highly conserved proline-rich region which is vital for protein maturation decreases enzyme activity (Ichinose & Wariishi, 2013).

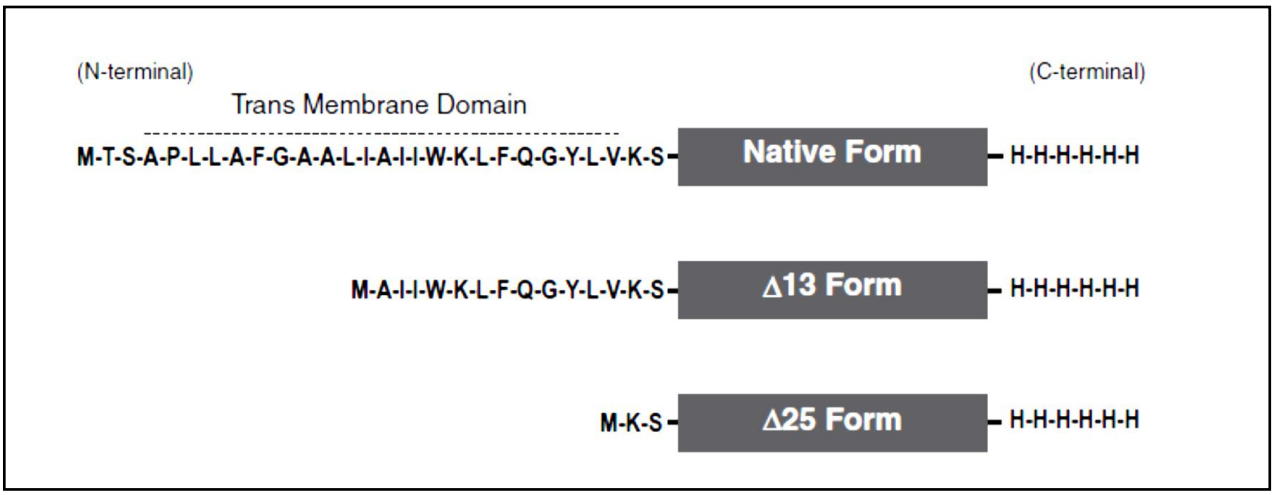


Figure 1.10. Transmembrane domain truncation on the N-terminus of a native eukaryotic CYP450 (Ichinose & Wariishi, 2012).

Other methods used to improve CYP450 expression in *E. coli* include alteration of culture media and growth conditions, supplementation of heme precursors, co-expression with molecular chaperones, induction of heat/cold shock responses and the use of microaerobic cultures (Bernhardt, 2006).

Extensively studied fungal CYP450s include the self-sufficient *Fusarium oxysporum* CYP505A1 (Kitazume *et al.*, 2008), CYP5150A2 (Ichinose & Wariishi, 2012) which was expressed using an *E. coli* host and CYP53A15 which was expressed both in *E. coli* and *S. cerevisiae* (Lah *et al.*, 2011). Some of the different vectors and hosts which have been successfully used for CYP450 expression are shown in Table 1.3.

Attempts have been made to heterologously express the numerous fungal CYP450 genes from a given organism and carry out functional analysis. The successful expression of 84 *A. oryzae* CYP450s in *S. cerevisiae* together with a CYP450 reductase was reported by Nazir and co-workers (2011). Hydroxylation activity towards 7-ethoxycoumarin was demonstrated with some of the expressed CYP450s, while CYP57B3 was specifically reported to hydroxylate genistein producing value added isoflavonoids. The hydroxylated products obtained was an indication of the likely natural substrates of the screened CYP450s.

Table 1.3. Fungal cytochrome P450 expression vectors and host.

CYP450	VECTOR	HOST	REFERENCE
CYP5150A2	pET22	*E. coli*	Ichinose & Wariishi, 2012
CYP53A15	pCWori+		Lah *et al*., 2011
$CYP505_{foxy}$			Kitazume *et al*., 2002a
CYP52A21			Kim *et al*., 2007
CYP51			Warrilow *et al*., 2010
CYP53A15			Podobnik *et al*., 2008
CYP55	pUC19		Takaya *et al*., 2002
CYP56	pSP19g10L		Melo *et al*., 2008
304 *P. chrysosporium* & *P. placenta* CYP450s	pET22 & pET19		Ichinose & Wariishi, 2013
$CYP505_{foxy}$	pYES2	*S. cerevisiae*	Kitazume *et al*., 2002b
CYP53A15	YEpGAL1		Lah *et al*., 2011
CYP52	YEp51		Zimmer *et al*., 1995
CYP57B3	pGYR		Nazir *et al*., 2011
CYP5061B5			Ichinose, 2009
70 *P. chrysosporium* CYP450s			Hirosue *et al*., 2011
84 *A. oryzae* CYP450s			Nazir *et al*., 2011
116 *P. placenta* CYP450s			Ide *et al*., 2012
CYP5136A3	pPICZB	*P. pastoris*	Syed *et al*., 2011
CYP505A1	pKM173 pKM118	*Arxula adeninivorans* *Yarrowia lipolytica*	Theron *et al*., 2014

Hirosue and co-workers (2011) reported the successful expression of 70 *P. chrysosporium* CYP450s in *S. cerevisiae*. Functional screening of expressed CYP450s indicated that they catalyse oxidation, hydroxylation or *O*-deethylation of

compounds such as steroids, petrochemicals, plant related compounds and pharmacochemicals. Seven hydroxylated products of testosterone were produced from 10 CYP450s which showed activity, while 8 CYP450s which exhibited activity towards testosterone were also active towards progesterone. Several *P. chrysosporium* CYP450s were reported to degrade aromatic petrochemicals and pharmacochemicals which include carbazole, dibenzofuran, dibenzothiophene, fluorene, biphenyl and naphthalene.

The role of *P. chrysosporium* CYP5136A2, CYP5145A3, CYP5144A7, CYP5136A3, CYP5142A3, CYP5144A5 genes in oxidation of polycyclic aromatic hydrocarbons (PAH) were investigated by Syed and co-workers (2010). The genes were expressed in *P. pastoris* and showed varying substrate specificity towards the different PAHs tested. CYP5136A3 was observed to display catalytic versatility towards alkylphenols and PAHs and was further studied by Syed and co-workers (2011).

Ide and co-workers (2012) reported the heterologous expression of 116 full length CYP450 genes, from the brown rot fungus *Postia placenta* using a *S. cerevisiae* host. Functional screening of expressed CYP450s was carried out using selected compounds which included 7-ethoxycoumarin, 4-ethoxybenzoic acid, dehydroabietic acid, testosterone, anthracene, carbazole, pyrene, phenanthrene, *trans*-stilbene, 3,5-dimethoxy-*trans*-stilbene and 3,5,4´-trimethoxy-*trans*-stilbene. The CYP5139 family showed activity towards 7-ethoxycoumarin, carbazole and phenanthrene. CYP512 family were active towards steroid compounds such as testosterone, while CYP5150, CYP502 and CYP5350 family had multifunctional activity towards the polycyclic aromatic hydrocarbons anthracene, carbazole, phenanthrene and pyrene. CYP53 family has been characterized for para hydroxylation of benzoate (Podobnik *et al.*, 2008). However, the subfamily member CYP53D2 identified in *P. placenta* displayed *O*-demethylation activity towards tested stilbene derivatives except the *trans*- stilbene (Ide *et al.*, 2012).

A total of 304 CYP450 genes were successfully cloned from *P. placenta* and *P. chrysosporium* into pET plasmids by Ichinose and Wariishi (2013). These genes were modified by TMD truncation or N-terminal replacement for heterologous expression in *E. coli*. N-terminal modification of the CYP5137 and CYP505 families was not required because they lacked a characteristic TMD. High level expression

was obtained with both N-terminally modified and non-modified CYP450s. The expression of some CYP450s was improved using their chimeric variants with junction fixed at a position before the proline rich region. The N-terminus of CYP5144C1 and CYP5139D7v1 were identified to be exchangeable with other CYP450s to improve expression levels. Improved expression levels obtained with the chimeric CYP450 variants indicated that substituting the N-terminus can significantly improve expression of fungal CYP450s in *E. coli*.

1.8. Conclusions

The filamentous fungi have adapted their survival with unique capacities to utilize carbon and nitrogen from various environmental sources. They are also involved in ecologically beneficial roles of nutrient recycling and xenobiotic detoxification, supported by the catalytic functions of numerous CYP450 enzymes. Fungal sequencing information revealed the presence of large numbers of CYP450s, however, many of their functions are unknown.

Functional studies of the numerous fungal CYP450s require heterologous expression of the individual genes. However, expression of fungal CYP450s with the versatile *E. coli* host requires N-terminal sequence modifications. Although suggestions have been made for N-terminal modifications, some fungal CYP450s may require specific modifications to improve expression levels in *E. coli*. Alternatively, fungal CYP450s can be expressed with a yeast host to resolve the requirement for sequence modification. Cytochrome P450 libraries expressed in *S. cerevisiae* and *E. coli* have been used to establish the probable functions of several fungal CYP450s. The successful expression of the numerous fungal CYP450s is essential to understanding their natural roles and possible activities in order to explore applications in drug designs, environmental remediation and biotechnology.

1.9. Aims of study

The versatility of CYP450 enzymes are constantly being explored for diverse applications. Catalytic functions of CYP450 enzymes to oxidize substrates requires

electrons which are conveyed from co-factors by the CYP450 reductase. Although several CYP450s have been identified within the fungal genome, very few have the reductase component fused to the enzyme. Within the sequenced fungal genomes, numerous CYP450s which holds potentials to catalyse several organic substrates have been identified. The investigations of these fungal CYP450s for the production of regio- and stereo-selective compounds are essential for various applications.

Cytochrome P450s in the microsomal fraction of *A. terreus* cells grown on glucose were reported to show activity towards alkanes, alkane derivatives, alcohols, aromatic compounds, organic solvents, and steroids. Heme staining of microsomal fractions from glucose grown cultures analysed on SDS-PAGE indicated the presence of a self-sufficient CYP450. Within the genome of this organism there are 125 CYP450s and only two could possibly be self-sufficient. One of the two possible self-sufficient CYP450 was identified as CYP505E3 while the other had a critical domain missing (Mabwe, unpublished results). The first aim of this study was to heterologously express the self-sufficient CYP505E3 from *A. terreus* in *E. coli* and investigate its substrate specificity (Chapter 2).

Fungal meningitis in immunocompromised patients is mainly caused by the opportunistic pathogen *Cryptococcus neoformans*. The organism is capable of producing the immunomodulatory eicosanoid, prostaglandin E_2, using an unclear cyclooxygenase like pathway. In humans, CYP450s catalyse conversions of arachidonic acid, a prostaglandin precursor, into other eicosanoids. The genome of *C. neoformans* include five CYP450 enzymes and a CYP450 reductase. The heterologous expression of these CYP450s in order to investigate their contributions to arachidonic acid metabolism was the second aim of this study (Chapter 3).

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CHAPTER 2

Heterologous expression and substrate hydroxylation by self-sufficient *Aspergillus terreus* cytochrome P450s

2.1. Abstract

A self-sufficient cytochrome P450 with terminal alkane hydroxylase activity was reported to be present in cell-free extracts of *Aspergillus terreus*. Two open reading frames (ORFs) encoding possible self-sufficient CYP450s were identified within the sequenced genome of the organism. One of the two ORFs had a crucial domain missing; hence it is not likely to be an active CYP450. The second CYP450 ORF, which encodes a CYP450 classified as CYP505E3, was investigated in this study. An N-terminal variant of CYP505E3, similar to the N-terminal variant described for self-sufficient CYP505A1, was constructed for heterologous expression in *Escherichia coli* BL21 (DE3) cells. Whole cell biotransformations were carried out with the expressed CYP505E3 variant using hexadecane, pristane, naphthalene, hexylbenzene, nonylbenzene, 4-hexylbenzoic acid and 4-nonyloxybenzoic acid as substrates. Soluble CYP450 recovery after cell disruption was improved from 0.26 nmol g^{-1} to 0.52 nmol g^{-1} wet weight (101%) using a Plackett-Burman experimental design. Hydroxylated products of hexylbenzoic acid were identified as ω-4 hydroxy hexylbenzoic acid and ω-2 hydroxy hexylbenzoic acid, while no hydroxylated products were produced from hexadecane. Hexylbenzoic acid, which is a substrate of self-sufficient sub-terminal fatty acid hydroxylases such as CYP102A1 and CYP505A1, is also hydroxylated by CYP505E3. We therefore concluded that CYP505E3 is probably also a sub-terminal fatty acid hydroxylase, rather than a terminal alkane hydroxylase.

2.2. Introduction

The filamentous fungi carry genes encoding an array of CYP450 enzymes essential in fungal pathogenesis, xenobiotic degradation and substrate utilization (Moktali *et*

al., 2012). The oxidation of substrates catalysed by CYP450s requires an additional reducing system for electron supply. However, the discovery of a naturally fused self-sufficient system in *Bacillus megaterium* (CYP102A1) solved the fundamental need of a separate redox partner protein (Li *et al.*, 2007). The first identified fungal self-sufficient system was a sub-terminal fatty acid hydroxylase (CYP505A1) from *Fusarium oxysporum* which catalyses ω-1 to ω-3 hydroxylation of medium chain fatty acids (Crešnar & Petrič, 2011). The CYP505 family has been identified to be fairly conserved within the filamentous fungi (Syed *et al.*, 2014). The phylogenetic relationship between CYP505s from *Aspergillus* and *Fusarium* species are shown in Fig. 2.1.

The genome of the filamentous fungus *Aspergillus terreus* comprises of 10,406 open reading frames with 125 annotated as CYP450 genes (Park *et al.*, 2008). Vatsyayan and co-workers (2008) investigated the substrate specificity of *A. terreus* CYP450s obtained from cells grown on glucose or *n*-hexadecane. CYP450 distribution from the *n*-hexadecane grown cells was contained mainly in the cytosol while the CYP450s obtained from the glucose grown cells were obtained in the microsomes. The CYP450 containing microsomal fractions showed activity towards alkanes, alkane derivatives, alcohols, aromatic compounds, organic solvents, and steroids.

SDS-PAGE analysis of the microsomal protein fraction after heme staining indicated that there was only a single heme protein present. The molecular weight of the heme stained protein band was approximately 110 kDa when compared with standard protein markers. It was reported that the size of the single heme stained protein band corresponded to the self-sufficient *Fusarium oxysporum* CYP505A1 reported by Nakayama and co-workers (1996). Hence, the protein band was presumed to be a self-sufficient CYP450 in *A. terreus*. Sequence information from the CYPome of *A. terreus* reveals that there are only two possible self-sufficient CYP450s within its genome. One of the identified self-sufficient CYP450, CYP505A19 lacks a crucial segment of the heme domain, while there is also a large deletion in the reductase domain (Fig. 2.2). It was postulated that this protein will not fold properly and would be inactive or alternatively not accept small molecules such as alkanes as substrates. The second of these self-sufficient CYP450s, designated as CYP505E3 was heterologously expressed and its substrate specificity investigated in this study.

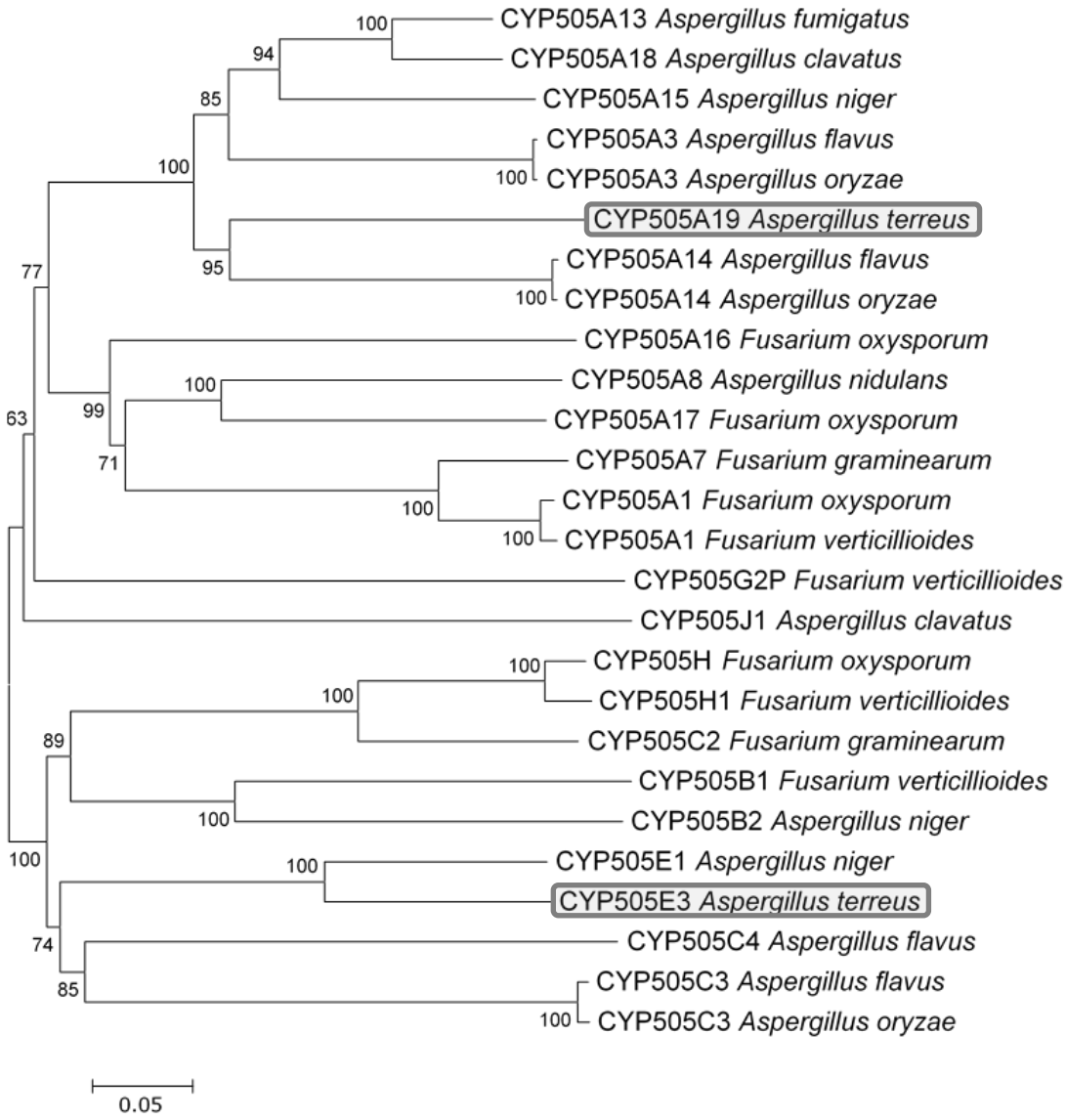
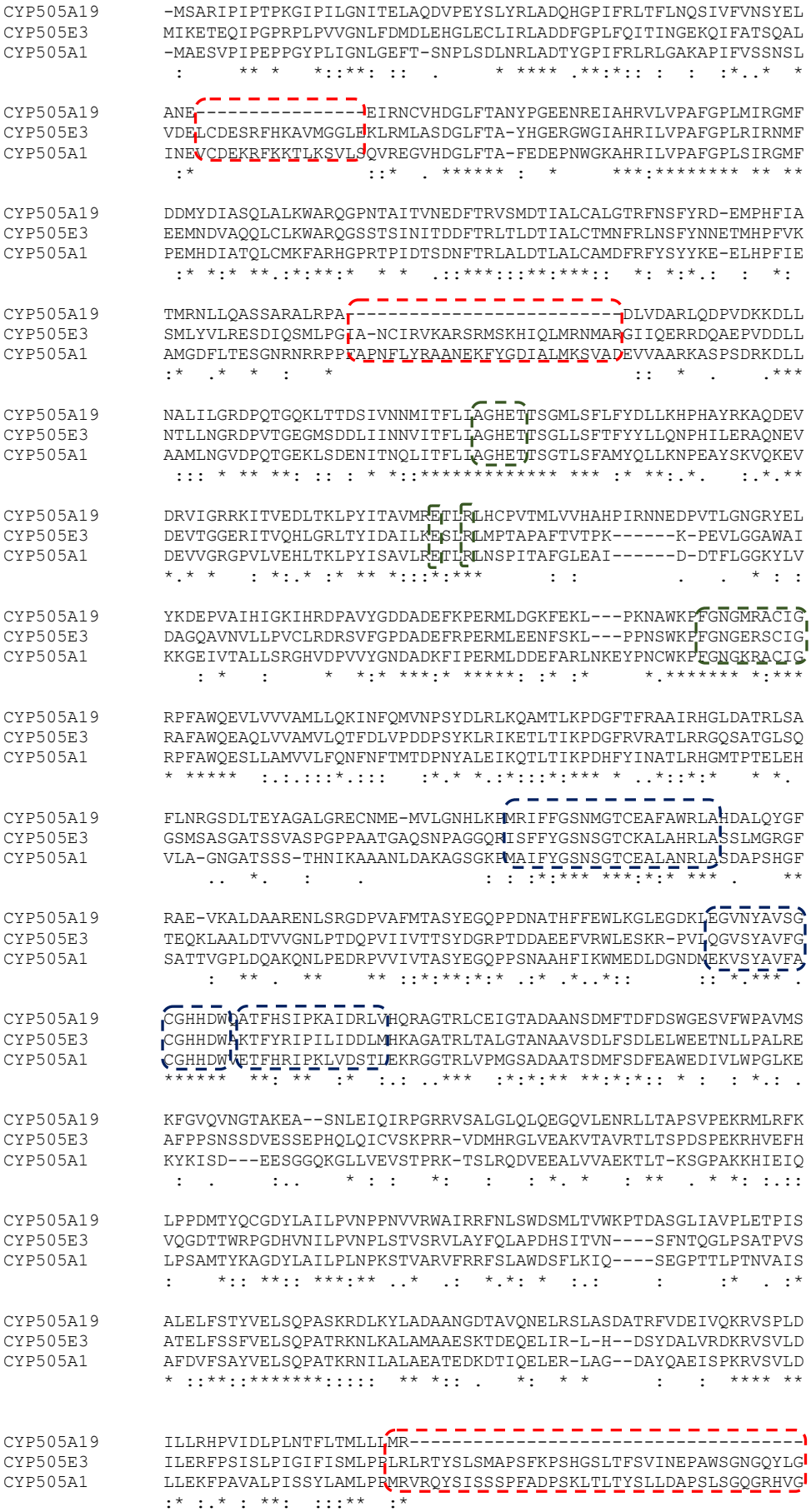


Figure 2.1. Phylogenetic relationship between 26 members of the CYP505 family from *Aspergillus* and *Fusarium* species. The analysis was conducted with Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) program. The relationships were inferred using Neighbor-Joining method while bootstrap consensus was inferred from 500 replicates. Evolutionary distances were computed using the p-distance method (Tamura *et al.*, 2013). *Aspergillus* terreus self-sufficient CYP450s are highlighted in grey.


CYP505A19 -MSARIPIPTPKGIPILGNITELAQDVPEYSLYRLADQHGPIFRLTFLNQSIVFVNSYEL
CYP505E3 MIKETEQIPGPRPLPVVGNLFDMDLEHGLECLIRLADDFGPLFQITINGEKQIFATSQAL
CYP505A1 -MAESVPIPEPPGYPLIGNLGEFT-SNPLSDLNRLADTYGPIFRLRLGAKAPIFVSSNSL
CYP505A19 ANE----------EIRNCVHDGLFTANYPGEENREIAHRVLVPAFGPLMIRGMF
CYP505E3 VDE----------LCDESRFHKAVMGGLFKLRMLASDGLFTA-YHGERGWGIAHRILVPAFGPLRIRNMF
CYP505A1 INE----------VCDEKRFKKTLKSVLSQVREGVHDGLFTA-FEDEPNWGKAHRILVPAFGPLSIRGMF
CYP505A19 DDMYDIASQLALKWARQGPNTAITVNEDFTRVSMDTIALCALGTRFNSFYRD-EMPHFIA
CYP505E3 EEMNDVAQQLCLKWARQGSSTSINITDDFTRLTLDTIALCTMNFRLNSFYNNETMHPFVK
CYP505A1 PEMHDIATQLCMKFARHGPRTPIDTSDNFTRLALDTLALCAMDFRFYSYYKE-ELHPFIE
CYP505A19 TMRNLLQASSARALRPA----------DLVDARLQDPVDKKDLL
CYP505E3 SMLYVLRESDIQSMLPGIA-NCIRVKARSRMSKHIQLMRNMARGIIQERRDQAEPVDDLL
CYP505A1 AMGDFLTESGNRNRRPPFAPNFLYRAANEKFYGDIALMKSVADEVVAARKASPSDRKDLL
CYP505A19 NALILGRDPQTGQKLTTDSIVNNMITFLIAGHETTSGMLSFLFYDLLKHPHAYRKAQDEV
CYP505E3 NTLLNGRDPVTGEGMSDDLIINNVITFLIAGHETTSGLLSFTFYYLLQNPHILERAQNEV
CYP505A1 AAMLNGVDPQTGEKLSDENITNQLITFLIAGHETTSGTLSFAMYQLLKNPEAYSKVQKEV
CYP505A19 DRVIGRRKITVEDLTKLPYITAVMFETLRLHCPVTMLVVHAHPIRNNEDPVTLGNGRYEL
CYP505E3 DEVTGGERITVQHLGRLTYIDAILKETLRLMPTAPAFTVTPK------K-PEVLGGAWAI
CYP505A1 DEVVGRGPVLVEHLTKLPYISAVLRETLRLNSPITAFGLEAI------D-DTFLGGKYLV
CYP505A19 YKDEPVAIHIGKIHRDPAVYGDDADEFKPERMLDGKFEKL---PKNAWKPFGNGMRACIG
CYP505E3 DAGQAVNVLLPVCLRDRSVFGPDADEFRPERMLEENFSKL---PPNSWKPFGNGERSCIG
CYP505A1 KKGEIVTALLSRGHVDPVVYGNDADKFIPERMLDDEFARLNKEYPNCWKPFGNGKRACIG
CYP505A19 RPFAWQEVLVVVAMLLQKINFQMVNPSYDLRLKQAMTLKPDGFTFRAAIRHGLDATRLSA
CYP505E3 RAFAWQEAQLVVAMVLQTFDLVPDDPSYKLRIKETLTIKPDGFRVRATLRRGQSATGLSQ
CYP505A1 RPFAWQESLLAMVVLFQNFNFTMTDPNYALEIKQTLTIKPDHFYINATLRHGMTPTELEH
CYP505A19 FLNRGSDLTEYAGALGRECNME-MVLGNHLKHMRIFFGSNMGTCEAFAWRLAHDALQYGF
CYP505E3 GSMSASGATSSVASPGPPAATGAQSNPAGGQHISFFYGSNSGTCKALAHRLASSLMGRGF
CYP505A1 VLA-GNGATSSS-THNIKAAANLDAKAGSGKHMAIFYGSNSGTCEALANRLASDAPSHGF
CYP505A19 RAE-VKALDAARENLSRGDPVAFMTASYEGQPPDNATHFFEWLKGLEGDKIEGVNYAVSG
CYP505E3 TEQKLAALDTVVGNLPTDQPVIIVTTSYDGRPTDDAEEFVRWLESKR-PVIQGVSYAVFG
CYP505A1 SATTVGPLDQAKQNLPEDRPVVIVTASYEGQPPSNAAHFIKWMEDLDGNDMEKVSYAVFA
CYP505A19 CGHHDWGATFHSIPKAIDRLVHQRAGTRLCEIGTADAANSDMFTDFDSWGESVFWPAVMS
CYP505E3 CGHHDWAKTFYRIPILIDDLMHKAGATRLTALGTANAAVSDLFSDLELWEETNLLPALRE
CYP505A1 CGHHDWVETFHRIPKLVDSTIEKRGGTRLVPMGSADAATSDMFSDFEAWEDIVLWPGLKE
CYP505A19 KFGVQVNGTAKEA--SNLEIQIRPGRRVSALGLQLQEGQVLENRLLTAPSVPEKRMLRFK
CYP505E3 AFPPSNSSDVESSEPHQLQICVSKPRR-VDMHRGLVEAKVTAVRTLTSPDSPEKRHVEFH
CYP505A1 KYKISD---EESGGQKGLLVEVSTPRK-TSLRQDVEEALVVAEKTLT-KSGPAKKHIEIQ
CYP505A19 LPPDMTYQCGDYLAILPVNPPNVVRWAIRRFNLSWDSMLTVWKPTDASGLIAVPLETPIS
CYP505E3 VQGDTTWRPGDHVNILPVNPLSTVSRVLAYFQLAPDHSITVN----SFNTQGLPSATPVS
CYP505A1 LPSAMTYKAGDYLAILPLNPKSTVARVFRRFSLAWDSFLKIQ----SEGPTTLPTNVAIS
CYP505A19 ALELFSTYVELSQPASKRDLKYLADAANGDTAVQNELRSLASDATRFVDEIVQKRVSPLD
CYP505E3 ATELFSSFVELSQPATRKNLKALAMAAESKTDEQELIR-L-H--DSYDALVRDKRVSVLD
CYP505A1 AFDVFSAYVELSQPATKRNILALAEATEDKDTIQELER-LAG--DAYQAEISPKRVSVLD
CYP505A19 ILLRHPVIDLPLNTFLTMLLLMR----------
CYP505E3 ILERFPSISLPIGIFISMLPLRLRTYSLSMAPSFKPSHGSLTFSVINEPAWSGNGQYLG
CYP505A1 LLEKFPAVALPISSYLAMLPRMRVRQYSISSSPFADPSKLTLTYSLLDAPSLSGQGRHVG

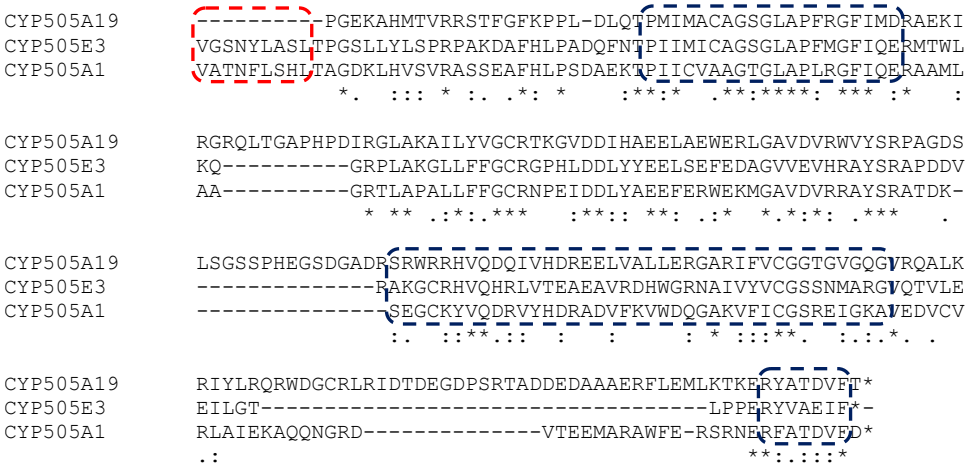


Figure 2.2. Amino acid alignment of self-sufficient *A. terreus* CYP505E3, CYP505A19 and *Fusarium oxysporum* CYP505A1. The analysis was conducted with the EMBL– EBI Clustal Omega multiple sequence alignment program version 1.2.1 (Goujon *et al.*, 2010). The heme-binding motif (F(G/S)XGX(R/H)XCXG), the consensus sequence ((A/G)GX(E/D)T) and other regions which are conserved in CYP450 are boxed in green. Conserved residues in the CYP450 reductase are boxed in blue while the missing regions in CYP505A19 are boxed in red (Kitazume *et al.*, 2000). The identical residues are shown by asterisks.

2.3. Materials and Methods

2.3.1. General experimental procedures

2.3.1.1. Chemicals and enzymes

Chemicals and reagents used in these experiments were analytical grade and were, unless otherwise stated, supplied by either Fluka, Sigma-Aldrich or Merck. Restriction endonucleases, T4 DNA ligase and molecular weight markers were obtained from Thermo Fisher Scientific.

2.3.1.2. Digestion and ligation of plasmids

Reaction mixtures for digestion of plasmid DNA with endonucleases comprised 4 U of the enzyme and 1x buffer and were incubated at 37 ^{o}C for 1-4 h. Digestion of plasmid DNA with two endonucleases (double digestions) were setup with required enzymes and buffer concentrations as described by the manufacturer. Reaction mixtures for ligation of insert DNA into plasmid backbone contained 5 U T4 DNA ligase and 1x DNA ligase buffer and were incubated overnight at 4 ^{o}C.

2.3.1.3. Transformation into *E. coli*, small scale plasmid isolation and agarose gel electrophoresis

Transformation of plasmid DNA into competent *E. coli* cells, was done with the heat shock treatment as described by Inoue *et al*., (1990). The transformed cells were plated on Luria-Bertani (LB) (5 g yeast extract, 10 g tryptone, 10 g NaCl and 15 g bacteriological agar L^{-1}) supplemented with 30 µg kanamycin ml^{-1} (pET28b) or 60 µg ampicillin ml^{-1} (pMA) and incubated for 16 h at 37 ^{o}C. Colonies were inoculated in 5 - ml LB broth supplemented with 50 µg kanamycin ml^{-1} and incubated overnight in a shaking incubator at 37 ^{o}C.

Plasmid extraction using the lysis by boiling small scale plasmid isolation method was carried out with freshly grown *E. coli* cells as described by Sambrook *et al*., (1989). Extracted plasmids were resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH8) containing 50 µg RNase ml^{-1}. Restriction analysis was used to confirm successful cloning and correct orientation of the inserts and selected positive clones were stored at -20 ^{o}C. Alternatively, positive constructs were extracted from *E. coli* using GenElute™ plasmid miniprep kit (Sigma).

Agarose gel analysis was performed with a 1% (w/v) gel prepared with TAE buffer (40 mM Tris, 2 mM EDTA, 20 mM glacial acetic acid and pH 8.5). GoldView™ nucleic acid stain (SBS Genetech) was added to the molten gel for visualization. The reference molecular ladder was O' GeneRuler™ DNA ladder mix (Thermo Scientific) and the gels ran constantly at 90 V for 35 min in TAE buffer. Gels were analysed with a Gel Doc™ (Bio-Rad) while fragments to be excised were viewed with a DarkReader transilluminator (Clare Chemicals). Excised fragments were purified with Biospin gel extraction kit (Bioer technology) according to manufacturer's instructions.

2.3.1.4. SDS-PAGE electrophoresis

SDS-PAGE gels were cast with 10% acrylamide as described by Laemmli (1970) using a 0.75 mm Mini-PROTEAN (Bio-Rad). Whole cells resuspended in buffer (whole cells) and cell-free extracts for SDS-PAGE analysis were mixed in a 1:1 (v/v) ratio with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) and boiled for 10 min to solubilize

the proteins. SDS-PAGE gels with wells containing the prepared samples were electrophoresed at 100 V together with a PageRuler™ Prestained or Unstained Protein ladder (Thermo Scientific). The resolved gel was developed with Coomassie staining as described by Fairbanks *et al.*, (1971) and analysed.

2.3.2. Expression plasmids

The CYP505E3 wild-type gene and modified N-terminal fragment were synthesized by GeneArt® and supplied in pMK and pMA vectors. The CYP505E3 wild-type gene was then cloned into a pET28b vector by Dr. D.J. Opperman. The pET28b vector containing the CYP505E3 wild-type gene was double digested with *Nde*I and *Dra*I (Fig. 2.3), to remove a 393 bp fragment from the N-terminal region. Agarose gel electrophoresis was carried out to separate the fragments while the backbone of 8068 bp was purified. The modified 393 bp N-terminal fragment in pMA was also digested with the same enzymes (Fig. 2.3) and purified after agarose gel electrophoresis.

The purified 8068 bp backbone construct containing the CYP505E3 wild-type gene and the 393 bp fragment of the DNA for the N-terminal variant were ligated together and transformed into chemically competent *E. coli* XL10-Gold strain. Small scale plasmid isolations of transformed colonies were digested with *Xba*I to identify positive clones. The clones were further confirmed by sequencing with a Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Sequences were aligned using Geneious v6.0.3 software (Biomatters). The final construct was designated as CYP505E3 variant.

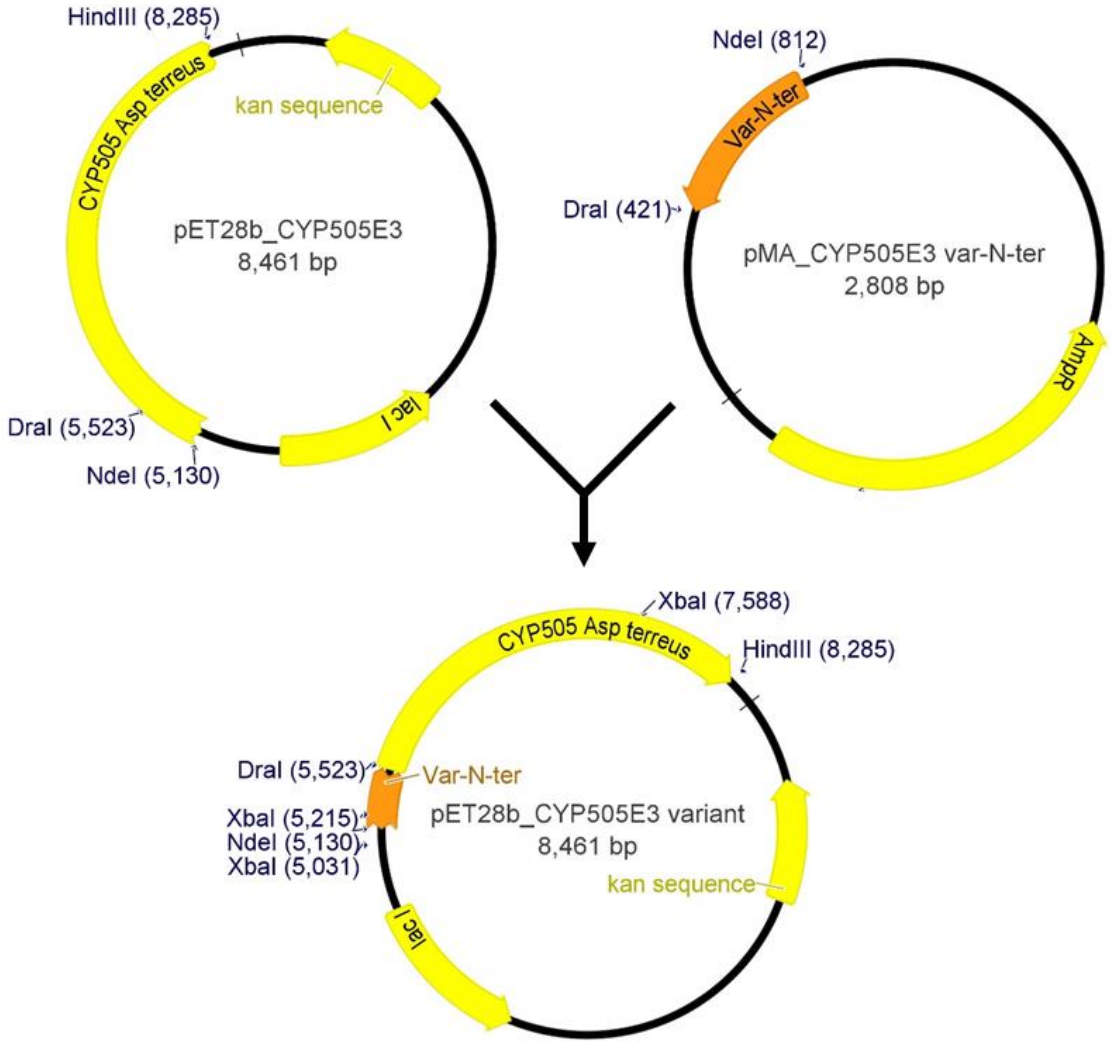


Figure 2.3. Graphical illustration of *A. terreus* CYP505E3 variant construction. The *A. terreus* CYP505E3 wild-type was cloned into pET28b with *Nde*I and *Hin*dIII while the modified N-terminal fragment was supplied in a pMA vector. The *Nde*I, *Dra*I and *Xba*I restriction sites used for cloning and confirmation are also indicated.

2.3.3. *Escherichia coli* expression of CYP505E3 variant

CYP505E3 variant was transformed into *E. coli* BL21 (DE3) strain and glycerol stocks of transformed colonies were stored at -20 ^{o}C. The ZY auto-induction media (Table 2.1) was used for expression of the CYP505E3 variant. Frozen stocks of *E. coli* isolates transformed with CYP505E3 variants were revived overnight in LB medium and used as pre-inoculum. Aliquots (100 ml) of ZY auto-induction media supplemented with 50 µg kanamycin ml^{-1}, 1 mM $FeCl_3$ and 1 mM 5-aminolevulinic

acid (ALA) were transferred into sterile 500 ml flasks. Each flask was inoculated with 2 ml of fresh LB-grown pre-inoculum and incubated at 25 ^{o}C on a rotary shaker shaking at 180 rev min^{-1} for 24 h.

Table 2.1. Composition of ZY auto-induction media (Studier, 2005).

Stock solutions			**ZY auto-induction media**
ZY medium	**20x NPS**	**50x 5052***	50 ml 20x NPS
10 g/l tryptone	0.5 M $(NH_4)_2SO_4$	250 g/l glycerol	20 ml 50x 5052
5 g/l yeast extract	1 M KH_2PO_4	25 g/l glucose	2 ml 1 M $MgSO_4$
	1 M Na_2HPO_4	100 g/l α-lactose	928 ml ZY-medium

*5052 - 0.5% glycerol, 0.05% glucose, 0.2% α-lactose

E. coli cells were harvested by centrifugation at 6000 *x g*, 4 ^{o}C for 10 min and resuspended in a 1:3 (w/v) ratio in 50 mM or 200 mM phosphate buffer (pH 8.0, 0.8% glycerol). The cells were lysed with a cell disruptor (Constant Systems) at 30 kpsi. Membrane and soluble fractions were separated by centrifugation at 20,000 *x g* for 20 min at 4 ^{o}C while the cell debris was resuspended to the initial volume. The whole cells and cell-free extracts were used for CO difference spectra and SDS-PAGE analysis.

2.3.4. CO difference spectra

The resuspended cells were further diluted with phosphate buffer in a 1:1 (v/v) ratio to record CO difference spectra. The assay was conducted as described by Choi *et al*., (2003) using 200 µl of the diluted cells transferred into microtiter strips (Thermo Scientific). Absorbance readings between 400 and 500 nm were measured with a SpectraMax® Microplate Reader (Molecular Devices). The CYP450 concentration was determined using an extinction coefficient of 0.091 nM^{-1} ml^{-1} (Omura & Sato, 1964) and a pathlength of 0.596 cm. Peak corrections were done and CYP450 concentrations calculated by using the equation A_{450} – ((0.375 * A_{470}) + (0.625 * A_{438})) / (0.091*0.596) (Johnston *et al.*, 2008).

2.3.5. Plackett-Burman experimental design

The Plackett-Burman experimental design investigated eight factors that could affect CYP450 expression, each with two options designated as low and high levels (Table 2.2). These were ratio of glycerol to lactose, temperature, optical density at the time of ALA and $FeCl_3$ addition, concentration of ALA, concentration of $FeCl_3$, culture volume, initial pH and harvest time. These factors were combined using SPC for Microsoft Excel program to obtain an experimental design of 12 different runs (Table 2.3). The response variables included wet biomass and CYP450 content in whole cells and cell-free extracts.

Table 2.2. Plackett-Burman factors.

Factor	Name	Low Level	High Level
A	Glycerol/Lactose ratio[§]	2.5	6
B	Temperature (°C)	20	25
C	OD[#] at ALA & $FeCl_3$ addition	0	0.6
D	Conc. ALA* (mM)	0.5	1
E	Conc. $FeCl_3$ (µM)	50	1000
F	Culture volume (ml)	100	200
G	pH	6.5	7.2
H	Harvest time (h)	28	40

[§] Glycerol (g/l) / Lactose (g/l) ratio – low level (250/100), high level (300/50)
[#]OD - optical density
*ALA - 5-aminolevulinic acid

Table 2.3. Combination of factors in individual Plackett-Burman runs.

Run Order	A	B	C	D	E	F	G	H
1	6	20	0.6	1	1000	100	6.5	28
2	2.5	20	0	1	50	200	7.2	28
3	6	25	0	0.5	50	200	6.5	40
4	6	20	0.6	1	50	200	7.2	40
5	6	25	0.6	0.5	50	100	7.2	28
6	2.5	20	0	0.5	50	100	6.5	28
7	2.5	25	0.6	1	50	100	6.5	40
8	2.5	25	0.6	0.5	1000	200	7.2	28
9	6	25	0	1	1000	200	6.5	28
10	2.5	20	0.6	0.5	1000	200	6.5	40
11	6	20	0	0.5	1000	100	7.2	40
12	2.5	25	0	1	1000	100	7.2	40

2.3.6. Substrate hydroxylation by CYP505E3 variant

Expressed CYP505E3 variant was screened for hydroxylation activity against a number of substrates using whole cell biotransformation. The required amount of substrates for the desired stock solution (Table 2.4) was measured and dissolved in diethyl ether. The solution was added to 20 ml 50 mM MOPS buffer (pH 7) containing 0.4 mg ml^{-1} Triton X-100 and sonicated till a homogeneous mixture was obtained. The biotransformation reaction mixture setup in amber bottles for each of the tested substrate included 500 µl 1:3 (w/v) resuspended cells, 500 µl of the prepared substrate stock solution and 22 mM glucose. The final concentration of the substrates in the biotransformation mixture are shown in Table 2.4.

Further biotransformation reactions using hexylbenzoic acid (HBA) as substrate were setup in amber bottles. Stock solutions for biotransformation reactions using HBA as substrate was prepared by dissolving 20 mg HBA in 300 µl dimethyl sulfoxide (DMSO). The reaction included 500 µl 1:3 (w/v) resuspended cells, 500 µl of phosphate buffer, 22 mM glucose and 20 µl of the substrate stock solution. The addition of 20 µl of the prepared HBA stock gave a final concentration of 5.72 mM

HBA in the reaction mixture. All biotransformation reaction mixtures were incubated on a 180 rev min^{-1} rotary shaker for 1-24 h at 25 ^{o}C. CYP102A1 HBA conversion has been reported by Gudiminchi & Smit (2011). To identify some of the products formed by CYP505E3, CYP102A1 in pET28a was expressed in *E. coli*. The expressed CYP102A1 was the positive control for the biotransformation reaction.

The reaction was stopped with the addition of 5 M HCl (70 µl) and transferred into 2 ml tubes. Extraction was done twice with the addition of 500 µl ethyl acetate containing 1 mM dodecanoic acid. The solution was vortexed for 8 minutes and centrifuged for 5 minutes, before the organic phase was collected. Five hundred microliters of the organic phase was evaporated to dryness and resuspended in ethyl acetate (50 µl).

Table 2.4. Concentration of substrates used for biotransformation.

Substrate	**Concentration (mM)**	
	Stock	Final in Reaction
Hexadecane	100	50
Pristane	100	50
Naphthalene	10	5
Nonylbenzene	25	12.5
Hexylbenzene	25	12.5
Nonyloybenzoic acid	10	5
Hexylbenzoic acid	10	5

The extract was analysed with thin layer chromatography (TLC) and gas chromatography–mass spectrometry (GC/MS). The TLC stationary phase was a silica gel 60 F_{254} TLC plate (Merck) which was spotted with 10 µl of the concentrated extract. The plate was developed in a TLC chamber containing dibutyl ether/ formic acid/ water (90:7:3, v/v) as the mobile phase.

For GC/MS the extract was methylated by adding 0.2 M trimethylsulfonium hydroxide (TMSH) dissolved in methanol in a 1:2 (v/v) ratio. The products were identified using a Finnigan Trace GC ultra attached to a TraceDSQ mass spectrometer. The analytical column employed was a Varian FactorFour VF-5ms with dimensions: 30 m x 0.25 mm x 0.25 µm (length x inner diameter x film thickness). Helium at 2 ml min^{-1}

was the carrier gas. One microliter of the methylated extract was injected with the injection port temperature at 250 ºC while the Injection mode was set to splitless for 2 minutes after which a split ratio of 1:20 was maintained. Initial column temperature was 100 °C held for 2 min, then raised at 5 °C min^{-1} to a final temperature of 250 °C held for 10 min.

The MS transfer line temperature was 280 °C and that of the source 200 °C. MS data was collected in TIC mode with a scan range of 40 to 450 amu. Instrument control and data collection and analysis software was Finnigan Xcalibur 1.4.

2.4. Results and Discussions

2.4.1. Cloning of CYP505E3 and construction of a N-terminal variant

The CYP505E3 wild-type gene sequence obtained from the fungal CYP450s database of the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) was synthesised and cloned into a pMK vector by GeneArt®. The gene was then digested with *Nde*I and *Hin*dIII and cloned into a pET28b vector by Dr. D.J. Opperman (Fig. 2.3).

N-terminal sequence modifications of eukaryotic CYP450s have been suggested as a requirement for improved expression levels in *E. coli*. Kitazume and co-workers (2002) reported improved expression of a N-terminal variant of CYP505A1. N-terminal nucleotides of the CYP505A1 variant were modified to obtain a more A / T rich sequence while the amino acid coding remained unchanged. Additionally, the proline codons were changed to the codon CCG which is more common in *E. coli* genes. Sequence modifications which include enriched A / T content and the alteration of the second codon to alanine was also described to generally enhance eukaryotic CYP450 expression in *E. coli* (Gillam, 2008). The improved expressions obtained in *E. coli* using N-terminal modified eukaryotic CYP450 motivated the construction of a CYP505E3 variant.

A small fragment of the CYP505E3 gene with the second and third codons changed so that the second amino acid was substituted with alanine and the lysine codon AAG replaced with the alternative AAA codon (Fig. 2.4) had also been synthesised by GeneArt®. These alterations improved the A / T content of the N-terminal

sequence and also allows the use of codons matching with *E. coli* preferences. These modifications were expected to minimize the formation of secondary structure in the transcript (Gillam, 2008).

Figure 2.4. Sequence alignment between CYP505E3 wild-type and variant indicating modified nucleotides.

The CYP505E3 variant was constructed by replacing the *Nde*I – *Dra*I fragment in the wild-type gene in pET28b with the corresponding fragment from a pMA plasmid containing the modified N-terminal fragment. The CYP505E3 variant construct in pET28b was digested with *Xba*I to confirm the ligation of the modified N-terminal fragment. The expected band sizes of approximately 6000 bp and 2500 bp indicating a completely cloned gene was observed on the agarose gel as show in Fig. 2.5. Addition of the modified N-terminal fragment in the desired orientation was confirmed by sequencing the completed construct.

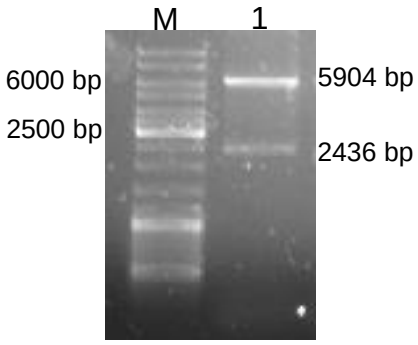


Figure 2.5. Agarose gel electrophoresis of CYP505E3 variant. Lane 1 is *Xba*I digested CYP505E3 variant while the DNA marker is labelled M.

2.4.2. Expression and SDS-PAGE analysis of CYP505E3 variant

The pET28b vector carrying the CYP505E3 variant was transformed into *E. coli* BL21 (DE3) strain. The gene cloned into the pET28b vector is under the control of a

T7 *lac* promoter while the vector also contains a lac repressor (*lacI*) to prevent leaky expression. The *lac* repressor (*lacI*) blocks the *lacUV5* promoter in the host from expressing the chromosomal T7 RNA polymerase gene and also blocks the T7 *lac* promoter on the vector. However, protein expression is induced with the addition of lactose or the analog isopropyl β-D-1-thiogalactopyranoside (IPTG) (Terpe, 2006). We used lactose containing auto-induction medium, a cheap and less toxic alternative to the popular IPTG addition, to induce expression of the cloned CYP505E3. It has been reported that the protein yield obtained with auto-induction medium is much higher than when using IPTG induction (Studier, 2005).

The presence of expressed CYP505E3 variant was indicated by the presence of a peak at 450 nm observed with CO difference spectra recorded using whole cells (Fig. 2.6). Expression levels obtained in the whole cells were 0.93 nmol g^{-1} wet weight while 0.26 nmol g^{-1} wet weight was recovered from the cell-free extract. The levels obtained in the cell free extract was higher than that reported for the self-sufficient CYP505A1 expressed by Kitazume and co-workers (2002) using IPTG induction. However, the recovered protein in the cell-free extract was 72% less than in the whole cells (Fig. 2.6). The low recovery in the cell-free extract might be due to the loss of unbroken whole cells which settles together with other debris during centrifugation.

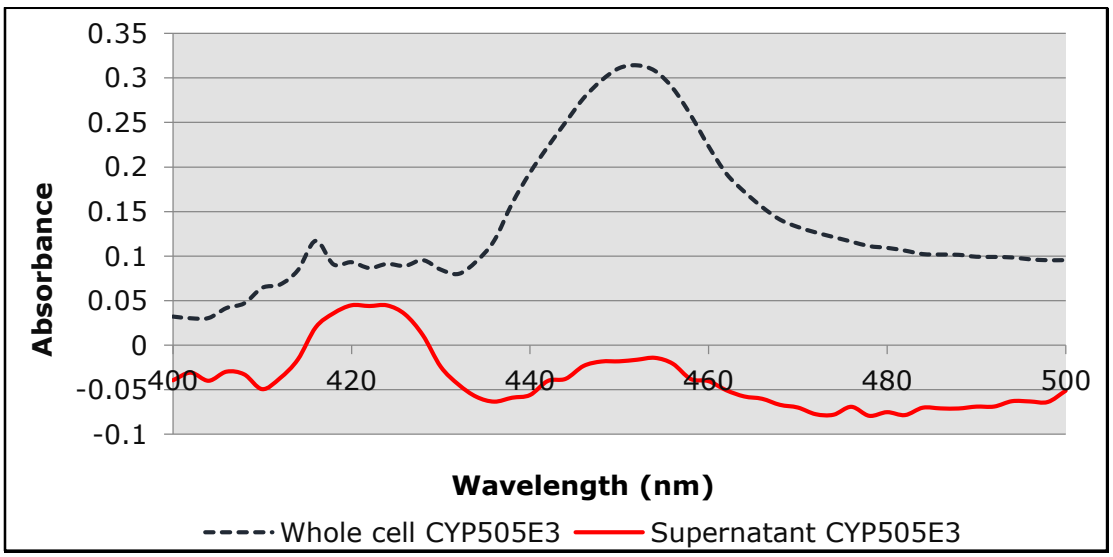


Figure 2.6. CO difference spectra detecting CYP505E3 variant in whole cells and cell free extract.

The expression of the CYP505E3 variant was further confirmed with SDS-PAGE analysis. The molecular weight of CYP505E3 variant was calculated as 116 kDa using the ExPASy ProtParam tool (http://web.expasy.org/protparam/). The expected

molecular weight of 116 kDa for CYP505E3 variant was confirmed with SDS-PAGE analysis as shown in Fig. 2.7 (a) and (b). The molecular weight obtained for CYP505E3 was similar to the 110 kDa protein band reported by Vatsyayan and co-workers (2008). The self-sufficient fungal CYP505A1 reported by Nakayama and co-workers (1996) had a molecular weight of 118 kDa. The similarity in molecular weight suggests that the heme containing band observed by Vatsyayan and co-workers (2008) could have been the self-sufficient CYP505E3.

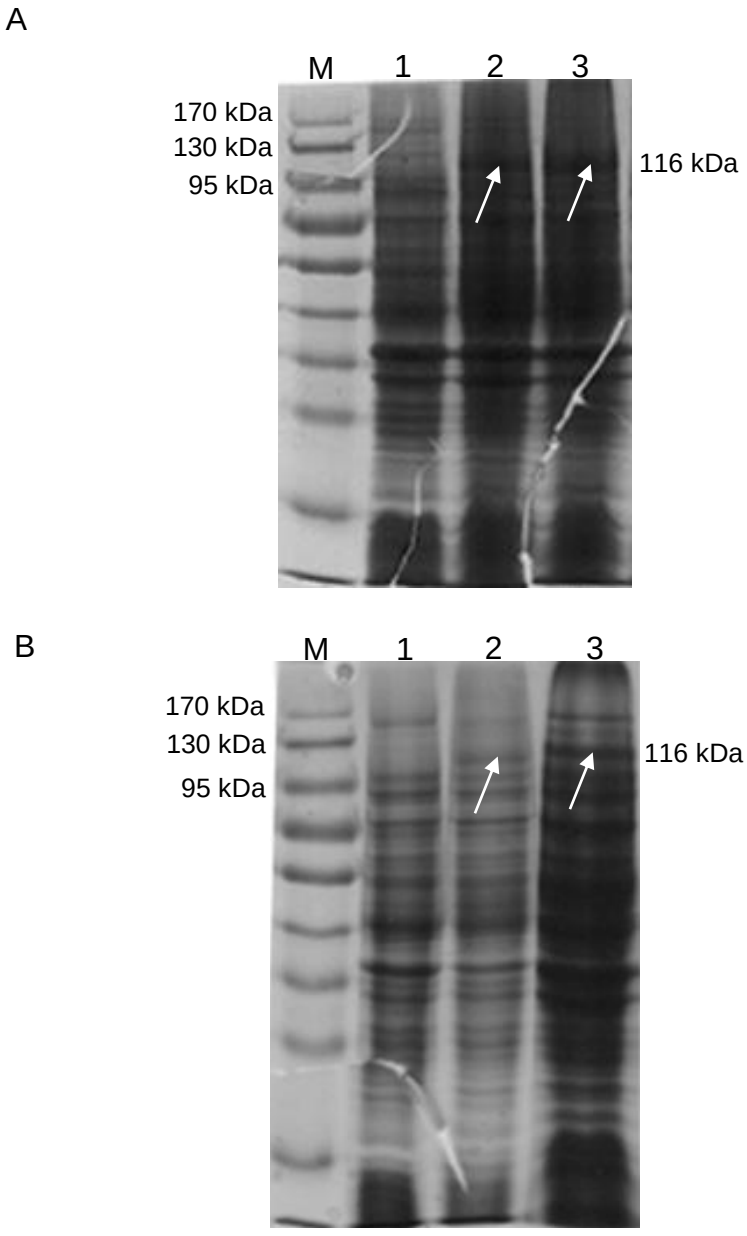


Figure 2.7. SDS-PAGE analysis of expressed CYP505E3. (A) Whole cell, (B) soluble fraction of cell free extract. Lane 1 is a blank pET28b vector negative control. Lane 2 is CYP505E3 wild-type. Lane 3 is CYP505E3 variant. The PageRuler™ prestained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

2.4.3. Plackett-Burman design experiments to improve expression

The Plackett-Burman experimental design is used to identify factors that influence an experimental process. This is done by varying the factors independently and then the effect of each factor can be studied individually (Plackett & Burman, 1946). A Plackett-Burman experiment was designed (Table 2.2 and Table 2.3) to improve recovery of the CYP505E3 variant in the cell-free extract. The factors investigated included glycerol to lactose ratio, growth temperature, optical density at ALA and $FeCl_3$ addition, concentration of ALA and $FeCl_3$ added, culture volume, initial pH and harvest time.

Glycerol to lactose ratio was increased to improve carbon and energy supply because lactose, which is an excellent source of carbon and energy (Santillán & Mackey, 2008), is also required to induce expression at high cell density (Studier, 2005). The temperature for expression was decreased to improve protein stability and solubility (Khow & Suntrarachun, 2012), while the concentrations of ALA and $FeCl_3$ as well as pH were increased to improve CYP450 expression levels. A later harvest time was used to improve the overexpression of CYP505E3 at high cell densities. The culture volume was increased to reduce aeration because high aeration inhibits induction at low lactose concentration (Studier, 2005) while low levels of aeration reportedly also favours CYP450 expression. The optical density at ALA and $FeCl_3$ addition were also varied to determine their influence on CYP505E3 expression.

The Plackett-Burman response variables which included wet biomass, whole cell CYP450 content and cell-free extract CYP450 content were statistically analysed. The analysis of variance (ANOVA) was used and a *p*-value of 0.05 or less was used to identify significant factors while values between 0.05 and 0.20 indicate possible significant factors. The factors contributing to CYP505E3 recovery from the soluble fraction of the cell free extract were analysed. The *p*-values of 0.15 obtained for temperature and 0.06 for concentration of $FeCl_3$ suggest that these factors may be significant (Supplementary Information, Fig. S1). The effects of these factors showed that reduced temperature and a low concentration of $FeCl_3$ would be favourable for improved recovery of CYP505E3 in the soluble fraction. The reduced temperature is

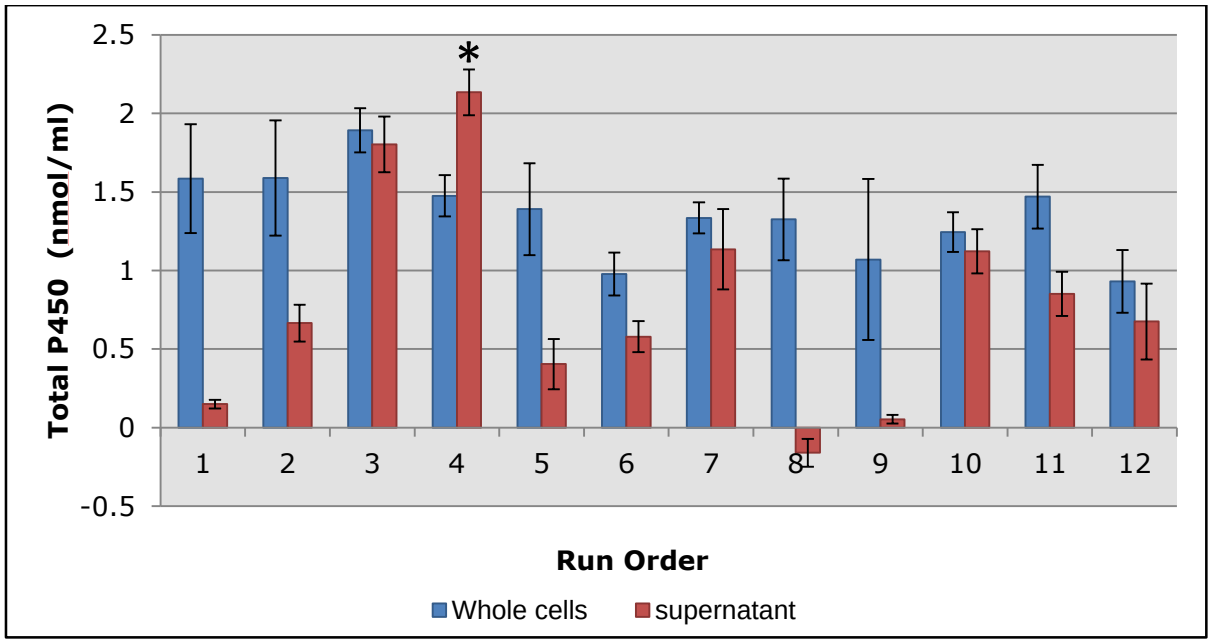
associated with production of correctly folded protein which reduces the formation of inclusion bodies.

Harvest time with, a *p*-value of 0.17, was identified as the only potentially significant factor from the analysis of whole cell CYP450 content (Fig. S2). The effect of this factor showed that an early harvest time would enhance whole cell CYP450 content. The significant factors influencing the amount of wet biomass obtained are temperature with a *p*-value of 0.02 and culture volume with a *p*-value of 0.04 (Fig. S3). The effect of these factors showed that low temperatures and low culture volumes would favour an increase in wet biomass.

The best recovery of expressed protein in the cell-free extract was obtained with conditions in run 4 (Fig. 2.8(a)). The combination of parameters in run 4 significantly improved recovery in the cell free extract from 0.26 nmol g^{-1} obtained in the initial experiment to 0.52 nmol g^{-1} wet weight (101%) (Fig. 2.9).

Improved recovery in the cell-free extract was also observed with SDS-PAGE analysis (Fig. 2.10–2.11) indicating the presence of higher concentrations of CYP505E3 variant in cell-free extract from run 4. However, a significant amount of expressed protein was also present within the insoluble membrane fraction. The conditions from the Plackett-Burman experiment run 4 was used in subsequent expression of CYP505E3 variant.

(a)



(b)

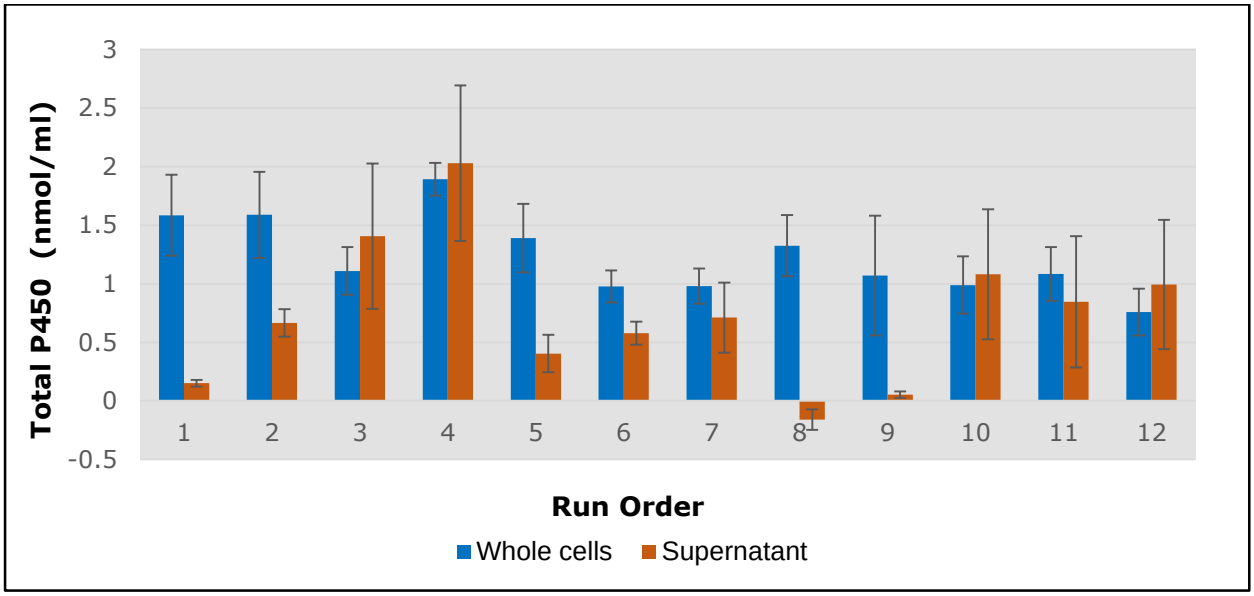


Figure 2.8. CYP505E3 variant Plackett-Burman CO difference spectra assay. (a) Maximum recovery obtained from the cell-free extract is indicated with the asterisk. (b) Recurrent negative concentration for run 8 observed in additional experiment.

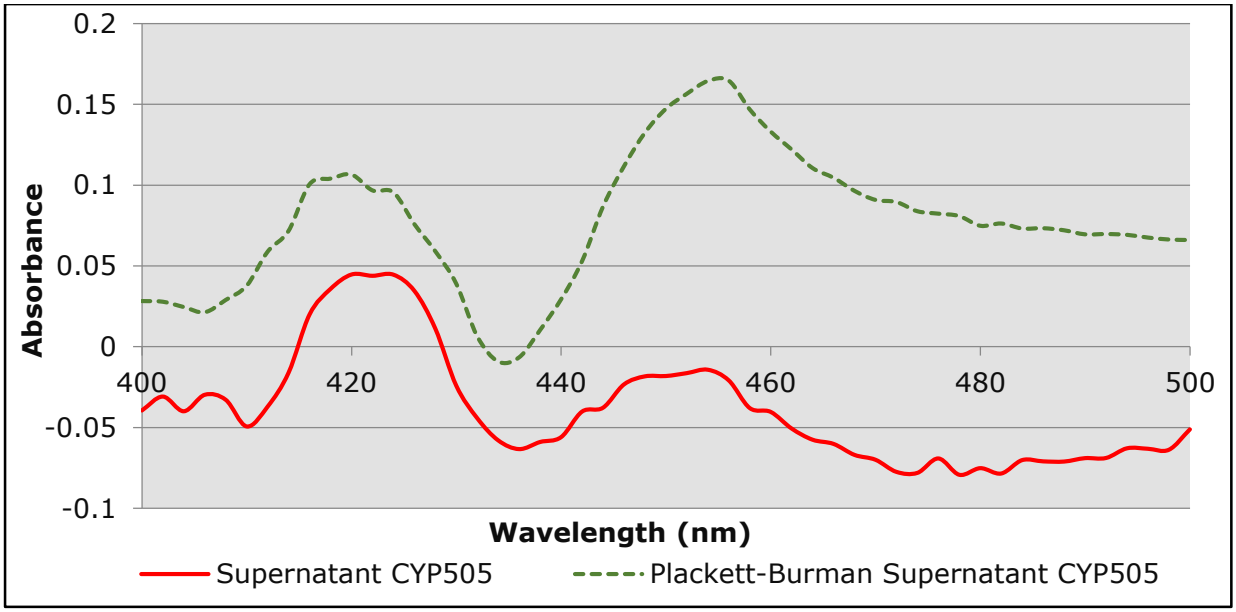
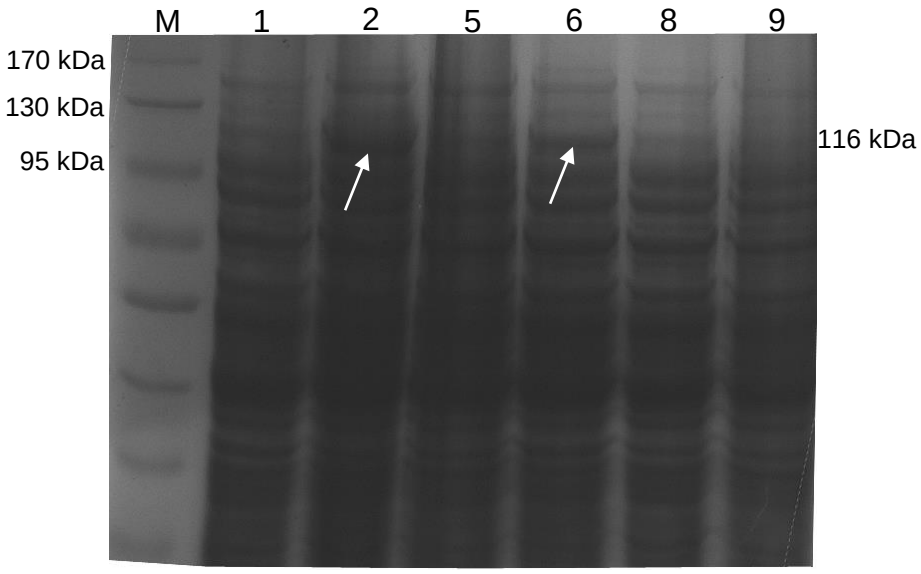


Figure 2.9. Cell-free extract CO difference spectra of the maximum A_{450} initially obtained and that of the Plackett-Burman run 4.

A



B

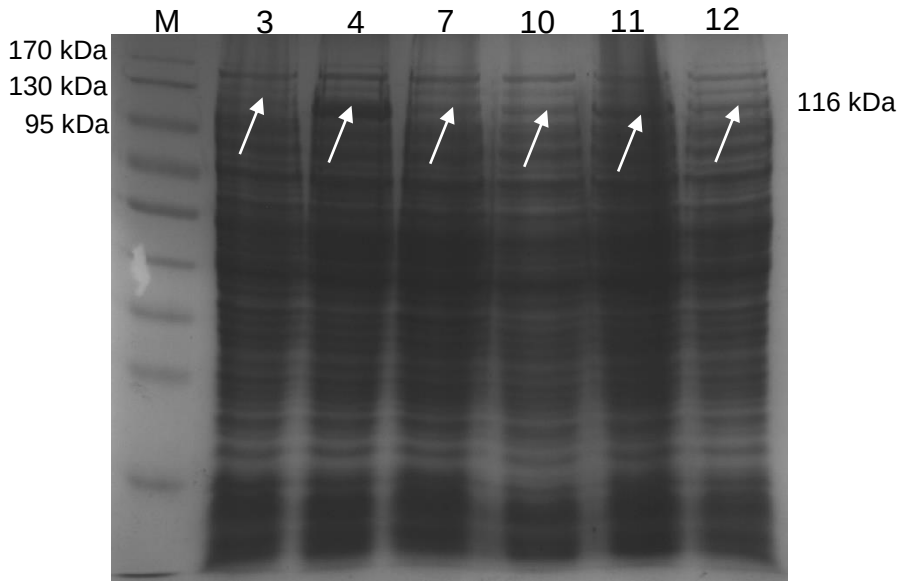
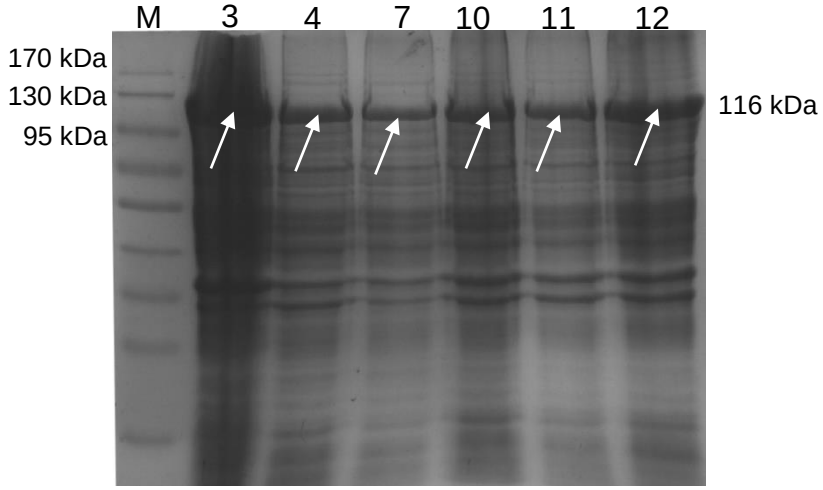


Figure 2.10. SDS-PAGE analysis of soluble fractions of cell free extracts from Plackett-Burman experiment on expression of CYP505E3 variant by *E. coli* cultures incubated for (A) 28 h; (B) 40 h. The lanes are labelled for the respective runs. The PageRuler™ prestained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

A



B

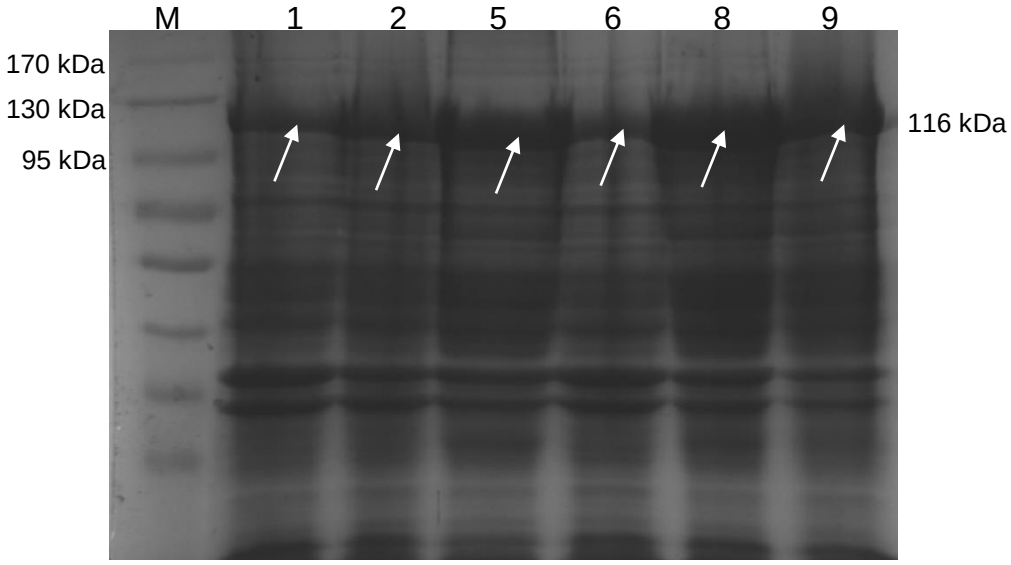


Figure 2.11 SDS-PAGE analysis of insoluble membrane fractions from Plackett-Burman experiment on expression of CYP505E3 variants by *E. coli* cultures incubated for (A) 40 h; (B) 28 h. The lanes are labelled for the respective run. The PageRuler™ prestained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

2.4.4. Substrate hydroxylation

Aspergillus terreus CYP450s obtained from microsomes showed activity towards alkanes, aromatic compounds and pristane (Vatsyayan *et al.*, 2008). The highest activity was observed with hexadecane (HD), pristane (PRI) and naphthalene (NA). These substrates as well as hexylbenzene (HB), nonylbenzene (NB), 4-hexylbenzoic

acid (HBA) and 4-nonyloxybenzoic acid (NOBA) were selected to test for activity of CYP505E3 in whole cell biotransformation experiments.

CYP505E3 variant showed activity towards HBA and NOBA indicated by spots observed on the TLC (Fig. 2.12). Multiple spots were observed on the TLC with HBA representing the formation of several products. A faint spot was observed on the TLC for NOBA indicating a product, but it was not followed up because the activity was very low. No activity could be detected for HB, NB, NA, HD, and PRI using TLC and GC analyses. HBA was used as substrate in further biotransformation experiments.

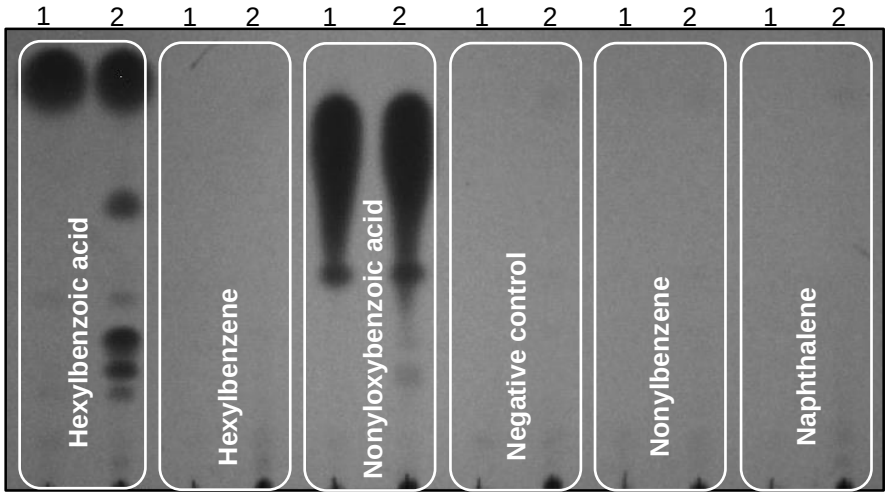


Figure 2.12. TLC results of tested substrates lane 1 is the control and lane 2 is the substrate tested for activity with CYP505E3 variant.

The effect of buffer concentration on product formation was tested by doing biotransformations using 50 mM and 200 mM phosphate buffer (pH 8) to determine the suitable buffer concentration for HBA biotransformation. The 200 mM phosphate buffer increased the concentration of HBA biotransformation products shown by the spot intensity when compared with the 50 mM (Fig. 2.13). Hence, 200 mM phosphate buffer was used in subsequent HBA biotransformation reactions.

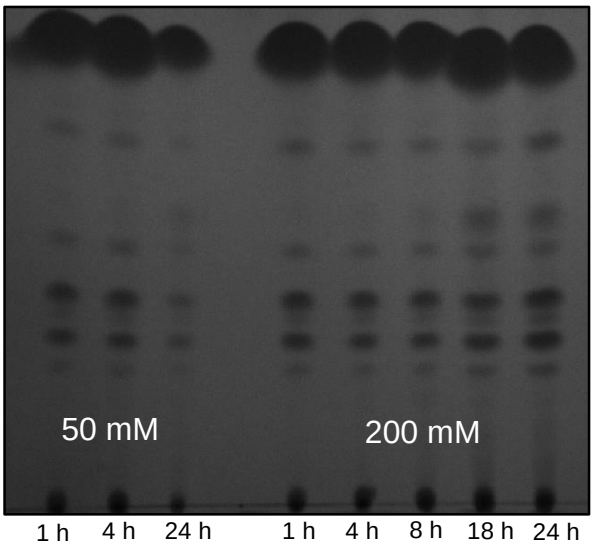


Figure 2.13. HBA hydroxylated with CYP505E3 variant tested in 50 mM and 200 mM phosphate buffer (pH 8).

Gudiminchi & Smit (2011) reported that the self-sufficient CYP102A1 converted HBA to ω-1 and ω-2 OH-HBA. This enzyme was used as a positive control with CYP505E3 variant for HBA hydroxylation. The CYP505E3 variant produced the same HBA hydroxylation products as the CYP102A1 (Fig. 2.14). However, CYP505E3 variant produced an additional hydroxylated product identified as ω-4 OH-HBA which was not produced by CYP102A1 (Fig 2.14). The GC-MS results for hydroxylated HBA produced by CYP505E3 variant and CYP102A1 are shown in Fig. 2.15 (GCs) and Fig. 2.16 (mass spectra).

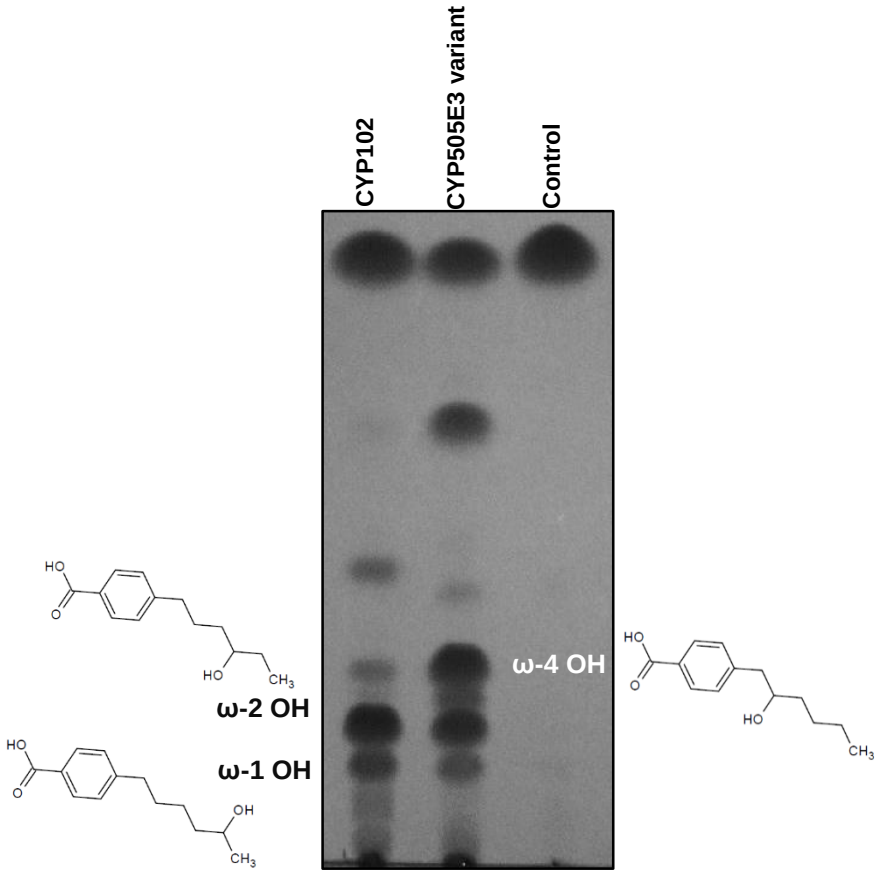


Figure 2.14. TLC comparison between CYP505E3 variant and CYP102A1 for HBA hydroxylation.

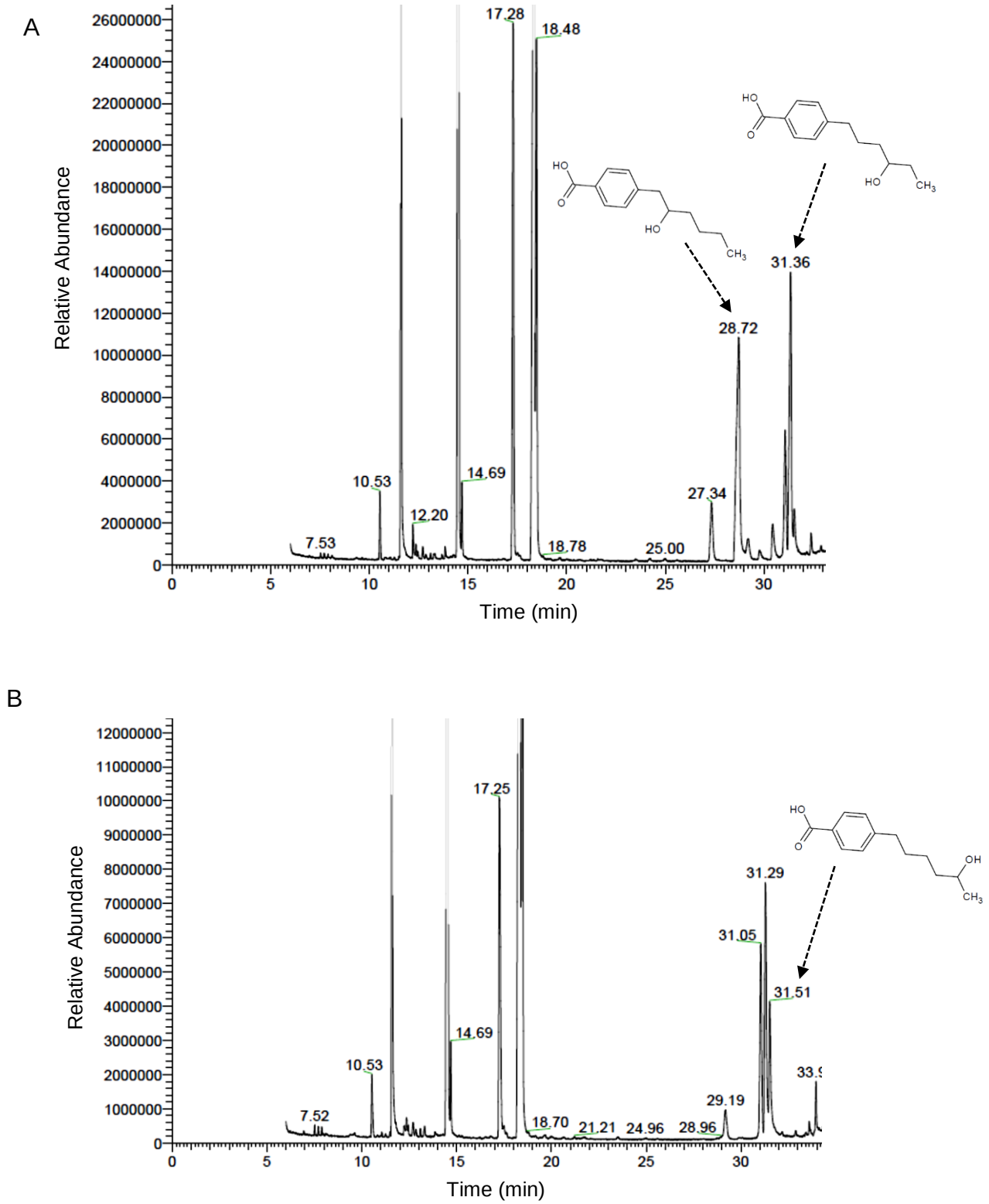
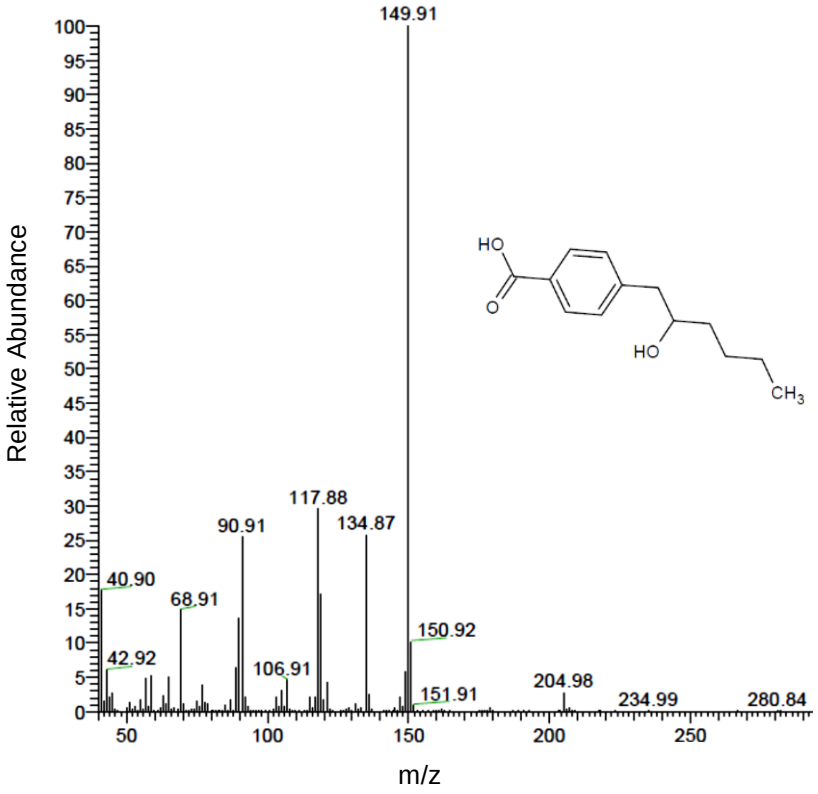
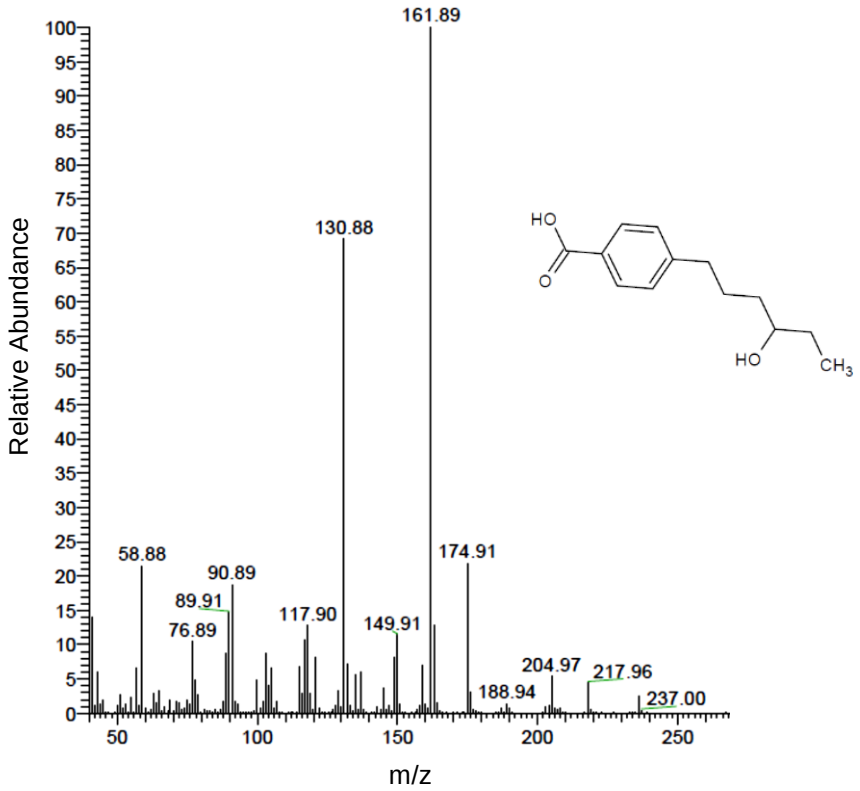


Figure 2.15. GC chromatograms of HBA conversion by CYP505E3 (A) and CYP102A1 (B) showing retention times of ω-4 OH-HBA, ω-2 OH-HBA and ω-1 OH-HBA.

A


Relative Abundance
m/z
149.91
117.88
90.91
134.87
40.90
68.91
150.92
42.92
106.91
204.98
151.91
234.99
280.84
HO
O
HO
CH3

B


Relative Abundance
m/z
161.89
130.88
58.88
90.89
89.91
76.89
117.90
149.91
174.91
204.97
217.96
188.94
237.00
HO
O
HO
CH3

C

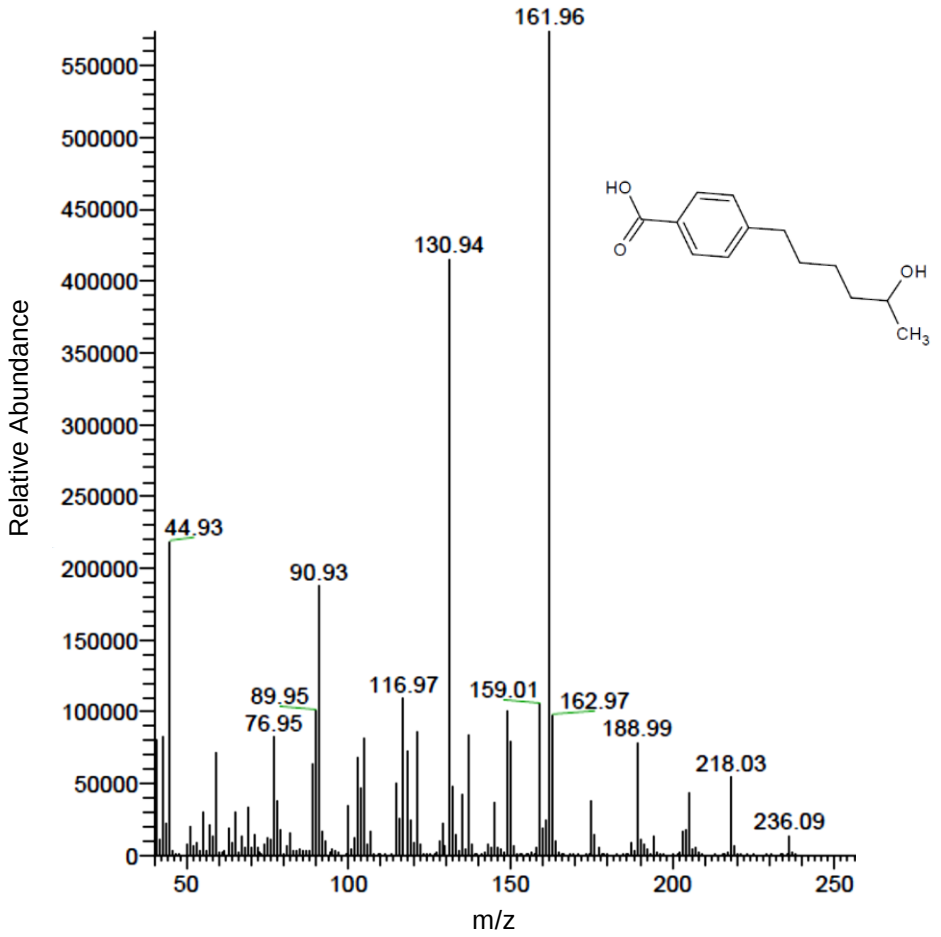


Figure 2.16. Mass spectra of (A) ω-4 OH-HBA (B) ω-2 OH-HBA (C) ω-1 OH-HBA.

2.4.5. Effect of growth conditions on HBA biotransformation

Whole cells containing CYP505E3 variant from the Plackett-Burman design experiment were used to hydroxylate HBA. The product concentration of ω-2 and ω-4 OH-HBA were estimated with a standard curve constructed with HBA. The production of more ω-4 OH-HBA as shown in Fig. 2.17 confirms that CYP505E3 variant prefers ω-4 hydroxylation and that the specificity is independent of expression conditions.

The Plackett-Burman statistical analysis showed that the concentration of $FeCl_3$ with a *p*-value of 0.03 was a significant factor which influenced CYP505E3 variant expression and activity in whole cells. The *p*-values of 0.05 for temperature, 0.10 for culture volume and 0.09 for the pH suggest that these factors may also be significant

in whole cell biotransformation (Fig. S4). The effects of these factors indicate low temperature, low $FeCl_3$, high culture volume and low pH would enhance whole cell biotransformation. Two of these factors namely low temperature and low $FeCl_3$ concentration also favoured recovery of CYP505E3 variant in the soluble fraction. The correlation between recovery of CYP505E3 variant in the soluble fraction and whole cell hydroxylation activity was however quite poor, indicating that factors other than CYP505E3 variant expression influences hydroxylase activity.

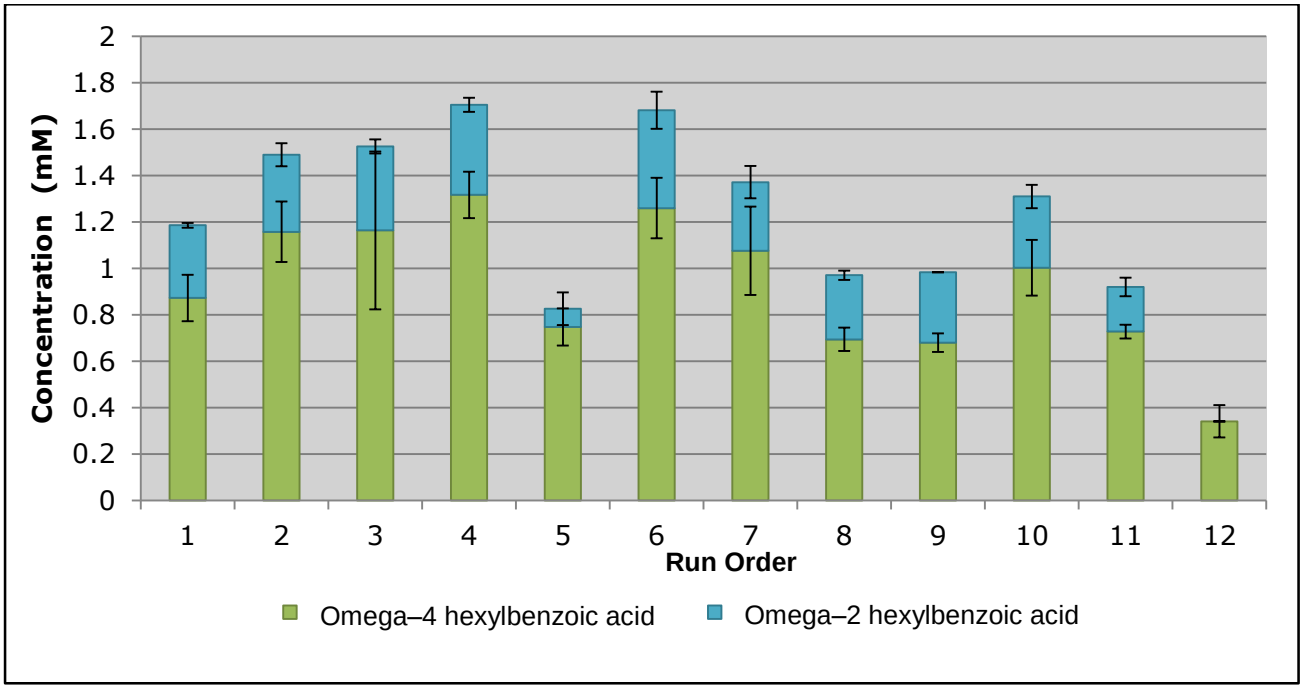


Figure 2.17. Bar chart representation of estimated ω-2 and ω-4 OH-HBA produced using whole cells containing CYP505E3 variant from the Plackett-Burman design experiment.

2.4.6. Comparison of wild-type and variant CYP505E3

In order to compare expression and activity of wild-type and variant CYP505E3, these proteins were expressed using the conditions of run 4 of the Plackett-Burman experiment. Comparison of the expression levels indicates that the whole cell concentration of the wild-type CYP505E3 was 35% higher than that of the variant CYP505E3. However, comparison of the concentration in cell-free extract indicate that recovery of the variant CYP505E3 in cell-free extract was 20% higher than that of the wild type CYP505E3 (Fig. 2.18). This confirms that the conditions of Plackett-

Burman run 4 improved recovery of the CYP505E3 variant in cell-free extract, but not necessarily that of the wild-type CYP505E3. Biotransformation activity and products obtained with the wild-type and variant were identical using HBA and NOBA as substrate (Fig. 2.19).

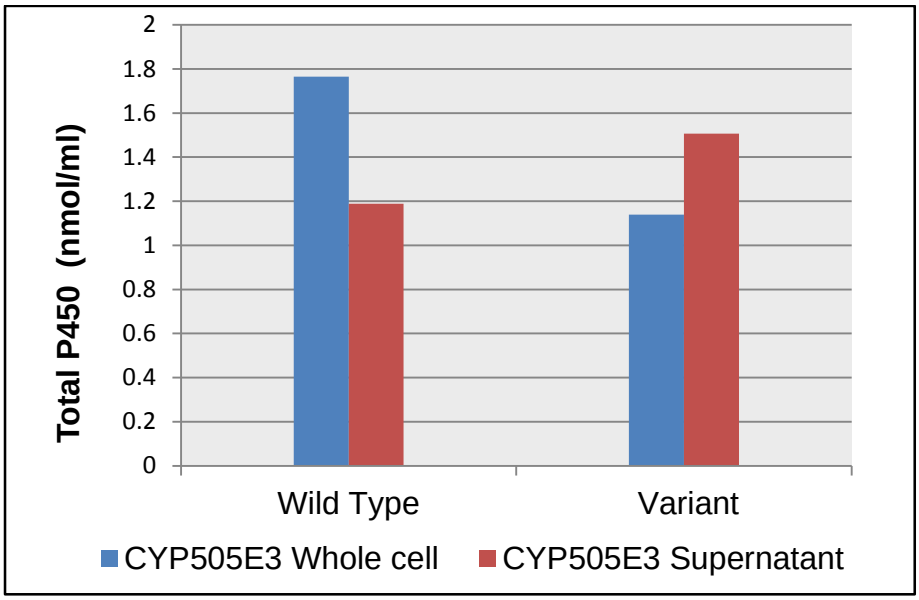


Figure 2.18. Comparison of CYP505E3 variant and wild-type expression using CO difference spectra assay. Expression was done using the conditions of run 4 of the Plackett-Burman experiment.

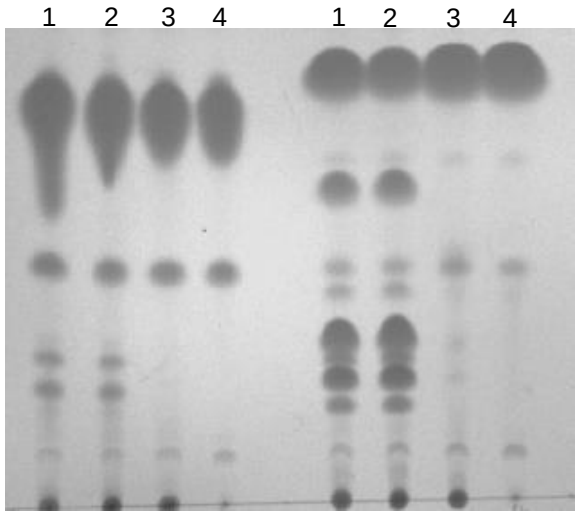


Figure 2.19. TLC of comparison of substrate hydroxylation by the wild-type and variant CYP505E3. Lane 1 is CYP505E3 wild-type, lane 2 is CYP505E3 variant, lane 3 is an empty pET28b vector and lane 4 is substrate control.

2.5. Conclusions

The discovery of the self-sufficient CYP450s has reduced the challenges encountered using CYP450s for substrate oxidation. Besides the well-known *Bacillus megaterium* CYP102A1 and *Fusarium oxysporum* CYP505A1, several other self-sufficient CYP450s in the bacterial and fungal kingdoms have been identified. The genome of *A. terreus* contains two self-sufficient CYP450s, however the homology between the CYP450s is evolutionarily distant.

Successful expression of *A. terreus* CYP505E3 variant was obtained with the whole cells. However, recovery in the cell-free extract was only 28%. The effect of expression conditions were investigated using statistically designed Plackett-Burman experiments. Cell-free extract recovery levels, were significantly improved with the use of a lower temperature, lower $FeCl_3$ concentration, longer harvest time and higher culture.

Aspergillus terreus CYP505E3 variant was able to catalyse the sub-terminal hydroxylation of HBA to produce ω-1 OH-HBA and ω-2 OH-HBA, similar to the products obtained for CYP102A1 (Gudiminchi & Smit, 2011) and CYP505A1 (Theron

et al., 2014). Additionally, CYP505E3 variant catalysed the oxidation of HBA to ω-4 OH-HBA. The comparison between ω-2 OH-HBA and ω-4 OH-HBA reveals that CYP505E3 is more specific towards ω-4 OH-HBA production.

Vatsyayan and co-workers (2008) reported high activity of *A*. *terreus* microsomal CYP450s towards HD, NA and PRI. The absence of CYP505E3 activity towards these substrates suggests other CYP450s in *A*. *terreus* are responsible for their oxidation. Comparison between expression levels and hydroxylated products indicates the expression conditions and composition could be a factor influencing concentration of the biotransformation products. The conversion of HBA by *A. terreus* CYP505E3 might indicate that CYP505E3 is also, like CYP102A1 and CYP505A1, a fatty acid hydroxylase.

2.6. References

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CHAPTER 3

Heterologous expression of *Cryptococcus neoformans* cytochrome P450s and their role in arachidonic acid metabolism

3.1. Abstract

The opportunistic yeast pathogen *Cryptococcus neoformans* was found to produce authentic immunomodulatory prostaglandin E_2 from arachidonic acid (AA). The biosynthetic pathway used by this organism has to be unusual given the absence of cyclooxygenase (COX) and lipoxygenase (LOX) genes within its genome. A linear pathway involving the laccase enzyme (Lac1) was proposed. However, enzymes upstream and downstream of this enzyme are still unknown. Five cytochrome P450 monooxygenase (CYP450) genes and a cytochrome P450 reductase (CPR) were identified within the genome of this organism and investigated for eicosanoid production. These genes were cloned in appropriate vectors and co-expressed with pRARE or groES-groEL molecular chaperones in *E. coli* to improve soluble expression. The expected peak at 450 nm was not observed in the CO difference spectra of all the cloned *C. neoformans* CYP450s. However, the SDS-PAGE analysis indicated probable expression with proteins bands which corresponds to the expected molecular weights. The co-expression of *C. neoformans* CYP450s with the groES-groEL molecular chaperones prevented the formation of low molecular weights protein bands earlier observed in two CYP450s and the CPR. Whole cell biotransformations were carried out with expressed *C. neoformans* CYP450s. However, they showed no activity towards tested substrates.

3.2. Introduction

Oxylipins constitute a large family of structurally related oxygenated polyunsaturated fatty acids (PUFA) (Tsitsigiannis & Keller, 2007) which include the highly important C20 PUFA group known as eicosanoids (Noverr *et al.*, 2003). Eicosanoids are involved in essential physiological processes and are mainly synthesised from free arachidonic acid (AA) via oxidation reactions. In mammals, the eicosanoid precursor

AA, is oxidized by cyclooxygenases (COX), lipoxygenase (LOX) and cytochrome P450s (CYP450) in different pathways to produce prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and epoxides (Harizi *et al.*, 2008). The physiological response of inflammation by living organisms to trauma, infections or immunological deficiencies is facilitated by different stimulators including eicosanoids. During inflammation, the level of eicosanoid production increases (Harizi & Gualde, 2004). However, besides the host, the pathogen is another source of eicosanoids (Noverr *et al.*, 2003). Fungal pathogens which includes *Aspergillus fumigatus* (Kupfahl *et al.*, 2012), *Candida albicans* (Erb-Downward & Noverr, 2007) and *C. neoformans* (Erb-Downward & Huffnagle, 2007) have been shown to synthesise eicosanoids.

Cryptococcus neoformans was identified to produce immunomodulatory prostaglandin E_2 from AA (Erb-Downward & Huffnagle, 2007), but the mechanism used by the organism is not completely understood. The inability of known COX inhibitors such as aspirin and indomethacin to inhibit Cryptococcal prostaglandin production indicates the absence of a COX pathway. In addition, the genome search of four *Cryptococcus* serotypes also revealed the absence of a COX or LOX homologue (Erb-Downward & Huffnagle, 2007; Erb-Downward *et al.*, 2008). Cryptococcal laccase (*Lac1*), a multicopper oxidase has been shown to contribute to prostaglandin production by metabolising prostaglandin G_2 (PGG_2) to PGE_2 and 15-keto PGE_2 (Fig. 3.1), but enzymes responsible for PGG_2 production and conversion of PGE_2 to $PGF_{2\alpha}$ are unknown (Erb-Downward *et al.*, 2008).

The CYP450 enzymes in mammals catalyse the oxidation of the eicosanoid precursor AA to produce several mid-chain hydroxyeicosatetraenoic acids (HETEs), ω-HETEs and epoxyeicosatrienoic acids (EETs) (Zeldin, 2001). The HETEs are produced in the ω-hydroxylase pathway by the CYP4A and CYP4F subfamily while the EETs are produced in the epoxygenase pathway by the CYP2C and CYP2J subfamily (Panigrahy *et al.*, 2010). These CYP450 derived eicosanoids are essential signalling compounds in renal tubular control and vascular functions (Sarkis *et al.*, 2005). The genome of the eicosanoid producing yeast *C. neoformans* lacks a LOX and COX homologue. However, it has five CYP450 genes and a CYP450 reductase

(CPR) gene within its genome. The aim of this chapter was to investigate the probable roles of these CYP450s in Cryptococcal prostaglandin production.

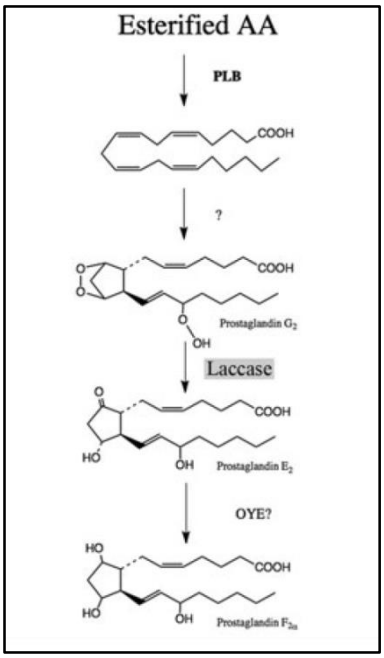


Figure 3.1. Suggested linear model for Cryptococcal prostaglandin synthesis involving the Lac1 enzyme (Erb-Downward *et al.*, 2008).

3.3. Materials and Methods

3.3.1. General experimental procedures

3.3.1.1. Chemicals and enzymes

Chemicals and reagents used were analytical grade and were, unless otherwise stated, supplied by either Fluka, Sigma-Aldrich or Merck. Restriction endonucleases, T4 DNA ligase, molecular weight markers and modification enzymes were obtained from Thermo Fisher Scientific.

3.3.1.2. Restriction digest and ligation

Plasmid DNA restriction endonuclease digestions and ligations were carried out as described in section 2.3.1.2. The sticky ends obtained were filled with nucleotide bases in a reaction mixture containing 1 U Klenow fragment, 0.5 mM dNTP and 1X Klenow buffer. The mixture was incubated at 37 ^{o}C for 10 min with further incubation at 75 ^{o}C for 10 min to deactivate the Klenow fragment.

3.3.1.3. Transformation into *E. coli*, small scale plasmid isolation and agarose gel electrophoresis

Transformation of plasmid DNA into chemically competent *E. coli* cells, small scale plasmid isolation and agarose gel electrophoresis were carried out as described in section 2.3.1.3.

3.3.1.4. SDS-PAGE electrophoresis

SDS-PAGE gels were cast and stained as described in section 2.3.1.4.

3.3.2. *Escherichia coli* expression of *Cryptococcus neoformans* CYP450s

Sequenced CYP450 genes of *C. neoformans* var. *grubii* H99 were supplied in pET28 vectors by Dr. R. Ells. The genes were transformed into chemically competent *E. coli* BL21 (DE3) strain and expressed as described in section 2.3.3. After expression, the cells were harvested by centrifugation at 6000 *x g* at 4 ^{o}C for 10 min and resuspended in a 1:3 (w/v) ratio in 20 mM MOPS buffer (pH 7.0, 10% glycerol, 0.1 M NaCl, 1 mM DDT). Whole-cell CO difference spectra were recorded as described in section 2.3.4.

3.3.3. Construction of pETDuet vector containing *C. neoformans* CYP450 genes

Recombinant pETDuet vectors with *C. neoformans* CYP450 gene in the first multiple cloning site (MCS) and CPR in the second MCS were constructed for co-expression. The CNAG_01003 gene (CPR) was modified by PCR to include an *Nde*I site on the 5` end and a *Mun*I site on the 3` end for insertion into the second MCS of pETDuet. The PCR reaction comprised 0.5 U KAPA Taq polymerase, 1X PCR buffer containing 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of primers 5´-GCATATGTTGAGCGCGGTAGACATCG-3´ (to introduce *Nde*I site) and 5´-GCAATTGCTAACTCCAGACATCGGTCATCAAC-3´ (to introduce *Mun*I site), DNA template and PCR grade water. The PCR protocol selected, comprised an initial denaturation at 95 °C for 3 min, followed by 30 cycles with denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 2 min and 25 s. This was then followed by a final extension at 72 °C for 2 min and 25 s.

3.3.4. Co-expression of *C. neoformans* CYP450 with pRARE or pGro7

Recombinant plasmids containing *C. neoformans* CYP450 genes were transformed into chemically competent *E. coli* BL21 (DE3) strains containing either the pRARE (Novagen, SA) plasmid or the pGro7 plasmid containing the *E. coli* groES-groEL molecular chaperone encoding genes (Takara, Japan). Expression of the molecular chaperones was induced with 0.5 mg arabinose ml^{-1} while 20 µg chloramphenicol ml^{-1} was added for selection. Expressed *C. neoformans* CYP450s and molecular chaperone proteins were analysed with SDS-PAGE.

3.3.5. Whole cell biotransformation of hexylbenzoic acid

CNAG_02841 and CNAG_04029 co-expressed with CNAG_01003 (CPR) in pETDuet were tested for hydroxylase activity towards hexylbenzoic acid. The reaction set up, extraction and TLC analysis were performed as described in section 2.3.6. However, the buffer used in this reaction was 20 mM MOPS (pH 7.0, 10% glycerol, 0.1 M NaCl, 1 mM DDT).

3.3.6. Whole cell biotransformation of arachidonic acid (AA)

CNAG_04029, CNAG_05942, CNAG_00040 and CNAG_06644 co-expressed with CNAG_01003 (CPR) in pETDuet were tested for activity towards AA. The biotransformation reaction was set up as described in section 2.3.6 using 1 ml 1:3 (w/v) resuspended cells and 1 ml of 20 mM MOPS buffer (pH 7.0, 10% glycerol, 0.1 M NaCl, 1 mM DDT) while AA was added to the reaction to a final concentration of 500 µM. After 24 h incubation, the cells were washed three times and resuspended to the original volume with 20 mM MOPS buffer, while 160 µl 1 N citric acid was added as an antioxidant. The cells were lysed with a cell disruptor (Constant Systems) at 30 kpsi, collected in glass tubes and placed on ice. Two millilitres of hexane : ethyl acetate (1:1, v/v) was added to the broken cells and the solution was vortexed for 1 min. The mixture was centrifuged at 1800 *g* for 10 min at 4 ^{o}C after which the organic phase was collected. Extraction from the lysed cells was done three times, after which the collected organic phase was dried under a stream of N_2 gas at room temperature. This was followed by resuspension in ethyl acetate and TLC analyses as described in section 2.3.6. The resolved TLC plate was sprayed with 20% phosphomolybdic acid dissolved in ethanol and incubated in a 60 ^{o}C oven for 15 min.

3.4. Results and Discussions

3.4.1. Identification of *C. neoformans* CYP450 genes

The amino acid sequences of *C. neoformans* CYP450s were obtained from the *C. neoformans var. grubii* H99 sequencing project webpage, managed by the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). The CYP450 families of *C. neoformans* CYP450s were obtained from sequence alignment scores on the DR Nelson CYP450 database (http://drnelson.uthsc.edu/CytochromeP450.html), while the probable function of *C. neoformans* CYP450s were predicted from the characterized functions of related CYP450 families.

CNAG_00040 and CNAG_06644 shared 56% and 61% similarity with CYP450 families from *P. chrysosporium* and *Ustilago maydis* respectively (Table 3.1) while the CYP450 family of CNAG_04029 is unidentified. The CYP450 with known activity

that CNAG_05842 shared the highest identity (35%) with is the β-carotene hydroxylase from the astaxanthin producing yeast *Xanthophyllomyces dendrorhous* (Álvarez *et al.*, 2006)**.** CNAG_02841 shows relatively high identity (31%) with CYP63A3 from *Phanaerochaete chrysosporium*, which belongs to the CYP52 clan that include alkane and fatty acid hydroxylases (Van Bogaert *et al.*, 2011).

The open reading frames coding for the *C. neoformans* CYP450 genes were amplified from an RNA template isolated from the organism and ligated into pET28 vectors by Dr. R. Ells. The earlier submitted sequence of CNAG_04029 which we used to design primers has been updated on the Broad Institute database to include a new start codon upstream the existing sequence and thus an additional 12 amino acids (Fig. 3.2). The CNAG_04029 gene amplified from the *C. neoformans* cDNA template did not include the additional 12 amino acids on the N-terminus.

Table 3.1. *C. neoformans* Cytochrome P450 genes with their predicted functions.

Accession number	Possible Function	CYP450 Family	Percentage identity
CNAG_05842	β-carotene hydroxylase	CYP5139B2	35% to product of *crtS* from *Xanthophyllomyces dendrorhous*
CNAG_02841	Fatty acid hydroxylase	CYP5216A2	31% to *P. chrysosporium* CYP63A3
CNAG_06644	C_{22} Sterol Desaturase	CYP61	61% to *Ustilago maydis* CYP61A1
CNAG_00040	Lanosterol 14α – demethylase	CYP51	56% to *P. chrysosporium* CYP51F1
CNAG_04029	Unknown	CYP5215	
CNAG_01003	CYP450 Reductase		

```
OldCNAG_04029      ------------MIGSILLALVILVVWLLHVFVYKSFTSALKNVPCPPGGNGSQGHIGEI
NewCNAG_04029      MMLKQLSFQPPTMIGSILLALVILVVWLLHVFVYKSFTSALKNVPCPPGGNGSQGHIGEI
                             **********************************************

OldCNAG_04029      MNLQGIEVHQWIRTYGSTFMVRGPFGVHHRIFTIDPRALSHVLQHTNIYTKSDLLRDLVR
NewCNAG_04029      MNLQGIEVHQWIRTYGSTFMVRGPFGVHHRIFTIDPRALSHVLQHTNIYTKSDLLRDLVR
                   ************************************************************

OldCNAG_04029      RYMKEGLIVAEGERHKVQRKVSQKLFSMGGLKSMGQIVQDKSNQLRDILLNLCANPTASN
NewCNAG_04029      RYMKEGLIVAEGERHKVQRKVSQKLFSMGGLKSMGQIVQDKSNQLRDILLNLCANPTASN
                   ************************************************************

OldCNAG_04029      PYSPVNRTLPPGSREVDVYSAASRCTFDLIGSIGVDHQFDSIADWEGSGGKLFHKYEQMQ
NewCNAG_04029      PYSPVNRTLPPGSREVDVYSAASRCTFDLIGSIGVDHQFDSIADWEGSGGKLFHKYEQMQ
                   ************************************************************

OldCNAG_04029      LLCPGAMGIRMLLSLTWPLVDRIWPSENTKRVNDAMGSLEKFAKEKMIERQQELLTMDSK
NewCNAG_04029      LLCPGAMGIRMLLSLTWPLVDRIWPSENTKRVNDAMGSLEKFAKEKMIERQQELLTMDSK
                   ************************************************************

OldCNAG_04029      KGDVPDRRDLLTLMLRHNMSRKISPADKLRDHEISGQLSTFMFAGSETTAGTISFGLYDL
NewCNAG_04029      KGDVPDRRDLLTLMLRHNMSRKISPADKLRDHEISGQLSTFMFAGSETTAGTISFGLYDL
                   ************************************************************

OldCNAG_04029      ARHPDVQSRLRAEIFECGDNLPFDQIDELPYLDAVVKEIMRINPSLPGTVRQAQKDDIIP
NewCNAG_04029      ARHPDVQSRLRAEIFECGDNLPFDQIDELPYLDAVVKEIMRINPSLPGTVRQAQKDDIIP
                   ************************************************************

OldCNAG_04029      LAEPVTLTNGKVGTDIHIQKGQLVHVPIEHLHTSEHIWGPTAKEFDPSRFISTSQPAAFS
NewCNAG_04029      LAEPVTLTNGKVGTDIHIQKGQLVHVPIEHLHTSEHIWGPTAKEFDPSRFISTSQPAAFS
                   ************************************************************

OldCNAG_04029      DQLTLASNPVATFSARRDAVPSYVPEGPGIWPNFMTFIDGPRRCIGYKLAVMEIKTVIFT
NewCNAG_04029      DQLTLASNPVATFSARRDAVPSYVPEGPGIWPNFMTFIDGPRRCIGYKLAVMEIKTVIFT
                   ************************************************************

OldCNAG_04029      LLREFEIEPVEGQHIFRWNMMSNRPFVANTLWSKGSRLPLHFKLYKGEQ
NewCNAG_04029      LLREFEIEPVEGQHIFRWNMMSNRPFVANTLWSKGSRLPLHFKLYKGEQ
                   *************************************************
```

Figure 3.2. Amino acid alignment of earlier deposited CNAG_04029 and the updated CNAG_04029. The analysis was conducted with the EMBL–EBI Clustal Omega multiple sequence alignment program version 1.2.1 (Goujon *et al.*, 2010). The identical residues are shown by asterisks.

3.4.2. Expression of *C. neoformans* CYP450 in pET28

The CO difference spectrum maximum A_{450} peak, which indicates the presence of correctly folded and active CYP450, was not observed in the expressed *C. neoformans* CYP450s. However, a peak at A_{420} was observed for all the tested *C. neoformans* CYP450s (Fig. 3.3) which might indicate a CYP450 form associated with the loss of the A_{450} (Guengerich *et al.*, 2009). However, the empty vector control cells also gave a peak at 420 nm, which makes it difficult to distinguish between lack of expression and inadequate folding.

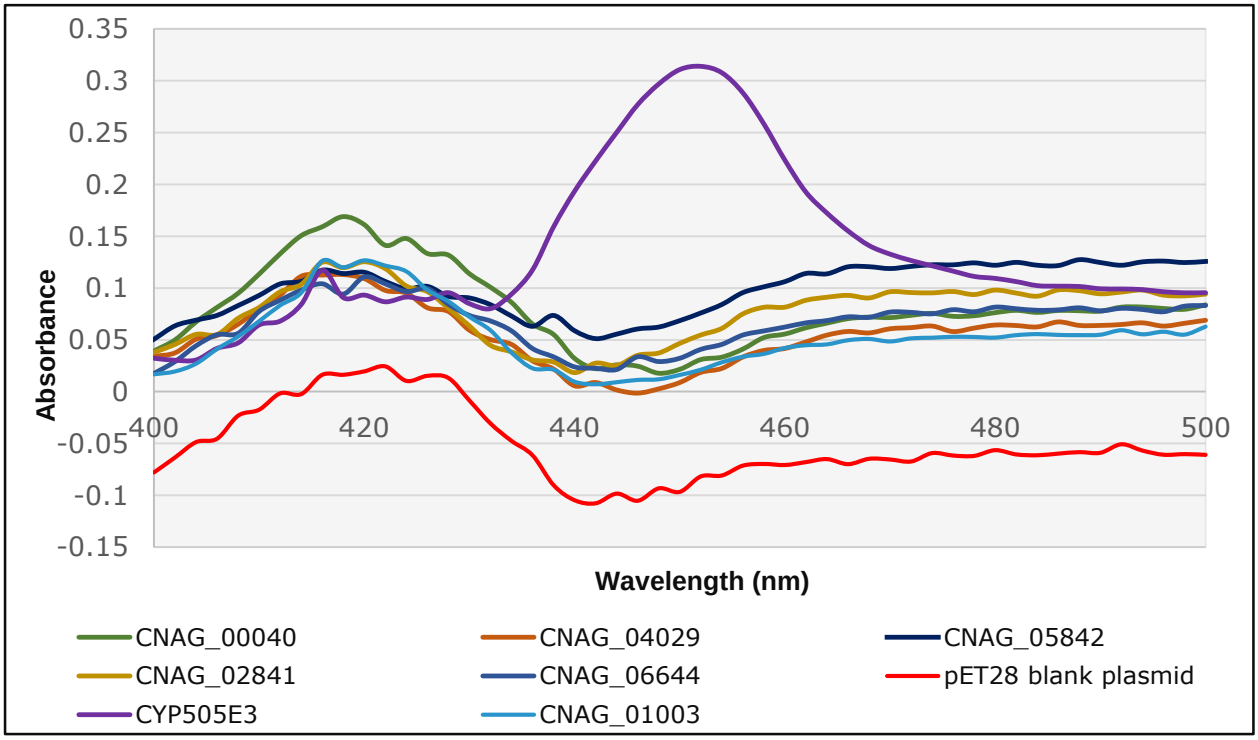


Figure 3.3. *C. neoformans* CYP450 CO difference spectra showing A_{420} peak obtained while the expected A_{450} peak is the CYP505E3 control in purple.

3.4.3. SDS-PAGE analysis of expressed *C. neoformans* CYP450 in pET28

Whole cell *C. neoformans* CYP450s expressed in pET28 vectors were subjected to SDS-PAGE analysis to confirm the presence of the expressed protein. The expected molecular weights for *C neoformans* CYP450s were predicted with the ProtParam tool on the ExPASy server (Gasteiger *et al*., 2005) and the expected sizes are shown in Table 3.2.

Table 3.2. Molecular weight of *C. neoformans* CYP450s.

Accession number	Molecular Weight (kDa)
CNAG_06644	59.9
CNAG_00040	61.7
CNAG_05842	64.6
CNAG_02841	64.8
CNAG_04029	65
CNAG_01003	82.2

Expressed proteins indicated by bands corresponding to the predicted molecular weight were observed for CNAG_04029, CNAG_05842 and CNAG_00040 as shown in Fig. 3.4. However, the highest expression was observed with CNAG_04029 which was indicated by the presence of a protein band of approximately 65 kDa. The expression of CNAG_01003, CNAG_06644 and CNAG_02841 did not produce protein bands which corresponded to the predicted molecular weights, but rather low molecular weight bands of approximately 30 kDa were observed. The low molecular weight bands observed were presumed to be due to *E. coli* codon bias or incorrect folding resulting in protein truncation.

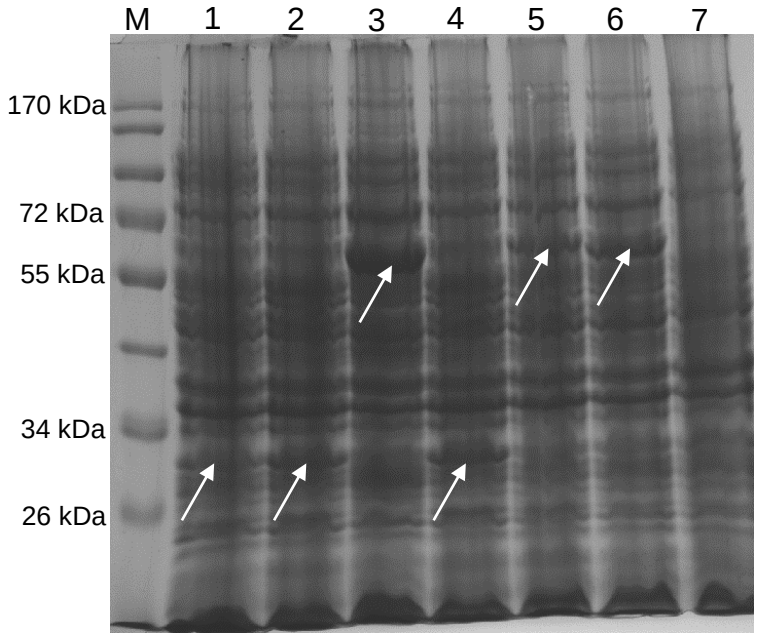


Figure 3.4. SDS-PAGE analysis of whole cells of *E. coli* expressing *C. neoformans* CYP450s cloned into pET28. Lane 1 is CNAG_01003 (82.2 kDa) - CYP450 Reductase. Lane 2 is CNAG_06644 (59.9 kDa) - C_{22} Sterol desaturase. Lane 3 is CNAG_04029 (65 kDa) - Unknown. Lane 4 is CNAG_02841 (64.8 kDa) - Fatty acid hydroxylase. Lane 5 is CNAG_05842 (64.6 kDa) – β-carotene hydroxylase. Lane 6 is CNAG_00040 (61.7 kDa) - Lanosterol 14α demethylase. Lane 7 is a blank pET28 vector - negative control. The PageRuler™ prestained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

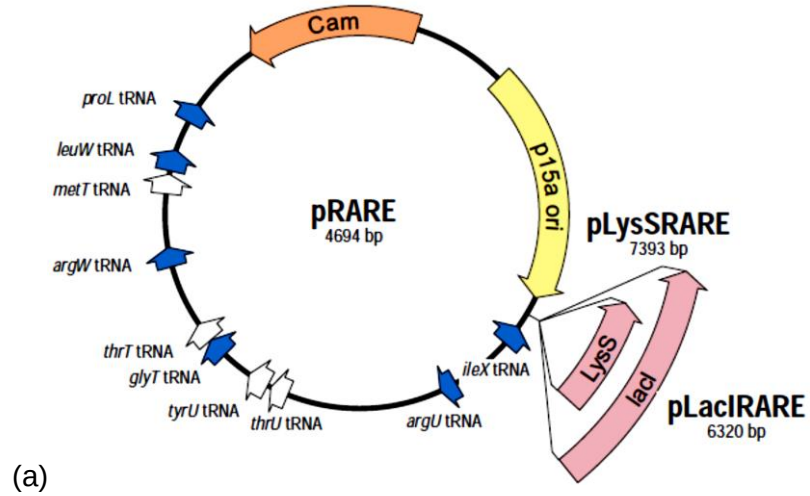
3.4.4. Co-expression of *C. neoformans* CYP450s cloned into pET28 with pRARE or groES-groEL molecular chaperones

Analysis of the *C. neoformans* CYP450 genes indicated the presence of a number of rare *E. coli* codons (Table 3.3). CNAG_04029, CNAG_05842 and CNAG_00040 which showed expression (Fig. 3.4), were co-expressed with the pRARE vector (Fig. 3.5(a)). These genes in pET28 were co-expressed with pRARE to supplement the rare *E. coli* codons during expression and improve expression levels of the proteins (Table 3.4).

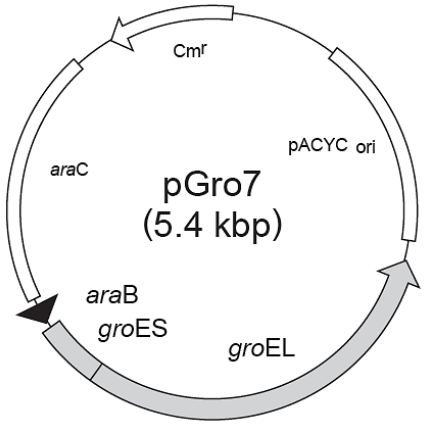
Table 3.3. Number of rare *E. coli* codons identified within *C. neoformans* CYP450 genes. The rare codons were determined using the NIH MBI Rare Codon Calculator (RaCC).

	Arginine	Leucine	Isoleucine	Proline	Total	Double Tandem Rare Codon
00040	17	-	-	19	36	3
01003	22	3	1	17	43	1
02841	24	11	10	10	55	-
04029	24	5	8	6	43	4
05842	16	5	2	12	35	-
06644	22	-	-	24	46	-

*Arginine- AGG, AGA, CGA Leucine- CTA Isoleucine- ATA Proline- CCC



(a)



(b)

Figure 3.5. (a) pRARE plasmid carrying the genes coding tRNA that are rare in *E. coli* (b) pGro7 plasmid carrying the genes for the groES-groEL molecular chaperones.

Table 3.4. *Cryptococcus neoformans* CYP450 genes in pET28 co-expressed with pRARE or groES-groEL molecular chaperones.

Accession number	Co-expression in pET28
CNAG_00040	pRARE
CNAG_05842	pRARE
CNAG_04029	pRARE
CNAG_06644	groES-groEL
CNAG_02841	groES-groEL
CNAG_01003	groES-groEL

There was no difference in the CO difference spectra obtained for *C. neoformans* CYP450 genes expressed alone in pET28 and those co-expressed with pRARE. SDS-PAGE analysis of the co-expressed CYP450 genes showed no significant improvement in expression level using pRARE. Additionally, the highly expressed CNAG_04029 gene had a much lower expression level when co-expressed with pRARE (Fig. 3.6).

Molecular chaperones contribute to *de novo* folding of recombinant proteins and prevent inclusion body formation hence, improving protein solubility (Khow & Suntrarachun, 2012). The *E. coli* molecular chaperones groES-groEL (Fig. 3.5(b)) were co-expressed with CNAG_01003, CNAG_06644 and CNAG_02841 genes (Table 3.6) which previously produced low molecular weight protein bands (Fig. 3.4). The earlier observed low molecular weight protein bands were absent in the SDS-PAGE analysis with molecular chaperone co-expression (Fig. 3.6). However, the protein bands for CNAG_06644 and CNAG_02841 was not distinct on the SDS-PAGE gel due to possible superimposition with groEL which has a similar molecular weight of approximately 60 kDa (Fig. 3.6). The comparison between the co-expressed CYP450 genes and the positive molecular chaperone control indicated the presence of higher protein concentration at the positions corresponding to the CYP450s. This suggests the likely presence of more than one expressed protein indicating that CNAG_06644 and CNAG_02841 were expressed in soluble form.

The cell free extract SDS-PAGE analysis showed no indication of expressed CNAG_01003 (CPR) with a molecular weight of 82.2 kDa. However, a protein band corresponding to the molecular weight of CNAG_01003 was observed within the insoluble fraction (Fig. 3.7). The presence of CNAG_01003 within the insoluble fraction could be due to limitation of groES-groEL active site to proteins with molecular weights less than 60 kDa as described by Martínez-Alonso and co-workers (2010).

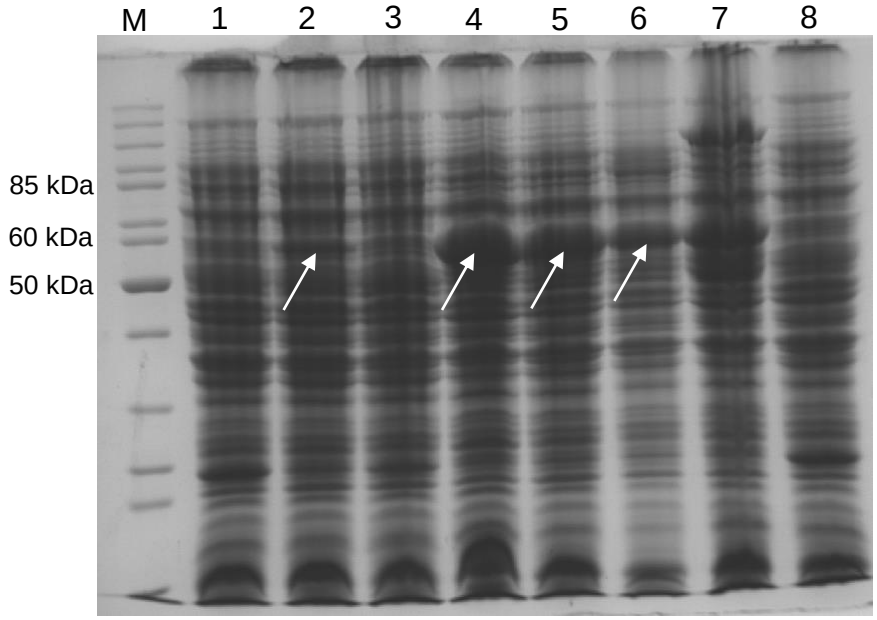


Figure 3.6. SDS-PAGE analysis of soluble fraction from disrupted *E. coli* cells expressing *C. neoformans* CYP450 cloned in pET28. Lanes 1-3 were co-expressed with pRARE while lanes 4-6 were co-expressed with groES-groEL molecular chaperones. Lane 1 is CNAG_00040 (61.7 kDa) - lanosterol 14α demethylase. Lane 2 is CNAG_04029 (65 kDa) - Unknown. Lane 3 is CNAG_05842 (64.6 kDa) – β-carotene hydroxylase. Lane 4 is CNAG_02841 (64.8 kDa) - fatty acid hydroxylase. Lane 5 is CNAG_06644 (59.9 kDa) - C_{22} sterol desaturase. Lane 6 is CNAG_01003 (82.2 kDa) - CYP450 reductase. Lane 7 is a groES-groEL molecular chaperones negative control. Lane 8 is a pRARE negative control. The PageRuler™ unstained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

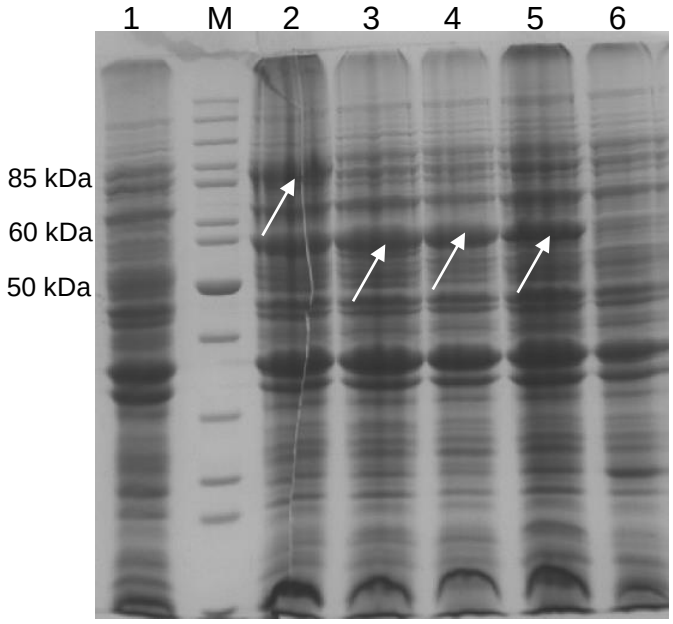


Figure 3.7. SDS-PAGE analysis of insoluble fractions from disrupted *E. coli* cells expressing *C. neoformans* CYP450s cloned in pET28. Lanes 1, 5 and 6 were co-expressed with pRARE while lanes 2-4 were co-expressed with groES-groEL molecular chaperones. Lane 1 is CNAG_05842 (64.6 kDa) – β-carotene hydroxylase. Lane 2 is CNAG_01003 (82.2 kDa) - CYP450 reductase. Lane 3 is CNAG_06644 (59.9 kDa) - C_{22} sterol desaturase. Lane 4 is CNAG_02841 (64.8 kDa) - fatty acid hydroxylase. Lane 5 is CNAG_04029 (65 kDa) - Unknown. Lane 6 is CNAG_00040 (61.7 kDa) - lanosterol 14α demethylase. The PageRuler™ unstained protein ladder is labelled M while the expressed proteins are indicated by white arrows.

3.4.5. Construction of pETDuet vectors containing *C. neoformans* CYP450 genes

A functional eukaryotic CYP450 requires a CYP450 reductase to supply electrons required for its catalytic reactions (Van Bogaert *et al.*, 2011). The *C. neoformans* CYP450s and *C. neoformans* CPR were co-expressed with a pETDuet vector (Fig. 3.8) which contains two multiple cloning sites. The co-expression was necessary to obtain a functional *C. neoformans* CYP450 having the CPR required for electron supply. The sequence length for the *C. neoformans* CYP450s are shown in Table 3.5.

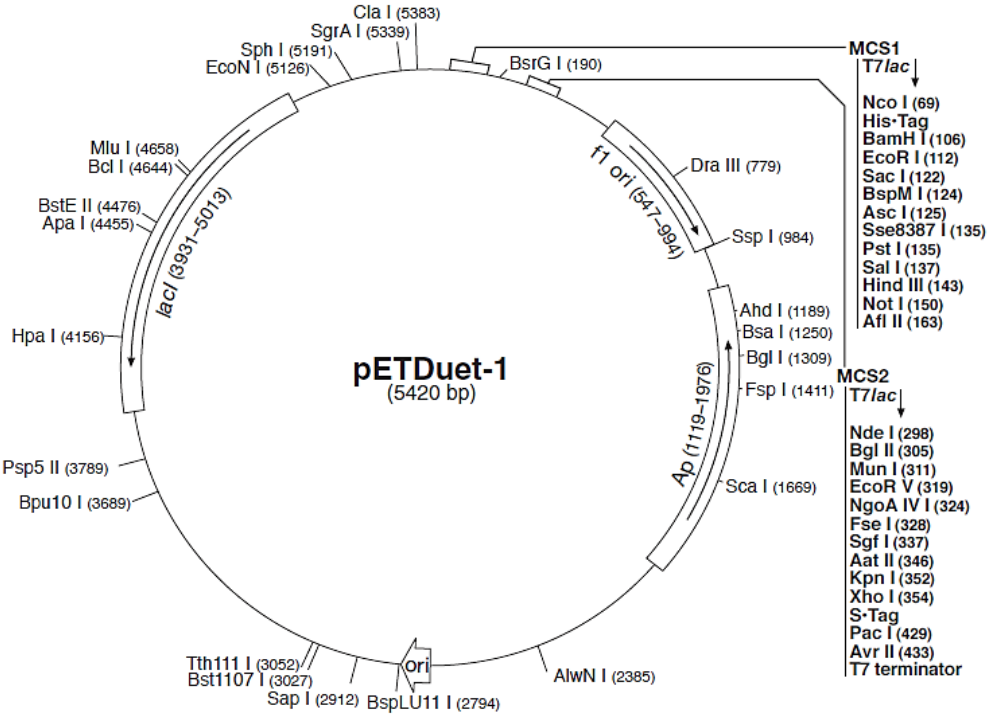


Figure 3.8. Plasmid diagram of a pETDuet plasmid showing the two multiple cloning sites.

Table 3.5. Nucleotide length of *C. neoformans* CYP450s genes.

Accession number	Length (bp)
CNAG_00040	1644
CNAG_05842	1743
CNAG_04029	1734
CNAG_06644	1590
CNAG_02841	1725
CNAG_01003	2226

The CNAG_01003 (CPR) gene was modified by PCR to include *Nde*I and *Mun*I restriction sites for insertion into the second MCS of pETDuet. The expected amplicon size of approximately 2200 bp was confirmed with agarose gel electrophoresis (Fig. 3.9) before ligation into pGEM® - T Easy vector.

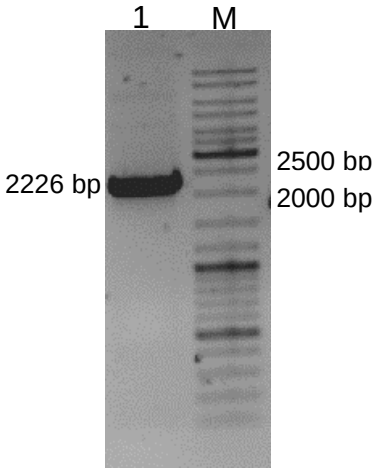


Figure 3.9. Agarose gel electrophoresis confirming the PCR amplification of *C. neoformans* CNAG_01003 (CPR) gene. Lane 1 is the PCR product while the DNA marker is labelled M.

The ligated construct was transformed into *E. coli* XL10-Gold strain while small scale plasmid isolations were carried out on transformed colonies. Positive pGEM® -T Easy vector containing the CNAG_01003 was double digested with *Mun*I and *Nde*I to excise the CPR gene. Agarose gel electrophoresis was used to separate the DNA fragments and the DNA band representing the CNAG_01003 gene was purified from the gel. The purified CNAG_01003 fragment, with *Mun*I and *Nde*I sticky ends, was ligated into a pETDuet vector linearized with the same enzymes. The ligated construct was transformed into *E. coli* XL10-Gold strain. Small scale plasmid isolations were performed on transformed colonies while the positive clones were screened with *Xho*I digestion (Fig. 3.10). Positive clones with the CNAG_01003 gene inserted had bands approximately 700 bp, 1500 bp and 5500 bp after *Xho*I digestion (Fig. 3.10).

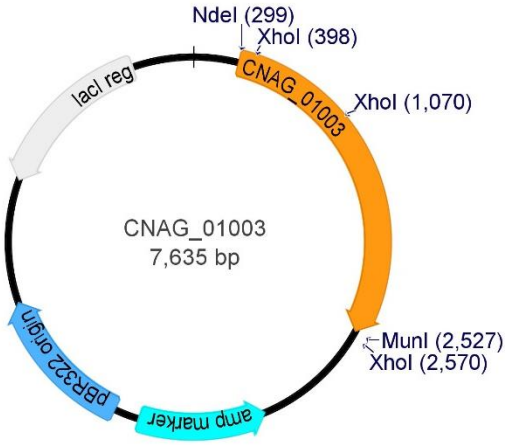


Figure 3.10. Plasmid map for *C. neoformans* CNAG_01003 gene in pETDuet. The *Mun*I and *Nde*I restriction sites used for cloning and *Xho*I restriction site used for confirmation are indicated.

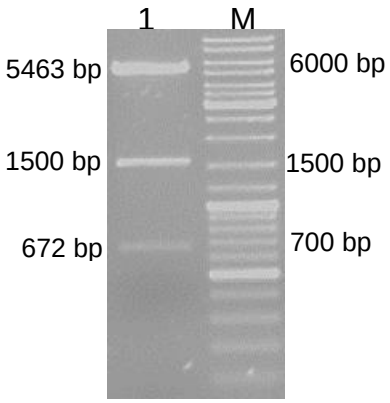
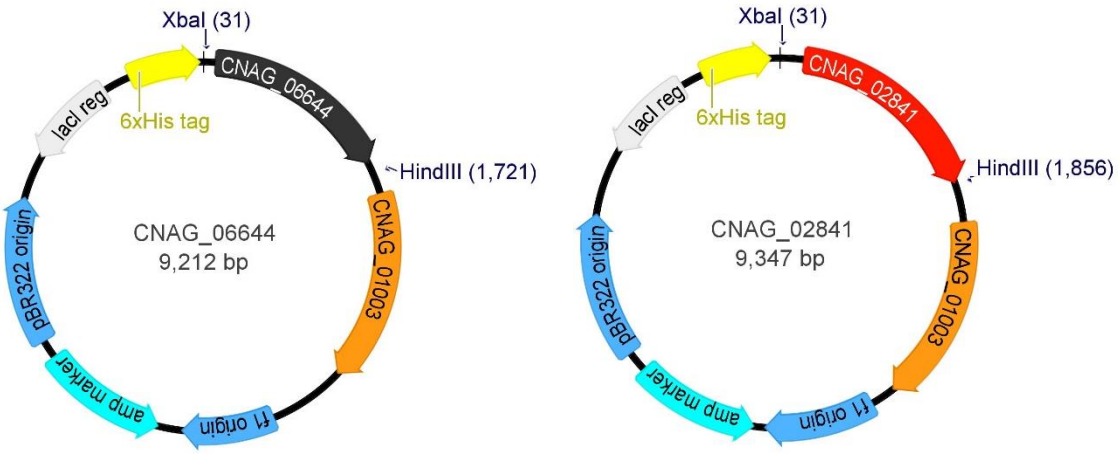


Figure 3.11. *Xho*I restriction digest of *C. neoformans* CPR gene (CNAG_01003) ligated into the second MCS of pETDuet. Lane 1 is the digested positive constructs while the DNA marker is labelled M.

The pETDuet vector containing *C. neoformans* CPR was used as a template for recombinant constructs containing CYP450 genes. The *C. neoformans* CYP450 genes, CNAG_06644 and CNAG_02841, in pET28 were digested with *Xba*I and *Hin*dIII to obtain the gene fragments. The gel purified fragments with sticky ends were ligated with a pETDuet construct containing the CPR with compatible sticky ends. The ligated construct was transformed into *E. coli* XL10-Gold strain and small

scale plasmid isolations were carried out on transformed colonies. Positive constructs were identified with *Xba*I and *Hin*dIII double digestion (Fig. 3.12). Positive clones with the CNAG_06644 gene inserted had bands approximately 1700 bp and 7500 bp while the CNAG_02841 had bands approximately 1800 bp and 7500 bp after *Xba*I/*Hin*dIII digestion (Fig. 3.13).



(a) (b)

Figure 3.12. Plasmid map for *C. neoformans* genes (a) CNAG_06644 (b) CNAG_02841 in pETDuet. The *Xba*I and *Hin*dIII restriction sites used for cloning and confirmation are also indicated.

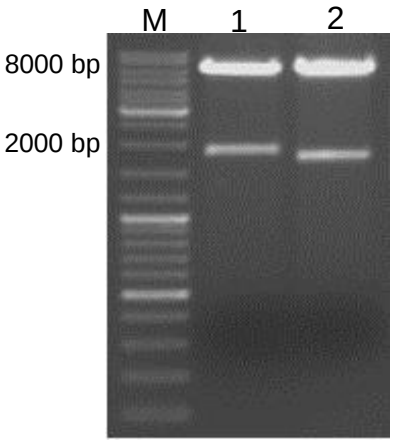
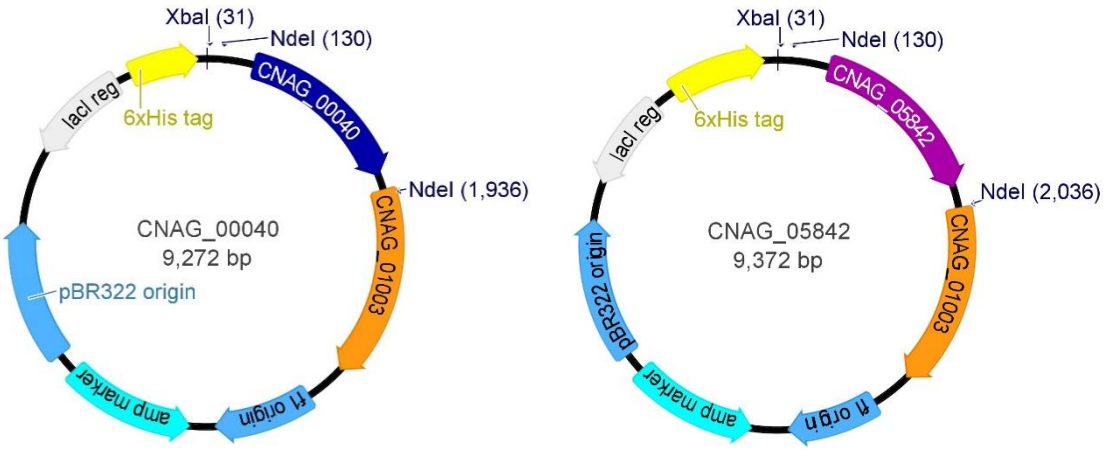


Figure 3.13. *Xba*I and *Hin*dIII double digestion of *C. neoformans* CPR genes in pETDuet. Lane 1 is CNAG_02841. Lane 2 is CNAG_06644. The DNA marker is labelled M.

The pET28 vectors containing the *C. neoformans* CYP450 genes CNAG_00040 and CNAG_05842, were linearized with *Sal*I, while pETDuet construct containing the CPR was linearized with *Hin*dIII. The blunted pET28 construct was digested with *Xba*I to excise the gene fragment with a 5` sticky end and 3` blunt end. The pETDuet construct containing the CPR was also digested with *Xba*I to obtain a 5` blunt end and 3` sticky end. The genes and pETDuet construct containing the CPR with compatible ends were ligated together and transformed into *E. coli* XL10-Gold strain. Small scale plasmid isolations were carried out on transformed colonies, while positive constructs were screened for with *Nde*I digestion (Fig. 3.14). Positive clones with the CNAG_00040 gene inserted had bands approximately 1800 bp and 7400 bp while the CNAG_05842 had bands approximately 1900 bp and 7400 bp after *Nde*I digestion (Fig. 3.15).



(a) (b)

Figure 3.14. Plasmid map for *C. neoformans* genes (a) CNAG_00040 (b) CNAG_05842 in pETDuet. The *Xba*I and *Nde*I restriction sites used for cloning and confirmation are also indicated.

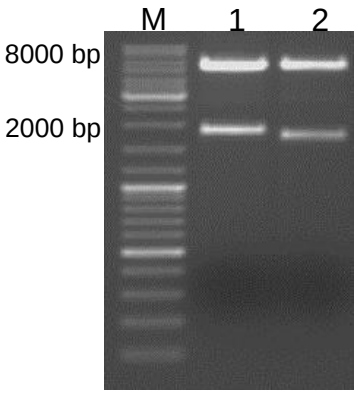


Figure 3.15. *Nde*I restriction digest of *C. neoformans* CPR genes in pETDuet. Lane 1 is CNAG_05842. Lane 2 is CNAG_00040. The DNA marker is labelled M.

CNAG_04029 gene in pET28 was digested with *Hin*dIII and *Mlu*I to excise the gene from the plasmid backbone while pETDuet construct containing the CPR was linearized with the same enzymes. The purified gene fragment and the pETDuet construct containing the CPR with compatible ends were ligated together. The ligated construct was transformed into *E. coli* XL10-Gold strain while small scale plasmid isolations were carried out on transformed colonies. Positive constructs were screened for with *Xba*I digestion (Fig. 3.16). Positive clones with the CNAG_04029 gene inserted had bands of approximately 890 bp and 8400 bp (Fig. 3.17).

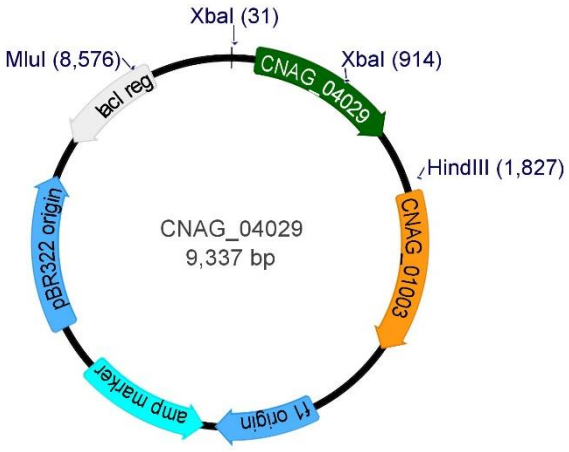


Figure 3.16. Plasmid map for *C. neoformans* gene CNAG_04029 in pETDuet. The *Xba*I, *Hin*dIII and *Mlu*I restriction sites used for cloning and confirmation are also indicated.

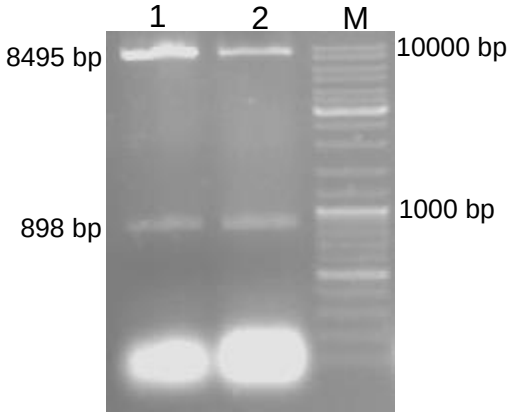


Figure 3.17. *Xba*I restriction digest of *C. neoformans* CNAG_04029 gene ligated into the first MCS of pETDuet. Lanes 1 and 2 are digested positive constructs while the DNA marker is labelled M.

3.4.6. Co-expression of *C. neoformans* CYP450s in pETDuet with pRARE or groES-groEL

Recombinant pETDuet plasmids containing *C. neoformans* CYP450s genes and the CPR were co-expressed with the groES-groEL molecular chaperones. The SDS-PAGE analysis showed superimposed protein bands of the molecular chaperone and the CYP450 genes similar to that earlier obtained. However, there were no bands present indicating expression of the CPR in either the cell free extracts or insoluble fraction as shown in Fig. 3.18. The CPR could have been expressed at very low concentrations which prevented visualization on the SDS-PAGE gel.

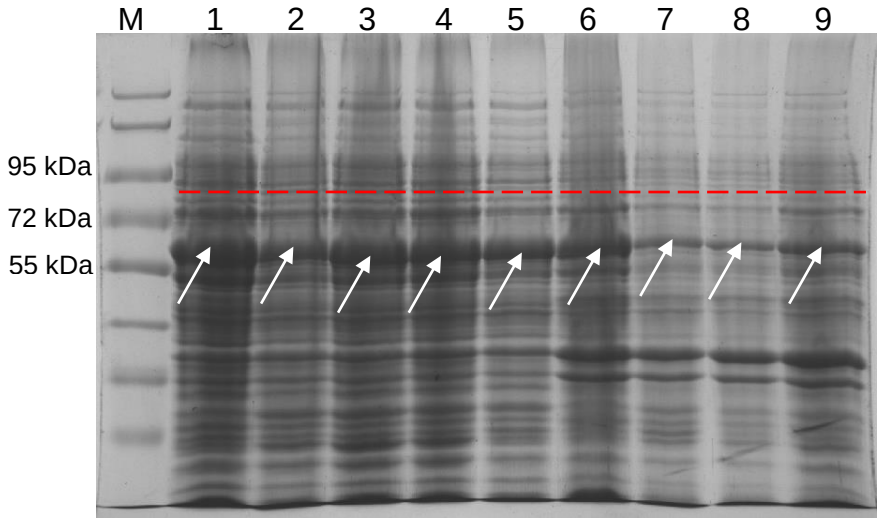


Figure 3.18. SDS-PAGE analysis of *C. neoformans* CYP450s and CPR in pETDuet co-expressed with groES-groEL molecular chaperones. Lane 1-5 are samples from the cell free extract while lanes 6-9 are samples from the insoluble membrane fraction. Lane 1 and 6 is CNAG_04029 (65 kDa) – Unknown. Lane 2 and 7 is CNAG_05842 (64.6 kDa) – β-carotene hydroxylase. Lane 3 and 8 is CNAG_00040 (61.7 kDa) - lanosterol 14α demethylase. Lane 4 and 9 is CNAG_06644 (59.9 kDa) - C_{22} sterol desaturase. Lane 5 is a blank pET28 vector negative control. The PageRuler™ prestained -protein ladder is labelled M while the expressed proteins are indicated by white arrows. The red line indicates the expected position for expressed CPR.

3.4.7. *Cryptococcus neoformans* CYP450s substrate specificity

The hydroxylation of 4-hexylbenzoic acid (HBA) by *Aspergillus terreus* CYP505E3 motivated the investigation of CNAG_04029 and CNAG_02841 for similar activity. The CO difference spectra (Fig. 3.19) and SDS-PAGE (Fig. 3.20) of CNAG_04029 and CNAG_02841 co-expressed with the CPR and groES-groEL tested for substrate specificity are identical to earlier results. The expressed CNAG_04029 and CNAG_02841 were tested for activity using HBA as substrate. However, the resolved TLC plate showed no conversion of HBA by CNAG_04029 and CNAG_02841 (Fig. 3.21). This result was anticipated given that the expression of the CPR could not be observed.

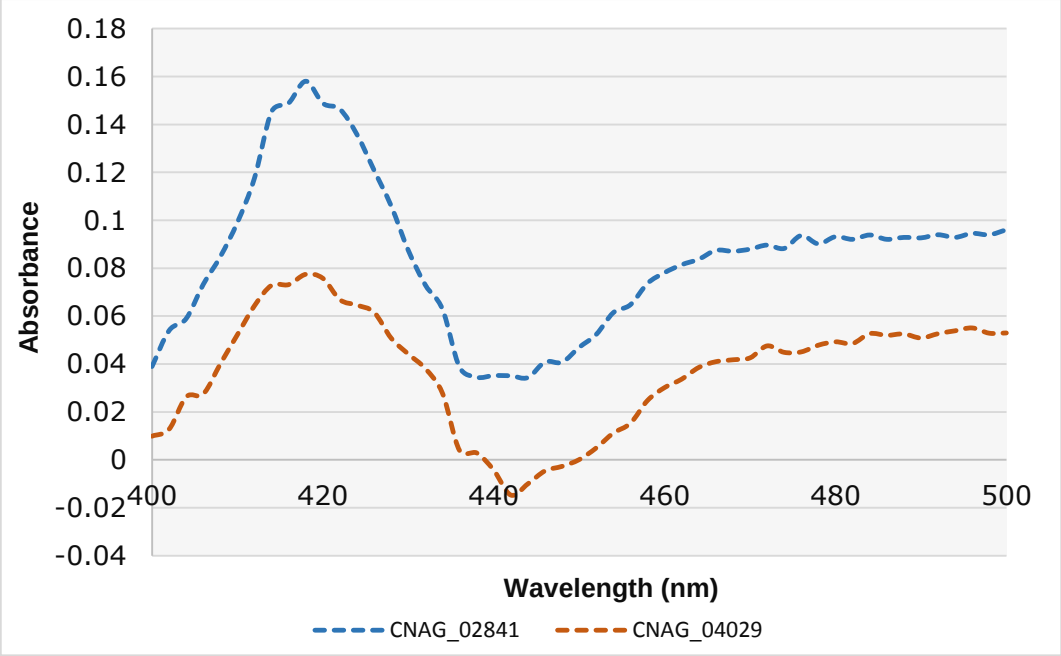


Figure 3.19. CO difference spectrum of expressed CNAG_02841 and CNAG_04029 tested for HBA specificity.

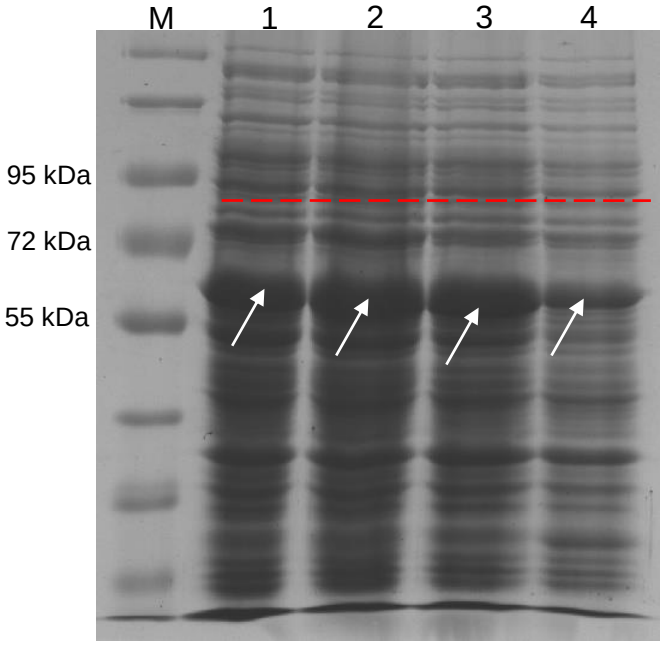


Figure 3.20. Cell free extract SDS-PAGE analysis of *C. neoformans* CYP450s and CPR in pETDuet co-expressed with groES-groEL molecular chaperones tested for HBA specificity. Lane 1 is CNAG_02841 (64.8 kDa) - fatty acid hydroxylase. Lane 2 is CNAG_04029 (65 kDa) – unknown. Lane 3 is CNAG_01003 (82.2 kDa) - CYP450 reductase. Lane 4 is a blank pET22 vector negative control. The Prestained protein ladder is labelled M while the expressed proteins are indicated by white arrows. The red line indicates the expected position for expressed CPR.

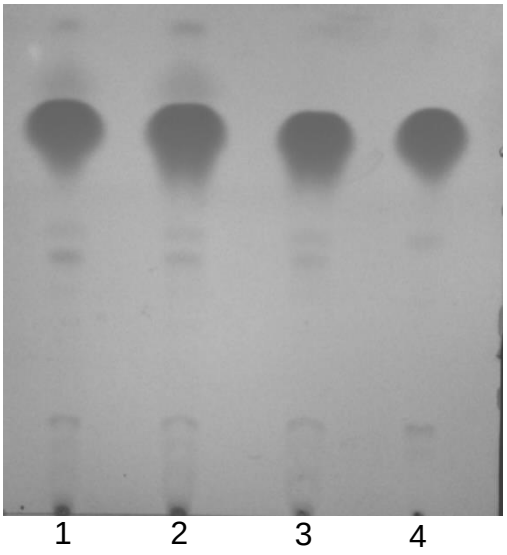


Figure 3.21. TLC results for *C. neoformans* CYP450 co-expressed with the CNAG_01003 (CPR) and groES-groEL biotransformation using HBA as substrate. Lane 1 is CNAG_02841 - Fatty acid hydroxylase. Lane 2 is CNAG_04029 – Unknown. Lane 3 is a blank pET22 vector negative control. Lane 4 is HBA substrate.

CNAG_04029, CNAG_05842, CNAG_06644 and CNAG_00040 co-expressed with the CPR and groES-groEL were tested for activity towards AA. There appeared to be some hydroxylation products formed as indicated by TLC, however this was not conclusive due to the low quality of the TLC plates and the low AA concentration used (Fig. 3.22). CO difference spectra of the expressed CYP450s are shown in Fig 3.23. No peaks were observed at 450 nm.

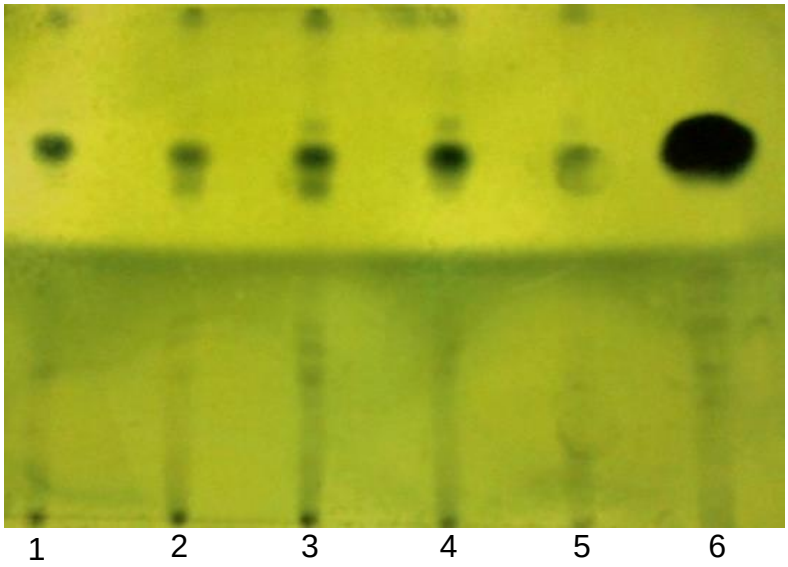


Figure 3.22. TLC results for *C. neoformans* CYP450 co-expressed with the CNAG_01003 (CPR) and groES-groEL biotransformation using AA as substrate. Lane 1 is CNAG_04029 – Unknown. Lane 2 is CNAG_05842 – β-carotene hydroxylase. Lane 3 is CNAG_06644 - C_{22} sterol desaturase. Lane 4 is CNAG_00040 - lanosterol 14α demethylase. Lane 5 is a blank pET22 vector negative control. Lane 6 is AA substrate.

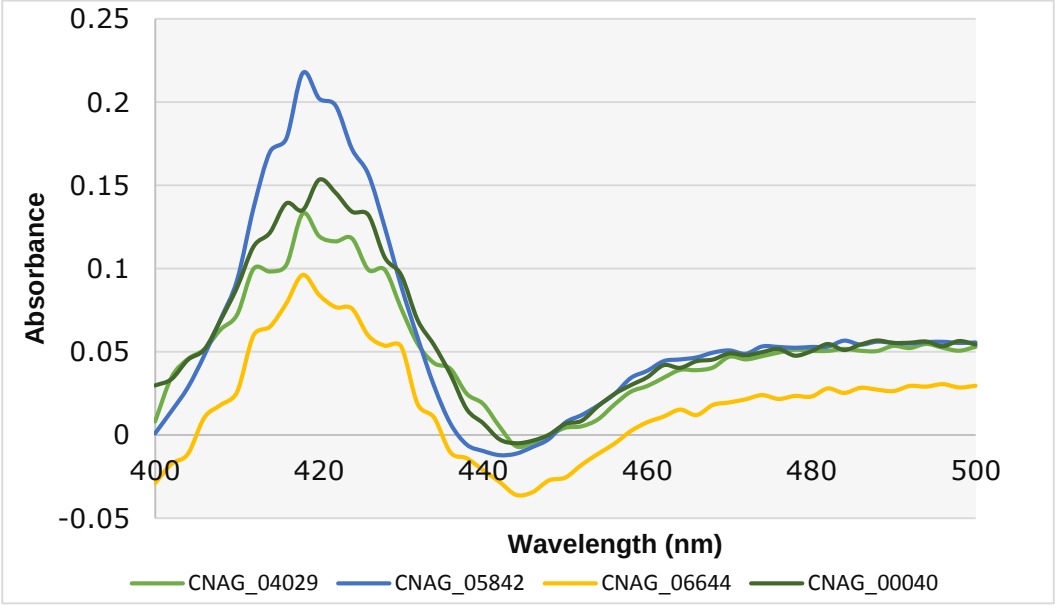


Figure 3.23. CO difference spectra of expressed CNAG_04029, CNAG_05842, CNAG_06644 and CNAG_00040 tested for HBA specificity.

3.5. Conclusions

The successful heterologous expressions of *C. neoformans* CYP450s which has been challenging with an *E. coli* host is essential to establish their possible roles in AA metabolism. Expression in *E. coli* with a pET28 vector was observed for CNAG_04029, CNAG_0542 and CNAG_00040 as indicated by SDS-PAGE protein bands. However, protein bands lower than the expected sizes were observed for CNAG_01003, CNAG_06644 and CNAG_02841. Additionally, CNAG_04029 had the highest expression level in *E. coli* using pET28 vector.

The expected CO difference spectra peak at A_{450} which indicates a correctly folded and catalytically active P450 was not observed for *C. neoformans* CYP450s expressed alone or co-expressed with groES-groEL molecular chaperones. The co-expression of groES-groEL molecular chaperones have prevented formation of truncated proteins with low molecular weights, however, this created another challenge of protein superimposition.

All expressed *C. neoformans* CYP450s including CNAG_04029 which had the highest expression level in pET28 did not show any activity towards tested substrates. The high expression of the CNAG_04029 gene could be as a result of the truncated N-terminus. The use of a yeast host such as *Saccharomyces cerevisiae* for *C. neoformans* CYP450s expression could resolve some of the challenges encountered with expression in *E. coli*.

3.6. References

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SUMMARY

Cytochrome P450 monooxygenases (CYP450) are heme-thiolate proteins which are present in all biological kingdoms. They catalyse the addition of a single oxygen atom to a non-activated carbon atom. The metabolic system of filamentous fungi is maintained by numerous enzymes catalysing the production of bioactive compounds. Genome sequencing projects have revealed the presence of a large collection of CYP450 families within the fungal kingdom. Although some fungal CYP450s are involved in essential reactions such as xenobiotic degradation and membrane ergosterol synthesis, there is still a large collection of uncharacterized fungal CYP450s. The aim of this study was to accomplish and optimize expression and then investigate the substrate specificity of the self-sufficient *Aspergillus terreus* CYP505E3 as well as five CYP450s from *Cryptococcus neoformans*. In the case of the *Cryptococcus* CYP450s the ultimate aim was to establish their possible role in Cryptococcal prostaglandin production.

Self-sufficient *A. terreus* CYP450s were reported by Vatsyayan and co-workers (2008) to show activity towards alkanes, alkane derivatives, alcohols, aromatic compounds, organic solvents and steroids. The genome of *A. terreus* comprises 125 CYP450s with two probable self-sufficient CYP450s namely CYP505A19 and CYP505E3. Omissions within the CYP505A19 sequence makes it unlikely to be active leaving the CYP505E3 as probably the only functional self-sufficient CYP450 in *A. terreus*. The nucleotides on the N-terminus of *A. terreus* CYP505E3 were modified to enhance the expression level in *Escherichia coli*. The recovery of CYP505E3 in the soluble fraction of cell free extract was significantly improved by using expression conditions established through a number of Plackett-Burman experiments. A low temperature, low $FeCl_3$, high culture volume and prolonged incubation time contributed to the improved recovery.

The CYP505E3 within the whole cells catalysed sub-terminal hydroxylation of hexylbenzoic acid (HBA) to produce ω-1 OH-HBA, ω-2 OH-HBA and ω-4 OH-HBA. However, no hydroxylated products were produced from the other substrates tested. The activity of self-sufficient *A. terreus* CYP505E3, towards HBA was similar to that of *Bacillus megaterium* CYP102A1 and *Fusarium oxysporum* CYP505A1 indicating that CYP505E3 is probably also a fatty acid hydroxylase.

The opportunistic yeast pathogen *Cryptococcus neoformans* is known to produce authentic immunomodulatory prostaglandin E_2 from arachidonic acid (AA). However, because of the absence of cyclooxygenase (COX) and lipoxygenase (LOX) homologues in its genome, the mechanism used by the organism to produce prostaglandins remains unclear. Five CYP450s and a CYP450 reductase (CPR) are present within the genome of the organism. The probable roles of these CYP450s in Cryptococcal prostaglandin production were investigated.

The *C. neoformans* CYP450 genes were cloned into pET28 and into a pETDuet plasmid into which the CPR had already been cloned. These plasmids were used to investigate the expression of the CYP450s as well as the CPR using *E. coli* BL21 (DE3) as well as strains carrying plasmids for the co-expression of tRNAs that are rare in *E. coli* or for the molecular chaperones groES and groEL. The CO difference spectra of all the cloned CYP450s did not produce the expected absorbance peak at 450 nm. However, SDS-PAGE analysis indicated possible expression with protein bands which corresponded with the expected sizes. Three of the CYP450s gave acceptable expression when they were expressed using just pET28 without co-expression of the additional tRNA or molecular chaperones. The co-expression of *C. neoformans* CYP450 with groES-groEL molecular chaperones eliminated low molecular weights protein bands produced in the case of the CYP450s and the CPR.

The *C. neoformans* CYP450s co-expressed with the CPR and groES-groEL molecular chaperones had barely detectable or no activity when tested with arachidonic acid and hexylbenzoic acid. The challenges encountered with heterologous expressions of *C. neoformans* CYP450s in an *E. coli* host, could be resolved using an alternative host such as *Saccharomyces cerevisiae*.

Keywords: *Aspergillus terreus, Cryptococcus neoformans*, cytochrome P450, self-sufficient CYP450, hexylbenzoic acid, groES-groEL, arachidonic acid, CYP505E3, hydroxylation

OPSOMMING

Sitochroom P450 monoöksigenases (CYP450's) is heem-tiolaat proteïene wat voorkom in alle biologiese koninkryke. Hulle kataliseer die toevoeging van 'n enkele suurstofatoom aan 'n nie-geaktiveerde koolstofatoom. Die metaboliese sisteem van filamentagtige fungi word in stand gehou deur menigte ensieme wat die produksie van bio-aktiewe verbindings kataliseer. Genoom basispaaropeenvolgingbepalings projekte het gedui op die teenwoordigheid van 'n groot versameling CYP450 families in die fungale koningryk.

Alhoewel sekere fungale CYP450's betrokke is in essensiële reaksies soos die afbreek van xenobiotiese produkte en membraan ergosterol sintese, is daar steeds 'n groot hoeveelheid ongekarakteriseerde fungale CYP450s. Die doel van hierdie studie was om die self-onderhoudende *Aspergillus terreus* CYP505E3, asook vyf CYP450's van *Cryptococcus neoformans,* uit te druk en die uitdrukking te optimiseer, sowel as om hul substraatspesifisiteit te bepaal. In die geval van die *Cryptococcus* CYP450's was die uiteindelike doel om hul moontlike rol in Kriptokokkale prostaglandien produksie te bepaal.

Vatsyayan en medewerkers (2008) het aangedui dat self-onderhoudende *A. terreus* CYP450's aktief was teenoor alkane, alkaan-afgeleide produkte, alkohole, aromatiese verbindings, organiese oplosmiddels en steroïdes. Die *A. terreus* genoom bevat 125 CYP450's met twee moontlike self-onderhoudende CYP450's, naamlik CYP505A19 en CYP505E3. Weglatings binne die basispaar volgorde van CYP505A19 laat dit heel waarskynlik onaktief, menende CYP505E3 is heel waarskynlik die enigste funksionele, self-onderhoudende CYP450 in *A. terreus.*

Die N-terminale nukleotides van die *A. terreus* CYP505E3 was verander om die uitdrukkingsvlak in *Escherichia coli* te verhoog. Die vlak van oplosbare CYP505E3 in die sel-vrye ekstrak was noemenswaardig verhoog deur die uitdrukking kondisies te optimiseer met behulp van Plackett-Burman eksperimente. 'n Lae temperatuur, lae $FeCl_3$, hoë kultuurvolume en verlengde inkubasie tyd het bygedra tot die verhoogde vlak van oplosbare CYP505E3.

Heelselekstrakte wat CYP505E3 bevat het, het die subterminale hidroksilasie van heksielbensoësuur (HBA) gekataliseer om ω-1 OH-HBA, ω-2 OH-HBA en ω-4 OH-HBA te produseer. Geen gehidroksileerde produkte is geproduseer van enige ander getoetse substrate nie. Die aktiwiteit van die self-onderhoudende *A. terreus* CYP505E3 teenoor HBA was vergelykbaar met die van *Bacillus megaterium* CYP101A1 en *Fusarium oxysporum* CYP505A1, wat daarop dui dat CYP505E3 heel waarskynlik ook ŉ vetsuur hidroksilase is.

Die opportunistiese gispatogeen *Cryptococcus neoformans* produseer die immuunmoduleerder prostaglandien E_2 vanaf arachidoonsuur (AA). As gevolg van die afwesigheid van siklooksigenase (COX) en lipoksigenase (LOX) homoloë in die genoom, is die meganisme van prostaglandien produksie deur die organisme onbekend. Die genoom bevat vyf CP450's en 'n CYP450 reduktase (CPR). Die waarskynlike rolle van hierdie CYP450's in prostaglandien produksie is ondersoek.

Die *C. neoformans* CYP450 gene was ingekloneer in die pET28 plasmied, asook in die pETDuet plasmied waarin die CPR geen reeds teenwoordig was. Die plasmiede was gebruik om die uitdrukking van die CYP450's, asook die CPR, in *E. coli* BL21(DE3) te ondersoek, asook stamme wat plasmiede bevat vir die gesamentlike uitdrukking van tRNA's wat skaars is in *E. coli*, en vir die molekulêre chaperones groES en groEL.

Die CO verskil spektra van die gekloneerde CYP450's het nie die verwagte absorbansiepiek by 450nm getoon nie. Tog het SDS-PAGE analise gedui op moontlike uitdrukking, aangesien proteïen bande teenwoordig was met die verwagte groottes. Drie van die CYP450's het aanvaarbare uitdrukkingsvlakke gehad deur slegs die pET28 vektor te gebruik, sonder gesamentlike uitdrukking met addisioneel toegevoegde tRNA molekules of molekulêre chaperones. Uitdrukking van die *C. neoformans* CYP450 saam met die groES-groEL molekulêre chaperones het die lae molekulêre massa proteïenbande wat geproduseer is, geëlimineer in die geval van die CYP450's en die CPR.

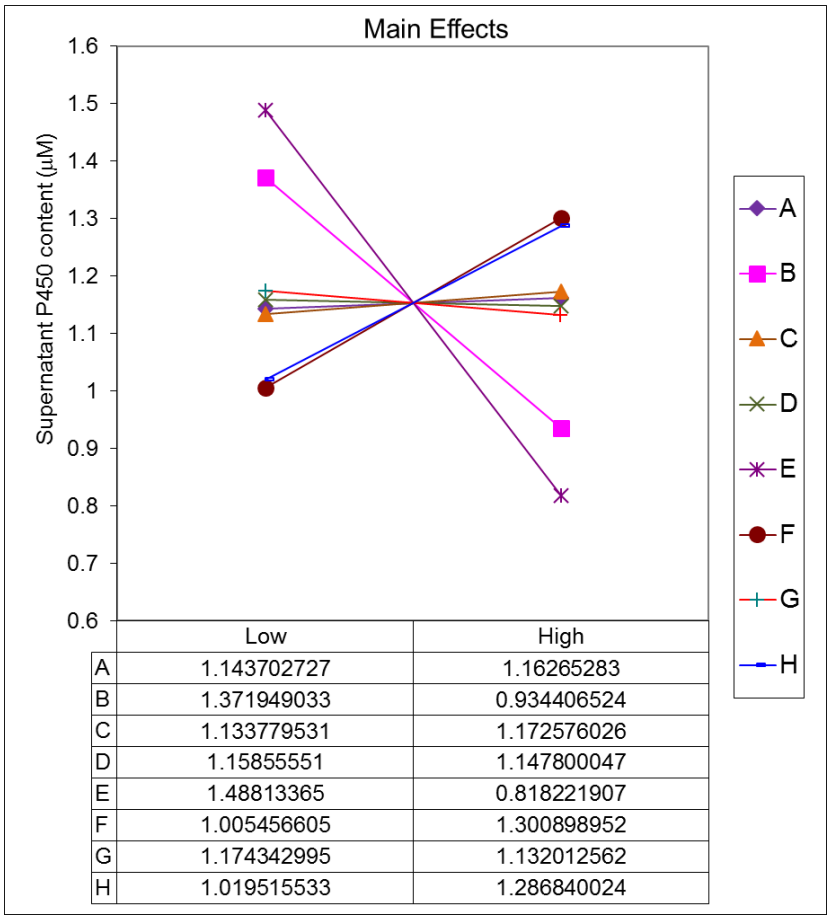
Die *C. neoformans* CYP450's wat saam uitgedruk is met die CPR en groES-groEL molekulêre chaperone, het baie lae of geen aktiwiteit getoon teenoor arachidoonsuur

en heksielbensoësuur nie. Die uitdagings wat gebied word deur die heteroloë uitdrukking van die *C. neoformans* CYP450s in *E. coli* kan moontlik oorkom word deur ’n alternatiewe gasheer te gebruik, soos *Saccharomyces cerevisiae.*

Sleutelwoorde: *Aspergillus terreus, Cryptococcus neoformans*, sitochroom P450, self-onderhoudende CYP450, heksielbensoësuur, groES-groEL, arachidoonsuur, CYP505E3, hidroksilasie

Supplementary Information

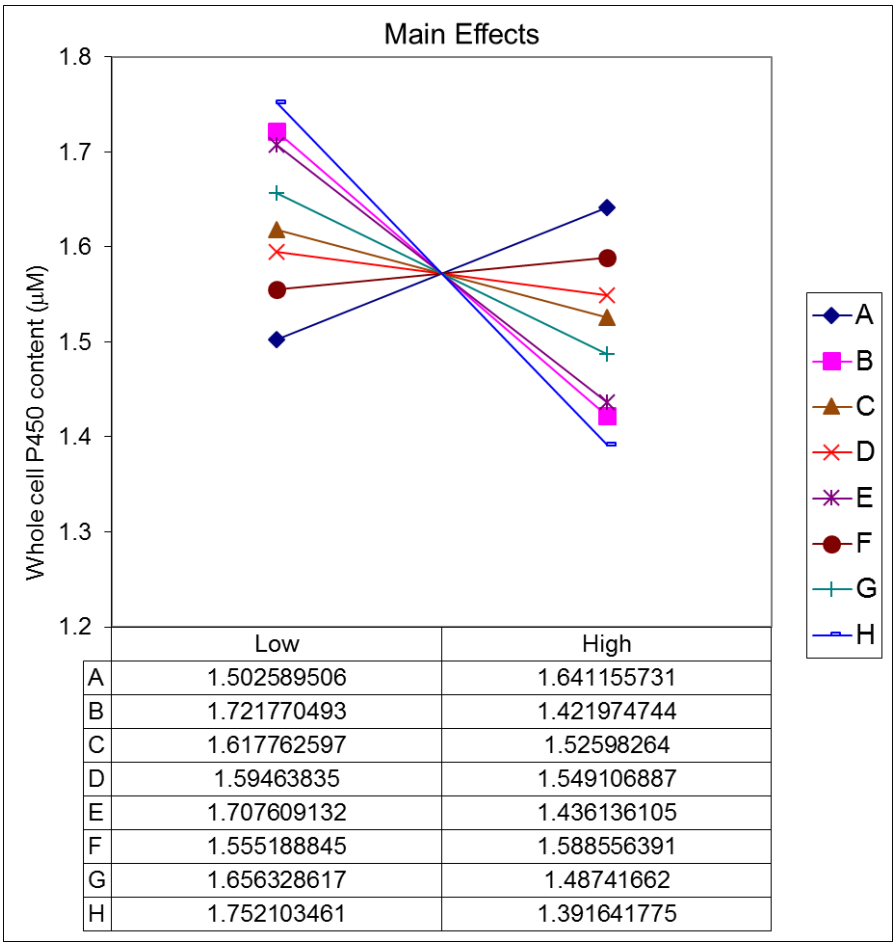
ANOVA Table Based on All Factors and Interactions							
Source	SS	df	MS	F	p value	%Cont	
A	0.00108	1	0.00108	0.007	0.9381	0.04%	
B	0.574	1	0.574	3.796	0.1465	20.07%	
C	0.00452	1	0.00452	0.030	0.8738	0.16%	
D	0.000347	1	0.000347	0.002	0.9648	0.01%	
E	1.346	1	1.346	8.898	0.0585	47.04%	***There are no signficant factors***
F	0.262	1	0.262	1.731	0.2798	9.15%	
G	0.00538	1	0.00538	0.036	0.8625	0.19%	
H	0.214	1	0.214	1.417	0.3195	7.49%	
Error	0.454	3	0.151			15.86%	
Total	2.862	11				100.00%	



	Low	High
A	1.143702727	1.16265283
B	1.371949033	0.934406524
C	1.133779531	1.172576026
D	1.15855551	1.147800047
E	1.48813365	0.818221907
F	1.005456605	1.300898952
G	1.174342995	1.132012562
H	1.019515533	1.286840024

Figure S1. Analysis of variance (ANOVA) for CYP450 content of soluble fraction of the cell free.

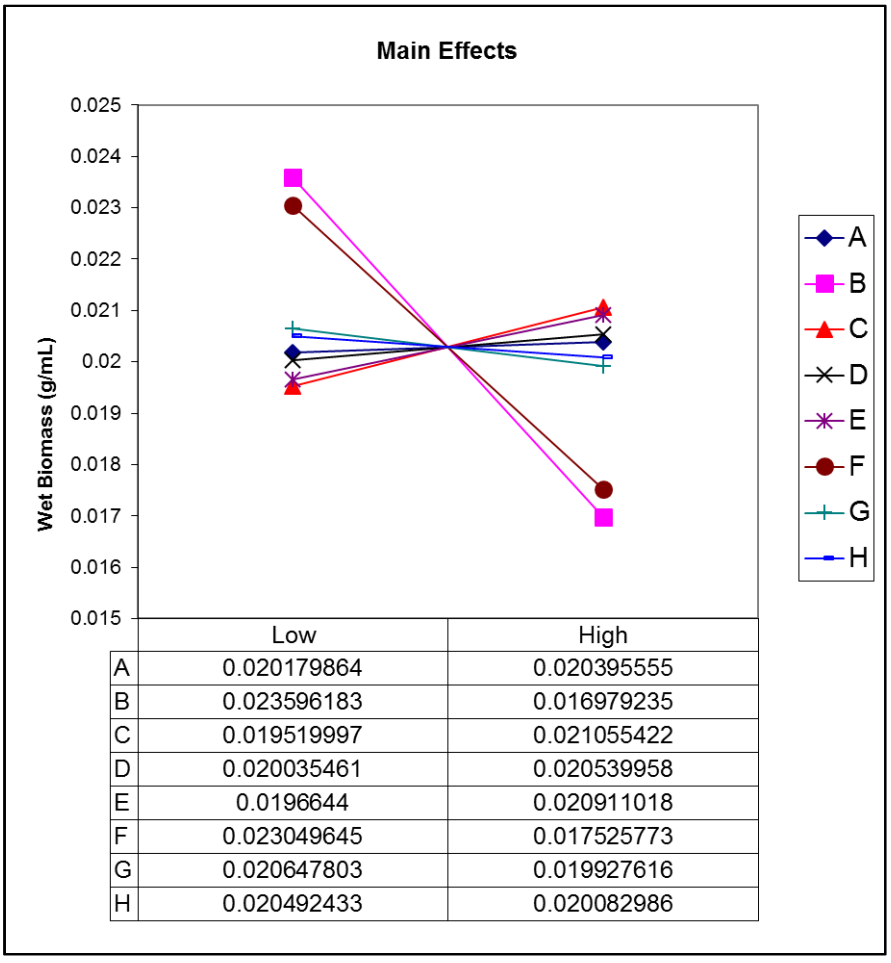
ANOVA Table Based on All Factors and Interactions							
Source	SS	df	MS	F	p value	%Cont	
A	0.0576	1	0.0576	0.465	0.5441	4.03%	
B	0.270	1	0.270	2.178	0.2365	18.86%	
C	0.0253	1	0.0253	0.204	0.6821	1.77%	
D	0.00622	1	0.00622	0.050	0.8371	0.43%	
E	0.221	1	0.221	1.786	0.2738	15.46%	***There are no signficant factors***
F	0.00334	1	0.00334	0.027	0.8800	0.23%	
G	0.0856	1	0.0856	0.691	0.4667	5.99%	
H	0.390	1	0.390	3.148	0.1741	27.26%	
Error	0.371	3	0.124			25.98%	
Total	1.430	11				100.00%	



	Low	High
A	1.502589506	1.641155731
B	1.721770493	1.421974744
C	1.617762597	1.52598264
D	1.59463835	1.549106887
E	1.707609132	1.436136105
F	1.555188845	1.588556391
G	1.656328617	1.48741662
H	1.752103461	1.391641775

Figure S2. Analysis of variance (ANOVA) for whole cell CYP450 content.

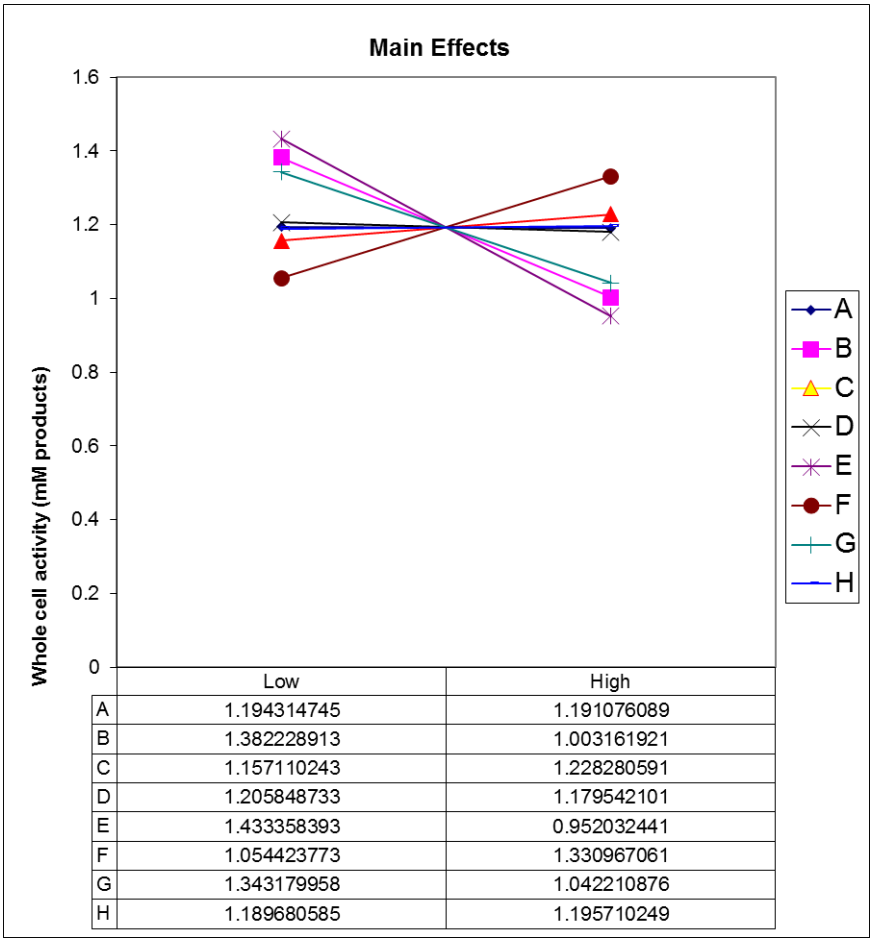
ANOVA Table Based on All Factors and Interactions							
Source	SS	df	MS	F	p value	%Cont	
A	0.000000140	1	0.000000140	0.019	0.8995	0.05%	
B	0.000131	1	0.000131	17.746	0.0244	50.56%	
C	0.00000707	1	0.00000707	0.956	0.4004	2.72%	***The signficant factors are in red (p <= 0.05). Factors in blue (0.05 < p <=0.20) may or may not be significant.***
D	0.000000764	1	0.000000764	0.103	0.7691	0.29%	
E	0.00000466	1	0.00000466	0.630	0.4854	1.79%	
F	0.0000915	1	0.0000915	12.367	0.0390	35.24%	
G	0.00000156	1	0.00000156	0.210	0.6778	0.60%	
H	0.000000503	1	0.000000503	0.068	0.8112	0.19%	
Error	0.0000222	3	0.00000740			8.55%	
Total	0.000260	11				100.00%	



	Low	High
A	0.020179864	0.020395555
B	0.023596183	0.016979235
C	0.019519997	0.021055422
D	0.020035461	0.020539958
E	0.0196644	0.020911018
F	0.023049645	0.017525773
G	0.020647803	0.019927616
H	0.020492433	0.020082986

Figure S3. Analysis of variance (ANOVA) for wet biomass.

ANOVA Table Based on All Factors and Interactions							
Source	SS	df	MS	F	p value	%Cont	
A	0.0000315	1	0.0000315	0.001	0.9801	0.00%	
B	0.431	1	0.431	10.042	0.0505	24.31%	
C	0.0152	1	0.0152	0.354	0.5938	0.86%	***The signficant factors are in red (p <= 0.05). Factors in blue (0.05 < p <=0.20) may or may not be significant.***
D	0.00208	1	0.00208	0.048	0.8401	0.12%	
E	0.695	1	0.695	16.191	0.0276	39.19%	
F	0.229	1	0.229	5.345	0.1039	12.94%	
G	0.272	1	0.272	6.330	0.0865	15.32%	
H	0.000109	1	0.000109	0.003	0.9630	0.01%	
Error	0.129	3	0.0429			7.26%	
Total	1.773	11				100.00%	



	Low	High
A	1.194314745	1.191076089
B	1.382228913	1.003161921
C	1.157110243	1.228280591
D	1.205848733	1.179542101
E	1.433358393	0.952032441
F	1.054423773	1.330967061
G	1.343179958	1.042210876
H	1.189680585	1.195710249

Figure S4. Analysis of variance (ANOVA) for whole cell biotransformation.