

Indian Institute of Technology Delhi



THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CYTOCHROME P450 (CYPs) FROM EXTREMOPHILIC SOURCES AND THEIR BIOTECHNOLOGICAL APPLICATIONS

SIMRAN KUNDRAL

INDIAN INSTITUTE OF TECHNOLOGY DELHI

&

THE UNIVERSITY OF QUEENSLAND

MAY 2026

© **Indian Institute of Technology Delhi (IITD), New Delhi, 2026**

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CYTOCHROME P450 (CYPs) FROM EXTREMOPHILIC SOURCES AND THEIR BIOTECHNOLOGICAL APPLICATIONS

by

SIMRAN KUNDRAL

M. Biotechnology, BSc (H) Biochemistry

Submitted

in fulfilment of the requirements for the joint degree of

DOCTOR OF PHILOSOPHY

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

&

THE UNIVERSITY OF QUEENSLAND

MAY 2026

Supervisor Certification

This is to certify that the thesis entitled "**Biochemical and Molecular Characterization of Cytochrome P450 (CYPs) from Extremophilic Sources and their Biotechnological Applications**", being submitted by **Ms Simran Kundral** to the Indian Institute of Technology Delhi and the University of Queensland for the award of the degree of **Doctor of Philosophy,** is a record of bonafide research work carried out by her. **Ms Simran** has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis, which, to our knowledge, has reached the requisite standard. The results contained in this thesis are original and have not been submitted, in part or full, to any other University or Institute for the award of any other degree or diploma.



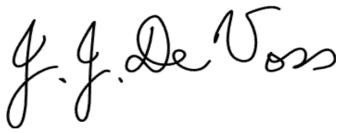
Prof. Sunil Kumar Khare

Professor

Department of Chemistry

Indian Institute of Technology Delhi

New Delhi-110016, India



Prof. James De Voss

Professor

The School of Chemistry and Molecular Biosciences

The University of Queensland

Brisbane, QLD-4072, Australia

Date: 12 May 2026

Place: New Delhi

Acknowledgements

As I bring this thesis to completion, I am filled with gratitude. I owe everything to the blessings of God and the unwavering love and support of my parents. Their encouragement has allowed me to reach heights that are still a dream for many. I hope I can use what I have learned to help young women see the world and achieve their dreams, just as I have been fortunate to do.

I am deeply thankful to both of my supervisors, Prof. James De Voss and Prof. Sunil Kumar Khare. I truly could not have completed this PhD without them. I want to especially thank James for always believing in me, giving me second chances and constantly looking out for my growth. There were moments when finishing this PhD felt impossible, but you never stopped believing in me, even when I doubted myself. Thank you for going above and beyond, not just for my research, but also for my future career. You have made me not just a better scientist, but a kinder human being. I admire your endless curiosity about science, and if I can be even half as curious and passionate as you are, I will consider it a big achievement. Thank you for being more than a supervisor, thank you for being a mentor and a friend.

A heartfelt thank you to my supervisor, Prof. Sunil Kumar Khare, for always supporting me even from afar. Your encouraging words after my milestone presentations are some of my most cherished memories. Thank you for giving me the freedom to grow into an independent scientist and for your patience and gentle guidance throughout this journey. Even from a distance, you always helped me find the right direction and I am truly grateful for that.

I would like to express my sincere thanks to Prof. Joanne Blanchfield for creating a home-like environment on Level 10 and for always being there when I needed guidance the most. My gratitude also goes to Prof. Stephen Bell for his thoughtful advice and continuous guidance throughout the PhD journey. A heartfelt thanks to Prof. Paul Bernhardt for allowing me to use your lab instruments and for your support with protein electrochemistry. I am also deeply thankful to Prof. Luke Guddat for teaching me how to solve crystal structures and for being patient during my learning phase. I am equally grateful to all the professional staff at SCMB, UQ, for their assistance and support.

A big thank you to Dr. Peter Giang for helping me settle into the lab and always offering a helping hand exactly when I needed it. You are someone I deeply trust, and I truly value your guidance and mentorship. I could not have asked for a better senior mentor to look up to. To my labmates Dr. Laura and Ms. Julia Buczynski, thank you for your friendship and for bringing humour and joy to the lab.

I owe special thanks to Dr. Huma Fatima for being like an elder sister to me. Your emotional support, whether during stressful lab days or difficult personal moments, has meant more than I can ever put into words and I know our bond will always stay strong. I am so grateful to have walked this path with you.

I also want to thank Dr. Ankita Maurya for constantly looking out for me and for being someone I truly admire. You have been my go-to person in every phase of the PhD journey, whether it was celebrating a good experiment or surviving a bad week. Thank you for being a true friend, for your honesty, humour and unwavering support. A big thanks to Dr. Nikky Goel for being my friend through every high and low. Your support has been a true blessing.

To my labmates at IIT Delhi, Ms. Deeksha, Ms. Saniya, and Mr. Bibhuti, thank you for your kind nature and encouragement. A special thanks to my junior, Ms. Tanisha, for your helpful and cheerful nature in the lab.

I don’t have enough words to express my gratitude to my partner, Mr. Nikhil Reddy. Thank you for lifting me up from my lowest moments and making me shine again. You have been there for me with no judgement, even when you had your own PhD battles to fight. Thank you for believing in me when I couldn’t believe in myself.

I am once again at a loss for words to thank my wonderful parents, Mrs. Savita Kundral and Mr. Raj Kumar Kundral. I am so blessed to be your daughter. Thank you for giving me wings and always being my strongest cheerleader. Mom, you are my backbone. I know you see a part of yourself in all my achievements, and I promise to continue making you proud.

My dad, you have always been so proud of me, even on the days when I woke up at 11 am at home. Thank you for giving me every comfort in life. I hope to one day give back even half of what you have given me, and then I will feel I have achieved something meaningful.

To my brother Tushar, you are a pillar of my strength. I have achieved what I have because you stood by me and took care of Mom and Dad in my absence. Your emotional support has meant more to me than I can express. A heartfelt thank you as well to my cousins Deeksha and Shubham for all the laughter, love and constant encouragement.

Simran Kundral
11 May 2026

Abstract

Cytochrome P450 monooxygenases (P450s) are versatile biocatalysts capable of catalyzing a wide range of oxidative transformations of diverse substrates. However, their practical application is often limited by intrinsic challenges, including reliance on auxiliary redox partner proteins and costly nicotinamide cofactors (NAD(P)H), as well as poor stability at the elevated temperatures typical of industrial processes. These limitations motivated the present research, which aimed to discover and engineer P450 enzymes to overcome these barriers, thereby creating more robust and self-sufficient biocatalysts suitable for industrial use.

To address the need for thermostable P450s, a metagenomic approach was employed. Microbial DNA from an Australian hot spring (Innot hot springs, ~71 °C) had been sequenced, revealing a database of over 200 putative P450 enzymes, many potentially thermotolerant. From careful analysis of this resource, a novel self-sufficient P450, CYP116B305, was identified and selected for detailed study. CYP116B305 originates from an uncultivated bacterium in the Geminicoccaceae family (order Geminicoccales, class Alphaproteobacteria), representing the first member of the CYP116 family reported from this lineage. Like other CYP116 enzymes, it contains a catalytic heme domain naturally fused to its own ferredoxin redox domains, obviating the need for external electron transfer partners.

CYP116B305 was heterologously expressed in *Escherichia coli* and shown to be highly thermostable. The enzyme retained activity at elevated temperatures, with thermal half-life (T_{50}) values far exceeding those of typical mesophilic P450s. Functionally, CYP116B305 binds and regioselectively hydroxylates 2-hydroxyphenylacetic acid (2-HPA), converting it to homogentisic acid. Notably, its catalytic activity (electron transfer rate and coupling efficiency) improved at 45 °C relative to 25 °C, presumably reflecting an adaptation to high-temperature conditions. To gain insight into the natural role of the larger CYP116 family of enzymes, a homologous self-sufficient P450, CYP116B234 from *Rhodococcus globerulus* was also examined. Proteomic analysis linked CYP116B234 to the catabolism of 2-HPA via the homogentisate pathway, providing evidence for the native physiological function of this class of P450s in aromatic compound degradation.

Further characterization of CYP116B305 was conducted at the domain level to understand its biophysical properties. The heme domain and the phthalate family oxygenase reductase (PFOR) domain (containing FMN and [2Fe–2S] cofactors) were expressed and purified separately, allowing assessment of their independent stability and function. Each domain retained its functionality: the isolated heme domain remained thermostable and capable of substrate binding, while the isolated reductase domain maintained NADPH-dependent electron transfer activity. Spectroelectrochemical measurements of the reductase domain revealed distinct redox potentials for the FMN (approximately -200 mV and -310 mV for the oxidized/semiquinone and semiquinone/reduced transitions, respectively) whilst the EPR analysis indicated a redox potential of the [2Fe–2S] centre (around -255 mV). This experiment provided the first comprehensive domain-level characterization of a thermophilic self-sufficient P450 and demonstrated that both domains can operate effectively in isolation, an observation that could inform future engineering of modular P450 systems.

In addition to CYP116B305, the metagenomic screening uncovered a second enzyme of interest, CYP108D18, associated with a *Novosphingobium* species. CYP108D18 is a Class I P450 (requiring external redox partners) that exhibits moderate thermostability. It was expressed in *E. coli* and purified to homogeneity for functional studies. Spectroscopic ligand-binding assays showed that CYP108D18 has a high affinity for aromatic hydrocarbons such as phenylcyclohexane and phenanthrene, inducing significant shifts to the high-spin state of the heme, whereas monoterpenes bound with lower affinity. Using surrogate redox partner proteins (spinach ferredoxin and ferredoxin reductase), CYP108D18 was reconstituted *in vitro* and demonstrated the ability to oxidize phenylcyclohexane to *trans*-4-phenylcyclohexanol, confirming its monooxygenase activity on an aromatic hydrocarbon substrate.

To elucidate the structural basis of its function, the crystal structure of CYP108D18 was solved both without substrate and with bound substrates (phenylcyclohexane and *p*-cymene) at high resolution. The structures revealed that the enzyme remains in an open conformation even when the substrate is bound, unlike many P450s, which undergo a transition to a closed conformation upon substrate binding. This persistent open state, together with spectroelectrochemical data showing only a modest increase in heme redox potential upon substrate binding (from -451 mV to -409 mV vs. NHE), suggests a structural rationale for the enzyme's low reactivity and its ineffective interaction with typical P450 electron transfer partners. The discovery and analysis of CYP108D18 expanded the known diversity of P450 enzymes from extreme environments

and provided insight into how protein structure can influence stability, redox and catalytic behaviour.

To obviate the dependency on expensive cofactors and auxiliary proteins, a protein engineering strategy was applied to convert these P450s into hydrogen peroxide-driven biocatalysts (peroxygenases). By introducing a pair of mutations in the highly conserved *I*-helix motif of the active site (replacing the native acid-alcohol residues with a glutamine and glutamate, i.e. "QE" mutations), both CYP116B305 and CYP108D18 were re-engineered to utilize H_2O_2 directly via the peroxide shunt pathway. This modification enables the generation of the key reactive intermediate (Compound I) using H_2O_2 as the oxidant, bypassing the normal requirement for NAD(P)H and redox partner proteins.

The QE-engineered variant of CYP116B305 heme domain retained its intrinsic thermostability and achieved near-complete (>96%) conversion of 2-HPA to homogentisate using only H_2O_2 as the oxygen donor. Similarly, the CYP108D18 QE mutant exhibited peroxygenase activity by hydroxylating phenylcyclohexane with H_2O_2, albeit with lower product yields than the CYP116B305 mutant with 2-HPA. Both engineered enzymes showed reduced susceptibility to heme bleaching (oxidative inactivation) in the presence of peroxide, especially when their substrates were present to help stabilize the active site. The introduction of an acid-base catalyst in the distal pocket broadened the catalytic repertoire of these enzymes, enabling oxidative transformations not observed in their native monooxygenase forms. These results illustrate a generalizable approach to eliminate cofactor dependence in P450 catalysis, creating simplified and cost-effective biocatalytic systems.

In summary, this thesis combines metagenomic discovery and directed enzyme engineering to develop P450 monooxygenases with enhanced thermostability and self-sufficiency. The identification of CYP116B305 expands the phylogenetic and functional scope of self-sufficient P450s, revealing an enzyme that is both robust at high temperatures and capable of efficient oxidation of aromatic hydrocarbons. The characterization of a related mesophilic enzyme (CYP116B234) provides insight into the ecological role of CYP116 family P450s in bacterial metabolism. The detailed domain-level analysis of CYP116B305 contributes a fundamental understanding of the independent stability and redox properties of its bi-modular architecture. Meanwhile, the discovery and structural characterization of CYP108D18 uncover previously unrecognized P450 diversity in thermally enriched environments and provide a valuable

framework for understanding how specific structural elements govern both thermal adaptation and the underlying thermodynamic properties of P450 enzymes. Finally, the successful engineering of both CYP116B305 and CYP108D18 into peroxide-driven catalysts demonstrates a viable strategy to overcome natural limitations of P450s as robust catalysts for large-scale bioprocesses, integrating thermostability with cofactor independence. Together, these findings advance the development of more robust, versatile P450 enzymes, with implications for sustainable industrial biocatalysis and environmental bioremediation applications.

सार

सायटोक्रोम P450 मोनोऑक्सीज़नेज़ (P450s) बहुपयोगी जैव-उत्प्रेरक हैं, जो विभिन्न प्रकार के सब्सट्रेट्स पर अनेक ऑक्सीडेटिव अभिक्रियाओं को उत्प्रेरित करने में सक्षम होते हैं। हालांकि, इनके व्यावहारिक उपयोग में कई अंतर्निहित चुनौतियाँ बाधा बनती हैं, जिनमें सहायक रेडॉक्स पार्टनर प्रोटीनों और महंगे निकोटिनामाइड कोफैक्टर्स (NAD(P)H) पर निर्भरता, तथा औद्योगिक प्रक्रियाओं में सामान्यतः पाए जाने वाले उच्च तापमान पर कम स्थिरता शामिल है। इन्हीं सीमाओं ने इस शोध को प्रेरित किया, जिसका उद्देश्य इन बाधाओं को दूर करने हेतु P450 एंज़ाइमों की खोज और अभियांत्रिकी करना था, ताकि औद्योगिक उपयोग के लिए अधिक सुदृढ़ और आत्मनिर्भर जैव-उत्प्रेरक विकसित किए जा सकें।

ताप-स्थिर (थर्मोस्टेबल) P450s की आवश्यकता को पूरा करने के लिए एक मेटाजीनोमिक दृष्टिकोण अपनाया गया। ऑस्ट्रेलिया के एक गर्म जलस्रोत (इनॉट हॉट स्प्रिंग्स, लगभग 71 °C) से प्राप्त सूक्ष्मजीवी डीएनए का अनुक्रमण किया गया, जिससे 200 से अधिक संभावित P450 एंज़ाइमों का एक डेटाबेस प्राप्त हुआ, जिनमें से कई के ताप-सहिष्णु होने की संभावना थी। इस संसाधन के सावधानीपूर्वक विश्लेषण से एक नया आत्मनिर्भर P450, CYP116B305, पहचाना गया और विस्तृत अध्ययन के लिए चुना गया। CYP116B305 जेमिनिकोकेसी (Geminicoccaceae) कुल के एक अशोधित (असंस्कृत) जीवाणु से उत्पन्न होता है (ऑर्डर Geminicoccales, वर्ग Alphaproteobacteria) और इस वंश से रिपोर्ट किया गया CYP116 परिवार का पहला सदस्य है। अन्य CYP116 एंज़ाइमों की तरह, इसमें एक उत्प्रेरक हीम डोमेन स्वाभाविक रूप से अपने ही फेरेडॉक्सिन रेडॉक्स डोमेनों से जुड़ा होता है, जिससे बाहरी इलेक्ट्रॉन ट्रांसफर पार्टनरों की आवश्यकता समाप्त हो जाती है।

CYP116B305 को *Escherichia coli* में विषम अभिव्यक्ति (heterologous expression) के माध्यम से व्यक्त किया गया और यह अत्यधिक ताप-स्थिर पाया गया। यह एंज़ाइम ऊँचे तापमान पर भी सक्रिय बना रहा, तथा इसकी थर्मल हाफ-लाइफ (T_{50}) मान सामान्य मेसॉफिलिक P450s की तुलना में कहीं अधिक थे। क्रियात्मक रूप से, CYP116B305 2-हाइड्रॉक्सीफेनिलएसेटिक अम्ल (2-HPA) से बंधता है और उसे क्षेत्र-विशिष्ट रूप से हाइड्रॉक्सीलेट कर होमोजेंटिसिक अम्ल में परिवर्तित करता है। उल्लेखनीय रूप से, इसकी उत्प्रेरक

सक्रियता (इलेक्ट्रॉन ट्रांसफर दर और कपलिंग दक्षता) 25 °C की तुलना में 45 °C पर बेहतर पाई गई, जो संभवतः उच्च तापमान के प्रति इसके अनुकूलन को दर्शाती है। CYP116 परिवार की प्राकृतिक भूमिका को समझने के लिए *Rhodococcus globerulus* से एक समजात आत्मनिर्भर P450, CYP116B234, का भी अध्ययन किया गया। प्रोटीओमिक विश्लेषण ने CYP116B234 को 2-HPA के अपचय (कैटाबोलिज़्म) से जोड़ा, जो होमोजेंटिसेट मार्ग के माध्यम से होता है, और इस प्रकार एरोमैटिक यौगिकों के अपघटन में इस P450 वर्ग की स्वाभाविक जैविक भूमिका के प्रमाण प्रदान किए।

CYP116B305 के जैव-भौतिक गुणों को समझने के लिए डोमेन-स्तरीय विश्लेषण किया गया। हीम डोमेन और फ्थैलेट फैमिली ऑक्सीज़नेज़ रिडक्टेज़ (PFOR) डोमेन (जिसमें FMN और [2Fe–2S] कोफैक्टर्स होते हैं) को अलग-अलग व्यक्त कर शुद्ध किया गया, जिससे उनकी स्वतंत्र स्थिरता और कार्यक्षमता का आकलन संभव हुआ। दोनों डोमेनों ने अपनी कार्यक्षमता बनाए रखी: पृथक हीम डोमेन ताप-स्थिर रहा और सब्सट्रेट बाइंडिंग में सक्षम था, जबकि पृथक रिडक्टेज़ डोमेन ने NADPH-निर्भर इलेक्ट्रॉन ट्रांसफर गतिविधि बनाए रखी। रिडक्टेज़ डोमेन के स्पेक्ट्रो-इलेक्ट्रोकेमिकल मापों से FMN के लिए विशिष्ट रेडॉक्स पोटेंशियल (लगभग −200 mV और −310 mV) निर्धारित किए गए, जबकि EPR विश्लेषण से [2Fe–2S] केंद्र का रेडॉक्स पोटेंशियल लगभग −255 mV पाया गया। यह अध्ययन एक ताप-प्रिय आत्मनिर्भर P450 का पहला व्यापक डोमेन-स्तरीय वर्णन प्रस्तुत करता है और दर्शाता है कि दोनों डोमेन स्वतंत्र रूप से प्रभावी ढंग से कार्य कर सकते हैं, जो भविष्य में मॉड्यूलर P450 प्रणालियों की अभियांत्रिकी के लिए महत्वपूर्ण हो सकता है।

CYP116B305 के अतिरिक्त, मेटाजीनोमिक स्क्रीनिंग से एक दूसरा महत्वपूर्ण एंज़ाइम, CYP108D18, भी पहचाना गया, जो गर्म जलस्रोत के नमूने में प्रचुर मात्रा में पाए जाने वाले *Novosphingobium* प्रजाति से संबद्ध है। CYP108D18 एक क्लास-I P450 है, जिसे बाहरी रेडॉक्स पार्टनरों की आवश्यकता होती है और यह मध्यम ताप-स्थिरता प्रदर्शित करता है। इसे *E. coli* में व्यक्त कर पूर्णतः शुद्ध किया गया। स्पेक्ट्रोस्कोपिक लिगैंड-बाइंडिंग अध्ययनों से पता चला कि CYP108D18 एरोमैटिक हाइड्रोकार्बनों (जैसे फेनिलसाइक्लोहेक्सेन और फेनैंथ्रीन) के प्रति उच्च अभिरुचि रखता है, जिससे हीम का हाई-स्पिन अवस्था की ओर उल्लेखनीय परिवर्तन होता है, जबकि मोनोटरपीन्स के प्रति इसकी अभिरुचि कम पाई गई। पालक से प्राप्त फेरेडॉक्सिन और फेरेडॉक्सिन रिडक्टेज़ जैसे सरोगेट रेडॉक्स पार्टनरों के साथ पुनर्संयोजन पर, CYP108D18 ने फेनिलसाइक्लोहेक्सेन को ट्रांस-4-फेनिलसाइक्लोहेक्सानॉल में ऑक्सीकृत करने की क्षमता प्रदर्शित की।

इसकी क्रियात्मक संरचना को समझने के लिए CYP108D18 की क्रिस्टल संरचना को सब्सट्रेट-मुक्त अवस्था तथा सब्सट्रेट-बंधित अवस्थाओं (फेनिलसाइक्लोहेक्सेन और *p*-साइमीने) में उच्च रिज़ॉल्यूशन पर हल किया गया। संरचनाओं से पता चला कि सब्सट्रेट बाइंडिंग के बाद भी एंज़ाइम खुली संरचना में बना रहता है, जबकि अधिकांश P450s सब्सट्रेट बाइंडिंग पर बंद संरचना ग्रहण करते हैं। यह स्थायी खुली अवस्था, तथा सब्सट्रेट बाइंडिंग पर हीम रेडॉक्स पोटेंशियल में केवल मामूली वृद्धि (−451 mV से −409 mV बनाम NHE) यह संकेत देती है कि इसकी कम प्रतिक्रियाशीलता और सामान्य P450 इलेक्ट्रॉन ट्रांसफर पार्टनरों के साथ कमजोर अंतःक्रिया का एक संरचनात्मक आधार है। CYP108D18 की खोज और विश्लेषण ने चरम पर्यावरणों से P450 विविधता के दायरे का विस्तार किया और यह समझ प्रदान की कि प्रोटीन संरचना किस प्रकार स्थिरता, रेडॉक्स गुणों और उत्प्रेरक व्यवहार को प्रभावित करती है।

महंगे कोफैक्टर्स और सहायक प्रोटीनों पर निर्भरता को समाप्त करने के लिए, इन P450s को हाइड्रोजन पेरॉक्साइड-चालित जैव-उत्प्रेरकों (पेरऑक्सीज़नेज़) में परिवर्तित करने की एक प्रोटीन अभियांत्रिकी रणनीति अपनाई गई। सक्रिय स्थल के अत्यधिक संरक्षित *I*-हेलिक्स मोटिफ में दो उत्परिवर्तन (मूल अम्ल-अल्कोहल अवशेषों को ग्लूटामाइन और ग्लूटामेट से प्रतिस्थापित करना; “QE” उत्परिवर्तन) प्रविष्ट कर CYP116B305 और CYP108D18 दोनों को H_2O_2 का प्रत्यक्ष उपयोग करने योग्य बनाया गया। इससे NAD(P)H और रेडॉक्स पार्टनरों की आवश्यकता को दरकिनार करते हुए पेरऑक्साइड शंट मार्ग के माध्यम से प्रमुख प्रतिक्रियाशील मध्यवर्ती (कम्पाउंड-I) का निर्माण संभव हुआ।

QE-अभियांत्रित CYP116B305 हीम डोमेन ने अपनी अंतर्निहित ताप-स्थिरता बनाए रखी और केवल H_2O_2 का उपयोग करते हुए 2-HPA से होमोजेंटिसेट में लगभग पूर्ण (>96%) रूपांतरण प्राप्त किया। इसी प्रकार, CYP108D18 का QE म्यूटेंट भी H_2O_2 के साथ पेरऑक्सीज़नेज़ गतिविधि प्रदर्शित करता है, यद्यपि उत्पाद उपज CYP116B305 म्यूटेंट की तुलना में कम रही। दोनों अभियांत्रित एंज़ाइमों में पेरॉक्साइड की उपस्थिति में हीम ब्लीचिंग (ऑक्सीडेटिव निष्क्रियता) के प्रति संवेदनशीलता कम पाई गई, विशेषकर तब जब सब्सट्रेट उपस्थित थे। डिस्टल पॉकेट में अम्ल-क्षार उत्प्रेरक के समावेशन से इन एंज़ाइमों की उत्प्रेरक क्षमता का विस्तार हुआ और ऐसी ऑक्सीडेटिव अभिक्रियाएँ संभव हुईं जो उनकी प्राकृतिक मोनोऑक्सीज़नेज़ अवस्था में नहीं देखी गई थीं। ये परिणाम P450 उत्प्रेरण में कोफैक्टर-निर्भरता को समाप्त करने के लिए एक सामान्यीकृत और किफायती रणनीति प्रस्तुत करते हैं।

संक्षेप में, यह शोध प्रबंध मेटाजीनोमिक खोज और निर्देशित एंज़ाइम अभियांत्रिकी को संयोजित कर उन्नत ताप-स्थिरता और आत्मनिर्भरता वाले P450 मोनोऑक्सीज़नेज़ विकसित करता है। CYP116B305 की पहचान आत्मनिर्भर P450s के वंशानुगत और क्रियात्मक दायरे का विस्तार करती है और एक ऐसे एंज़ाइम को उजागर करती है जो उच्च तापमान पर सुदृढ़ है तथा एरोमैटिक हाइड्रोकार्बनों के कुशल ऑक्सीकरण में सक्षम है। संबंधित मेसॉफिलिक एंज़ाइम CYP116B234 का अध्ययन बैक्टीरियल चयापचय में CYP116 परिवार की पारिस्थितिक भूमिका पर प्रकाश डालता है। CYP116B305 का विस्तृत डोमेन-स्तरीय विश्लेषण इसकी द्वि-मॉड्यूलर संरचना की स्वतंत्र स्थिरता और रेडॉक्स विशेषताओं की मूलभूत समझ प्रदान करता है। वहीं, CYP108D18 की खोज और संरचनात्मक व्याख्या ताप-समृद्ध पर्यावरणों में P450 विविधता को उजागर करती है और यह समझने का ढाँचा प्रदान करती है कि विशिष्ट संरचनात्मक तत्व तापीय अनुकूलन और P450 एंज़ाइमों के ऊष्मागतिक गुणों को कैसे नियंत्रित करते हैं। अंततः, CYP116B305 और CYP108D18 दोनों को पेरॉक्साइड-चालित उत्प्रेरकों में सफलतापूर्वक रूपांतरित करना यह दर्शाता है कि P450s की प्राकृतिक सीमाओं को पार कर बड़े-पैमाने की जैव-प्रक्रियाओं के लिए अधिक सुदृढ़, किफायती और टिकाऊ जैव-उत्प्रेरक विकसित किए जा सकते हैं, जिनका उपयोग औद्योगिक जैव-उत्प्रेरण और पर्यावरणीय जैव-उपचार में महत्वपूर्ण हो सकता है।

Declaration by author

This thesis is composed of my original work and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that the copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis and have sought permission from co-authors for any jointly authored works included in the thesis.

Publications included in this thesis

1. **Kundral, S.,** Beamish, H., Giang, P.D., Salisbury, L.J., Nouwens, A.S., Khare, S.K., Bernhardt, P.V., Harmer, J.R., Bell, S.G., De Voss, J.J. Characterization of the self-sufficient cytochrome P450 CYP116B234 from *Rhodococcus globerulus* and its suggested native role in 2-hydroxyphenylacetic acid metabolism. *Microb Biotechnol* 18, e70125 (2025). https://doi.org/10.1111/1751-7915.70125

2. **Kundral, S.,** Giang, P.D., Grundon, L.R., Supper, J.M., Khare, S.K., Bernhardt, P.V., Evans, P., Bell, S.G., & De Voss, J.J. Characterization of the thermophilic P450 CYP116B305 identified using metagenomics-derived sequence data from an Australian hot spring. *Appl Microbiol Biotechnol* 109, 133 (2025). https://doi.org/10.1007/s00253-025-13521-2

Manuscripts in preparation

1. **Kundral, S.,** Giang, P.D., Khare, S.K., Bernhardt, P.V., Harmer, J.R., Bell, S.G. and De Voss, J.J. *In vitro* reconstitution and domain-level characterization of the thermophilic CYP116B305 enzyme.

2. **Kundral, S.,** Giang, P.D., Khare, S.K., Bernhardt, P.V., Bell, S.G. and De Voss, J.J. Functional and structural characterization of CYP108D18 from *Novosphingobium* sp. revealed in the metagenomic database of an Australian hot spring.

3. **Kundral, S.,** Khare, S.K., Bell, S.G. and De Voss, J.J. Engineering cytochrome P450 to enable the switch from monooxygenase to peroxygenase.

Contributions by others to the thesis

Electrochemical experiments were supervised by Dr. Peter Giang and Professor Paul Bernhardt (The University of Queensland, St Lucia, QLD).

Molecular dynamics simulations and ONIOM calculations were performed by Dr. Alicia Kirk (The University of Queensland, St Lucia, QLD).

Hannah Beamish performed the biochemical characterization of the CYP116B234 enzyme and contributed to drafting the early versions of the CYP116B234 manuscript.

Protein mass spectral analysis was performed by Dr. Amanda Nouwens (The University of Queensland, St Lucia, QLD).

Organic synthesis of *trans*-4-phenylcyclohexanol was performed by Julia Buczynski (The University of Queensland, St Lucia, QLD).

The final refinement and validation of the crystal structures were undertaken with the guidance of Prof. Luke Guddat (The University of Queensland, St Lucia, QLD).

The CYP108D1 plasmid construct was provided by Associate Professor Stephen Bell (Adelaide University, Adelaide, SA).

Project conception and supervision were led by Prof. James De Voss (The University of Queensland, St Lucia, QLD) and Prof. Sunil Kumar Khare (Indian Institute of Technology Delhi).

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research involving human or animal subjects

No animal or human subjects were involved in this research.

Financial support

This research was supported by the Australian Government Research Training Program Scholarship and the Council of Scientific & Industrial Research Junior Research Fellowship. Funding for conference travel was provided by the UQ School of Chemistry and Molecular Biosciences and the Project Enhancement Award fund of UQ-IITD Research Academy (UQIDRA).

Keywords

Biochemistry, Cytochrome P450, Enzymes, Microbiology, Metabolism, Catalysis, Biocatalyst, Electrochemistry, X-ray crystallography

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060101, Analytical Biochemistry, 20%

ANZSRC code: 060107, Enzymes, 70%

ANZSRC code: 060112, Structural Biology, 10%

Fields of Research (FoR) Classification

FoR code: 0601, Biochemistry and Cell Biology, 80%

FoR code: 0604, Genetics, 10%

FoR code: 0605, Microbiology, 10%

Table of Contents

Chapter 1 Introduction 1

1.1 Rationale of the work 1

1.2 Objectives of the thesis 5

1.3 Scope of the thesis 5

1.4 Review of literature 10

1.4.1 History and background 10

1.4.2 Structure of P450s 12

1.4.3 Redox systems 14

1.4.4 Catalytic mechanism of P450s 17

1.4.5 Spectroscopic properties of P450s 19

1.4.6 Redox characterization 23

1.4.7 P450s as biocatalysts 25

1.4.8 Limitations of P450s in biocatalysis 26

1.4.9 The thermophilic advantage: A strategic solution 27

1.4.10 Discovery and characterization of thermostable P450s 28

1.4.10.1 The Pioneers of thermostable P450s: CYP119As, CYP175 and their 30

1.4.10.2 Structural basis of P450 thermostability 32

1.4.10.3 The second wave of thermostable P450 discoveries 34

1.4.10.4 The metagenomic era: Culture-independent P450 discovery 35

1.5 Conclusion 37

Chapter 2 Characterization of the CYP116B enzymes 38

2.1 Characterization of the thermophilic P450 CYP116B305 identified using metagenomics-derived sequence data from an Australian hot spring 38

2.1.1 Introduction 39

2.1.2 Materials and Methods 42

2.1.2.1 Chemicals 42

2.1.2.2 Sample collection and DNA extraction 42

2.1.2.3 Metagenome sequencing, assembly, and annotation 42

2.1.2.4 Identification and phylogenetic analysis....43
2.1.2.5 Protein expression, purification and spectroscopic characterization....43
2.1.2.6 Determination of thermal stability....45
2.1.2.7 Substrate binding analysis....45
2.1.2.8 *In silico* modelling and docking studies....46
2.1.2.9 Cofactor Preference and kinetics....46
2.1.2.10 *In-vitro* catalytic turnovers....47
2.1.2.11 Redox characterization....47
2.1.3 Results and Discussion....48
2.1.3.1 Discovery of self-sufficient CYP116B305 from Geminicoccaceae....48
2.1.3.2 Heterologous expression, purification and spectroscopic characterization....50
2.1.3.3 Thermal stability of the CYP116B305 holoenzyme....54
2.1.3.4 Binding studies....56
2.1.3.5 Cofactor preference and kinetics....62
2.1.3.6 *In vitro* analysis of CYP116B305 and coupling efficiency measurements....64
2.1.3.7 Redox characterization....70
2.1.4 Conclusion....73
2.2 Elucidating the native function of CYP116B234 from *R. globerulus* in 2-hydroxyphenylacetic acid metabolism....75
2.2.1 Introduction....76
2.2.2 Materials and Methods....78
2.2.2.1 Phylogenetic sequence analysis and structure prediction....78
2.2.2.2 Growth of *R. globerulus*....78
2.2.2.3 SWATH-MS analysis on the cell-lysate....78
2.2.2.4 P450 purification and trypsin digestion....79
2.2.2.5 Proteomic analysis....79
2.2.3 Results and Discussion....80
2.2.3.1 Phylogenetic analysis, sequence alignment and structure prediction of....80
2.2.3.2 Determination of CYP116B234 biological function and proteome....83
2.2.4 Conclusion....86

Chapter 3 Analysis and reconstitution of enzyme activity using the individual domains of CYP116B305 88

3.1 Introduction 88

3.2 Materials and Methods 91

3.2.1 Cloning, heterologous expression and purification of heme and PFOR 91

3.2.2 Determination of thermal stability 93

3.2.3 Substrate binding analysis 93

3.2.4 NADPH consumption, coupling efficiency and H_2O_2 production 94

3.2.5 Hydrogen peroxide formation assay 94

3.2.6 Redox characterization 95

3.3 Results and Discussion 96

3.3.1 Heterologous expression, purification and spectroscopic characterization 96

3.3.2 Thermal stability of the heme and PFOR domains 98

3.3.3 Substrate binding analysis 100

3.3.4 Redox characterization of isolated domains 101

3.3.5 Reconstituting heme and PFOR domain catalysis 107

3.4 Conclusion 110

Chapter 4 Structural and functional characterization of CYP108D18 112

4.1 Introduction 112

4.2 Materials and Methods 114

4.2.1 Identification and phylogenetic analysis of CYP108D18 114

4.2.2 Heterologous expression and purification of CYP108D18 114

4.2.3 Determination of thermal stability 115

4.2.4 Substrate binding 116

4.2.5 Redox characterization 116

4.2.6 NAD(P)H consumption assays 117

4.2.7 *In vitro* turnover experiments 117

4.2.8 Crystallization and structure determination 118

4.2.9 Molecular dynamics simulations 119

4.2.10 ONIOM calculations 120

4.3 Results and Discussion 121

4.3.1 Identification of CYP108D18 from *Novosphingobium* sp. 121
4.3.2 Heterologous expression and purification of CYP108D18 122
4.3.3 Thermal stability of CYP108D18 124
4.3.4 Binding studies 125
4.3.5 Redox characterization 127
4.3.6 NAD(P)H consumption assay 129
4.3.7 *In vitro* turnover assays 130
4.3.8 Overall structure of CYP108D18 131
4.3.9 Molecular dynamics simulations and ONIOM transition states 140
4.4 Conclusion 143

Chapter 5 Engineering cytochrome P450 monooxygenases into peroxygenases 145

5.1 Introduction 145
5.2 Materials and Methods 148
5.2.1 Site-directed mutagenesis of CYP116B305 HD and CYP108D18 enzymes 148
5.2.2 Protein expression and purification of QE mutants 149
5.2.3 Spectroscopic characterization and substrate binding of QE mutants 149
5.2.4 Thermal stability 150
5.2.5 Heme bleaching assay 150
5.2.6 H_2O_2 driven peroxygenase assays and time course experiments 150
6.2.7 Substrate scope of mutant enzymes 151
5.3 Results and Discussion 152
5.3.1 Heterologous expression, purification, and spectroscopic characterization 152
5.3.2 Thermal stability of CYP116B305 HD QE mutant 155
5.3.3 Hydrogen peroxide stability of QE mutants 156
5.3.4 Peroxygenase activity and time course analysis 163
5.4 Conclusion 172

Chapter 6 Conclusions and Future Directions 174

Chapter 7 Appendices 179

7.1 Chapter 2.1 179
7.1.1 CYP116B305 protein sequence 179

7.1.2 Amino acid sequence alignment of CYP116B enzymes 181
7.1.3 Spectroelectrochemistry – inorganic mediator structures 185
7.2 Chapter 2.2 186
7.2.1 Amino-acid sequences 186
7.2.1.1 CYP116B234 186
7.2.1.2 Phenylalanine-4-hydroxylase (Incomplete sequence) 186
7.2.1.3 Putative pterin-4-alpha-carbinolamine dehydratase 186
7.2.1.4 Aromatic-amino-acid aminotransferase 187
7.2.1.5 4-hydroxyphenylpyruvate dioxygenase 187
7.2.1.6 Homogentisate 1,2-dioxygenase[a] 187
7.2.1.7 Ureidoglycolate lyase 188
7.2.1.8 Homogentisate 1,2-dioxygenase[b] 188
7.3 Chapter 4 189
7.3.1 Amino-acid sequence of CYP108D18 189
7.3.2 Computational details of molecular dynamics simulations 189
7.3.3 Amino acid sequence alignment 192
7.3.4 Structural characterization of CYP108 enzymes 195
7.3.5 Molecular dynamics simulations and ONIOM transition states 198
7.4 Media composition 200
7.4.1 Lysogeny broth media (1 L) 200
7.4.2 Terrific broth media (1 L) 200
7.4.3 M9 minimal media (1 L) 200
7.4.4 Enrichment media 200
8 Bibliography 202
Biodata 225

List of Figures

Figure 1.1 Modern P450 nomenclature with CYP101A1 as example showing prefix.....12

Figure 1.2 Crystal structure of substrate-bound (D-camphor) CYP101A1 (PDB: 2CPP).....13

Figure 1.3 Graphical representation of the domain architectures of the ten P450 classes.....16

Figure 1.4 Schematic representation of the canonical cytochrome P450 catalytic cycle 19

Figure 1.5 Representative UV-visible spectrum (250 – 700 nm) of a purified P450 20

Figure 1.6 Representative UV-visible CO difference spectrum of P450, showing the 21

Figure 1.7 (a) Representative UV-visible spectra (300 – 600 nm) of P450 in both 22

Figure 2.1.1 Phylogenetic analysis of the amino-acid sequence of CYP116B305 with.....49

Figure 2.1.2 UV-visible spectra (250 – 700 nm) of purified CYP116B305. The observed. ..51

Figure 2.1.3 UV-visible spectra (300 – 700 nm) of purified CYP116B305 are as follows....52

Figure 2.1.4 CO-complex formation of purified CYP116B305 (1 μM) following. 54

Figure 2.1.5 Thermal stability of CYP116B305 following the incubation of holoenzyme....55

Figure 2.1.6 (a) Structures of compounds tested for binding with CYP116B305. Type.....57

Figure 2.1.7 UV-visible spectra of purified CYP116B305 (black) and in the presence.....59

Figure 2.1.8 The predicted structure of CYP116B305 using AlphaFold 3 and visualized.....61

Figure 2.1.9 Kinetic parameters of CYP116B305 determined using substrate-dependent 62

Figure 2.1.10 Kinetic parameters of CYP116B305 investigated using cytochrome *c* as 63

Figure 2.1.11 *In vitro* reactions catalyzed by the CYP116B305 enzyme. 65

Figure 2.1.12 (a) GC Chromatogram of *in vitro* turnover of 2-HPA by CYP116B305 in.....66

Figure 2.1.13 GC Chromatogram of products following *in vitro* NADPH oxidation of.....67

Figure 2.1.14 (a) GC Chromatogram of *in vitro* turnover of HPPA by CYP116B305 in 68

Figure 2.1.15 Mass spectrum of BSTFA-TMS derivatised **(a)** turnover product.....68

Figure 2.1.16 UV-visible spectra of spectroelectrochemical titration of **(a)** substrate. 71

Figure 2.2.1 Proposed pathway for the involvement of CYP116B monooxygenase in 77

Figure 2.2.2 Phylogenetic tree of characterized CYP116 family enzymes. Amino-acid 81

Figure 2.2.3 The predicted structure of CYP116B234 using AlphaFold 3 and visualised..... 82

Figure 2.2.4 Focused image showing the five key residues involved in the proposed..... 83

Figure 2.2.5 Pathway for the catabolism of phenylalanine adapted from Arias-Barrau..... 84

Figure 3.1 UV-visible difference spectrum (400 – 500 nm) of ferrous·CO versus ferrous.... 97

Figure 3.2 SDS-PAGE (NuPAGE 4 – 12%) analysis of (A) heme and (B) PFOR 97

Figure 3.3 UV-visible spectra (250 – 700 nm) of **(a)** the oxidised heme domain of..... 98

Figure 3.4 (a) Thermal stability plots of the CYP116B305 heme domain after 15 min 99

Figure 3.5 UV-visible spectra (300 – 700 nm) of the oxidised heme domain of 100

Figure 3.6 Substrate binding analysis for the heme domain of CYP116B305 (4 μM)..... 101

Figure 3.7 (a) UV-visible spectra (350 – 700 nm) of spectroelectrochemical titration of ... 102

Figure 3.8 (a) UV-visible spectra (350 – 800 nm) of the spectroelectrochemical..... 104

Figure 3.9 (a) X-band (ca. 9.56 GHz) CW EPR spectra recorded at 17.5 K of PFOR..... 106

Figure 3.10 Temperature- and ratio-dependent NADPH consumption by the 108

Figure 3.11 GC chromatograms of *in vitro* turnovers catalyzed by the reconstituted. 109

Figure 4.1 Phylogenetic analysis of the amino-acid sequence of CYP108D18 with 121

Figure 4.2 UV-visible spectra (250 – 700 nm) of purified CYP108D18. The observed 122

Figure 4.3 SDS-PAGE (NuPAGE 4 - 12%) analysis of CYP108D18 protein purified..... 123

Figure 4.4 (a) UV-visible spectra (300 – 700 nm) of CYP108D18 (~4 μM) displaying..... 124

Figure 4.5 UV-visible spectra (300 – 700 nm) of purified CYP108D18 in its oxidised 126

Figure 4.6 Binding curves for CYP108D18 (1 μM) with **(a)** phenylcyclohexane and..... 126

Figure 4.7 UV-visible spectra (350 – 800 nm) of spectroelectrochemical titration of 128

Figure 4.8 (Left) GC-MS traces of *in vitro* turnovers of phenylcyclohexane by 131

Figure 4.9 (a) Overall structure of CYP108D18, with α-helices shown in cyan 132

Figure 4.10 (a) Focused view of the orientation of the region from residues Asp371 135

Figure 4.11 (a) Active-site view of CYP108D18 co-crystallized with..... 138

Figure 4.12 Active-site views of the two CYP108D18 monomers in the asymmetric..... 140

Figure 4.13 Distribution of C-H-O and Fe-O-H angles (°) and H-O distances (Å) for..... 142

Figure 4.14 Structures of **a)** reactant complex, **b)** TS of Hγax abstraction, and..... 143

Figure 5.1 Ferrous-CO versus ferrous difference spectra (CO-difference assay) used 152

Figure 5.2 SDS-PAGE (NuPAGE 4 – 12%) analysis of **(a)** CYP116B305 HD QE and..... 153

Figure 5.3 UV-visible absorption spectra (250 – 700 nm) of purified QE mutants 154

Figure 5.4 Thermal stability profiles of CYP116B305 HD QE mutant showing..... 155

Figure 5.5 Comparison of the heme bleaching rates of the CYP116B305 HD QE..... 157

Figure 5.6 Comparison of the heme bleaching rates of the CYP116B305 HD QE..... 158

Figure 5.7 Comparison of the heme bleaching rates of the CYP108D18 QE mutant. 161

Figure 5.8 Comparison of the heme bleaching rates of the CYP108D18 QE mutant 162

Figure 5.9 GC chromatograms of products from H_2O_2 -driven (2 mM) oxidations 164

Figure 5.10 (a) Products of H_2O_2 -driven oxidation of HPPA by the CYP116B305..... 166

Figure 5.11 GC chromatograms of products from H_2O_2 -driven (10 mM) oxidations..... 167

Figure 5.12 GC chromatograms of products from H_2O_2-driven (20 mM) oxidations 169

Figure 5.13 (a) Products of H_2O_2-driven oxidation of (*R*)-limonene by the..... 170

Figure 5.14 (a) GC chromatograms of 2 h reactions of 3 μM CYP108D18 QE mutant 172

Figure 7.1 The amino acid sequence alignment of characterized CYP116B subfamily..... 183

Figure 7.2 SDS-PAGE (NuPAGE 4 – 12%) analysis of CYP116B305 protein purified 184

Figure 7.3 UV-visible spectra (250 – 600 nm) of FMN released after heating 184

Figure 7.4 UV-visible spectra (300 – 700 nm) of purified CYP116B305 (1 μM) in its 185

Figure 7.5 Overlay of crystal structure (yellow) and AlphaFold3 predicted structure 191

Figure 7.6 The amino acid sequence alignment of characterized enzymes from 193

Figure 7.7 Secondary structure comparison between CYP108D18 (wheat), CYP108D1 ... 195

Figure 7.8 Secondary structure comparison of CYP108D18 (wheat), CYP108D1 195

Figure 7.9 A proposed short proton delivery pathway in CYP108D18. A chain of 196

Figure 7.10 Electrostatic potential of the proximal face of (a) CYP101A1 197

Figure 7.11 Breakdown of the clusters identified for the MD simulations of. 198

Figure 7.12 Energy decomposition comparison between TS for Hγax and Hδeq 199

List of Tables

Table 1.1 Overview of characterized thermophilic P450s, highlighting key information.....29

Table 2.1.1 Comparison of the ratio of charged residues (Asp, Glu, Lys, His, Arg) to.....50

Table 2.1.2 The dissociation constant (K_D) and heme-spin shift change of compounds.....58

Table 2.1.3 Activity parameters determined for substrates investigated with 64

Table 2.2.1 SWATH-MS analysis of cell lysate of *R. globerulus* grown on.....85

Table 2.2.2 SWATH-MS analysis of cell lysate of *R. globerulus* grown on.....85

Table 3.1 Activity parameters determined for substrates investigated with the. 110

Table 4.1 X-ray crystallography data collection and refinement statistics. 119

Table 4.2 The heme-spin shifts and dissociation constants (K_D) of potential substrates.....127

Table 4.3 NAD(P)H consumption rate (μM min^{-1} per μM CYP108D18) of CYP108D18 ..130

Table 5.1 Primers used for constructing QE double mutants of the CYP116B305 HD 148

Table 5.2 Rates of heme bleaching for the CYP116B305 HD QE mutant and wild 159

Table 5.3 Rates of heme bleaching for the CYP108D18 QE mutant and wild-type.....163

Table 7.1 Comparison of the active-site residues of CYP116B enzymes, including.....180

Table 7.2 Identification of key binding interactions between CYP116B305 and the.....180

Table 7.3 Comparison of heme propionate interactions with key residues in 193

Table 7.4 Comparison of the access channel residues of CYP108D18, CYP108D1.....194

Table 7.5 Comparison of the active site residues of CYP108D18, CYP108D1 and 194

Glossary

AF3.....AlphaFold 3

Å.....Angstrom

AGRF.....Australian Genome Research Facility

ALA.....δ-Aminolevulinic acid

BMR.....*Bacillus megaterium* reductase domain

BMP.....*Bacillus megaterium* P450 domain

BSTFA-TMS....*N*,*O*-bis(trimethylsilyl)trifluoroacetamide

Cpd I.....Compound I

CPR.....Cytochrome P450 reductase

CW-EPR.....Continuous Wave Electron Paramagnetic Resonance

CYP.....Cytochrome P450

DFT.....Density Functional Theory

DNA.....Deoxyribonucleic acid

DTT.....Dithiothreitol

ϵ.....Extinction coefficient

EPR.....Electron Paramagnetic Resonance

ET.....Electron Transfer

EDTA.....Ethylenediaminetetraacetic Acid

E. coli.....*Escherichia coli*

FAD.....Flavin adenine dinucleotide

FdR.....Ferredoxin Reductase

Fdx.....Ferredoxin

FMN.....Flavin mononucleotide

GC-MS.....Gas Chromatography Mass Spectrometry

h.....hour(s)

HPPA.....3-(2-hydroxyphenyl)propionic acid

2-HPA.....2-hydroxyphenylacetic acid

IPTG.....Isopropyl β-D-1-thiogalactopyranoside

K_D.....Dissociation constant

KPi.....Potassium Phosphate buffer

K_m.....Michaelis constant

L.....Litre(s)

LB media.....Lysogeny Broth media

min.....minute(s)

mg.....milligram

mL.....millilitre

mM.....millimolar

mmol.....millimole

µmol.....micromole

m/z.....mass to charge ratio

MD.....Molecular Dynamics

MS.....Mass Spectrometry

NAD(P)H.....Reduced Nicotinamide Adenine Dinucleotide (Phosphate)

$NAD(P)^+$.....Oxidised Nicotinamide Adenine Dinucleotide (Phosphate)

NIST.....National Institute of Standards and Technology

NHE.....Normal Hydrogen Electrode

nm.....nanometre

ONIOM.....Our own N-layered Integrated molecular Orbital and molecular Mechanics

P450(s).....Cytochrome P450 Enzyme(s)

PCR.....Polymerase chain reaction

PFOR.....Phthalate Family Oxygenase Reductase domain

PMSF.....Phenylmethylsulfonyl Fluoride

P. putida.....*Pseudomonas putida*

R. globerulus…*Rhodococcus globerulus*

RMSD.....Root-Mean-Square Deviation

rpm.....Revolutions per Minute

ROS.....Reactive Oxygen Species

RZ.....Reinheitszahl value

SDS-PAGE.....Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

SWATH-MS.....Sequential Window Acquisition of All Theoretical Mass Spectra

SRS.....Substrate recognition sites

TB.....Terrific broth

UV-Vis.....Ultraviolet-Visible Spectroscopy

x *g*.....times gravity