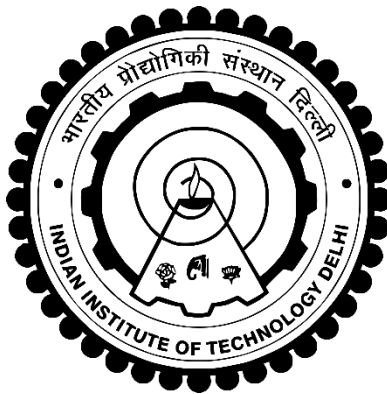


**MOLECULAR BASIS OF ASPHALTENE
BIOTRANSFORMATION BY A MICROBIAL
CONSORTIUM**

NIDHI NITIN PATIL



**DEPARTMENT OF BIOCHEMICAL ENGINEERING
AND BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI
DECEMBER 2025**

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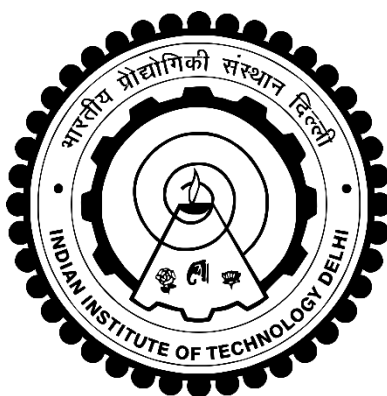
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DEPARTMENT OF BIOCHEMICAL ENGINEERING AND
BIOTECHNOLOGY

Submitted

In fulfilment of the requirements of the degree of Doctor of Philosophy
to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

DECEMBER 2025

CERTIFICATE

This is to certify that the thesis titled “**Molecular basis of asphaltene biotransformation by a microbial consortium**” being submitted by **Ms. Nidhi Nitin Patil** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** is a record of bonafide research work carried out by her under our supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi, New Delhi.

The results presented in this thesis have not been submitted in part or full to any other University or Institute for the award of any other degree or diploma.

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Date-

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ABSTRACT

The ever-increasing demand of crude oil is leading to its rapid consumption, causing a scarcity in the conventional fuels available. Hence, newer and alternative energy resources are needed. Even with the emerging renewable energy resources, yet today, the maximum demand of energy is met with coal and crude oil. Thus, before we are completely able to shift to green and clean energy, efficient utilisation of the existing non-renewable energy resource is necessary. Most of the fuel products such as petrol, diesel, gasoline, etc are derived from light crude oils. It is because, the high density and viscosity of heavy crude oils makes them unsuitable to be used for commercial production using the available conventional methods. The number of heavy crude oil reservoirs in the world are about seven times more in number than the rapidly utilised light crude oil reservoirs. This makes the heavy crude oil reserves a large untapped source of energy. The reason for such heaviness of these oils is the presence of asphaltenes in them. Biotransformation of asphaltene can lead to a breakdown of its structure resulting in a decrease in viscosity and density of heavy oil. By targeting asphaltene breakdown, the trapped energy from heavy oil reservoirs could be harnessed to meet with the world's energy demand. A nine membered microbial consortium has been reported to biotransform about 75% asphaltene in 21 days. The members have been reported to secrete enzymes which can act on asphaltene (Zargar 2021). The study carried out in this thesis works on the focused biotransformation of asphaltene using those enzymes. This provides a biological, ecofriendly and cost-effective method of asphaltene biotransformation.

In the present study, the genome of all members of the consortium were assessed to find the presence of genes acting on aromatic hydrocarbons and alkane chains. The various enzymes secreted extracellularly by the consortium during biotransformation were analysed. Based on the genome and secretome data, certain enzymes known to act on sulphur and nitrogen were

investigated to check for their role in asphaltene biotransformation. The genes were cloned, and the protein was overexpressed in a heterologous host. The action of the enzymes on asphaltene was assessed. An enzyme thiol peroxidase from four different strains of the consortium was tested for its asphaltene biotransformation efficiency. The biotransformation achieved by the several thiol peroxidases overexpressed from a heterologous host, ranged between 45 to 63.83%, the highest being from the thiol peroxidase of *Micrococcus* sp. IITD107. The enzyme was purified, and its enzyme activity and kinetics were studied. The action of the enzyme was studied on asphaltene and characterization of transformed asphaltene was done. The asphaltene fraction was analysed by GC-MS and it was found that during enzymatic biotransformation, several peaks corresponding to PAHs were found to reduce in size with respect to control. The FTIR and NMR spectra indicated changes in the functional group and chemical bonds of treated asphaltene. Change in elemental composition was checked and a reduction in sulphur and nitrogen content was observed whereas the carbon and hydrogen remained largely unaffected. The change in aromaticity levels of asphaltene and model polyaromatic hydrocarbon (PAH) compounds due to the action of the enzyme was studied to find a reduction in their levels. Change in surface morphology was assessed by Scanning Electron Microscopy (SEM). The role of the enzyme on asphaltene biotransformation was confirmed by overexpressing the gene in its native host. The recombinant *Micrococcus* showed increased rate of biotransformation as compared to wild type. The gene was also successfully deleted by homologous recombination in the native host. The deletion of gene led to a major drop in the asphaltene biotransformation capability of the host strain as compared to wild type. The purified enzyme was immobilised for creation of a packed bed column which could be applied for asphaltene biotransformation. Model oil when passed through this column was found to have a 34 % reduction in weight of asphaltene in just 6 hours of treatment whereas asphaltene from crude oil reduced by 23.5% in weight in 48 hours. Due to action of the enzyme

on asphaltene, the smooth surface of asphaltene was converted into a porous structure. This led to the development of a novel and rapid enzymatic process for development of porous carbon from asphaltene. The process can lead to successful valorization of asphaltene into useful porous carbons.

सार

कच्चे तेल की लगातार बढ़ती मांग के कारण इसकी खपत तेजी से बढ़ रही है, जिससे उपलब्ध पारंपरिक ईंधन की कमी हो रही है। इसलिए, नए और वैकल्पिक ऊर्जा संसाधनों की आवश्यकता है। उभरते हुए नवीकरणीय ऊर्जा संसाधनों के बावजूद, आज भी ऊर्जा संसाधन की अधिकतम मांग कोयले और कच्चे तेल से पूरी होती है। इसलिए, इससे पहले कि हम पूरी तरह से हरित और स्वच्छ ऊर्जा में बदल सकें, मौजूदा गैर-नवीकरणीय ऊर्जा संसाधन का कुशल उपयोग आवश्यक है। पेट्रोल, डीजल, गैसोलीन आदि जैसे अधिकांश ईंधन उत्पाद हल्के कच्चे तेलों से प्राप्त होते हैं। ऐसा इसलिए है, क्योंकि भारी कच्चे तेलों का उच्च घनत्व और चिपचिपापन उन्हें उपलब्ध पारंपरिक तरीकों का उपयोग करके वाणिज्यिक उत्पादन के लिए अनुपयुक्त बनाता है। दुनिया में भारी कच्चे तेल के भंडारों की संख्या तेजी से उपयोग किए जाने वाले हल्के कच्चे तेल के भंडारों की तुलना में लगभग सात गुना अधिक है। यह भारी कच्चे तेल के भंडारों को ऊर्जा का एक बड़ा अप्रयुक्त स्रोत बनाता है। इन तेलों के इतने भारी होने का कारण उनमें डामर की उपस्थिति है। डामर के जैव-रूपांतरण से इसकी संरचना टूट सकती है, जिसके परिणामस्वरूप भारी तेल की चिपचिपाहट और घनत्व में कमी आ सकती है। डामर के टूटने को लक्षित करके, भारी तेल भंडारों से फंसी हुई ऊर्जा का उपयोग दुनिया की ऊर्जा मांग को पूरा करने के लिए किया जा सकता है। नौ सदस्यों वाले माइक्रोबियल संघ ने 21 दिनों में लगभग 75% डामर को जैव-रूपांतरित करने की सूचना दी है। सदस्यों को एंजाइम सावित करने की सूचना मिली है जो डामर पर कार्य कर सकते हैं (जरगर 2021)। इस थीसिस में किया गया अध्ययन उन एंजाइमों का उपयोग करके डामर के केंद्रित जैव-रूपांतरण पर काम करता है। यह डामर जैव-रूपांतरण की एक जैविक, पर्यावरण-अनुकूल और लागत-प्रभावी विधि प्रदान करता है।

वर्तमान अध्ययन में, सुगंधित हाइड्रोकार्बन और एल्केन श्रृंखलाओं पर कार्य करने वाले जीन की उपस्थिति का पता लगाने के लिए संघ के सभी सदस्यों के जीनोम का मूल्यांकन किया गया। जैव-रूपांतरण के दौरान संघ द्वारा बाह्य रूप से स्रावित विभिन्न एंजाइमों का विश्लेषण किया गया। जीनोम और सीक्रेटोम डेटा के आधार पर, सल्फर और नाइट्रोजन पर कार्य करने वाले कुछ एंजाइमों की जांच की गई ताकि एस्फाल्टीन बायोट्रांसफॉर्मेशन में उनकी भूमिका की जांच की जा सके। जीन को क्लोन किया गया, और प्रोटीन को विषम मेजबान में अधिक व्यक्त किया गया। एस्फाल्टीन पर एंजाइमों की क्रिया का आकलन किया गया। संघ के चार अलग-अलग उपभेदों से एक एंजाइम थिओल पेरोक्सीडेज को इसके एस्फाल्टीन बायोट्रांसफॉर्मेशन दक्षता के लिए परीक्षण किया गया था। विषम मेजबान से अधिक व्यक्त किए गए कई थिओल पेरोक्सीडेज द्वारा प्राप्त बायोट्रांसफॉर्मेशन 45 से 63.83% के बीच था, जिसमें सबसे अधिक माइक्रोकॉकस एसपी के थिओल पेरोक्सीडेज से था। IITD107। एंजाइम को शुद्ध किया गया, और इसकी एंजाइम गतिविधि और गतिकी का अध्ययन किया गया। एंजाइम की क्रिया का एस्फाल्टीन पर अध्ययन किया गया और रूपांतरित एस्फाल्टीन का लक्षण वर्णन किया गया। जीसी-एमएस द्वारा एस्फाल्टीन अंश का विश्लेषण किया गया और पाया गया कि एंजाइमेटिक जैवपरिवर्तन के दौरान, पीएच से संबंधित कई चोटियों का आकार नियंत्रण के संबंध में कम हो गया था। एफटीआईआर और एनएमआर स्पेक्ट्रा ने उपचारित एस्फाल्टीन के कार्यात्मक समूह और रासायनिक बंधों में परिवर्तन का संकेत दिया। तत्व संरचना में परिवर्तन की जाँच की गई और सल्फर और नाइट्रोजन की मात्रा में कमी देखी गई जबकि कार्बन और हाइड्रोजन काफी हद तक अप्रभावित रहे। एंजाइम की क्रिया के कारण एस्फाल्टीन और मॉडल पॉलीएरोमैटिक हाइड्रोकार्बन (पीएच) यौगिकों के एरोमैटिकिटी स्तरों में परिवर्तन का अध्ययन किया गया ताकि उनके स्तरों में कमी का पता लगाया जा सके। स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी (एसईएम) द्वारा सतह की आकृति विज्ञान में परिवर्तन का मूल्यांकन किया गया। एस्फाल्टीन जैवपरिवर्तन पर एंजाइम की भूमिका की पुष्टि इसके मूल मेजबान में जीन को अधिक अभिव्यक्त करके की गई। पुनः संयोजक माइक्रोकॉकस ने जंगली प्रकार की तुलना में जैवपरिवर्तन की दर में वृद्धि दिखाई। मूल मेजबान

में समजातीय पुनर्संयोजन द्वारा जीन को सफलतापूर्वक हटा दिया गया। जीन के विलोपन के कारण मेजबान स्ट्रेन की एस्फाल्टीन जैवरूपांतरण क्षमता में जंगली प्रकार की तुलना में बड़ी गिरावट आई। पैक्ड बेड कॉलम के निर्माण के लिए शुद्ध किए गए एंजाइम को स्थिर किया गया, जिसे एस्फाल्टीन जैवरूपांतरण के लिए लागू किया जा सकता है। जब मॉडल तेल को इस कॉलम से गुजारा गया तो पाया गया कि उपचार के केवल 6 घंटे में एस्फाल्टीन के वजन में 34% की कमी आई, जबकि कच्चे तेल से प्राप्त एस्फाल्टीन के वजन में 48 घंटे में 23.5% की कमी आई। एस्फाल्टीन पर एंजाइम की क्रिया के कारण, एस्फाल्टीन की चिकनी सतह एक छिद्रपूर्ण संरचना में परिवर्तित हो गई। इससे एस्फाल्टीन से छिद्रपूर्ण कार्बन के विकास के लिए एक नई और तीव्र एंजाइमेटिक प्रक्रिया का विकास हुआ। इस प्रक्रिया से एस्फाल्टीन को उपयोगी छिद्रपूर्ण कार्बन में सफलतापूर्वक परिवर्तित किया जा सकता है।

LIST OF CONTENTS

Certificate	i
Acknowledgments	ii
Abstract	vi
Contents	xii
List of Figures	xvii
List of Tables	xxi
Abbreviations and Symbols	xxiii
Chapter 1 Introduction and Objectives	1
Chapter 2 Literature Review	
2.1 Crude oil and its types	5
2.1.1 Formation	5
2.1.2 Classification	6
2.2 Asphaltene	10
2.3 Physical methods for degradation of asphaltenes	15
2.4 Chemical degradation of asphaltene	16
2.5 Asphaltene biodegradation	16
2.6 Enzymes for asphaltene biotransformation	21
2.7 Enzymes for PAH degradation	23
2.7.1 Peroxidases	24
2.7.2 Dioxygenase and monooxygenase	26
A) Dioxygenase	27
B) Monooxygenase	29
2.7.3 Other enzymes	31
2.8 Thiol peroxidase	31
2.9 Genetic engineering for bioremediation	33
2.10 Heavy oil upgradation	39
2.10.1 Microorganisms for enhanced oil recovery	41
2.10.2 Enzymes used for upgradation of heavy oil	42
2.11 Valorization of asphaltene	43
2.11.1 Porous carbon	43
2.11.2 Synthesis of porous carbon	44
2.12 Application of porous carbon	46
2.12.1 Adsorbent	46
2.12.2 Porous carbon in carbon capture	48
2.12.3 Graphene carbon dots	48
2.12.4 Carbon fibres	49
2.12.5 Asphaltene as sensors	51
2.12.6 Asphaltene as supercapacitors	51

Chapter 3	Materials and Methods	
3.1	List of strains, plasmids and primers used in the study	52
3.2	Media used in the study for growth along with growth conditions	55
3.3	Growth kinetics	57
3.4	Asphaltene biotransformation	57
	a. Model oil preparation	57
	b. Experimental setup – Biphasic experiment	57
	c. Determination of asphaltene biotransformation	58
3.5	Genomic DNA isolation	59
3.6	Whole genome sequencing	60
3.7	Bioinformatic analysis of consortium members	61
3.8	Secretome analysis	62
3.9	Molecular cloning techniques	62
3.9.1	Plasmid isolation (manual method)	62
3.9.2	Plasmid isolation (kit method)	63
3.9.3	Agarose gel electrophoresis	64
3.9.4	Polymerase chain reaction	65
3.9.5	Restriction digestion	66
3.9.6	DNA extraction from agarose gel	66
3.9.7	Ligation	67
3.9.8	Preparation of chemically competent <i>E. coli</i> cells	68
3.9.9	Transformation in the prepared chemically competent cells	68
3.9.10	Molecular cloning of genes in heterologous host	69
3.10	Overexpression of protein from a heterologous host	70
3.11	Sample preparation for visualisation of protein bands	71
3.12	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE)	71
3.13	Determination of solubility of protein	72
3.14	Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI ToF) of protein expressed by pNP4	73
3.15	Asphaltene biotransformation using overexpressed enzyme	73
3.16	Characterization of asphaltene	
3.16.1	Fourier Transform Infrared Spectroscopy (FTIR)	74
3.16.2	Nuclear Magnetic Resonance (NMR)	74
3.16.3	Gas Chromatography Mass Spectrometry (GC-MS)	74
3.16.4	Elemental Analysis (CHNS)	75
3.16.5	Scanning electron microscopy (SEM)	75
3.17	To determine the effect of overexpression and deletion of gene encoding thiol peroxidase on asphaltene biotransformation	75
3.17.1	Construction of <i>Ptac.tpx</i> (pNP11)	75
3.17.2	Construction of shuttle vector (pNP12)	76

3.17.3	Preparation of electrocompetent cells and electroporation	76
3.17.4	Plasmid segregational stability	76
3.17.5	Deletion of <i>tpx</i> from native host	77
3.17.6	Effect of deletion and overexpression on asphaltene biotransformation by <i>Micrococcus</i> sp. IITD107	77
3.17.7	Morphological changes due to engineering of <i>Micrococcus</i> sp. IITD107	78
3.18	Development of a method for rapid biotransformation of asphaltene	78
3.18.1	Protein purification	78
3.18.2	Enzyme kinetics	79
3.18.3	Optimum pH and temperature determination along with stability analysis	79
3.18.4	Determination of effect of metal ions and organic solvents on enzyme activity	80
3.19	Effect of enzyme on pure PAH compounds	80
3.20	Action of pure protein on aromaticity	81
	a) Reduction of aromaticity of asphaltene	81
	b) Reduction of aromaticity of pure model PAH	81
	c) Action of enzyme on lignin	81
3.21	Immobilisation of pure protein	82
3.22	Effect of immobilized enzyme on model oil containing asphaltene	82
3.23	Effect of immobilized enzyme on heavy crude oil	82
3.24	Molecular docking of asphaltene with protein	83
Chapter 4	Results and Discussion	
4.1	To isolate and identify the enzymes for asphaltene biotransformation	84
4.1.1	Biphasic experiment using the nine membered microbial consortium resulted in about 66% asphaltene biotransformation in 14 days	84
4.1.2	Whole genome sequencing of the consortium members	
i	Whole genome sequencing of <i>Arthrobacter</i> sp. IITD100	85
ii	Whole genome sequencing of <i>Arthrobacter</i> sp. IITD101	90
iii	Whole genome sequencing of <i>Rhodococcus</i> sp. IITD102	96
iv	Whole genome sequencing of <i>Rhodococcus</i> sp. IITD103	104
v	Whole genome sequencing of <i>Lysinibacillus</i> sp. IITD104	111
vi	Whole genome sequencing of <i>Sporosarcina</i> sp. IITD105	116
vii	Whole genome sequencing of <i>Micrococcus</i> sp. IITD107	120
viii	Whole genome sequencing of <i>Paenibacillus</i> sp. IITD108	125
4.1.3	Determination of antibiotic resistance genes	129
4.1.4	Determination of metal resistance in the consortium members	132

4.1.5	Proposed target sites for the action of enzymes on asphaltene for biotransformation	133
4.1.6	Analysis of extracellular enzymes	138
4.1.7	Secretome analysis	140
4.2	To determine the efficacy of asphaltene biotransformation by recombinant bacteria	146
4.2.1	Shortlisting of enzymes based on genome and secretome analysis	146
4.2.2	Multiple sequence alignment of gene and protein sequences of thiol peroxidase	146
4.2.3	Cloning of genes and their expression in heterologous host	148
4.2.4	Overexpression of proteins in heterologous host	159
4.2.5	Biotransformation of asphaltene using overexpressed enzymes	161
4.3	To study asphaltene biotransformation by thiol peroxidase from <i>Micrococcus</i> sp. IITD107	163
4.3.1	Multiple sequence alignment of thiol peroxidases of the consortium	163
4.3.2	Asphaltene biotransformation by <i>Micrococcus</i> sp. IITD107	164
4.3.3	Deletion of thiol peroxidase from <i>Micrococcus</i> sp. IITD107	165
4.3.4	Screening of deletion mutant	166
4.3.5	Deletion mutant resulted in reduced asphaltene biotransformation	167
4.3.6	Construction of a shuttle vector for overexpressing enzyme in native host	168
4.3.7	Stability of the shuttle vector in <i>Micrococcus</i> sp. IITD107	171
4.3.8	Improved asphaltene biotransformation by recombinant <i>Micrococcus</i> due to overexpression of thiol peroxidase	173
4.3.9	Growth, colony and cell morphology	174
4.3.10	Comparative halotolerance and peroxide tolerance of the strains	177
4.3.11	Asphaltene biotransformation by crude cell lysate from recombinant <i>E. coli</i>	180
4.3.12	Characterization of biotransformed asphaltene	181
a	FTIR analysis	181
b	NMR analysis	182
c	Analysis of the enzymatic biotransformation products by GC-MS	182
d	Elemental analysis	187
e	Scanning Electron Microscopy (SEM)	188
4.4	To develop a method for rapid biotransformation of asphaltene	
4.4.1	Insights into the structure and function of thiol peroxidase	192
4.4.2	Purification of thiol peroxidase	193
4.4.3	Enzyme kinetics of purified protein	194
4.4.4	Biochemical characterization of the recombinant protein	195

4.4.5	Effect of metal ions and organic solvents on the activity of enzyme	198
4.4.6	Stability enhancement of purified enzyme	200
4.4.7	Action of purified enzyme on asphaltene	200
4.4.8	Molecular docking of thiol peroxidase with asphaltene	201
4.4.9	Action of purified enzyme on pure PAH compounds	204
4.4.10	Action of purified enzyme on lignin	206
4.4.11	Immobilization of purified enzyme on agarose beads	207
4.4.12	Biotransformation of asphaltene in a packed bed column	209
a)	Biotransformation of asphaltene present in model oil	209
b)	Biotransformation of asphaltene present in crude oil	210
4.4.13	Mechanism of action of thiol peroxidase	211
4.4.14	Application of thiol peroxidase for valorization of asphaltene	213
Chapter 5	Summary, Conclusions and Future Prospects	
	Summary	215
	Conclusions	220
	Future Prospects	222
	References	223
	Appendix	249
	Curriculum Vitae	262

LIST OF FIGURES

Figure No	Title	Page No
2.1	Formation of crude oil over a period of time	6
2.2	World heavy oil and bitumen resources in billion barrels	9
2.3	Stages of asphaltene precipitation	12
2.4	General mechanism of Thiol Peroxidase	33
2.5	Sources of Global energy consumption	40
2.6	Valorization of asphaltene into porous carbon	46
4.1	Asphaltene biotransformation by the microbial consortium	84
4.2	GC skew circular map of <i>Arthrobacter</i> sp. IITD100	86
4.3	Phylogenetic tree of <i>Arthrobacter</i> sp. IITD100 marked as 2Y	86
4.4	RAST analysis of <i>Arthrobacter</i> sp. IITD100	87
4.5	GC skew circular map of <i>Arthrobacter</i> sp. IITD101	91
4.6	Phylogenetic tree of <i>Arthrobacter</i> sp. IITD101 marked as 2YMTCC	92
4.7	RAST analysis of <i>Arthrobacter</i> sp. IITD101	92
4.8	GC skew circular map of <i>Rhodococcus</i> sp. IITD102	97
4.9	Phylogenetic tree of <i>Rhodococcus</i> sp. IITD102 marked as 2R	97
4.10	RAST analysis of <i>Rhodococcus</i> sp. IITD102	98
4.11	GC skew circular map of <i>Rhodococcus</i> sp. IITD103	104
4.12	Phylogenetic tree of <i>Rhodococcus</i> sp. IITD103 marked as Q3R	105
4.13	RAST analysis of the strain <i>Rhodococcus</i> sp. IITD103	105
4.14	GC skew circular DNA map of <i>Lysinibacillus</i> sp. IITD104	112
4.15	Phylogenetic tree of <i>Lysinibacillus</i> sp. IITD104 marked as NP4	112
4.16	RAST analysis of the strain <i>Lysinibacillus</i> sp. IITD104	112
4.17	GC skew circular map of <i>Sporosarcina</i> sp. IITD105	116
4.18	Phylogenetic tree of <i>Sporosarcina</i> sp. IITD105 marked as NP#5	117
4.19	RAST analysis of the strain <i>Sporosarcina</i> sp. IITD105	117
4.20	GC skew circular map of <i>Micrococcus</i> sp. IITD107	120
4.21	Phylogenetic tree of <i>Micrococcus</i> sp. IITD107 marked as NP7	121
4.22	RAST analysis of the strain <i>Micrococcus</i> sp. IITD107	121
4.23	GC skew circular map of <i>Paenibacillus</i> sp. IITD108	126
4.24	Phylogenetic tree of <i>Paenibacillus</i> sp. IITD108 marked as 8	126
4.25	RAST analysis of the strain <i>Paenibacillus</i> sp. IITD108	126
4.26	The various antibiotic resistance elements present in the members of the microbial consortium	131
4.27	Possible target sites of the enzymes encoded by the consortium members on the molecular structure of asphaltene	138

4.28	SDS PAGE of aqueous medium to detect presence of extracellular enzymes	139
4.29	Various enzymes detected in the secretome of the biphasic asphaltene biotransformation experiment at days 7, 14 and 21	141
4.30	The multiple sequence alignment of gene sequence of <i>tpx</i> encoding enzyme thiol peroxidase	147
4.31	The multiple sequence alignment of protein sequence of enzyme thiol peroxidase between all members of the consortium	147
4.32	Cloning strategy of pNP4 from pAK3 and <i>tpx</i> gene from <i>Micrococcus</i> sp. IITD107	149
4.33	Construction of pNP4	150
4.34	Cloning strategy of pNP6 from pAK3 and <i>tpx</i> gene from <i>Sporosarcina</i> sp. IITD105	151
4.35	Construction of pNP6	152
4.36	Cloning strategy of pNP7 from pAK3 and <i>tpx</i> gene from <i>Bacillus</i> sp. IITD106	152
4.37	Construction of pNP7	153
4.38	Cloning strategy of pNP8 from pAK3 and <i>tpx</i> gene from <i>Paenibacillus</i> sp. IITD10	154
4.39	Construction of pNP8	155
4.40	Cloning strategy of pNP9	156
4.41	Construction of pNP9	157
4.42	Cloning strategy of pNP10	158
4.43	Construction of pNP10	158
4.44	Overexpression of heterologous expressed proteins on SDS PAGE	160
4.45	Asphaltene biotransformation by the different overexpressed proteins	162
4.46	The multiple sequence alignment and percentage identity matrix of protein sequence of enzyme thiol peroxidase from the <i>Micrococcus</i> sp. IITD107	163
4.47	Asphaltene biotransformation by wild type <i>Micrococcus</i> sp. ITID107	164
4.48	Deletion strategy for <i>tpx</i>	166
4.49	Asphaltene biotransformation by the deletion mutant of <i>Micrococcus</i> sp. IITD 107 lacking the <i>tpx</i> gene.	167
4.50	Cloning strategy of pNP11	169
4.51	Construction of pNP11	170
4.52	Cloning strategy of pNP12	170
4.53	Construction of pNP12	171
4.54	Replica plating for checking plasmid stability	172
4.55	Number of generations for which the plasmid is stable in <i>Micrococcus</i> sp. IITD107	172

4.56	Asphaltene biotransformation by the overexpression strain of <i>Micrococcus</i> sp. IITD107.	173
4.57	Asphaltene biotransformation efficiency of the three strains of <i>Micrococcus</i>	174
4.58	Growth curves of wild type and mutant strains of <i>Micrococcus</i> sp. IITD107	175
4.59	SEM imaging of a) Wild type <i>Micrococcus</i> sp. IITD107; b) Overexpression strain with gene <i>tpx</i> present in extrachromosomal plasmid, c) deletion mutant without presence of <i>tpx</i> gene in chromosome	176
4.60	Halotolerance in <i>Micrococcus</i> strains	178
4.61	Tolerance to peroxide stress	178
4.62	MALDI score of the thiol peroxidase overexpressed from pNP4	180
4.63	Asphaltene biotransformation by crude lysate overexpressing thiol peroxidase from pNP4	180
4.64	FTIR spectra of control and test asphaltene samples	181
4.65	NMR spectroscopy of asphaltene control and treated test asphaltene	182
4.66	GC MS of control asphaltene A) raw asphaltene at day 0 time point; time point B) Asphaltene, treated with crude lysate of only wild type <i>E. coli</i> cells	183
4.67	GC-MS spectra of asphaltene treated with crude lysate at various time points, A). Control asphaltene post 18 days, untreated with enzyme, B) Asphaltene fraction post 7 days of treatment with overexpressed enzyme, C) Asphaltene fraction post 14 days of treatment with overexpressed enzyme, D) Asphaltene fraction post 18 days of treatment with overexpressed enzyme	184
4.68	Elemental analyses of treated asphaltene fraction as compared to control analyzing the changes in elemental composition of Carbon, Hydrogen, Nitrogen and Sulphur. A) comparative elemental analysis with respect to Sulphur and Nitrogen, B) with respect to carbon and hydrogen	188
4.69	SEM images of untreated and treated asphaltene fraction	190
4.70	Pores developed on the surface of asphaltene	191
4.71	A) Analysis of the protein sequence on InterPro revealed the presence of three conserved domains. B) The protein sequence of thiol peroxidase from <i>Micrococcus</i> sp. IITD107 highlighted with the peroxidatic and resolving cysteine residues which have a key part in the peroxidative activity of the enzyme, the thiol peroxidase family signature domain and the conserved residues which are a part of the enzyme's catalytic triad	192

4.72	SDS PAGE silver stained image showing purified protein and overexpression of protein in crude lysate at 18kDa	193
4.73	Michaelis Menten plot for enzyme thiol peroxidase	194
4.74	Relative enzyme activities	197
4.75	A) Effect of metal ions on enzyme activity. B) Effect of several detergents and organic solvents on enzyme activity	199
4.76	The stability of purified enzyme improved after addition of PEG	200
4.77	Fluorescence emission spectra obtained from asphaltene	201
4.78	A. Interaction between ligand asphaltene and Thiol peroxidase from <i>Micrococcus</i> sp. IITD 107; B. Interaction between ligand asphaltene and Thiol peroxidase from <i>E. coli</i> (3HVS)	203- 204
4.79	Reduction in aromaticity of small pure PAH compounds A) naphthalene and B) phenanthrene.	205
4.80	HPLC chromatograms of untreated and thiol peroxidase treated PAHs- naphthalene and phenanthrene.	205
4.81	The action of purified enzyme on lignin	206
4.82	Enzyme entrapped on agarose gel matrix and creation of small equal size discs	208
4.83	Creation of a lab scale packed column using alternate agarose gel discs and sand	208
4.84	Enzyme activity at multiple time points post enzyme immobilization on agarose gel	209
4.85	Biotransformation of Asphaltene in model oil of treated (green) and untreated (red) samples	210
4.86	Asphaltene concentration determination in crude oil	211
4.87	Proposed mechanism of action of thiol peroxidase on asphaltene	212
4.88	Proposed application of the enzyme thiol peroxidase for valorization of asphaltene generated in the oil industry	214

LIST OF TABLES

Table No	Title	Page No
2.1	Differences between heavy and light crude oil	8
2.2	Asphaltene biodegradation by various microorganisms	17
2.3	Enzymes reported for asphaltene biotransformation	22
2.4	Genetically engineered microorganisms for PAH removal	36
2.5	Protein Engineering for PAH removal	38
3.1	Description of strains of bacteria and their source	52
3.2	List of plasmids utilized in the present study	53
3.3	List of primers utilised in the present study	54
3.4	Composition of medium for asphaltene biotransformation	56
3.5	Composition of trace elements stock	56
3.6	Sample specific octamers used in the study	61
3.7	PCR reaction mixture components	65
3.8	Conditions of PCR	65
3.9	Components of SDS polyacrylamide gel	72
3.10	Buffers used to determine optimum pH	79
4.1	Number of genes in the various subsystems determined in <i>Arthrobacter</i> sp. IITD100	87
4.2	List of various genes of <i>Arthrobacter</i> sp. IITD100 which might play a role in asphaltene biotransformation	88
4.3	Number of genes present in various subsystems in <i>Arthrobacter</i> sp. IITD101	92
4.4	The various genes present in <i>Arthrobacter</i> sp. IITD101 which might play a role in Asphaltene biotransformation	93
4.5	The subsystems present in <i>Rhodococcus</i> sp. IITD102	98
4.6	The list of genes that could play a role in biotransformation of asphaltene in <i>Rhodococcus</i> sp. IITD102	99
4.7	The number of genes present in various subsystems in <i>Rhodococcus</i> sp. IITD103	106
4.8	The probable genes playing a role in asphaltene biotransformation in <i>Rhodococcus</i> sp. IITD103	106
4.9	The RAST analysis indicated the presence of genes in the various subsystems of <i>Lysinibacillus</i> sp. IITD104	113
4.10	List of genes which might play a role in asphaltene biotransformation in <i>Lysinibacillus</i> sp. IITD104	113
4.11	Number of genes present in various subsystems in <i>Sporosarcina</i> sp. IITD105	117
4.12	Various genes present in <i>Sporosarcina</i> sp. IITD105	118
4.13	The number of genes present in various subsystems of <i>Micrococcus</i> sp. IITD107	122
4.14	The list of genes likely to be involved in asphaltene biotransformation from <i>Micrococcus</i> sp. IITD107	122

4.15	The number of genes present in various subsystems in <i>Paenibacillus</i> sp. IITD108	127
4.16	The various genes that might play a role in asphaltene biotransformation in <i>Paenibacillus</i> sp. IITD108	127
4.17	Strict hits obtained in the genome of the consortium members corresponding to antibiotic resistance	131
4.18	Metal resistance present in the members of the consortium	133
4.19	Number of genes related to various metabolic pathways of interest present in the nine members of the consortium	134
4.20	Presence of some PAH acting genes present in all the members of the consortium	135
4.21	List of enzymes detected in the secretome which might play a role in PAH degradation	144
4.22	The different peroxidases present throughout the genome of the consortium members	146
4.23	The various metabolites detected along with their retention times and peak height at different time points	185
4.24	Change in weight of asphaltene present in model oil and crude oil post enzymatic treatment in the packed bed column	211

ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
$^{\circ}\text{C}$	Degree Celsius
μg	Microgram
μl	Microlitre
μm	Micrometer
μM	Micromole
Δ	Deletion or Delta
ε	Epsilon
%	Percentage
ACN	Acetonitrile
API	American Petroleum Institute
APS	Ammonium persulfate
ANI	Average Nucleotide Identity
BLAST	Basic Local Alignment Search Tool
bp	base pair
CHNS	Carbon Hydrogen Nitrogen Sulphur
CFU	Colony forming unit
Cyt	cytochrome
DBT	Dibenzothiophene
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EB	Elution buffer
EDTA	Ethylene diamine tetra acetic acid
EEOR	Enzyme Enhanced Oil Recovery
EtBr	Ethidium bromide
Fig.	Figure
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
gm	Gram
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
IPTG	Isopropyl β -D thio galactopyranoside
Kan	Kanamycin
Kan ^R	Kanamycin resistance
kb	Kilobase
Kcat	Catalytic constant
kDa	kilo Dalton
Km	Michaelis constant
LA	Luria Agar
LB	Luria Broth
LC	Liquid chromatography

MALDI	Matrix Assisted Laser Desorption Ionization
MEOR	Microbial Enhanced Oil Recovery
min	minute
ml	millilitre
mM	millimolar
MS	Mass Spectroscopy
MSA	Multiple Sequence Alignment
MSM	Minimal Salt Medium
MTCC	Microbial Type Culture Collection
m/z	mass to charge ratio
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NaOH	Sodium Hydroxide
NEB	New England Biolab
NCBI	National Centre for Biotechnology Information
ng	nano gram
nm	nanometer
NMR	Nuclear Magnetic Resonance
OD	Optical Density
Ori	Origami
PAGE	Polyacrylamide gel electrophoresis
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PMSF	Phenylmethane sulfonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Room Temperature
SARA	Saturates Aromatic Resins and Asphaltenes
SDS	Sodium Dodecyl Sulphate
Sec	Second
SRA	Sequence Read Archive
TAE	Tris glacial acetic acid EDTA
TEMED	N N N' N' tetramethylene diamine
TLC	Thin Layer Chromatography
Tpx	Thiol Peroxidase
Tris	Tris (hydroxymethyl)amino methane
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UV	Ultraviolet
w/v	weight per volume
WGS	Whole Genome Sequencing