MOLECULAR BASIS OF ASPHALTENE BIOTRANSFORMATION BY A MICROBIAL CONSORTIUM

NIDHI NITIN PATIL

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

NIDHI NITIN PATIL

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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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.

Dr Preeti Srivastava
Professor
Department of Biochemical Engineering
and Biotechnology
Indian Institute of Technology Delhi
New Delhi – 110016

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 स्रावित 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, पीएएच 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 (पीएएच) 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।

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ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
°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