WIRED FOR ACTION: TAILORING LACCASES FOR BIOREMEDIATION AND BIOSENSING

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by

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CERTIFICATE

This is to certify that the thesis entitled "**Wired for Action: Tailoring Laccases for Bioremediation and Biosensing**" being submitted by **Ms. Stanzin Lzaod** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** in the Department of Chemistry is a record of bonafide research work carried out by her. Ms. Stanzin Lzaod has worked under my guidance and supervision and has fulfilled the requirements for the submission of the thesis, which, to my knowledge, has reached the requisite standard. The results contained in this dissertation have not been submitted in part or in full to any other university or institute for the award of any degree or diploma.

Date:

Place: New Delhi, India

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Abstract

Laccases belong to the family of multi-copper oxidoreductases and have gained prominence due to their broad substrate specificity, making them valuable in bioremediation, organic synthesis, biosensing, and other industrial and biotechnological applications. However, despite their promising potential, several limitations exist that hinder their widespread use. Among all laccases, bacterial and fungal laccases are the most widely studied, and they offer contrasting properties. For instance, fungal laccases typically exhibit higher enzymatic activity but lower tolerance to adverse conditions such as extreme pH, temperature, and salinity. In contrast, even though bacterial laccases present poorer catalytic efficiency, they are more robust and show superior stability under harsh operational conditions. Considering these differences, this study explores bacterial and fungal laccases through different approaches to exploit their respective advantages.

Initially, we employed two distinct immobilization methods – polyacrylamide gel-encapsulated laccase and cross-linked laccase aggregates – assessing their effects on catalytic efficiency, stability, and reusability of fungal laccase from *T. versicolor* in the biotransformation of two phenolic contaminants – 4,4'-dihydroxybiphenyl and dienestrol. This approach successfully improved the stability of the fungal laccase, thereby enhancing their potential for industrial applications. We then shifted our focus to a bacterial laccase, CotA laccase from *Bacillus licheniformis*, given their inherent robustness and stability. Hence, Chapter 3 and 4 of this study focused on cloning and overexpressing CotA laccase, followed by site-directed mutagenesis (SDM) to improve its enzymatic performance. SDM targeting residues in the substrate-binding pocket successfully improved the catalytic efficiency of *B. licheniformis* CotA laccase, leading to enhanced dye decolorization efficiency.

Furthermore, we explored the potential of recombinant *Bacillus licheniformis* CotA laccase in the fabrication of an electrochemical biosensor for the detection of catechol, an

environmental pollutant. The biosensor was constructed by modifying a screen-printed electrode with CotA encapsulated in a conducting polymer (PEDOT:PSS)/chitosan film. CotA can oxidize catechol, and this step enabled the detection of catechol through amperometric measurements. The biosensor demonstrated competitive analytical features to fungal laccases with a low limit of detection, high sensitivity, and excellent storage stability. Moreover, it successfully detected catechol in spiked tap and river water samples making it an effective and efficient solution for monitoring catechol in real environmental samples.

Unlike fungal laccases, which are typically secreted extracellularly under well-characterized conditions, the regulation and induction of bacterial laccases remain poorly understood. To address this gap, we explored the *in vivo* expression of CotA laccase in *Bacillus licheniformis* under different growth conditions. While no induction was observed in the presence of exogenous copper, phenolics, or oxidative stress, CotA expression and enzymatic activity significantly increased during sporulation. Additionally, we observed an increase in melanin production in sporulating cultures, which indicates towards a possible role for CotA in sporulation-linked melanin synthesis.

सारांश

लैकेस मल्टी-कॉपर ऑक्सीडोरेडक्टेस परिवार से संबंधित हैं और अपनी व्यापक सब्सट्रेट विशिष्टता के कारण प्रमुखता प्राप्त कर चुके हैं, जो उन्हें बायोरेमेडिएशन, कार्बनिक संश्लेषण, बायोसेंसिंग और अन्य औद्योगिक और जैव प्रौद्योगिकी अनुप्रयोगों में मूल्यवान बनाता है। हालांकि, उनकी आशाजनक क्षमता के बावजूद, कई सीमाएँ मौजूद हैं जो उनके व्यापक उपयोग में बाधा डालती हैं। सभी लैकेस में, जीवाणु और कवक लैकेस सबसे व्यापक रूप से अध्ययन किए गए हैं, और वे विपरीत गुण प्रदान करते हैं। उदाहरण के लिए, कवक लैकेस आमतौर पर उच्च एंजाइमेटिक गतिविधि प्रदर्शित करते हैं, लेकिन अत्यधिक पीएच, तापमान और लवणता जैसी प्रतिकूल परिस्थितियों के प्रति कम सहनशीलता रखते हैं। इसके विपरीत, भले ही जीवाणु लैकेस कमज़ोर उत्प्रेरक दक्षता प्रस्तुत करते हैं, वे अधिक मजबूत होते हैं और कठोर परिचालन स्थितियों के तहत बेहतर स्थिरता दिखाते हैं। इन अंतरों को ध्यान में रखते हुए, यह अध्ययन विभिन्न तरीकों के माध्यम से जीवाणु और कवक लैकेसेज़ की खोज करता है ताकि उनके संबंधित लाभों का फायदा उठाया जा सके।

प्रारंभ में, हमने दो अलग-अलग स्थिरीकरण विधियों – पॉलीएक्रिलामाइड जेल-एनकैप्सुलेटेड लैकेस और क्रॉस-लिंक्ड लैकेस एग्रीगेट्स – का उपयोग किया और दो फेनोलिक संदूषकों – 4,4’-डाइहाइड्रॉक्सीबाइफेनिल और डायनेस्ट्रोल के जैवरूपांतरण में टी. वर्सीकलर से प्राप्त कवक लैकेस की उत्प्रेरक दक्षता, स्थिरता और पुन: प्रयोज्यता पर उनके प्रभावों का आकलन किया। इस दृष्टिकोण ने कवक लैकेस की स्थिरता में सफलतापूर्वक सुधार किया, जिससे औद्योगिक अनुप्रयोगों के लिए उनकी क्षमता में वृद्धि हुई। फिर हमने अपना ध्यान एक जीवाणु लैकेस, बैसिलस लाइकेनिफॉर्मिस से प्राप्त CotA लैकेस पर केंद्रित किया, क्योंकि उनमें अंतर्निहित मजबूती और स्थिरता है। इसलिए, इस अध्ययन के अध्याय 3 और 4 में CotA लैकेस की क्लोनिंग और अतिअभिव्यक्ति पर ध्यान केंद्रित किया गया, जिसके बाद इसके एंजाइमी प्रदर्शन को बेहतर

बनाने के लिए साइट-निर्देशित उत्परिवर्तन (SDM) का उपयोग किया गया। सब्सट्रेट-बाइंडिंग पॉकेट में अवशेषों को लक्षित करने वाले SDM ने बैसिलस लाइकेनिफॉर्मिस CotA लैकेस की उत्प्रेरक दक्षता में सफलतापूर्वक सुधार किया, जिससे रंग-विरंजन दक्षता में वृद्धि हुई।

इसके अलावा, हमने पर्यावरण प्रदूषक, कैटेचोल, का पता लगाने के लिए एक विद्युत-रासायनिक बायोसेंसर के निर्माण में पुनः संयोजक बैसिलस लाइकेनिफॉर्मिस CotA लैकेज़ की क्षमता का पता लगाया। बायोसेंसर का निर्माण एक स्क्रीन-प्रिंटेड इलेक्ट्रोड को संशोधित करके किया गया था जिसमें CotA को एक चालक बहुलक (PEDOT:PSS)/चिटोसन फिल्म में संपुटित किया गया था। CotA कैटेचोल का ऑक्सीकरण कर सकता है, और इस चरण ने एम्परोमेट्रिक मापों के माध्यम से कैटेचोल का पता लगाना संभव बनाया। बायोसेंसर ने कम पहचान सीमा, उच्च संवेदनशीलता और उत्कृष्ट भंडारण स्थिरता के साथ कवक लैकेज़ के लिए प्रतिस्पर्धी विश्लेषणात्मक विशेषताओं का प्रदर्शन किया। इसके अलावा, इसने स्पाइक किए गए नल और नदी के पानी के नमूनों में कैटेचोल का सफलतापूर्वक पता लगाया, जिससे यह वास्तविक पर्यावरणीय नमूनों में कैटेचोल की निगरानी के लिए एक प्रभावी और कुशल समाधान बन गया।

कवक लैकेस के विपरीत, जो आमतौर पर सुस्पष्ट परिस्थितियों में बाह्यकोशिकीय रूप से स्रावित होते हैं, जीवाणु लैकेस का नियमन और प्रेरण अभी भी पूरी तरह से समझा नहीं गया है। इस कमी को पूरा करने के लिए, हमने विभिन्न वृद्धि परिस्थितियों में बैसिलस लाइकेनिफॉर्मिस में CotA लैकेस की इन विवो अभिव्यक्ति का अध्ययन किया। हालाँकि बाह्य ताँबे, फेनोलिक्स या ऑक्सीडेटिव तनाव की उपस्थिति में कोई प्रेरण नहीं देखा गया, लेकिन बीजाणुजनन के दौरान CotA अभिव्यक्ति और एंजाइमी गतिविधि में उल्लेखनीय वृद्धि हुई। इसके अतिरिक्त, हमने बीजाणुजनन संवर्धन में मेलेनिन उत्पादन में वृद्धि देखी, जो बीजाणुजनन से जुड़े मेलेनिन संश्लेषण में CotA की संभावित भूमिका की ओर संकेत करता है।

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List of Abbreviations

Abbreviation	Full Form
4-AP	4-Aminoantipyrine
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACN	Acetonitrile
Ace	Acetone
BME	Beta-mercaptoethanol
bp	Base Pair
CA	Catechol
CD spectroscopy	Circular Dichroism Spectroscopy
cDNA	Complementary DNA
CE	Counter Electrode
CECs	Contaminants of Emerging Concern
CLEA-laccase	Cross-linked Laccase Aggregates
CS	Chitosan
CV	Crystal Violet
DCP	2,4-dichlorophenol
DEPC	Diethyl Pyrocarbonate
DHBP	4,4’-dihydroxybiphenyl
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DP	Dopamine
DS	Dienestrol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EDCs	Endocrine Disrupting Chemicals
EPR	Electron Paramagnetic Resonance
ET Route	Electron Transfer Route
EtBr	Ethidium Bromide
EtOH	Ethanol
GUA	Guaiacol
HAT Route	Hydrogen Abstraction Route
HBT	1-Hydroxybenzotriazole
HPI	N-Hydroxyphthalimide
HPLC	High Performance Liquid Chromatography
HQ	Hydroquinone
IPOH	Isopropanol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB broth	Luria-Bertani Broth
LB plot	Lineweaver–Burk Plot
LOD	Limit of Detection
LOQ	Limit of Quantification
MCO	Multi-Copper Oxidase
MeOH	Methanol

Abbreviation	Full Form
MG	Malachite Green
MM plot	Michaelis-Menten Plot
Ni-NTA	Nickel-Nitrilotriacetic Acid
NB	Nutrient Broth
NTC	No Template Control
OD	Optical Density
PAG-laccase	Polyacrylamide-Encapsulated Laccase
PBS	Phosphate-Buffered Saline
PEDOT:PSS	Poly(3,4-ethylenedioxythiophene):polystyrene sulfonate
q-PCR	Quantitative Polymerase Chain Reaction
RE	Reference Electrode
RSD	Relative Standard Deviation
SB	Sporulation Broth
SDM	Site-Directed Mutagenesis
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SPE	Screen-Printed Electrode
SY	Syringic Acid
TBE	Tris-Borate-EDTA
t-BHP	Tert-Butyl Hydroperoxide
TEM	Transmission Electron Microscopy
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TNC	Trinuclear Cluster
UV-Vis	Ultraviolet-Visible
VLA	Violuric Acid
WE	Working Electrode
WT	Wild-type