TREATMENT OF COMPLEX DYES AND EFFLUENTS USING ENGINEERED LACCASE 1 OF *Cyathus bulleri*

MANJU

DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2022

TREATMENT OF COMPLEX DYES AND EFFLUENTS USING ENGINEERED LACCASE 1 OF *Cyathus bulleri*

by

MANJU

DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY

Submitted

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

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2022

DEDICATION

In loving memory of my Mom and Dad...

CERTIFICATE

This is to certify that the thesis entitled "**Treatment of complex dyes and effluents using engineered Laccase 1 of *Cyathus bulleri"*** being submitted by **Ms. Manju** to the Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, for the award of the degree of **'DOCTOR OF PHILOSOPHY'**, is a record of the bonafide research work carried out by her, which has been prepared under our supervision and guidance in conformity with the rules and regulations of the 'Indian Institute of Technology Delhi'. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Professor Saroj Mishra

Professor

Department Of Biochemical Engineering & Biotechnology

Indian Institute of Technology Delhi

Date:

Place:

Dr. Preeti Srivastava

Associate Professor

Department Of Biochemical Engineering & Biotechnology

Indian Institute of Technology Delhi

Date:

Place:

Acknowledgement

PhD, a journey in which every day has four seasons, with some sunny and some gloomy moments, the progress with day-to-day variation is really fantastic. The challenge during this phase is finding strength for keeping calm and remaining motivated during moments of hardship when things don't work as expected. But we all know, "truly meaningful journeys are hard". Here invaluable support along with best possible advice of the people we are associated with is the best antidote. I am loving to acknowledge these people at this moment as without their support I would not have witnessed this day.

First and foremost, I am extremely grateful to my supervisors, ***Prof. Saroj Mishra*** *and* ***Dr. Preeti Srivastava*** *for their invaluable mentorship, patience, and continuous support during my PhD study. Their plentiful experience and immense knowledge have encouraged me through all this phase. The ease of approach, and very much comfortable and motivational experimental result discussion sessions have encouraged me to bring best out of myself. Ma'am your calmly diligent nature and graceful conduct has always been inspiring to me, not only on science front but in personal life too. I would love to imbibe many of the qualities that you both possess. I am also thankful to my student's research committee members for their valuable feedbacks.*

During PhD years, one is never really off work, I remember how before actually falling asleep I used to use those leisure moments to analyse the results obtained through the day and defining certain points which may be put forth in upcoming discussion sessions. Science had always been my passion and I must say that I really have lived these years. This phase became more beautiful with the company of my fellow lab-mates, I thank Dr. Sumbul Afreen, Dr. Nitu Maity, Dr. Sakshi Aggarwal (alike elder sisters), and Rohit Khandelwal for the stimulating scientific discussions in the field of molecular biology over lunch hours and tea breaks. I would also like to convey my sincere thanks to my juniors, Aakanksha Ahalwat and Avijeet Jaiswal for their readiness to help whenever I was in need of it. My special thanks go to Dar Tafazul Islam who sincerely helped me in conducting my experiments on membrane reactor. He is a knowledgeable fellow and was always available at the time of need. I wish him good luck in all his future endeavours. I would extend my thanks to Dr. Sumeet Kapoor (Lab in charge) and Rehman Ji for helping me set up my experiments without any hindrance by providing all equipments.

The unconditional love of my family is truly acknowledgeable. I thank my Mom and Dad with whose true efforts I could achieve this academic accomplishment. My Dad, a man of principle, always taught me to face challenges without failure. His efforts had always been inspirational to me. I dedicate my thesis to my Mom (my whole world) who supported me through out this journey and always extended a helping hand whenever I was in need, without her help it would never have been possible for me to reach this stage. I thank my siblings, Upender, Devender, Sunita, and Neetu and their children Sanskaar, Swarnnim, Sanskriti, and Shashwat for their unconditional love and affection. My sincere thanks go to my Husband (my true soulmate), Jag Parvesh Dahiya, who supported me throughout this phase with a true heart. His continuous help has allowed me to complete this document. He is a person with golden heart who has always boosted me up with confidence whenever I felt low. My dear daughter, Manya Dahiya, and my sweet son (still to be named...) also deserve my true thanks for giving me time enough to complete my thesis writing. Smile on their face keeps me going.

I thank Almighty for his blessings throughout my journey till here and I wish it continues……

…Manju

ABSTRACT

Textile industry accounts for nearly 14% of the total manufacturing output in India and is highly water intensive using 50-100 liters/kg textiles in wet processing. According to a recent World Bank report, 17-20% percent of global water contamination is contributed solely by finishing and coloring procedures. Fungal laccases have high biotechnological potential for bioremediation processes owing to their ability to degrade phenolics, aminophenols, aryldiamines and polyamines. In the present study, the efficiency of engineered Laccase1 isoform (LCC1) of *Cyathus bulleri* is reported in decolorizing and detoxification of Indigo Carmine (IC), effluent containing this dye as well as real effluent from a textile mill. The gene (*LCC1*) encoding wild type (WT) LCC1, was modified through site-directed mutagenesis at predefined positions generating three catalytically superior High-Activity-Variants (HAVs), viz., [LCC1-35; ($Gly_{463}Arg$), LCC1-61; ($Ser_{318}Thr$), LCC1-62; ($Ile_{490}Met$)]. The extracellularly produced laccases in *Pichia pastoris* were partially purified and exhibited 2-9 X times higher decolorization of real effluents than the WTLCC1 even in the absence of a mediator. More than 90% decolorization (~1.6 times higher than that of the WTLCC1) was shown of IC in 18 h by the three HAVs without the addition of any mediator. To increase the rate of decolorization, several natural and synthetic mediators were screened which dramatically increased the rates and reduced the treatment time to 20 min. Maximum increase in the rate (23 X relative to the WTLCC1) was obtained with the HAV LCC1-62 with the natural mediator 2,6-Dimethoxy Phenol (DMP) making it an attractive option for mediator-assisted laccase treatment. Examination of the mediator-laccase variant combination on decolorization of IC at molecular level, through *in-silico* studies, provided evidence that His_{132}, Ser_{134}, Leu_{475}, and Glu_{476} were associated with the hot-spot regions in the active pocket of LCC1. Apart from identifying the binding residues, the binding efficiency, strength of the hydrogen bonds, conformation of the ligand, and size of the active size pocket were identified as important factors contributing to the

higher catalytic efficiency of the HAVs. Identified laccase variant, LCC1-62, was very effective in treating real effluent in the membrane reactor in batch as well as continuous mode giving 94% decolorization without any mediator. Our findings strongly suggest the feasibility of application of LCC1-62 at larger scale for textile effluent treatment.

सारांश

कपड़ा उद्योग भारत में कुल विनिर्माण उत्पादन का लगभग 14% हिस्सा है और गीले प्रसंस्करण में 50-100 लीटर/किलोग्राम वस्त्रों का उपयोग करके अत्यधिक पानी की खपत होती है। विश्व बैंक की एक हालिया रिपोर्ट के अनुसार, वैश्विक जल प्रदूषण का 17-20% प्रतिशत पूरी तरह से परिष्करण और रंग भरने की प्रक्रियाओं से होता है। फंगल लैकेसेस में बायोरेमेडिएशन प्रक्रियाओं के लिए उच्च जैव-प्रौद्योगिकी क्षमता होती है, जो फेनोलिक्स, एमिनोफेनोल्स, एरिलडायमाइन और पॉलीमाइन को नीचा दिखाने की उनकी क्षमता के कारण होती है। वर्तमान अध्ययन में, *सायथस बुलेरी* के इंजीनियर लैकेस1 आइसोफॉर्म (एलसीसी1) की दक्षता को इंडिगो कारमाइन (आईसी) के रंगहीन और विषहरण में बताया गया है, इस डाई से युक्त अपशिष्ट के साथ-साथ एक कपड़ा मिल से वास्तविक अपशिष्ट भी। जीन (LCC1) जंगली प्रकार (WT) LCC1 को कूटबद्ध करता है, जिसे साइट-निर्देशित उत्परिवर्तजन के माध्यम से पूर्वनिर्धारित पदों पर संशोधित किया गया था, जो तीन उत्प्रेरक रूप से बेहतर उच्च-गतिविधि-वेरिएंट (HAVs) उत्पन्न करता है, अर्थात, [LCC1-35; ($Gly_{463}Arg$), LCC1-61; ($Ser_{318}Thr$), LCC1-62; ($Ile_{490}Met$)]। *पिचिया पेस्टोरिस* में बाह्य रूप से उत्पादित लैकेसेस को आंशिक रूप से शुद्ध किया गया था और एक मध्यस्थ की अनुपस्थिति में भी WTLCC1 की तुलना में वास्तविक अपशिष्टों के 2-9 X गुना अधिक विवर्णीकरण का प्रदर्शन किया गया था। 90% से अधिक विरंजन (WTLCC1 की तुलना में ~ 1.6 गुना अधिक) बिना किसी मध्यस्थ को शामिल किए तीन एचएवी द्वारा 18 घंटे में आईसी का दिखाया गया था। रंग बदलने की दर को बढ़ाने के लिए, कई प्राकृतिक और सिंथेटिक मध्यस्थों की जांच की गई, जिन्होंने नाटकीय रूप से दरों में वृद्धि की और उपचार के समय को घटाकर 20 मिनट कर दिया। दर में अधिकतम वृद्धि (WTLCC1 के सापेक्ष 23 X) HAV LCC1-62 के साथ प्राकृतिक मध्यस्थ 2,6-डाइमेथॉक्सी फिनोल (DMP) के साथ प्राप्त की गई, जिससे यह मध्यस्थ-सहायता प्राप्त लैकेस उपचार के लिए एक आकर्षक विकल्प बन गया। *इन-सिलिको* अध्ययनों के माध्यम से आणविक स्तर पर आईसी के रंग बदलने पर मध्यस्थ-लैकेस प्रकार के संयोजन की जांच ने इस बात का सबूत दिया कि His_{132}, Ser_{134}, Leu_{475}, और Glu_{476} LCC1 के सक्रिय पॉकेट में हॉट-

स्पॉट क्षेत्रों से जुड़े थे। बाध्यकारी अवशेषों की पहचान करने के अलावा, बाध्यकारी दक्षता, हाइड्रोजन बांड की ताकत, लिगैंड की संरचना, और सक्रिय आकार की जेब के आकार को एचएवी की उच्च उत्प्रेरक दक्षता में योगदान करने वाले महत्वपूर्ण कारकों के रूप में पहचाना गया। पहचाने गए लैकेस वैरिएंट, LCC1-62, बैच में झिल्ली रिएक्टर में वास्तविक बहिःस्राव के उपचार के साथ-साथ निरंतर मोड में बिना किसी मध्यस्थ के 94% विवर्णीकरण देने में बहुत प्रभावी था। हमारे निष्कर्ष कपड़ा अपशिष्ट उपचार के लिए बड़े पैमाने पर एलसीसी 1-62 के आवेदन की व्यवहार्यता का दृढ़ता से सुझाव देते हैं।

CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percent
~	Approximately
°C	Degree Celsius
€	Exposed geometry of amino acid residue
µM	Micromolar
µg	Microgram
µl	Microliter
3D	Three-dimensional
Λmax	Wavelength at which there is max absorbtion
Å	Angstrom
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BME	β Mercaptoethanol
BMGY	Buffered complex glycerol medium
BMMY	Buffered complex methanol medium
Bp	Base pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
2,6-DMP	2,6 Dimethoxyphenol
EDTA	Ethylenediaminetetraaceticaci
FA	Ferulic acid
G	Gram

H	Hour/hours
HAV	High Activity Variants of laccase isoform-1
HRT	Hydraulic Retention Time
HOBt	1-Hydroxybenzotriazole
IC	Indigo carmine
Kb	Kilobase pair
K_{cat}	Turn over number (Catalytic rate constant)
kDa	Kilodalton
K_m	Michaelis Menten constant
LA	Luria Agar
LB	Luria Broth
LCC1-35	Laccase mutant 35
LCC1-61	Laccase mutant 61
LCC1-62	Laccase mutant 62
M	Molar
Mg	Milligram
Min	Minute
Ml	Millilitre
mM	Millimolar
E	Molar extinction coefficient
Ng	Nanogram
nm	Nanometer
OD_{600}	Optical density measured at 600 nm
PCR	Polymerase Chain Reaction

rpm	Revolution per minute
RB 21	Reactive Blue 21
SDS	Sodium dodecyl sulphate
sec	Second
Tris	Tris(hydroxymethyl)aminomethane
U	Enzyme activity Unit
mU	Milli unit
UV	Ultra violet
v/v	Volume/volume
V_{max}	Maximum velocity of reaction
w/v	Weight/volume
WTLCC1	Wild type recombinant laccase isoform 1
E^o	Redox potential
YPD	Yeast Peptone Dextrose