

**ENGINEERING OF LACCASE OF *Cyathus bulleri*
FOR DECOLORIZATION OF COMPLEX TEXTILE
DYES**

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**ENGINEERING OF LACCASE OF *Cyathus bulleri*
FOR DECOLORIZATION OF COMPLEX TEXTILE
DYES**

by

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Submitted

in fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the



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DEDICATION

In loving memory of my father.....

CERTIFICATE

This is to certify that the thesis entitled “**Engineering of laccase of *Cyathus bulleri* for decolorization of complex textile dyes**” being submitted by **Ms. Tenzin Kenzom** to the **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.

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Laccase is a multi-copper containing polyphenol oxidase that catalyzes oxidation of a variety of phenolics with concomitant reduction of oxygen to water. Due to this, it finds applications in various industrial sectors. Previous research has suggested that the oxidative ability of laccase can be changed by manipulation of some amino acids at the C-terminus of the enzyme. No definite regions in laccase are identified which can extend this oxidizing ability. Through the present work, it is reported that the second half of the protein, towards the C-terminus, can be mutagenized to give laccase variants that exhibit high catalytic activity and ability to degrade dyes with high redox potential. For this, the cDNA encoding laccase (*Lcc*) from a white rot fungus *Cyathus bulleri* was cloned and expressed in the culture supernatant of *Pichia pastoris* under the control of the alcohol oxidase (*AOXI*) promoter. Since the expression was low, effect of addition of Cu salts, previously reported to enhance heterologous expression, was investigated. This strategy was found to increase the extracellular levels of laccase to ~7000U/L. The experimental data strongly suggested that the final yield of the functional laccase could be limited due to absence of metal ion (in this case, copper) and therefore under Cu limiting condition apo-laccases are produced. This finding bears considerable significance when trying to express metallo-proteins in the *P. pastoris* system. Both the recombinant *Pichia* expressed laccase (WtLcc) and the native laccase (nLcc) were purified to homogeneity. A comparison of their properties indicated the WtLcc to be of higher molecular weight and this was attributed to a difference in the glycosylation pattern of the two laccases, as determined from MALDI-TOF analysis. The WtLcc also exhibited increased tolerance towards chloride ions and EDTA. The C-terminus of laccase (comprising the 816-bp region towards the C-terminus) was subjected to random mutagenesis and a library of mutants created in *P. pastoris*. The mutants were selected, first,

for higher catalytic activity on ABTS using a modified microtitre based method. In the second screening, active variants were selected based on decolorization of a high redox dye, namely, Reactive Blue 21 (RB21). This dye has not been reported to be acted upon by laccase. The degradation products of RB21 were characterized by mass spectrometry, which indicated a mode of breakdown, quite analogous to that of peroxidases. The structural genes of these laccase variants (Lcc35, Lcc61 and Lcc62) were sequenced to locate the mutation sites and these were further mapped on the 3-D model of laccase. Contribution of the changed amino acids to catalytic activity or substrate binding was investigated and the data indicated residues both near and far from the T1Cu site to affect catalytic efficiency of laccase. The mutated proteins were also biochemically characterized to understand structure-function relationship. Importance of geometry as well as electronic changes was indicated in reactivity of laccases.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
~	Approximately
°C	Degree Celsius
μM	Micromolar
μg	Microgram
μl	Microliter
3D	Three-dimensional
λ _{max}	Wavelength at which there is max absorbtion
Å	Angstrom
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
APS	Ammonium per sulfate
AV 17	Acid violet 17
BLAST	Basic Local Alignment Search Tool
BME	β Mercaptoethanol
BMGY	Buffered complex glycerol medium
BMMY	Buffered complex methanol medium
bp	Base pair
BSA	Bovine serum albumin
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraaceticaci
FPLC	Fast protein liquid chromatography

List of abbreviations and symbols

g	Gram
GFC	Gel filtration chromatography
h	Hour/hours
IC ₅₀	Concentration to be Inhibitory to the 50 % of cells
kb	Kilobase pair
K _{cat}	Turn over number (Catalytic rate constant)
kDa	Kilodalton
K _m	Michaelis Menten constant
KB 9	Kiton blue 9
LA	Luria Agar
LB	Luria Broth
Lcc 35	Laccase mutant 35
Lcc 61	Laccase mutant 61
Lcc 62	Laccase mutant 62
M	Molar
MALDI	Matrix assisted laser desorption ionization
MEA	Malt extract agar medium
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
ε	Molar extinction coefficient
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid

List of abbreviations and symbols

nm	Nanometer
nLcc	Native laccase
OD ₆₀₀	Optical density measured at 600 nm
PCR	Polymerase Chain Reaction
PDB	Protein data bank
RB 5	Reactive black 5
RB 21	Reactive blue 21
RR 198	Reactive red 198
RO 16	Reactive orange 16
RY 42	Reactive yellow 42
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
sec	Second
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	Thin layer chromatography
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
WtLcc	Wild type recombinant laccase
E ⁰	Redox potential
YPD	Yeast Peptone Dextrose