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

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**by**


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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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Place:

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# ABSTRACT

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 (*AOX1*) 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 ABBREVATIONS AND SYMBOLS

| | |
|---|---|
| % | Percent |
| ~ | Approximately |
| °C | Degree Celsius |
| 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) |
| 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 |

| | |
|---|---|
| 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 | Laccse 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 |

| | |
|---|---|
| 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 |
| $E^o$ | Redox potential |
| YPD | Yeast Peptone Dextrose |