BIOCHEMICAL CHARACTERIZATION AND GENETIC STUDIES OF LACCASE IN *Cyathus bulleri*

By

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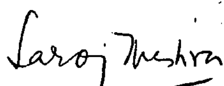
Dedicated to

my dear parents and brother

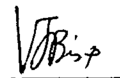
for their everlasting love and patience....

CERTIFICATE

This is to certify that the thesis entitled **"BIOCHEMICAL CHARACTERIZATION AND GENETIC STUDIES OF LACCASE IN *Cyathus bulleri*"** being submitted by **Ms. SALONY** 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 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.



Prof. Saroj Mishra



Prof. V.S. Bisaria

Acknowledgement...

The fascinating and mysterious world of science. A huge ocean with unfathomable secrets. Obviously not easy to sail across without a guiding hand to teach the tricks of sailing.

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The time I spent from childhood in the campus, the day I wrote my first alphabet till this day when I am writing this doctoral thesis, brings back all the memories and emotions of happiness, sorrow, success and failures. A little girl's dream of studying one day in this premium Institute has been fulfilled.

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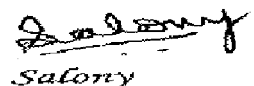

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ABSTRACT

Laccases are multicopper oxidases which catalyse the oxidation of a broad range of substrates such as polyphenols, methoxy-substituted phenols and diamines. The physisological roles postulated for them are diverse and these are based on their capability to oxidize recalcitrant aromatic compounds with redox potentials exceeding their own. *Cyathus bulleri*, a white rot fungus and a good producer of laccase, was taken up for detailed biochemical and genetic studies.

The fungus *C.bulleri* produced extracellular laccase in malt extract medium and basal liquid medium. The highest level of laccase was produced in the presence of 0.1 mM 2,6 dimethylaniline as the inducer. The most suitable carbon and nitrogen source identified for its production was glucose and ammonium tartrate respectively with a C/N ratio of 14. The enzyme was purified 82-fold by using a combination of ultrafiltration, ion exchange and gel filtration methods. The molecular mass of the monomeric laccase was 56.6 ± 5 kDa. The amino acid composition of *C. bulleri* also showed a high mol % of acidic amino acids, comparable to that reported for generic laccases. The sequences of three internal peptides (obtained from MS/MS nanospray) were matched with other laccases and strong homology was obtained. The laccase was identified to contain both Type 1 and Type 2/ Type3 copper atoms. The Far-UV CD spectrum for the determination of the secondary structure of laccase showed that it belonged to the $\alpha + \beta$ family.

The biochemical and kinetic properties of the purified laccase were investigated in detail. It was optimally active at 30 °C and pH of 5.5 on guaiacol. The *C.bulleri* laccase, like other fungal laccases, non-specifically oxidized a wide range of substrates with the highest activity on ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid).

Catalytic efficiency (k_{cat}/K_M) in the order of increasing efficiency was: guaiacol > ABTS > pyrogallol > hydroquinone > veratryl alcohol. However, the maximum V_{max} value of 15 x 10^{-2} μmol min^{-1} was obtained with ABTS. The purified LAC was strongly inhibited by copper chelating agents and was also found to be sensitive to 0.05 mM sodium azide, the most effective inhibitor. Unfolding and refolding studies were done using EDTA (1M), DTT (100μM) and guanidinium hydrochloride (4M). The refolding of the protein was achieved in alkaline conditions i.e. at a pH of 8.0 with the addition of copper. The process was complete within 10 min and the activity regained to 100 %. The folding and activity regain was also obtained with zinc, indicating the flexibility of the reaction centre.

Chemical modification studies with the group specific modifying agents suggested participation of Asp/Glu residues, more for maintaining structural integrity of the enzyme. The pattern of inactivation by EDC (1-ethyl-3- (3-dimethylaminopropyl) carbodiimide) showed it to be bi-phasic and the determination of the order of the reaction showed that that one molecule of EDC binds to one molecule of enzyme. The structural role of Cys and Trp was also indicated as their modification caused complete loss of the activity of laccase.

The culture filtrate of the fungus and purified laccase was tested for decolorization of various acidic, basic and reactive dyes. The most effective decolorization was obtained for Malachite green, Inco Sky blue and Texacid Fast Red. In the presence of mediators like ABTS at a concentration of 50 μM, the enzyme was able to decolorize reactive dyes as well. The enzyme was also tested for the decolorization of black liquor from a paper industry. The rate of decolorization and time for 50 % decolorisation was also obtained.

The cloning of laccase was done using the ligation anchored PCR and successful expression of the laccase gene was obtained in *E.coli.* The activity staining method confirmed synthesis of laccase in recombinant *E.coli*. Since this is the first report of the expression of laccase in *E.coli*, it is quite significant for further studies. The cDNA library was also constructed in λTriplEx2 vector.

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