GENERAL DISCUSSION

In the first chapter, screening of extracellular ligninolytic enzyme production during the dye decolorization processes and production optimization of *Pleurotus florida* NCIM 1243 laccase enzyme were carried out. *Pleurotus florida* NCIM 1243 produced laccase as the dominant ligninolytic enzyme during the dye decolorization. Statistically-based experimental designs were applied to optimize the production of laccase by *Pleurotus florida* NCIM 1243. Eleven components were screened for their significant effect on laccase production using Plackett-Burman design. Glucose (carbon source), asparagine (nitrogen source), CuSO$_4$ (inducer) and incubation period were found to have highest positive influence on the laccase production. The combined effect of these factors on laccase production was studied using central composite design of Response surface methodology. The optimal point of variables for maximum (4.8 U/ml) laccase production are glucose (15.21 g/l), asparagine (6.40 g/l), CuSO$_4$ (91.78 μM) and incubation period (178.55 h), respectively. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and results showed that the predicted value agreed with the experimental values well.

In the second chapter, *Pleurotus florida* NCIM 1243 laccase isoenzyme production was studied and the isoenzyme which is dominantly involved in the dye decolorization process was purified and characterized. *Pleurotus florida* NCIM 1243 laccase produces two extracellular laccase isoenzymes (L1 and L2) and the L1 isoenzyme is dominantly involved in the dye decolorization process. L1 isoenzyme was successfully purified by ultrafiltration, ammonium sulphate precipitation, anion exchange and size-exclusion chromatography. At the end of
the purification process, L1 isoenzyme was purified to 6.4 fold with a yield of 36% and had a specific activity of 52.6 U/mg of protein. The purified laccase was monomeric with an apparent molecular mass of ≈54 kDa. The optimum pH and temperature of the L1 isoenzyme was found to be around 5.5 and 50 °C, respectively. L1 isoenzyme showed a half-life of 2 h at 60 °C and at 4 h it retained around 25% residual activity. The kinetic parameters suggest that the order of affinity towards the tested substrates was syringaldazine > ABTS > DMP > guaiacol. Potential laccase inhibitor sodium azide completely inhibited *Pleurotus florida* laccase (L1 isoenzyme) at 0.1 mM concentration. L-cysteine completely inhibits the enzyme activity at 10 mM concentration, whereas EDTA was slightly inhibited at the same concentration. Purified laccase (L1 isoenzyme) activity was significantly stimulated by Cu⁺ by 3, 16 and 21% at 0.1, 1.0 and 10 mM concentrations, respectively. Other metals such as Ca⁺, Fe⁺, and Ni⁺ did not inhibit the enzyme activity up to 10 mM concentration, however, Mn⁺ and Hg⁺ inhibited 23% and 16% of L1 isoenzyme activity at 10 mM concentration, respectively. Interestingly, L1 isoenzyme of *Pleurotus florida* NCIM 1243 laccase was not significantly inhibited by chloroform and benzene, whereas above 50% of laccase activity was inhibited by acetone, dimethyl sulfoxide and methanol.

Third chapter mainly deals with the bioremediation processes such as textile important synthetic dye decolorization and also industrial effluents such as textile and paper effluent decolorization by *Pleurotus florida* NCIM 1243 laccase. Decolorization of Malachite green (MG), Remazol brilliant blue R (RBBR), Reactive black 5 (RB5) and Reactive Red 35 (RR 35) were carried out using *Pleurotus florida* NCIM 1243 laccase (L1 isoenzyme). *Pleurotus florida* NCIM 1243 laccase (L1 isoenzyme) effectively decolorized MG and RBBR dyes,
whereas RB 5 and RR 35 were not effectively decolorized. The mechanism behind MG decolorization was identified to be demethylation which was carried out by laccase enzyme. Oxidation of MG dye amino group followed by radical-radical coupling could be attributed for the observed precipitation, which can be removed from the dye solution. In the case of RBBR decolorization, oxidation of amino group followed by the formation of anthraquinone is the main mechanism. However, the presence of redox mediator, HBT greatly enhanced the RBBR dye decoloration and anthraquinone was disappeared. *In vitro* decolorization of textile and paper industrial effluents were assessed by crude filtrate and purified laccase from *Pleurotus florida* NCIM 1243. The textile industry effluent was decolorized by crude filtrate and purified laccase upto 14 and 21% respectively, after 2 h incubation. Among the three structurally different redox mediators (AHP, HBT and HPB) tested for effluent decolorization, HBT showed the highest decolorization activity, whereas AHA, HPB did not enhance the textile industry effluent decolorization. In the presence of 1mM HBT, purified laccase reveal efficient decolorization of textile industry effluent and the rate of decolorization was increased upto a maximum of 58 and 70% within 1 and 2h incubation, respectively. Maximum decolorization (69%) of paper industry effluent by crude filtrate of *Pleurotus florida* NCIM 1243 was obtained after 5 h incubation, whereas 27% of decolorization was observed using purified laccase alone. This difference in effluent decolorization between crude and purified laccase could be attributed to the ligninolytic enzyme present in the crude filtrate of *Pleurotus florida* NCIM 1243. In the presence of AHA, *Pleurotus florida* NCIM 1243 laccase (L1 isoenzyme) showed decolorization activity of paper industry effluent by 61, 77 and 82% within 1, 2 and 3h incubation, respectively.
In the fourth chapter, RBBR (as a model dye) decolorization was optimized. Response surface methodology (RSM) was applied in this study to optimize the RBBR decolorization. The variables namely, dye (RBBR), enzyme (laccase), redox mediator (HBT) and time (h) played a significant role in RBBR dye decolorization. The optimum parameters for maximum decolorization of RBBR dye (85%) were found to be RBBR dye (76.86 ppm), laccase (1.667 U/ml), HBT (1.707 mM) and time (6.12 h). The value of $R^2$ (0.9994) indicates good relation between the experimental and predicted values of the response.

Final chapter in thesis, mainly deals with the cloning and expression of *Pleurotus florida* NCIM 1243 laccase in *Saccharomyces cerevisiae* CEN.PK2-1C. A cDNA was synthesized by reverse transcription polymerase chain reaction (RT-PCR) from the total RNA isolated from *Pleurotus florida* NCIM 1243 mycelium. The isolated laccase nucleotide sequence has 1602 base pairs and was predicted to have 533 amino acid residues. The nucleotide sequence of the laccase gene reveals 98% similarity to the previously characterized nucleotide sequence of POXAlb from *Pleurotus ostreatus* and shares 95% similarity with nucleotide sequence of *Pleurotus eryngii* laccases PEL4 and ERY4. Laccase production from the recombinant host cell was identified on agar plate amended with 5 mM ABTS as laccase specific substrate. Expression of laccase in the recombinant yeast *Saccharomyces cerevisiae* CEN.PK2-1C was confirmed by zymogram analysis and found to be L1 isoenzyme.

In conclusion, because of the high yield, trouble free purification procedure, thermostability, synthetic dyes and effluent decolorization efficiency, *Pleurotus florida* NCIM 1243 laccase can be used for bioremediation processes.