Chapter 5

SUMMARY
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Macro fungal species growing on the littoral layer of three forest bed areas, Bethuadahari Wildlife Sanctuary, Ranaghat Forest and Zafarnagar Forest, of Nadia district, West Bengal were studied, collected, sampled; many of the collections were brought to cultures and screened for their ability to produce lignolytic enzymes and their potential in bioremediation of different high molecular weight dyes.

A total of 41 species of fungi belonging to 20 genera in 13 basidiomycetous and one Ascomycetous families were collected among which some were found to be inherently litter decomposing macrofungi (such as the different spp. of Agaricus, Macrolepiota, Lepiota, Marasmius, Coprinus and Podoscypha, etc), while some other species (i.e. Lentinus, Hypholoma, Hypocrea and two species of Schizophyllum etc.) were found associated with both the littoral layer and the wood growing habitats. The later fungal species, thus, represented the overlapping group between the litter decomposing macrofungi and the wood rot fungi. Among the collected species, however, the identity of some fungi (such as two species of Agaricus and Coprinus each, one species each of Marasmius, Agrocybe, Podoscypha, Lycoperdon and Schizophyllum) has not yet been confirmed up to the species level. Moreover, two fungi of Boletaceae family have not been identified.

All species collected were found associated with corresponding their fruiting body or stroma. However, the two species of Schizophyllum were collected as clearly visible fungal mycelia in the rotten leaf litter. This is the first report of association of Schizophyllum in littoral layer.

The diversity of the fungal species was studied in three different forest areas and it was found that the species composition, diversity of fungal flora, frequency of fungal species- all varied differently among the three forest floors. In term of species composition Bethuadahari Wildlife Sanctuary was highest (30 spp.) followed by Ranaghat and Zafarnagar Forests. In terms of genera, Agaricus was found to be the most dominant in all the three forests. In terms of diversity indices it was found that
Bethuadahari Wildlife Sanctuary is the most diverse forest, since the Shannon Index Value (2.057) and the Brillouin Index Value (1.966) were high, followed by Ranaghat Shannon Index Value= 1.9457; Brillouin Index Value= 1.8521) and Zafarnagar Forest (Shannon Value= 1.546714; Brillouin Value= 1.461). The species composition varied differently in different seasons. In the District of Nadia the Monsoon season starts from mid June and lasts up to the mid of September. The species composition was found higer during the time period between July-August, while the species composition of pre-Monsoon and post-Monsoon period were significantly lesser. Since the month of September is generally associated with flooding of the forest floors, there was a significant decrease in the number of LDFs.

The total number of fungi that were successfully isolated was only 20. Bacterial contamination played the major role in obtaining pure culture. Out of the 20 isolates, the detail morphological studies were done in case of 18. Since, Schizophyllum spp. fruiting bodies were not collected from the forest floor; the two species were not described.

The isolates showed varying degrees of lignolytic enzymes, laccase and manganese peroxidase. It was found that none of the isolates, including the overlapping species, exhibited any lignin peroxidase activity indicating that laccase and MnP are the two main lignolytic enzymes that are produced by the litter decomposing macrofungal species.

Of the 20 isolates, 14 showed laccase activity and 9 showed MnP activities. MnP activity was found to be low in the isolates indicating laccase as the mazor lignolytic enzyme in these species. Laccase activity was found to be higher in HNHC medium among the 6 media studied. All the 14 fungi, based on their laccase activity in HNHC medium, were grouped into 3 categories such as: (1) fungi having Very low laccase activity; (2) Medium laccase activity and (3) High laccase activity. The laccase activity of Agaricus sylvaticus, Agaricus bernardii, Agaricus campestris and Macrolepiota mastoidea were grouped into the category 1 (Very low), while Marasmius siccus, Coprinus comatus, Lentinus tigrinus, Mycena maculate and Hypholoma capnoides showed medium laccase activity. Thus, based on initial screening, only four species, Podoscypha petalodes, Schizophyllum commune,
Schizophyllum sp. and Hypocrea lixi, exhibiting high laccase activity, were selected in the detail studies of lignolytic enzymes and dye decolourization.

The different cultural conditions showed varying effects on laccase activity of the four species. *P. elegans* showed optimum laccase activity at 30°C pH-6.5 and veratryl alcohol supplementation at concentration of 0.6 mM showed maximum laccase activity (57.9 nkat/ml) at 28d in HNHC medium. In case of *S. commune* and *Schizophyllum* sp. 30°C, pH-4.5 and 0.2 mM VA and 30°C, pH-5.5 and 0.4mM VA were found to be the optimum conditions respectively and maximum activity of the enzyme was recorded as 56.54 nkat/ml and 53.71 nkat/ml in *S. commune* and *Schizophyllum* sp respectively. In *H. lixii*, 30°C temperature, pH-4.5 without any VA was the optimum condition for laccase enzyme and VA was found to be negatively affecting the laccase activity.

Among the 12 high molecular weight dyes, the four fungal isolates showed varying degree of decolourization percentages. It was found that in all cases the higher concentrations of dye affected the process of decolourization and 0.1% dye showed maximum decolourisation.

In case of *P. elegans*, maximum of 98.65% decolourization was noted in RBB at 28d and the minimum decolourization of 26.67% was noted in MG at 28d. An interesting phenomenon was observed in case of TB, where the deep blue colour of TB changed after 7th day to a reddish one. *S. commune* decolourized 0.1% RBB at its maximum value of 98.9% at 28d and the minimum decolourization was noted in MG (26.9%). In this case also the colour change of TB occurred after 21st day. *Schizophyllum* sp. showed maximum decolourization percentage in 0.1% RBB (91.46%) followed by OG (79.98%) and RB (77.5%) at 28d. Lowest decolourisation percentage was recorded in MG and the dye was not decolourized up to 14d, however, after incubation for more than 14d, a slight reduction in the optical peak was observed indicating decolourization of the dye. The decolourisation percentages were recorded 3.14 and 4.13 at 21 and 28d respectively. In *H. lixii*, the maximum decolourization percentage at 0.1% dye concentration was noted in RB (89.53%) and minimum decolourization percentages were recorded in MG (20.09%). In this case no colour change in TB was recorded up to 28d and the maximum decolourization in TB was recorded as 72.82% at 1% dye concentration at 28d.
Thus it was noted that either RBB or RB showed maximum decolourization at 0.1% dye concentration and MG was the least utilized dye in almost all cases. This may be due to the fact that molecular weight of RBB was the lowest among all the dyes used and it is not an azo dye and belongs to the group of anthraquinone dye. RB is also not an azo dye and it belongs to the xanthene “acid” dye, though the molecular weight is very high. On the contrary MG is a triarylmethane dye widely used in aquaculture as a parasiticide and in food, health, textile and other industries for one or the other purposes and it is known to control fungal attacks, protozoan infections etc (Srivastava et al 2004). It may be due to this reason the dye was least utilized by the four isolates.

The four isolates were identified based on the 18S rRNA gene sequencing and NCBI BLAST analysis and the molecular affinity of all the species excepting *H. lixii* was detected by MEGA 6.

The identity of *P. elegans* was confirmed by molecular studies. Among the stipitate steroid fungi, *Podoscypha* Pat. can be distinguished by its dimitic hyphal structure, presence of gloeocystidia, and amyloid spores. *Podoscypha* is the type genus of Podoscyphaceae D.A. Reid and is widespread both in tropical and temperate zones (Reid 1965). *P. elegans* showed taxonomic dilemma in literature studies. Phylogenetic analysis confirmed close relation with *Abortiporus* and the result was in validation of the previous report (Moreno et al. 2011).

The identity of *Schizophyllum commune* was confirmed in molecular studies and the species showed that it is closely related to *Fistulina hepatica* and *F. antarctica* in accordance with the previous reports (Hibbett et al 1997; Binder and Hibbett 2001; Moncalvo et al 2002). The identity of the *Schizophyllum* sp. was not confirmed up to the species level, but the generic taxon was confirmed. It also showed close relation with the *Schizopnyllum* spp. and *Fistulina* spp.

Blast analysis showed 99% identity (*Trichoderma harzianum*, *Trichoderma* sp., *Trichoderma reesei*, *Hypocrea atrovioliris*, *Hypocrea rufa*, *Hypocrea nigricans*, *Trichoderma koningiopsis*, *Hypocrea muroiana* and *Hypocrea lixii* etc) all having Max Score of 1572 at Quary Cover of 100% making it difficult to identify the specimen from the analysis. *Hypocrea lixii* is the teleomorph genus and the anamomorph genus
is known as *Trichoderma harzianum* (Jaklitsch 2009) and is found on diverse habitats.

According to recent morphological studies *H. lixii* is identified as having stromata green to black, lenticular, pulvinate to irregular (Jaklitsch 2009). The species of *Hypocrea* used in the study showed greenish to black stromata of about 7mm in maturity which was irregularly lenticular in shape. From both morphological and molecular studies the isolate may be accepted as *H. lixii* and it may be concluded that the 18S rRNA gene homology is not a strong support to identify the *Trichoderma/Hypocrea* group.