5. DISCUSSION

Lignocellulose is the predominant component of woody plant and dead plant materials and the most abundant biomass on earth (Ohkuma et al., 2001). Lignin is a heterogenous and irregular arrangement of phenylpropanoid polymer that resists chemical or enzymatic degradation to protect cellulose. Kirk and Farrell (1987) noted that basidiomycetes which cause white rot decay, are able to degrade lignin in wood. These fungi are called white rot fungi. Lignin degradation by white rot fungi has been extensively studied, and results revealed that three kinds of extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase are responsible for initiating the depolymerization of lignin. Ruiz-Duenas et al. (2009) reported that lignin is the second most abundant constituent of the cell wall of vascular plants, where it protects cellulose towards hydrolytic attack by saprophytic and pathogenic microbes. Its removal represents a key step for carbon recycling in land ecosystems, as well as a central issue for industrial utilization of plant biomass. The lignin polymer is highly recalcitrant towards chemical and biological degradation due to its molecular architecture, where different non phenolic phenylpropanoid units from a complex three-dimensional network linked by a variety of ether and carbon-carbon bonds. Ligninolytic microbes have developed a unique strategy to handle lignin degradation based on unspecific one electron oxidation of the benzenic rings in the different lignin sub structures by extracellular heamperoxidases acting synergistically with peroxide generating oxidases (Martinez et al., 2005). Kersten and Cullen (2007) reported that microbial degradation of lignin is the key step for closing the carbon cycle since removal of the lignin barrier enabled the subsequent use of plant carbon hydrates by other microorganisms.

Hedawoo and Mohite (2008) stated that collecting fungi should not be considered a haphazard pursuit. It is to emphasize that the task identification and proper record of larger fungi is of prime importance and can only be fulfilled by extensive surveys of different zones of the country. Priyadarsini (2011) observed that natural sample such as decay wood was collected from various parts of Hosur, Tamil Nadu in India. Collected in sterile plastic covers were brought to the laboratory without exposing to the external environment further.
Kirk, (1971) reported that more than 600 species have been reported to the lignin degraders. But the process of lignin degradation was elucidated only in a few fungi like *Phanerochaete chrysosporium* and *Trametes versicolor*. Tien and Kirt (1971) and Glenn *et al.* (1983) isolated an unique enzyme ligninase from the culture filtrate of *P. chrysosporium* later ligninases have been reported from other ligninolytic fungi like *T. versicolor* and *P. radiata* (Dodson *et al.*, 1986). Hassan *et al.* (2010) reported that mycorrhiza survey in Kashmir forests several macrofungi were collected from lower forest range to alpine zone. Grand and Vernia (2007) reported that specimens were placed in paper bags in the field with a sample of decayed wood with most collections and field notes for all collections. In the present study fifty six fungal species were collected and isolated from western ghats region of Tamilnadu and Karnataka in India from various sources like decayed woods, fallen woods, tree trunks, leaf litters and Grassland (Table 1; Plates 1-7).

The isolated fungi were maintained on 2 per cent malt agar plates (Roy Watling, 1971). Feniksova *et al.* (1972) recommended fukazumi medium supplemented with wood flour for maintenance of wood decaying fungi. Priyadarsini (2011) reported that potato dextrose agar was used for fungi isolation and malt extract medium either broth or solid medium were used. In the present study 2 per cent malt agar medium was used for the maintenance of wood rot fungi.

Gilbertson and Ryvarden (1986); Larsen and Lombard (1988); Overholts (1983) reported that specimens were examined in the laboratory and identified using existing taxonomic treatments. Johnson (1940) reported that the morphological features of the fungi were recorded from fresh specimen. For the anatomical study, the basidicarps were fixed in Formalin Acetic Acid (FAA), processed in TBA series, embedded in wax and blocks were prepared. Kaul (1999) reported that morphological observations were based on fresh specimens. The microscopic observations and spore prints were done immediately. Pacioni (1985) observed that identification was made on the basis of critical observation of the specimens and perusal of relevant literature and Mycokey. Priyadarsini (2011) noted that white rot fungus was isolated from decay wood sample and was subjected to Phylogenetic analysis by using basidiomycetes-specific internal transcriber spacer primers for identification.

The isolated fungi were screened for lignolytic activity. Kirk *et al.* (1987) reported that the lignolytic activity of *P. chrysosporium* by the ability of fungus to metabolize synthetic $^{14}$C lignins
to $^{14}$CO$_2$. Janshekar *et al.* (1982) screened white rot fungi based on their ability to degrade crude and fractional straw lignin. Orth *et al.* (1993) screened nine species of the wood rot fungi on oak saw dust substrate supplemented with wheat bran. Glenn *et al.* (1983) reported the lignolytic enzyme system generates ethylene from 2-keto-4-thiomethyl butyric acid (KTBA); hence it could also be used as an indicator for the presence of lignolytic system in fungi. Gold (1988) stated that use of $^{14}$C radio labeled and unlabelled substrates to screen for lignolytic activity which are relatively slow and often require synthetic substrates which are not commercially available. Moreover the assays are not well suited either for screening organism for lignin degrading system. Glenn and Gold (1983) reported that, the polymeric dyes B-XII, Poly R- 481 and Poly R- 606 could be used as alternatives to the radio labeled lignin compounds for lignin biodegradation assay.

The polymeric dyes are inexpensive, obtained commercially in high purity, stable and readily soluble having high extinction co-efficient and low toxicity towards fungi, they are preferred for screening ligninolytic fungi. The decolourizations of polymeric dyes are correlated with the onset of secondary metabolism and ligninolytic activity. Vyas and Molitoris (1995) reported that white rot fungus *Pleurotus ostreatus* produced on extracellular H$_2$O$_2$ referring Remazol brilliant blue R (RBBR) decolourizing enzymatic activity along with manganese dependent peroxidase, phenol oxidase activity and ligninolytic capability. Kuwahara *et al.* (1984) reported two H$_2$O$_2$ dependent oxidases in the extracellular medium of the white rot fungus *P.chrysosporium*, these enzymes catalyse the oxidation of phenol red, O-dianisidine Poly R and variety of other dyes. The enzyme fractions generated ethylene from KTBA in the presence of alcohol. Kelly and Reddy (1986) established that assay for ethylene production form $\alpha$-oxo-$\gamma$-methylthiobutyric acid is a simple sensitive measure of ligninolytic activity by *P. chrysosporium*. To confirm the ligninolytic activity of the fungi the ability of the fungi to grow and to degrade lignin was studied in lignin amended basal medium (Kirk *et al.*, 1978). Dominguez *et al.* (2001) and Couto *et al.* (2000) noted that 19 per cent decolourization of poly R-478, after 15 min of dye incubation higher than 95 per cent decolourization of poly-478 dye under optimal conditions. Ang *et al.* (2011) reported that *sajor-caju* may be a suitable fungus in bioconversion of lignocelluloses because it maintained guiacol oxidation and ramazol brilliant blue-R decolourization activities at 35°C. Moreira *et al.* (2001) explained that *Pleurotus ostreatus* is able to decolourize Remazol brilliant blue-R on agar plate. Vaithanomsat *et al.* (2010) observed that decolourizatoin of two
reactive dyes, Ramazol brilliant blue R (RBBR) and reactive black 5 (RB 5), by selected white-rot fungus Datronia sp. KAPI0039. A highly significant correlation was found between two ligninolytic indicators, ethylene formation from α-keto-γ-methylthiolbutyric acid and the decolourization of a polymeric dye, poly-R, and H2O2 - requiring enzyme system was found in the extracellular medium of ligninolytic cultures of P. chrysosporium (Jong et al., 2009).

In the present study fungi were screened for ligninolytic activity based on their ability to decolourize the polymeric dyes, Poly R and Remazol brilliant blue to degrade native lignin and to generate ethylene from KTBA; for dye degradation studies, C-limited medium of Janskekar and Fiechter (1988) was used. The results revealed that all the fungi tested were capable of oxidizing the dyes indicating that all of them possessed ligninolytic activity (Table 2). The lowest degradation (10.4 %) of Poly-R dye was observed in Phellinus Sp.2; and the highest (38.80%) in P. hirsutus. In Remazol brilliant blue, the lowest degradation (8.80%) was observed in Phellinus sp.2 and the highest (71.5%) degradation was observed in P. hirsutus. The growth was measured in terms of increase in mycelial dry weight (mg) per day (Table 3). The mycelial growth rate was observed in the range of 1.24 to 3.67 mg/day; minimum growth was observed (1.24 mg/day) in Ganoderma sp.7 and maximum 3.67mg/day in Phellinus sp.1; the percent lignin degradation was higher (68.4%) in P. hirsutus and then lower (20.4%) in phellinus sp.3. The ligninolytic activities of the fungi were further confirmed by their ability to release ethylene from KTBA. P. hirsutus released high amounts of ethylene (2.832 ppm) from KTBA and least in Daldenia sp.1 (1.210 ppm). Among the fifty six fungi P. hirsutus, D. flavida and Phillnus sp.1 was found to be more efficient lignin degraders.

The optimum culture parameters for growth and ligninase production by the fungi were well studied. The culture 0condition like incubation period, pH, temperature, carbon and nitrogen source greatly influence the growth and enzyme production of lignin degrading fungi. Pleurotus florida produced high amount of laccase (4.60 U/ml) after twelve days of growth in malt extract broth (Dhaliwal et al., 1992). Bose et al. (2007) reported that Termitomyces clypeatus, a white rot fungus was found to produce maximum MnP activity after six days of incubation using lignocellulosic substrates. MnP enzyme produced by P. chrysosporium peaked on the seven days of cultivation (Zahamatkesh et al., 2010) and white-rot fungus Datronia sp. KAPI0039 produced maximum laccase and MnP after four and eight days of cultivation, respectively (Vaithanomsat et
Irshad and Asgher (2011) reported that *S. commune* IBL-06 was observed as 3745 U/ml for manganese peroxidase (MnP), 2700 U/ml for lignin peroxidase (LiP) and 345 U/ml for laccase after three days incubation. Maximum production of MnP (998 U/ml), LiP (620 U/ml), and laccase (49.7 U/ml) was observed by *T. versicolor* IBL - 04 after five days in the SSF medium (Iqbal, et al., 2011). Selvam et al., (2007) observed that maximum growth was observed by *Thelephora* sp., on sixth day.

In the present study, it was observed (Table 4) that in shaken condition, the maximum growth was observed by *P. hirsutus* on ninth day (17.0 mg mycelial dry weight); In *D. flavida* and *Phellinus sp.* the maximum growth was observed on tenth day (20.0 mg mycelial dry weight) and eleventh day (20.0 mg mycelial dry weight) of incubation period respectively. In ligninase enzyme production, LiP and MnP were produced in higher amounts on seventh day and laccase on fourth day in all the three fungi.

Niku paavola et al. 1998 reported that in *P. chrysosporium* the optimum pH for ligninase production was 3.5. *Phlebia radaia* enzyme showed optimal activity at pH 3.0 - 4.0 and were stable in pH range of 5.0 - 6.0. Optimal pH was found between 4.5 and 5.0 for the production of ligninolytic enzymes by *Phanerochaete sortid.*2 had grown in milled wood lignins extracted in solid state medium (Ruttiman et al., 2008). Motomura et al. (2003) reported that the lignolytic enzymes production (LiP and MnP) by *P. ostreatus* in the medium containing liquid culture aflatoxin as substrate was also maximum at pH 4.0. Radha et al. (2005) explained that the maximum activities of MnP (1,205 U/ml), LiP (1,110 U/ml), and laccase (52.8 U/ml) were noted in the SSF medium processed at pH 4.0. Maximum LiP and MnP activities were produced by *P. ostreatus* in the pH range 4.0 to 5.0 at 25°C (Motomura et al., 2003). The optimum ligninase production by *Coriolus hirsutus* and *Trametes villosa CCB176* has been reported at pH 4.0 and 5.0, respectively (Shin and Lee 2000; Yamanaka et al., 2008). Maximum LiP, MnP and laccase production were 0.03, 0.26 and 9.3 U/ml, respectively (Galhaup et al., 2002; Jang et al., 2002; Chen et al., 2003).

In the present study it was observed (Table 5) that the optimum pH for growth of *P. hirsutus, D. flavida* and *Phellinus sp.* were 4.5. Kirk et al. (1978) and schimidt et al. (1989) reported that the optimum pH for LiP and MnP were found to be slightly lower than that of growth. Iqubal et al. (2011) established that *T. versicolor* IBL - 04’s Maximum MnP (1775 U/ml),
LiP (1663 U/ml), and laccase (99 U/ml) were produced at pH 4.0. Selvam et al., (2007) observed that optimum pH for the production of LiP, MnP and laccase producing *Thelephora sp* was 4.5 and 7.0 respectively. In *P. hirsutus* the optimum pH for the production of LiP was 4.5; MnP was 4.0; laccase was 5.0. In *D. flavida* and *Phellinus sp.* LiP, MnP and laccase production were optimum at pH 4.5.

Like pH, temperature also influences the enzyme production. At higher temperature (39°C and 28°C) LiP production was dominant and at 23°C MnP was dominant (Vyas et al., 1994). Tran and Chamleers (1987) reported that ligninase production by *P. chrysosporium* was maximum at 38°C; *Phlebia radiate* produced ligninase in an optimum temperature of 40°C. LiP of *P. chrysosporium* required a slightly higher temperature of about 45°C (Niku-Paavola et al., 1988). The temperature ranging from 25 to 37°C have been found to be optimum for ligninase production by different WRF (Zadrazil et al., 1999; Arora and Gill 2001; Tekere et al., 2001; Tripathi et al., 2008). Tripathi et al. (2008) reported that higher temperatures denature the metabolic enzymes of microorganisms leading to inhibition of growth and enzyme formation. Koroleva et al. (2002) observed that *Coriolus hirsutus* has also been found to excrete a considerable amount of laccase and MnP at 28°C. An incubation temperature of 32°C was optimum for laccase production by *streptomyces psammoticus*, and considerable activity was also observed at 30°C (Niladevi et al., 2007).

In the present study it was observed (Table 6) that the temperature 45°C favoured the growth of *P. hirsutus*, *D. flavida* and *Phellinus sp.* growth was favoured by 40°C. In *P. hirsutus* LiP production was optimum at 45°C; MnP and laccase production was maximum at 40°C. In *D. flavida*, LiP and laccase was produced at 40°C; MnP was produced at optimum temperature of 50°C. In *Phellinus sp.* LiP, MnP and laccase enzyme productions were maximum at 40°C.

The growth and extracellular ligninase production were enhanced at carbon levels of 10 g/l. The increase in ligninase production at higher carbon concentration was maintained at the transcriptional level (Collins and Dobson, 1995). Gurusamy (1997) reported that the glucose at 3.0 g/l concentration favoured mycelial growth and aryl alcohol oxidase production in *P. chrysosporium*, LiP and MnP production were maximum at 2.5 g/l concentration lignin at 3.5 g/l concentration favoured mycelial growth and at lower concentration (2.0 to 2.5 g/l) favoured enzyme production. Iqubal et al. (2011) stated that *T. versicolor* IBL-04 produced
maximum MnP (1775 U/ml), LiP (1663 U/ml), and laccase (99 U/ml) in the present of maltose (1% w/w) as carbon source. Revankar and Lele (2006) obtained the highest laccase yields by using a combination of glucose and starch as carbon and yeast extract as nitrogen source.

In the present study, it was observed (Tables 7a, b, c) that in *P. hirsutus*, glucose at 3.0 g/l favoured mycelial growth LiP, MnP and laccase production. In *D. flavida* and *Phellinus sp*.1 mycelial growth LiP, MnP and laccase production were favoured at 3.0 g/l glucose concentration. In cellulose amendments maximum mycelial growth LiP, MnP and laccase was observed at 3.0 g/l for *D. flavida* and *Phellinus sp*.1, whereas in *P. hirsutus* maximum growth LiP, MnP and laccase were produced in higher amounts at 3.0 g/l substrate concentration.

In lignin amendments maximum mycelial growth and ligninase enzyme production of all the three fungi was observed at 3.0 g/l concentration. Janshekar *et al*. (1982) stated that enhancement of ligninolytic activity by nitrogen depletion cannot be regarded as a general principle. Schmidt *et al*. (1989) reported that both low and high nitrogen media induce secondary metabolism involving lignin degradation by *P. chrysosporium*. Katagiri *et al*. (1995) reported that both low nitrogen high carbon (LN-HC) and high nitrogen carbon (HN-HC) media enhanced the ligninase production in *P. chrysosporium* and *T. versicolor*. *In Fomes sp*, no ligninase was detected in nitrogen limiting medium; the production was observed only in nitrogen sufficient glucose peptone medium (Kaal *et al*., 1995). Bonnarme *et al*. (1991) reported that optimum C: N ratio is necessary for good LiP, MnP and laccase production because some fungi grow better under carbon and nitrogen limitations, but others perform better in carbon and nitrogen sufficient culture medium. *Trematosphaeria mangrovei* showed a final specific activity of 82.51 U/mg proteins with higher laccase activity 14.03 U/ml when grown in low nitrogen (LN) medium with half-strength sea water. Pointing *et al*. (2000) explained enzyme production was increased 50 fold in the present of 20 µM xylindine to a maximum of 1360 U/l in high carbon low nitrogen medium. In *Thelephora sp.*, LiP and laccase production was favoured at 0.44 g/l diammonium tartrate and MnP was favoured at 0.22 g/l (Selvam *et al*., 2007).

In the present study, diammonium tartrate, urea and peptone were used as nitrogen sources. The results showed that (Table 8 a-c), in *P. hirsutus* diammonium tartrate at 0.44 g/l favoured mycelial growth. But LiP, MnP and laccase production were favoured at 0.66 g/l concentration in *D. flavida* and *Phellinus sp*.1, mycelial growth LiP, MnP and laccase production
were favoured at 0.66 g/l concentration of diammmonium tartrate. In urea amendments maximum growth of *P. hirsutus* was observed at 0.66 g/l where as the enzyme LiP and laccase maximum production was 0.66 g/l, but MnP was maximum at 0.44 g/l. In *D. flavida* and *Phellinus sp.1*, maximum growth of maximum LiP, MnP and laccase production were observed at 0.66 g/l of urea. Peptone at 0.66 g/l concentration yielded maximum mycelial growth in all fungi. In *P. hirsutus* the concentration optima for LiP, MnP and laccase was 0.66 g/l. In *D. flavida* LiP and laccase optimum production level was 0.66 g/l amendments; but for MnP production was optimum at 0.44 g/l concentration. In *Phellinus sp.1*, LiP and MnP production level was optimum at 0.66 g/l but laccase was produced in higher amount at 0.44 g/l peptone.

The ligninolytic enzymes produced by the test fungi were purified from the culture filtrate by acetone precipitation, dialysis and sephadex G 100 column chromatography (Tables 9-11; Fig. I, II and III). *Phellinus sp.1* produced high amount of LiP than *P. hirsutus* and *D. flavida*; when the enzyme preparations were subjected to sephadex G 100 column chromatography. *P. hirsutus*, *D. flavida* and *Phellinus sp.1* showed three fractions with LiP activity (Table 9; Fig. I); MnP was produced in higher amounts by *P. hirsutus* and *Phellinus sp.1* than *D. flavida* and its showed only one active fraction (Table 10; Fig. I, II and III) laccase was produced in higher amount by *Phellinus sp.1* than *D. flavida* and *P. hirsutus* the laccase activity was observed in three fractions (Table 11; Fig. IV, V and VI).

*P. chrysosporium* LiP had an optimal pH of 4.5 (Gelpke et al., 1999). In the ascomycetes fungus, *Chrysoniliasitophila* LiP was active in the pH range of 3.0 - 5.0 (Ferrer et al., 1992) LiP of *P. chrysoporium* the optimal pH was around 3.0 and it varies for different izsoenzymes (Cui et al. 1993). Selvam (2000) reported that, optimum pH for LiP of *F. lividus, Thelephorce sp* and *T. versicolor* was 3.5. In the present study, LiP of all the three fungi pH was observed to be 3.5 (Table 12; Fig. VII). The peroxidase of *Coprinus friessi* was active upto 60°C for 60 min of incubation (Heinzkill et al., 1998). Niku-paavola et al. (1988) observed that *Phlebia radiate* and *P. chrysosporium* LiP required slightly higher temperature of 45°C for optimal activity. Optimum temperature for LiP of *P. Chrysdosporium* was 40°C, *Ganodema Collosum* was 45°C and *Polyporus leucospongia* was 30°C (Gurusamy, 1997). In the present study the optimum temperature for LiP activity of *P. hirsutus* was 45°C and the optimum temperature for *Phellinus sp.1* was 50°C (Table 12; Fig. VIII).
Kirkpatrick and Palmer (1989) reported that $V_{\text{max}}$ of *P. chrysosporium* LiP isoenzymes were 3.1, 2.7 and 1.6 U/ml against veratly alcohol and the corresponding Km values were 108, 121 and 269 µM. Recombinant LiP of *P. chrysosporium* had a Km (µM) value of 89.4 - 92.8 µM for veratyl alcohol (Gelpke et al., 1999). In the present study, $V_{\text{max}}$ and Km values of LiP were determined against veratyl alcohol LiP of *P. hirsutus* had a $V_{\text{max}}$ of 1050 U/mg protein and Km of 148 µM; *D. flavida* had a $V_{\text{max}}$ of 1000 and Km of 100 U/mg protein; $V_{\text{max}}$ and Km values of *Phellinus sp*.1 Lip were 780 U/mg protein and 120 µM respectively (Table 12; Fig. IX, X and XI).

Heinrkill et al. (1998) reported that the optimum pH for the basidiomycetes fungi *Panaeolus sphinctrinus* and *P. chrysosporium* manganese dependent peroxidase was in the range of 4.0 to 5.0. pH of 4.5 to 5.0 as optimum for MnP of *P. chrysosporium* (Kuan et al., 1993, Chung et al., 1993 and Ruttiman et al., 1994). Selvam (2000) reported that optimum pH for MnP of *F. lividus*, *Thelephora sp* and *T. versicolor* was 4.5. Tekano et al. (2004) reported that, the optimal pH of *P. crassa* WD 1694 MnP was 3.0 to 4.0. The present study optimum pH for MnP obtained from all the three fungi were 4.5, 5.0 and 4.5 respectively (Table 12; Fig. VII).

Maltseva et al. (1991) and Golovleva et al. (1993) reported that optimum temperature for MnP activity was 25°C. Gurusamy (1997) reported temperature of 40°C to 45°C as optimum for MnP activities of *P. chrysosporium*, *G. colossum* and *P. leucospongia*. In the present study the optimum temperature for MnP activity was observed to be in the range of 45 - 50°C. For MnP from *P. hirsutus* and *D. flavida* the optimum temperature were 45°C and *Phellinus sp*.1 the optimum temperature was 50°C (Table 12; Fig. VIII).

Leontievsky et al. (1990) reported a Km of 4.35 X 10$^5$ M for MnP against H$_2$O$_2$; for ABTS the Km values were 5.4 x 10$^2$ and 5.26 X 10$^3$M (Golovelva et al., 1993). MnP of basidiomycetes fungi, *P. sphinctrinus* and *P. chrysosporium* had Km values of 53.1 and 50 µM for H$_2$O$_2$ (Heinzkill et al., 1998). In the present study $V_{\text{max}}$ were observed in 5.2, 4.8 and 4.6 U/mg protein (for H$_2$O$_2$), respectively for MnPs of *P. hirsutus*, *D. flavida*, and *Phellinus sp*.1 and the corresponding Km values were 4.8, 6.2 and 45.3 µM (Table 12; Fig. XII, XIII and XIV).

Youn et al. (1995) reported that the optimum pH for laccase activity in *P. ostreatus* was 6.0 to 6.5. Laccase from a new basidiomycetes strain PAS1 (CEAT 2971) was stable in a pH
range of 3.0 to 9.0 and its optimum pH was 4.5 (Coll et al., 1993). Purified laccase of *Pycnoporus cinnabarinus* was stable below pH 4.0 to 7.0 (Eggert et al., 1996). Selvam (2000) reported that the optimum pH of the laccase producing *F. lividus* and *T. versicolor* sp were 5.0 and pH of the *Thelephora sp* was 6.0. In the present study also the optimum pH of laccase was slightly acidic range. Optimum pH of all the three fungi was found to the 5.0 (Table 12; Fig. VIII).

The optimum temperature of laccase activity for basidiomycete strain PMZ (CECT 2971) was reported to be 80°C (Coll et al., 1993). The optimum temperature for *P. ostreatus* producing laccase activity was 30 to 35°C (Youn et al., 1995). In the present study optimum temperature of laccase activity producing all the three fungi were 50°C (Table 12; Fig. VIII). The fungal laccase had Km value of 48 µM and 89 µM for ferulic acid and syringic acid (Youn et al., 1995). Saida and Asgher (2011) observed that a V_max and Km value of *P. ostreatus* was 3.87 U/ml and 62 µM respectively. In the present study V_max values for the laccase producing *P. hirsutus*, *D. flavida*, and *Phellinus sp*.1 were 42, 40 and 34 U/mg protein and Km values for the respected fungi were 34.4, 36.4 and 38.4 µM (Table 12; Fig. XV, XVI and XVII).

Ligninolytic fungi *P. hirsutus*, *D. flavida*, and *Phellinus sp*.1 were tried for various biotechnological processes like biobleaching of HWKP. According to the Pratima Bajpai et al. (2006) the laccase enzyme from *T. versicolor* showing the highest delignification efficiency was selected and used in the elemental chlorine free biobleaching sequences for improving the pulp bleach ability. Tim et al. (2001) established that, cellobiose dehydrogenase (CDH) deficient strains of the basidiomycete *T. versicolor* were produced by transforming protoplasts of strain 52J with a plasmid carrying the *T. versicolor* CDH gene. It biobleached and delignified industrial unbleached kraft pulp as efficiently as did wild type *T. versicolor*, indicating that CHD is not required for the degradation and biobleaching of kraft lignin. Jian Zhao et al. (2006) reported that, modification of bleached pulp with enzymes of 41 U/g (on oxylanase) form *Asperigillus* L22 decrease pulp kappa number by 6.29 and 12.07 per cent and increased pulp brightness.

Eugenio et al. (2010) reported that kraft pulp from *Eucalyptus globules* was treated at 40°C or pH 3.0 and 60°C or pH 5.0 for 1 hour using an extracellular fluid enriched in laccase produced by *Pycnoporus sanguineus* and *Acetosyringone* as mediator, pretreatment with laccase Acetosyrin done at 40°C and pH 3.0 reduces that kappa number and hexenuromic acids, increase pulp viscosity, lowers hydrogen peroxide consumption down to an 87.4 per cent and enhances
brightness up to 59 per cent ISO (51% ISO without L). Veronica et al., (2010) examined that loblolly pine kraft pulp was bleached in a totally chlorine free sequence that involved treatment with culture supernatants from white rot fungus *Trametes troggi* followed by a peroxide stage.

In the present study, the wood rot fungi *P. hirsutus, D. flavida* and *Phellinus sp.1* were used for biobleaching and delignification of HWKP (Table 13; Fig. XVII). The fungus *P. hirsutus* reduced the pH of the reaction mixture from 7.30 to 4.52. Kappa number was reduced from 26.80 to 13.80 ISO units after ten days of incubation period brightness was increased by 46.90 per cent. The fungi *D. flavida* and *Phellinus sp.1* reduced the pH of the reaction mixture from 7.30 to 4.66 and 4.78 respectively. The kappa number was reduced from 26.80 to 12.90 by *D. flavida* where as in *Phellinus sp.1* the kappa number was reduced to 12.0 from 26.80 and the maximum increase in brightness (46.80 % and 46.50%) was achieved after ten days of incubation period by *D. flavida* and *Phellinus sp.1*.

The pulp and paper industry is one of the major industries in India causing water pollution. The manufacture of paper yields a significant quantity of waste water. It is estimated that 273 - 450 m³ of water is required to produce 1 ton of paper and about 60-300 m³ of waste water is discharged (Subraminum, 1976; Thomspon et al., 2001) According to Selvam. (2000) maximum decolourization of paper mill effluent 63.9 per cent was achieved by *T. versicolor* on the fourth day in the laboratory scale level. Inorganic chloride at a concentration of 765 mg/l which corresponded to 227.0 per cent of that in an untreated effluent was liberated by *F. lividus* on the tenth day. The chemical oxygen demand (COD) was also reduced to 1984 mg/l (59.3%) by each of the two fungi. On the pilot scale level a maximum decolourization of 68.0 per cent was obtained with the sixth day incubation by *T. versicolor*, inorganic chloride 475 mg/l (103%) was liberated on the seventh day by *T. versicolor* and the COD was reduced to 1984 mg/l corresponding to 59.32 per cent by *F. lividus* and the result suggested that *F. lividus* was the efficient candidate for dechlorination of waste water. Ragunathan and Swaminathan (2004) reported that the ability of *Pleurotus sp*, *P. sajor caju*, *P. platupus* and *P. citrinopileatus* in the treatment of pulp and paper mill effluent on a laboratory and pilot scale were studied. On the laboratory scale treatment *P. sajor caju* decolourized the effluent by 66.7 per cent on the sixth day of incubation period. Inorganic chloride content was 230.9 per cent (814.0 mg/l), COD was reduced by 61.3 per cent (1302.0 mg/l) on tenth day treatment. In the pilot scale treatment
maximum decolourization obtained by *P. sajor-caju* was 60.1 per cent inorganic chloride content was increased by 524.0 mg/dl (113.0%) and COD was reduced by 1442.0 mg/l (57.2%) on seventh day. A new mucoralean fungus *Rhizomucor pusillus* removed the colour of bleach plant effluent by 41.0-48.9 per cent (Christor and Van Driessel, 2003). Two basidiomycetous fungi (*Merulius aureus*, *Phlebia sp* and an unidentified genus) and a deuteromycetous fungi (*Fusarium sambercimum Fuckel MTCC 3788*) were immobilized and were used for bioremediation of pulp and paper mill effluent in a continuously treated beach top bioreactor. The treatment resulted in the reduction of colour, lignin and COD of the effluent in the order of 78.6 per cent, 79.0 per cent and 89.4 per cent in four days (Piyush and Rathore, 2007). Five white rot fungi *P. chrysosporium*, *P. ostreatus*, *Lentimus edodes*, *T. versicolor* used to treat black liquor from a pulp and paper mills over 71 per cent of lignin and 48 per cent of COD were removed from the waste water (Juan Wu *et al.*, 2005). Luciana *et al.*, (2003) reported that some white rot strain such as *Ceriopsis subvermispora* could decolourized kraft bleaching effluent at 90.0 per cent of also resulted in reduction of COD upto 45.0 per cent.

In the present study, the ligninolytic fungi were used for treatment of paper mill effluents in lab scale as well as pilot scale experiments. The study (Table 14 and 15; Fig. XIX and XX) revealed that in lab scale experiments, after tenth day of incubation reduced the effluent colour from 36.4 to 37.8 per cent and ten days incubation leads to the liberation of chlorine content from 165.0 to 189.7 per cent and reduced the COD by 42.1 to 43.0 per cent. But in pilot scale experiments were not as efficient as lab scale. In pilot scale, colour removal was only at the level of 31.0 to 33.7 per cent, liberation of inorganic chlorine was 89.0 to 101.0 per cent and COD was 37.3 to 40.1 per cent. From the above results lab scale experiments were highly efficient than pilot scale. In paper mill effluent treatments with *P. hirsutus* was found to the more efficient than two fungi.

Azo dyes are potential health hazards as they may be converted to toxic and or carcinogenic products under anaerobic conditions. White rot fungi have attracted a lot of attention due to their ability to attack a wide variety of recalcitrant compound including dyes (Rabinovich *et al.*, 2004 and Kaushik and Malik, 2009). Chhavi Rani *et al.* (2011) reported that the white rot fungus *D. flavida* decolourized azo dyes, amaranth, metanil yellow, trypton blue and chlorazole black. Saparat *et al.* (2008) reported that and the fungus *Grammothele subargentea* LPSC No 436
strain efficiently decolourized different dyes, being the highest levels of laccase activity in cultures with brilliant green. Selvam et al. (2003) first demonstrated that ligninolytic culture of *Fomes lividus* could decolourize azo dyes such as orange G, congo red and amido black. Neelamegam et al. (2004) conducted a study to assess the degree of decolourization of different dyes such as malachite green, indigo carmine, xylidine ponceau, bismark brown, congo red and methyl orange by *P. ostreatus*. Selvam et al. (2002) established that, white rot fungus *Thelephora sp* was capable of decolourizing azo dyes such as orange G, congo red and amido black 10B. The maximum decolourization (98%) of synthetic azodyes was achieved by the white rot fungus *P. chrysosporium* under normal condition (Senthil kumar et al., 2011).

In the present study, the ligninolytic fungi *P. hirsutus*, *D. flavida* and *Phellinus sp*.1 were used for the removal of congo red, methyl orange and orange G. The result showed in Table 16, 17 and 18 explained that, maximum decolourization of orange G was 37.9 per cent by *P. hirsutus* treatment after fourth day of incubation. For *D. flavida* and *Phellinus sp*.1 maximum decolourization was 36.9 and 37.0 per cent at fourth day of incubation respectively. In Congo red a maximum of 98.0 per cent removal was observed in *P. hirsutus* cultures on fourth day of incubation; *D. flavida* removed 97.6 per cent of the dye during fourth day; *Phellinus sp*.1 was removed 94.6 per cent of the dye on fourth day of incubation. In methyl orange the *P. hirsutus* required four days for 97.7 per cent dye removal. The per cent colour removal was found to be efficient in methyl orange. The per cent of the dye removal by *D. flavida* was observed upto 94.3 per cent within four days of incubation. *Phellinus sp*.1 removed a maximum of 97.0 per cent of the dye at fourth day of incubation.

Shaul et al. (1991) reported that the dye effluents are poorly decolourized by connectional biological waste water treatment. According to the Cripps et al. (1990), Paszezynbki and Crawford (1985) the white rot fungi are the most efficient ligninolytic microorganisms. They are able to degrade a wide variety of recalcitrant pollutants including various types of dyes. Poonam Dayaram et al. (2007) reported that, white rot fungus *Polyporus rubidus* degraded that effluent from textile industries greater than 80.0 per cent within five days under stationary incubation conditions. Rodriguez et al. (1999) reported that, several industrial dyes were decolourized biocatalytically by extra cellular enzymes from different strains of white rot fungi and the decolourization capacity was correlated with laccase activity levels. This enzymatic system was
selected as a good candidate for immobilization and used as a bioreactor for effluent treatment from the dyes and printing industries. Swamy and Ramsay (1999) stated that in decolourization process, identification of the decolourizing enzyme is useful for optimizing process parameter and medium composition to maximize enzyme production detected the production of extra cellular MnP and laccase but not LiP during decolourizing process. *Pleurotus calyptatus* strain produced high amount of laccase, MnP and AAO and they decolourized the textile dye effluent in liquid culture (Ivana Eichlerova et al., 2006). Laccase from *Pleurotus florida* decolourized the textile effluent (Krishnaveni and Kowsalya 2011).

In the present study, the dye industry effluents were treated with ligninolytic fungi for conventionally in batch mode and continuous mode. The results revealed (Table 19; Fig. XXIV) that in batch mode treatment, the maximum removal of colour from the effluent was obtained after five days of incubation. *P. hirsutus* removed 80.7 per cent of the effluent colour after five days in *D. flavida* and *Phellinus sp*.1 treatment, the maximum colour reduction were 80.0 and 78.6 per cent respectively on fifth day, where as in continuous flow mode maximum colour removal was observed in *P. hirsutus*. The decolourization were found to be were 67.8 and 66.7 per cent, so batch mode was found to be very efficient in colour removal than continuous flow mode.