4. RESULTS

4.1. Collection of fungi

In the present study, fifty six wood rot fungi were collected and isolated from the Western Ghats area of Tamilnadu and Karnataka in India and screened for ligninolytic activity. Among them three fungi *Polyporus hirsutus*, *Daedalea flavida* and *Phellinus sp.* were higher ligninolytic activity and further studies were carried out on those organisms. Most of the fungi were isolated from wooden logs of fallen trees in the forest, the reference fungus *Phanerochaeta chrysosporium* - 787 was obtained from Microbial type culture collection (MTCC) Chandigarh. The list of fungi collected, isolated and their source were given in Table 1 and Plate 1-7.

4.1.1. Isolation and maintenance

The fruit body was cut into small pieces and sterilized with 1 per cent mercuric chloride solution, repeatedly washed with sterile distilled water (Roy Watling, 1971) and inoculated on 2 per cent malt agar medium in petriplates. Plates were incubated at 37°C for six days the fungal growth occurred on the plates sub culture and maintained on malt agar slant.

4.1.2. Inoculation

The fungi were grown in malt agar plates for 6 days at 37°C. Then the plates were flood with sterile distilled water and brushed with camel-hair brush smoothly without disturbing the mycelial growth. The spore suspension was filtered over a sterile filter system to remove the mycelial fragments. Concentration of the filtered spores suspension was adjusted to 10^5 spores/ml and used as inoculums for further studies.

4.2. Screening of wood rot fungi for ligninolytic activity

Primary screening of wood rot fungi for ligninolytic activity was carried out by dye reduction method. Poly R dye (0.02%) and Remazol brilliant blue (3 mg/ml) dye were used as test dyes. The ability of the wood rot fungi to degrade these dyes was studied in the C-limited medium (M14) of Janshekar and Fiechter (1988) and the results were presented in Table 2.
The results revealed that in Poly R dye maximum degradation (36.4 %) was brought by *P. hirsutus*. The reference fungus *P. chrysosporium* - 787 degraded 31.2 per cent of the dye. Minimum degradation (10.4%) was observed in *Phellinus sp.2* treatment.

In Remazol brilliant blue maximum degradation (71.5%) was observed in *P. hirsutus* treatment. Through *Phellinus sp.2* (8.80%) minimum degradation was observed. The reference fungus *P. chrysosporium* - 787 degraded remazol brilliant blue 55 per cent only.

### 4.2.1. Degradation of lignin by wood rot fungi in synthetic medium

For confirmation of ligninolytic activity of the fungi, the ability of the fungi to grow and to degrade lignin was studied in lignin amended basal medium. The growth was measured in terms of increase in mycelial dry weight (mg) per day. The results revealed that (Table 3) all the fungi were capable of utilizing lignin as growth substrate. The mycelial growth rate was observed in the range of 1.34 mg/day to 2.832 mg/day. The growth rate of the reference fungus *P. chrysosporium* - 787 was 2.12 mg/ day.

The results revealed that all the fungi were capable of degrading lignin synthetic medium. Among the fungi *Phellinus sp.3* was the weakest lignin degrader, the per cent of lignin degradation was only 20.4 per cent; whereas *P. hirsutus* and *D. flavida* exhibited as maximum degradation of 68.4 and 68.1 per cent respectively. In reference fungus the lignin degradation was 42.0 per cent.

### 4.2.2. Production of ethylene from 2-keto-4-thiomethyl butyric acid (KTBA)

The ligninolytic activities of fungi were further confirmed by their ability to release ethylene from KTBA. The data is presented in the Table 3. Once again confirmed that all the test fungi were lignin degraders, but the efficiency differ from fungus to fungus. For instance the ethylene production from KTBA was maximum 2.832 ppm in *P. hirsutus*, whereas in *Phellinus sp.2* very low level of ethylene was produced (1.21). The reference fungus produced 1.822 ppm of ethylene next to *D. flavida* (2.421 ppm).
4.3. Optimization of culture condition on growth and ligninase production

4.3.1. Incubation period

The effect of incubation period on culture condition and ligninase production by *P. hirsutus*, *D. flavida* and *Phellinus sp.* were presented in Table 4. The results showed that maximum growth was observed by *P. hirsutus* on ninth day, tenth day and eleventh day (17.0 mg mycelial dry weight). Initial production of LiP on first day was 36 U/ml; it has increased up to 139 U/ml on seventh day of incubation period.

Initially MnP production by *P. hirsutus* was 0.02 U/ml and maximum production was observed on seventy day (0.13U/ml). After seventh day LiP and MnP production were decreased. On the first day, the laccase enzyme production was 0.39 U/ml, the maximum production (1.32 U/ml) was achieved on fourth day, after that the enzyme production was reduced.

In *D. flavida* maximum growth (20.0 mg mycelial dry weight) was observed on tenth day of incubation. LiP (136 U/ml) and MnP production (0.14 U/ml) were maximum on seventh day; on fourth day, the laccase production was 1.40 U/ml and then it was decreased.

In *Phellinus sp.* maximum growth (20.0 mg mycelial dry weight) was observed on eleventh day; LiP and MnP production were maximum on seventh day and it was found to be 192 U/ml and 0.14 U/ml; laccase production was maximum (1.62 U/ml) on fourth day; but after the optimum period the enzyme productions were gradually decreased.
4.3.2. pH

Effect of pH on culture conditions and ligninase production were studied in the pH range from 3.0 to 9.0. The results revealed (Table 5) that, in \textit{P. hirsutus} maximum (25.0 mg dry weight) growth was observed at pH 4.5, LiP production also favoured (174 U/ml) at pH 4.5. At pH 4.0 maximum level of MnP was produced (0.21 U/ml). In laccase production, the maximum level of 1.22 U/ml was observed at pH 7.0.

In \textit{D. flavida}, maximum growth (22.0 mg dry weight) was observed at pH 4.5; LiP, MnP and were maximum at pH 4.5 and the production was found to be 187.0, 0.21. For laccase maximum enzyme production 1.15 U/ml was observed on pH 7.0.

In \textit{Phellinus sp}.1 maximum growth (27.0 mg dry weight) was observed at pH 4.5. LiP, MnP productions were maximum at pH 4.5, laccase production was a pH of 6.5. LiP MnP was produced up to 174.0 U/ml and 0.24 U/ml respectively. In laccase maximum production was up to 1.00 U/ml was observed at pH 6.5.
4.3.3. Temperature

The effect of temperature on culture condition and ligninase production was studied in the temperature ranges from 30 to 80°C. The results (Table 6) showed that, 45°C favoured mycelial growth (29.0 mg dry weight) of *P. hirsutus*. LiP production was favoured by 45°C and the production was 165 U/ml. MnP and laccase enzyme productions were optimum at 40°C and the productions were 0.15 and 1.85 U/ml respectively.

In *D. flavida*, mycelial growth (21.0 mg) was favoured at 40°C. At 40°C, LiP was produced up to 136 U/ml. MnP was produced at a maximum of 40°C and the production was found to be 0.16 U/ml. Laccase production was favoured by 40°C temperature with a maximum production of 1.90 U/ml.

In *Phellinus sp.* growth and LiP, MnP and laccase enzyme productions was found to be maximum at 40°C, at 40°C the growth was 23.0 mg dry mycelium; LiP, MnP and laccase productions were 158.0, 0.17 and 1.35 U/ml respectively.
4.3.4. Carbon sources

In the present study, glucose, cellulose and lignin were supplemented as carbon sources at various concentrations ranging from 0 to 4.0 g/l in the culture medium and their effects on mycelial growth and ligninase production were determined (Tables 7a, b and c).

4.3.4.1. *Polyporus hirsutus*

In *P. hirsutus* control cultures (medium destitute of any carbon source) mycelial growth and enzyme production were very low. The mycelial dry weight on seventh day was 20.0 mg and its dry weight on fourth day was 16.0 mg. The production of LiP, MnP and laccase were found to be 30.0, 0.02 and 0.65 U/ml respectively. But supplementation with carbon sources the growth and enzyme production was increased.

In glucose modification, 3.0 g/l substrate concentration favoured mycelial growth (32.0 mg dry mycelium) and production of LiP (150 U/ml) MnP (0.17) and laccase (1.80 U/ml) enzymes.

In cellulose amendment, an increase in growth (31.0 mg dry mycelium) was observed up to 3.0 g/l and LiP, MnP and laccase production were maximum up to 3.0 g/l substrate concentration. The production level of LiP, MnP and laccase were found to be 175, 0.13 and 1.55 U/ml respectively.

In lignin supplementation, mycelial growth, LiP, MnP and laccase production were maximum at 3.0 g/l concentration. (34.0 mg dry mycelium and 169, 0.15 and 1.65 U/ml respectively) (Table 7a).

4.3.4.2. *Daedalea flavida*

Effect of carbon sources on growth and ligninase production by *D. flavida*. In control the mycelial growth was 15.0 mg dry weight at 3.0 g/l lignin substrate concentration. The enzyme productions were 50.0 U/ml in LiP 0.05 U/ml in MnP, 0.73 U/ml in laccase. In glucose amendment, maximum growth (25.0 mg) was observed at 3.0 g/l glucose; LiP (120 U/ml), MnP (0.23 U/ml) and laccase (1.52 U/ml) productions were maximum at 3.0 g/l concentration.
In cellulose supplement mycelial growth and LiP, MnP and laccase productions were maximum at 3.0 g/l concentration (29 mg dry mycelium and 160.0, 0.21 and 2.25 U/ml respectively).

In lignin modification, mycelial growth (36.0 mg) was maximum at 3.0 g/l concentration. LiP, MnP and laccase productions were favoured at 3.0 g/l concentration (165.0, 0.20 and 2.84 U/ml respectively) (Table 7b).

4.3.4.3. *Phellinus sp.* 1

Data presented in Table 7c revealed the effects of carbon source on growth and ligninase production by *Phellinus sp.*1. In control the mycelial growth was 20.0 mg dry weight, the enzyme production were 21 U/ml in LiP, 0.02 U/ml in MnP and 0.35 U/ml in laccase.

In glucose amendment, maximum growth (39.0 mg) was observed at 3.0 g/l, LiP, MnP and laccase production level was maintained up to 3.0 g/l substrate concentration (172.0, 0.15 and 2.10 U/ml respectively).

In cellulose supplement the mycelial growth (38.0 mg) was observed at 3.0 g/l substrate concentration. LiP, MnP and laccase production were maximum at 3.0 g/l (179.0, 0.13 and 2.55 U/ml) substrate concentration.

In lignin amendment, 3.0 g/l substrate concentration favoured mycelial growth (37.0 mg dry mycelium) and production of LiP (165.0 U/ml), MnP (0.17 U/ml), and laccase (1.25 U/ml) was favoured by 3.0 g/l substrate concentration.
4.3.5. Nitrogen sources

Nitrogen source used in the present study were diammonium tartrate, urea and peptone. The results showed that (Table 8a-c) the growth and enzyme production was increased by the amendment of those substrates in the range from 0.22 to 1.10 g/l.

4.3.5.1. Polyporus hirsutus

In P. hirsutus, the control culture (medium devoid of nitrogenous substrates) showed a mycelial growth of 21.0 mg; LiP, MnP and laccase production were 25.0, 0.02 and 0.92 U/ml respectively.

In diammonium tartrate amendment, maximum growth (32.0 mg) was observed at 0.44 g/l concentration. But, LiP production was favoured up to 185.0 U/ml at 0.66 g/l concentration. The production of MnP and laccase were found to be 0.10 and 2.97 U/ml at 0.66 g/l substrate concentration.

Amendment of medium with urea, growth (32.0 mg) and LiP production (163 U/ml) were maximum at 0.66 g/l MnP production was maximum (0.09 U/ml) at 0.44 g/l concentration. This level was maintained up to 0.66 g/l substrate concentration. Laccase production was maximum (1.44 U/ml) at of 0.66 g/l concentration.

In peptone, maximum growth (39.0 mg) LiP, MnP and laccase production were favoured by 0.66 g/l concentration. At 0.66 g/l concentration LiP, MnP and laccase production were 172.0, 0.09 and 1.11 U/ml respectively (Table 8a).

4.3.5.2. Daedalea flavida

The data presented in Table 8b represents the effect of nitrogen source on growth and ligninase production by the fungus D. flavida. Control showed 19.0 mg growth and the enzyme productions in the controls were 25.0, 0.03 and 0.92 U/ml respectively for LiP, MnP and laccase. Diammonium tartrate at 0.66 g/l brought a maximum level of growth (30.0 mg). LiP, MnP and laccase were produced (164, 0.13 and 4.00 U/ml) maximum at 0.66 g/l concentration. Increased substrate concentration decreased enzyme production.
In urea amendment, mycelium growth (30.0 mg) LiP, MnP and laccase production level were maximum at 0.66 g/l substrate concentration. The production level was found to be 200.0, 0.13 and 1.93 U/ml respectively.

In peptone supplementation, mycelium growth (37.0 mg) and LiP (212.0 U/ml) production level were maximum at 0.66 g/l substrate concentration. MnP production (0.09) was favoured by 0.44 g/l concentration and maximum production (1.43 U/ml) of laccase was achieved at 0.66 g/l concentration.

4.3.5.3. Phellinus sp.1

In *Phellinus sp.1* culture, control showed 17.0 mg growth and LiP, MnP and laccase in the control were 45.0, 0.03 and 0.62 U/ml respectively.

In diammonium tartrate amendment, maximum growth (31.0 mg) and LiP, MnP (172.0, 0.13 U/ml) productions were achieved at 0.66 g/l concentration, laccase production was maximum (3.10 U/ml) at 0.66 g/l concentration.

In urea amended medium, maximum growth (32.0 mg) was observed at 0.66 g/l concentration. All the three enzymes, LiP, MnP and laccase were produced at maximum level (177, 0.11 and 2.10 U/ml respectively) at 0.66 g/l concentration of urea.

In peptone amendment, maximum growth (36.0 mg) and LiP (209.0 U/ml), MnP (0.14 U/ml) production level were favoured at 0.66 g/l substrate concentration. Laccase (1.08 U/ml) at 0.66 g/l concentration (Table 8c).
4.4. Purification of ligninolytic enzymes

4.4.1. Lignin peroxidase (LiP)

Data obtained in the purification of lignin peroxidase were presented in Table 9 and figures I, II and III. The crude culture filtrate of *P. hirsutus* had a total activity of 7500 U/ml with a protein content 0.08 mg/ml; the specific activity was 187.5 U/mg protein. When the culture filtrate was concentrated by acetone precipitation method, the total activity was reduced to 1360 U/ml, but the protein content and specific activity were increased. The acetone concentrated enzyme preparation had a protein content of 0.10 mg/ml and the specific activity was 680 U/mg protein, showing a purification fold of 3.62. The enzyme recovery at this step was 18.13 per cent. When the acetone concentrate was passed through sephadex G 100 column, the fraction 12 exhibited LiP activity in which the protein content was 0.06 mg/ml; the specific activity of this fraction was 3916 U/mg protein (Fig.I) at this step, the purification fold was 20.88 and recovery was 15.66 per cent. Another fraction 20, exhibited LiP activity; protein content of this fraction was 0.04 mg/ml and the specific activity was 5025 U/mg protein; at this step, purification factor was 26.8 and recovery was 13.4 per cent. In fraction 26, the specific activity was 4650 U/mg and protein content was 0.04 mg/ml, the recover yield and purification were 12.4 per cent and 24.8 respectively concentration.

This culture filtrate of *D. flavida* had a total activity of 5000 U/mg and specific activity of 166.6 U/mg protein; the protein content was 0.06 mg/ml. In acetone concentrate, the total activity was reduced to 840 U/mg and the specific activity was 350 U/mg protein; the protein content was 0.12 mg/ml and the purification factor was 2.10 with a recovery yield of 16.80. In sephadex G 100 column chromatography, the fractions 12, 20 and 27 exhibited the LiP activity (Fig.II). In fraction 12, the specific activity was 2300 U/mg protein; the purification fold and recovery yield were 18.4. In fraction 20, the specific activity was 2650 U/mg protein and protein content was 0.08 mg/ml; the recovery yield and the purification. In fraction 27, the specific activity was 3166 U/mg protein and protein content was 0.06 mg/ml; recovery yield and purification factor were 19.0 per cent and 25.3 respectively.

*Phellinus sp.*1 culture filtrate contained 0.12 mg/ml protein and the specific activity was 116.66 U/ml. In acetone concentrate, the protein content was 0.14 mg/ml and the specific activity was 771.42 U/mg protein; the purification fold was 6.61 with 30.85 per cent recovery.
In sephadex G 100 column chromatography, fraction 10 exhibited LiP activity (Fig. III). In fraction 10, the specific was 1766 U/mg protein purification factor was 15.13 with a yield of 15.4. In fraction 21, the protein content was 0.12 mg/ml and specific activity was 1700 U/mg protein; the purification factor and recovery yield were 14.57 and 14.5 per cent respectively. In fraction 28, the specific activity was 1720 U/mg protein content was 0.10 mg/ml; the purification factor was 14.74 with a recovery 22.28 per cent.

4.4.2. Manganese dependent peroxidase (MnP)

Data obtained in the purification of manganese dependent peroxidase were presented in Table 10 and figures I, II and III. The results showed that the culture filtrate of *P. hirsutus* had a MnP total activity of 15 U with a protein content 0.12 mg/ml; the specific activity was 1.25 U/mg protein. When concentrated by acetone, the specific activity was decreased to 0.28 U/mg protein with a purification factor of 1.12. The protein content was increased to 0.14 mg/ml. The enzyme recovery was 7.46 per cent. When passed through sephadex G 100 column, the specific activity and purification factor were further increased respectively to 2.0 U/mg protein and 1.6. Here the protein content was 0.08 mg/ml and the recovery yield was 5.33 per cent (Fig .I).

In *D. flavida*. The culture filtrate had a total activity of 10 U and specific activity of 0.125 U/mg protein with a protein content of 0.14 mg/ml. In acetone concentrate, the protein content and specific activity were increased respectively to 0.18 mg/ml and 0.27 U/mg protein showing a purification factor of 2.16 and recovery of 10.0 per cent. Sephadex G 100 fraction 16 contained 0.07 mg/ml of protein with the specific activity of 2.0 U/mg protein (Fig. II); purification factor was 16.6 with a recovery of 7.0 per cent.

*Phellinus sp.*1 culture filtrate had a protein content of 0.15mg/ml and MnP specific activity of 0.4 U/mg protein. Acetone concentration increased the protein content (0.18 mg/ml) and the specific activity (0.44 U/mg protein) with a purification fold of 1.10; the enzyme recovery was 5.33 per cent. In sephadex G 100 column chromatography, the fraction 21 showed MnP activity (Fig. III). The protein content of the fraction was 0.08 mg/ml and the enzyme specific activity was 2.00 U/mg protein; the purification factor and enzyme recovery were 5.00 and 2.6 per cent respectively.
4.4.3. Laccase

The data presented in Table 11 and figures IV, V, and VI, revealed that the culture filtrate of *P. hirsutus* has 0.08 mg/ml protein. The total activity and the specific activity of the laccase were 70 and 1.75 U/mg protein respectively. In acetone precipitate, the protein, revealing a purification fold of 2.97. The enzyme recovery was 14.85 per cent. In sephadex G 100 column, fractions 7, 9 and 11 exhibited laccase activity (Fig. IV). In fraction 7, the enzyme activity was 4.61 U/mg protein and protein content was 0.13 mg/ml. The recovery yield and purification fold were 4.20 and 2.63 respectively. In fraction 9, the enzyme activity, protein content, purification fold and recovery were 5.14 U/mg protein, 0.14 mg/ml, 2.93 and 5.14 per cent respectively. In fraction 11, the enzyme activity was 3.56 U/mg protein and protein content was 0.16 mg/ml; recovery yield (4.07%) and purification factor (2.03) were low when compared to fraction 9.

The culture filtrate of *D. flavida* had a total laccase activity of 50 U the specific activity was 1.66 U/mg protein with a protein content of 0.16 mg/ml. When the culture filtrate was concentrated by acetone, the protein content was 0.12 mg/ml and the total and specific activity were 15.6 and 6.5 U/mg protein respectively; the purification fold was 3.90 and recovery was 31.1 per cent. When the acetone precipitate was passed through sephadex G 100 column, the fraction 4, 7 and 9 exhibited laccase activity (Fig. V). In fraction 4, the protein content was 0.12 mg/ml and the specific activity was 6.0 U/mg protein; enzyme recovery and purification fold at this step were increased to 5.14 per cent and 3.42 per cent. In fraction 7, protein content was 0.14 mg/ml, specific activity was 6.07 U/mg protein, purification factor was 3.46 and recovery yield was 6.70 per cent. In fraction 9, the specific activity was 5.84. U/mg protein and protein content was 0.13 mg/ml; the purification factor was 3.33 and the yield was 5.42 per cent.

In *phellinus sp.1*, the culture filtrate had 110 U of total activity and 1.83 U/mg protein specific activity of laccase; the protein content was 0.12 mg/ml. when concentrated by acetone, the specific activity was increased to 6.14 U/mg protein and purification factor was 3.35. The enzyme recovery was 15.6 and the protein content was 0.14 mg/ml. When through sephadex G 100 column, fraction 5, 7 and 9 exhibited laccase activity (Fig. VI). In fraction 5, the specific activity was 8.50 U/mg protein with a protein content 0.10 mg/ml; purification
factor was 4.85 with recovery yield of 6.70 per cent. The specific activity in fraction 4, 6 was 8.08 U/mg protein with a protein content of 0.12 mg/ml. This fraction has 4, 6 purification fold and 6.92 per cent recovery. In fraction 9, the protein content was 0.12 mg/ml with specific activity of 7.66 U/mg; the purification fold and recovery yield were 4.37 and 6.57 per cent respectively.
4.5. Characteristics of ligninolytic enzyme

4.5.1. Optimum pH

To determine the optimum pH for maximum enzyme activity, the specific activities of the enzyme at various pH levels (pH 2.0 to 7.0) were determined. The results (Fig. VII) revealed that initially, increase in pH favoured the activity of LiP MnP and laccase of all the three fungi; but the optimum pH level differ for each enzyme.

For LiP of the three fungi, the optimum pH was observed to be 3.5. In *P. hirsutus* the enzyme was active in the pH range of 2.0 to 7.0; whereas in *D. flavida* and *phellinus sp.1* it was active up to pH 7.0; beyond that the enzyme became inactive at pH 3.5, LiP of *P. hirsutus* showed maximum activity (1090 U/mg protein) followed by *D. flavida* (1013 U/mg protein) and *phellinus sp.1* (943 U/mg protein).

The optimum pH level for MnP of ligninolytic fungi was found to be 4.5 and an increase in pH beyond 4.5 decreased the enzyme activity. However, the enzyme was active in the pH range of 2.0 to 5.0. Here again maximum activity was observed in *P. hirsutus* (5.55 U/mg protein), followed by *D. flavida* (4.54 U/mg protein) and *phellinus sp.1* (3.63 U/mg protein).

In laccase, the enzyme preparation from *P. hirsutus* and *D. flavida* had an optimum pH of 5.0; whereas, laccase of *Phellinus sp.1* had a slightly higher pH optimum, that is 5.5; however, like MnP, laccase was also active in the pH range of 2.0 to 7.0. Maximum laccase activity was observed in *P. hirsutus* (29.09 U/mg protein) followed by *D. flavida* (25 U/mg protein) and *Phellinus sp.1* (26.66 U/mg protein).
4.5.2. Optimum temperature

To determine the optimum temperature for maximum enzyme activity, the effect of temperature on enzyme activity was determined in the temperature range of 30 to 80°C. The results (Fig. VII) revealed that all the enzymes were active in the temperature range of 30 to 60°C. The optimum temperature for LiP was in the range of 45 to 50°C. LiP of *P. hirsutus* had an optimum temperature 50°C; whereas for LiP of *D. flavida* and *Phellinus sp.*1, it was 45°C. At 45°C LiP of *P. hirsutus* showed a maximum activity of 1312 U/mg protein); which was observed to be higher than that of LiP of *D. flavida* (1257 U/mg protein) and *Phellinus sp.*1 (925 U/mg protein) at these optimum temperature levels. Even at 80°C, the LiP of the three fungi showed little activity (33.33 to 75.0 U/mg protein).

In MnP, the enzyme preparations from *P. hirsutus* and *D. flavida* had an optimum temperature of 45°C; whereas *Phellinus sp.*1 required a slightly elevated temperature of 55°C. Here again, MnP from *P. hirsutus* showed higher activity (8.57 U/mg protein) when compared to MnP of *D. flavida* and *Phellinus sp.*1 showed activity even at 80°C; whereas enzyme from *P. hirsutus* was inactivated at 80°C and that of *D. flavida* 70°C. Laccase preparations from all the three fungi have an optimum temperature of 35°C and they exhibit more or less equal levels of activity (12.00 to 15.55 U/mg protein). Laccase of *P. hirsutus* and *D. flavida*, showed slight activity even at 80°C but laccase of *Phellinus sp.*1 was inactivated at 70°C.
4.5.3. Optimum substrate concentration

To determine the optimum substrate concentrations for maximum enzyme activities, \( V_{\text{max}} \) (maximum enzyme velocity in U/mg protein) and \( K_{\text{m}} \) (substrate concentration at which enzyme activities were half maximal) values for each enzyme was estimated. For the estimation of \( V_{\text{max}} \) and \( K_{\text{m}} \) values, the data on enzyme kinetics were plotted in a graph taking substrate concentration (\( \mu \)M) in X axis and enzyme activity (U/mg protein) in Y axis. The \( V_{\text{max}} \) and \( K_{\text{m}} \) values were extrapolated from the graphs. For confirmation, double reciprocal plots and Eadie-Hostee plots for each enzyme were plotted.

For the estimation of \( V_{\text{max}} \) and \( K_{\text{m}} \) values of LiP, veratryl alcohol in sodium tartrate buffer (100 mM : pH 3.0) was used as substrate; from the figure IX it could be interpreted that \( V_{\text{max}} \) for \textit{P. hirsutus} was 1050 U/mg protein and \( K_{\text{m}} \) was 148 \( \mu \)M. Figure X revealed that LiP of \textit{D. flavida} had a \( V_{\text{max}} \) of 1000 U/mg protein and \( K_{\text{m}} \) of 100 \( \mu \)M; whereas LiP of \textit{Phellinus sp.1} had a \( V_{\text{max}} \) of 780 U/mg protein and \( K_{\text{m}} \) of 120 \( \mu \)M (Fig. XI).

For the determination of \( V_{\text{max}} \) and \( K_{\text{m}} \) values of MnP, \( \text{H}_2\text{O}_2 \) in succinate buffer (20 mM; pH 4.5) was used as substrate. MnP of \textit{P. hirsutus} had a \( V_{\text{max}} \) of 5.20 U/mg protein; \( K_{\text{m}} \) was 41.08 \( \mu \)M (Fig. XII); for \textit{D. flavida} MnP, the \( V_{\text{max}} \) was 4.08 U/mg protein and \( K_{\text{m}} \) was 62.2\( \mu \)M (Fig. XIII); for MnP of \textit{Phellinus sp.1}, \( V_{\text{max}} \) and \( K_{\text{m}} \) values were 4.06 U/mg protein and 45.3 \( \mu \)M respectively (Fig. XIV).

\( V_{\text{max}} \) and \( K_{\text{m}} \) values of laccase were estimated by using guaicol in phosphate buffer (0.1M; pH 7) as substrate. For laccase of \textit{Phellinus sp.1}, the \( V_{\text{max}} \) was 42.0 U/mg protein and \( K_{\text{m}} \) was 34.4 \( \mu \)M (Fig. XV); \( V_{\text{max}} \) and \( K_{\text{m}} \) values for \textit{Phellinus sp.1} laccase were 40.0 U/mg protein and 36.4 \( \mu \)M respectively (Fig. XVI). Laccase of \textit{Phellinus sp.1} had a \( V_{\text{max}} \) of 34.0 U/mg protein and \( K_{\text{m}} \) of 38.2 \( \mu \)M (Fig. XVII).
4.6. Properties of ligninolytic enzymes

4.6.1. Lignin peroxidase

The properties of lignin peroxidase of *P. hirsutus, D. flavida* and *Phellinus sp.*1 were summarized in Table 12. The data revealed that the optimum pH and temperature for *P. hirsutus* LiP activity was 3.5 and 45°C respectively. The $V_{\text{max}}$ against veratryl alcohol was 1050 U/mg protein and Km was 148.0 µM.

For LiP of *D. flavida* the optimum pH was 3.5 and the temperature was 45°C. $V_{\text{max}}$ for veratryl alcohol was 1000 U/mg protein and Km was 100.0 µM.

In *Phellinus sp.*1 LiP, the optimum pH and temperature were 3.5 and 50°C respectively. The $V_{\text{max}}$ for veratryl alcohol was 780 U/mg protein and Km was 120.0 µM.

4.6.2. Manganese dependent peroxidase

The properties of MnP were summarized in Table 12. The optimum pH for all the three enzyme preparations was 4.5. The optimum temperature for *P. hirsutus, D. flavida* MnP was 45°C and *Phellinus sp.*1 was 50°C. $V_{\text{max}}$ and Km values of *P. hirsutus* were 5.20 U/mg protein and 41.08 µM against H$_2$O$_2$; for *D. flavida* these values were 4.08 U/mg protein and 62.2 µM; for *Phellinus sp.*1 MnP, $V_{\text{max}}$ was 4.06 U/mg protein and Km was 45.3 µM.

4.6.3. Laccase

The properties of laccase of *P. hirsutus, D. flavida* and *Phellinus sp.*1 were summarized in Table 12. The laccase enzyme optimum pH was 5.0 for *P. hirsutus, D. flavida* and *Phellinus sp.*1 laccase of all three fungi had optimum temperature of 35°C. For laccase of *P. hirsutus*, the $V_{\text{max}}$ against guiacol was 42.0 U/mg proteins and Km was 34.4 µM. For laccase of *D. flavida* the $V_{\text{max}}$ against guiacol was 40.0U/mg protein and Km 36.4 µM. In *Phellinus sp.*1 the $V_{\text{max}}$ against guiacol was 34.0 U/mg protein and Km was 32.2 µM.
4.7. Application of wood rot fungi

In the present study wood rot fungi were exploited for various biotechnological applications like (i) Biobleaching and delignification of hardwood kraft pulp (HWKP) (ii) Biological treatment of paper mill effluent (iii) Azo dyes decolourization (iv) Decolourization of dye industry effluent.

4.7.1. Biobleaching and delignification of hardwood kraft pulp (HWKP) by wood rot fungi

The results presented in Table 13 and figure XVIII, revealed the efficiency of wood rot fungi in bleaching and delignification of HWKP. HWKP has an initial pH of 7.30; the kappa number was 26.80 and brightness was 35.05 ISO units.

*P. hirsutus* treatment after ten days incubation period brought down the pH 4.52 and also reduced the kappa number by 48.5 per cent; the brightness increased by 32.1 per cent.

In *D. flavida* treatment, HWKP pH has reduced to 4.66 after 10 days of incubation period and also reduced the kappa number by 51.86; the brightness was increased 31.8 per cent after ten days.

In *Phellinus sp.*1 treatment, pH of the hard wood kraft pulp solution had reduced to 4.78 after ten days whereas maximum reduction kappa number (55.22 %) and besides increased brightness (30.9 %) were obtained after ten days of treatment period.
4.7.2. Biological treatment of paper mill effluent by using wood rot fungi (lab scale)

A preliminary work was carried out to treat the paper mill effluents using wood rot fungi in lab scale. The results (Table 14 and Figure. XIX) revealed that the raw effluent was slightly brown in colour (OD at 465 nm: 0.74). The chloride content of the effluent was 283 mg/l and the COD was 5200 mg/l. Treatment with *P. hirsutus* reduced the colour by 36.4 per cent; *D. flavida* reduced the colour by 36.4 per cent and *Phellinus sp.1* reduced by 37.8 per cent after four days treatment period.

Maximum increase in chloride content was observed on tenth day; in *P. hirsutus* the chloride content was increased by 165.0 per cent and in *D. flavida* and *Phellinus sp.1* treatment was 177.0 and 189.7 per cent respectively.

Similarly, maximum reduction in COD was observed on tenth day. *P. hirsutus* treatment reduced the COD by 43.0 per cent; *D. flavida* reduce the COD by 42.6 per cent and *Phellinus sp.1* by 42.1 per cent.
4.7.3. Biological treatment of paper mill effluent by using wood rot fungi (pilot scale)

Paper mill effluent was treated for colour, organic chloride and COD removal in rotating biological contactor. The results were presented in Table 15 and figure XX.

*P. hirsutus*, *D. flavida* and *Phellinus sp.* treatment reduced the effluent colour by 33.7 per cent, 32.4 per cent and 31.0 per cent respectively on tenth day; Treatment on tenth day was found to be optimum for the removal of organic chlorides from the effluent. On tenth day treatment with *P. hirsutus* increased the chloride content by 89.0 per cent, whereas in *D. flavida* and *Phellinus sp.* treatments increased chloride content was obtained 101.0 and 105.0 per cent respectively.

Similar to organic chlorides removal, COD reduction on tenth day treatment was found to be optimum. After tenth day, COD reduction of 40.1, 38.2 and 37.3 per cent were observed in *P. hirsutus*, *D. flavida* and *Phellinus sp.* respectively.
4.7.4. Decolourization of azo dyes by wood rot fungi

The ability of the fungi to remove the azo dyes, congo red, methyl orange and orange G from the aqueous solutions, the per cent of dye removal and the effect of dye concentration were determined.

4.7.4.1. Congo red

In the present study three fungi were studied for the removal of congo red from aqueous solution. The results were tabulated in Table 16.

The fungus *P. hirsutus* removed 98.1 per cent of 50 µM dye from the aqueous solution within fourth day of incubation period. Mycelial growth also increased up to 61.0 mg on fourth day. Data in figure XXI showed that at 25.0 µM concentration 92.2 per cent of the dye was removed.

In *D. flavida* fourth day of incubation period, increased the mycelial dry weight (54.0 mg) removed 97.6 per cent of congo red from aqueous solution. In Data in figure XXI showed that at 25 µM concentration 90.3 per cent of the dye was removed.

*Phellinus sp.* treatment maximum (94.6 %) dye removal was observed at four days of incubation period. The mycelial dry weight was observed to be 53.0 mg on fourth day of incubation. Maximum at 100 µM concentration of dye become toxic to the mycelium growth.
4.7.4.2. Methyl orange

The removal of methyl orange (50 µM) from the aqueous solution was presented in Table 17.

In *P. hirustus* treatment maximum of 97.91 per cent dye was removed after fourth day of incubation period. The initial dry weight of the mycelium was 47.0 mg, at fourth day the dry weight was 54.0 mg; whereas the mycelial dry weight was increased along with increased the dye removal. Data in figure XXII showed that at 25 µM concentration 90.6 per cent of the dye was removed, the maximum mycelium growth was 57.0 mg. Dye above 100 µM concentration became toxic to the mycelium growth.

In *D. flavida* treatment, 94.3 per cent of methyl orange was removed from the aqueous solution within fourth day of incubation; the dry weight of the mycelium was 56.0 mg at fourth day. Data in figure XXII showed that at 25 µM concentration, 90.6 per cent of dye was removed maximum mycelium growth was 56.0 mg. Dye concentration maximum at 100 µM become toxic to the mycelium growth.

The per cent of methyl orange removal from aqueous solution by *Phellinus sp.*1 was observed up to 97 per cent at fourth day of incubation. The growth of mycelium was increased up to 53 mg at fourth day. Data in figure XXII showed, among 91.3 per cent of dye removed at 25 µM concentration, the mycelial growth was maximum at 54.0 mg. Dye above 100.0 µM concentration become toxic to the mycelium growth.
4.7.4.3. Orange G

The results tabulated in Table 18 and figure XXIII showed that the removal of orange G (50 µM) from aqueous solution.

In *P. hirsutus* treatment, four days incubation resulted in 37.9 per cent dye removal and the mycelial growth was increased along with incubation period. At fourth day the mycelial dry weight was found to the 53.0 mg. At 25 µM dye concentration, per cent removal was 42.5 and mycelia growth was observed 52.0 mg dry weight.

*D. flavida* removal, 36.9 per cent dye at fourth day of incubation period and increased mycelial growth was observed (50 mg). At 25 µM dye concentration, per cent removal was 41.7 and mycelia growth was observed 25.0 mg dry weight and after that there was slight decline in dye removal.

In *Phellinus sp.1* treatment, the per cent of dye removal was observed 37 per cent at fourth day along with mycelial dry weight was increased up to 50 mg. At 25 µM dye concentration, per cent removal was 38.0 and mycelia growth was observed 52.0 mg dry weight.
4.7.5. Decolourization of dye industry effluent by wood rot fungi

The dye industry effluent was decolourization by wood rot fungi in batch and continuous flow mode and the results were presented in the Table 19 and figure XXIV.

The results revealed in batch mode, the fungi took five days for maximum removal of colour from the effluent. After fifth day, *P. hirsutus* removed 80.7 per cent of the effluent colour, in *D. flavida* and *Phellinus sp.*1 treatments, the maximum colour reduction were 80.0 and 78.6 per cent respectively on fifth day.

According to the results, continuous flow mode was not as effective as batch mode. In continuous mode maximum colour removal was observed in *P. hirsutus* on fifth day (68.9 %). *D. flavida* decolourize the effluent 67.8 per cent on fifth day. The colour removed by *Phellinus sp.*1 was observed to the 66.7 per cent.