

## Characterization of Lignin Peroxidase from *Paecilomyces* Species for Decolorisation of Pulp and Paper Mill Effluent

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Native fungal isolates F1 - F8 and control fungi *Phanerochaete chrysosporium* were capable of degrading pulp and paper industry effluent and also efficient producers of cellulolytic and lignolytic enzymes. F3 (*Paecilomyces* sp) strain showed higher enzyme activity as compared to other native fungal isolates. Weight loss, cellulose loss and organic carbon contents were found maximum in F3. Reducing sugar, protein content and colour removing potential was also higher in F3. Color reduction initiated very fast with microbial enzyme treatment (initiated only after 2h of incubation) and reached maximum reduction after 24h. Lignin peroxidase fraction I and II resulted in 53% and 34% removal of colour and 36% and 38% lignin removal. The culture extract of F3 strain grown on pulp and paper effluent consists of five protein fractions and out of them two fractions of 38 and 40 kDa molecular weight showed lignin peroxidase activity. The pH and temperature optimum for lignin peroxidase activity were 2 to 3, and 20 - 30 °C, respectively. Maximum activity was observed at 6 mM to 48 mM veratryl alcohol concentration and 256 mM H<sub>2</sub>O<sub>2</sub>, however, sodium azide inhibits the enzyme activity. Different metals (CoCl<sub>2</sub>, HgSO<sub>4</sub>, CaCl<sub>2</sub>, SnCl<sub>2</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub> and ZnSO<sub>4</sub>) also affected the lignin peroxidase activity.

Keywords: Cellulolytic activity, Lignin peroxidase, *Paecilomyces*, Sequential bioreactor, Xylanase, Enzyme activity.

### Introduction

Pulp and Paper industry releases huge amount of waste material into the environment together with effluent containing high biological and chemical oxygen demands (BOD and COD), intense colour, lignin compounds etc<sup>1</sup>. Major contaminants of Pulp and Paper mill are lignosulphonic acid, chlorinated resin acid, chlorinated phenol, dioxins and chlorinated hydrocarbon<sup>2</sup>. Aerobic treatment including fungi and bacteria secreted degrading enzymes helps in removing large amount of colour, COD, lignin and phenol. Most well known fungal isolates responsible for lignin degradation were *Fusarium* sp., *Populaspora*, *Chaetomium* sp. *Hyoxylon*, *Xylaria*, *Callybia*, *Mycena*, *Trametes*, *Phanerochaete*, *Gleophyllum*, *Poria*<sup>3</sup>. Lignin degradation by fungal isolates are due to three extracellular phenol oxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) which are responsible for initiating the depolymerization of lignin<sup>2</sup>. Therefore, the present study was focused on screening of native fungal isolates capable of producing different enzymes. Purification and characterization of lignin peroxidase from the culture

filtrate of best fungal strains is carried out for significant removal of colour from pulp and paper mill effluent.

### Materials and Methods

#### Isolation of native strains

Eight fungal strains (F1-F8) were isolated from a sediment core and degraded wood of pulp and paper mill effluent<sup>2</sup>. The isolated fungal strains were maintained on potato dextrose agar<sup>8</sup>. *Phanerochaete chrysosporium* (F9) obtained from Institute of Microbial Technology, Chandigarh, India was considered as control in the study. These fungal isolates were then grown on MSP medium<sup>2</sup>.

#### Growth and Enzyme assay

Culture filtrate was withdrawn from the flasks and filtered through G-2 sintered glass. Residues of the pulp was dried at 80 °C for 48 h and used to estimate the loss in dry weight during degradation<sup>3</sup>. Cellulose content of the pulp before and after degradation was determined according to Updegraff<sup>4</sup> using microgranular cellulose as a positive control. Protein content was estimated using bovine serum albumin as standard<sup>1</sup>. Reducing sugar was estimated using glucose as standard. Xylanases was determined by monitoring the release of reducing sugar from oat

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spelt xylem (Sigma) by the dinitrosalicylic acid method. CMCase was assayed as recommended by Commission on Biotechnology, IUPAC using 2% CMC in sodium citrate buffer (pH 4.8, 0.05 M) as substrate<sup>5</sup>. FPase, activity was determined according to IUPAC<sup>5</sup>. Lignin peroxidase activity of the culture supernatant was assayed using 2, 4 di-chlorophenol assay based on reaction of A Li P-P<sub>3</sub> oxidized 2,4 dichlorophenol with 4 amino antipyrine that strongly absorbed at 510nm<sup>5</sup>.

#### Production and purification of enzyme

The culture filtrate was precipitated using solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (80% saturation) with continuous stirring for 12 h at 4°C. The content was centrifuged at 8000 x g for 15 min, precipitate thus obtained was dissolved in Tris buffer (1M, pH 7.8) and the ammonium sulphate fraction was dialyzed against Tris buffer in a dialysis bag of convenient length. The enzyme preparation was fractionated by ion exchange chromatography DEAE cellulose ion exchange chromatography column (size 2.0× 20cm) was prepared by filling Tris buffer (pH 7.8). Desalted lyophilized protein was eluted from the column at a flow rate of 30 ml/h with a linear gradient of NaCl (0-0.5 ml). The absorbances of fractions were measured at 280 nm. For further purification studies gel filtration chromatography was used. Glass column of 2×60 cm size was packed with Sephadex G-100 and the column was washed extensively. Enzyme preparation after ion exchange column was loaded on to Sephadex G-100 column. The column was washed with Tris buffer (150 ml) at a flow rate of 20 ml/h. Absorbance of each fraction was measured at 280 nm, and fractions that gave higher absorbance were checked for enzyme activity as described earlier. Molecular weight determination was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)<sup>5</sup>. In this method 10% acrylamide gel was used, and electrophoresis was performed. Gels were stained for protein with Coomassie brilliant blue R-250.

#### Effect of pH, Temperature, substrate concentration and chemicals on enzyme activity

To study the effect of pH on lignin peroxidase activity reaction mixture contained 1.5 mM H<sub>2</sub>O<sub>2</sub> in sodium tartarate buffer of pH 2.0 – 5.5. Temperature stability for enzyme was determined by incubating sample at different temperature (20 to 100°C). The veratryl alcohol oxidase activity was then initiated by adding 0.098 μM lignin peroxidase and initial rates

were determined. Incubation was performed for 1 min. Different substrate (veratryl alcohol) concentration (1.5-48 mM) and H<sub>2</sub>O<sub>2</sub> (1-256 mM) were mixed with lignin peroxidase enzyme at 50°C. Enzyme activity was determined for each concentration. Lignin peroxidase (0.98 μM) was mixed with Veratryl alcohol (15 mM) and H<sub>2</sub>O<sub>2</sub> (1.5 mM) and incubated at 50°C for 10 min after addition of various chemicals (5 mM). The chemicals used for test were EDTA, NaCl, ZnSO<sub>4</sub>.7H<sub>2</sub>O, CuSO<sub>4</sub>, and MgSO<sub>4</sub>.7H<sub>2</sub>O.

#### Decolorization studies

Effect of enzyme on decolorisation of pulp and paper mill effluent was monitored by adding 100-unit/mg enzyme to the effluent having pH and kept on rotatory shaker at 150 rpm. Colour, lignin, change in pH were determined at 2 h interval up to 12 h and finally after 24h.

#### Analysis

Color reduction was measured using a spectrophotometer as described by Bajpai and Bajpai<sup>5</sup>. The sample was centrifuged at 10,000 rpm for 30 min to remove the suspended matter. The pH of supernatant was adjusted to 7.6, and absorbance was measured at 465 nm. The absorbance value was transformed into colour units (CUs). The lignin of the effluent was estimated using the method of Pearl and Benson<sup>6</sup>. In these methods, the sample was centrifuged at 10,000 rpm for 30 min to remove all the suspended matter. Supernatant pH was adjusted to 7.6 with 2 M NaOH. The sample (50 ml) was mixed with 1 ml CH<sub>3</sub>COOH (10%) and 1 ml NaNO<sub>2</sub> (10%). After 15 min, 2 ml of NH<sub>4</sub>OH was added. The mixture was left for 5 min and absorbance was measured at 430 nm. For blank, 1 ml CH<sub>3</sub>COOH (10%) was added to 50 ml distilled water and 2 ml NH<sub>4</sub>OH. After 15 min, 1 ml of NaNO<sub>2</sub> (10%) was added and measure OD at 430nm after 5 min.

#### Results and Discussion

The degrading efficiency of eight native fungal isolates and *Phanerochaete chrysosporium* was studied to understand degradation of cellulose, carbon and weight loss. All the nine strains were grown on MSP medium found to produce cellulolytic, hemicellulolytic and lignolytic enzymes (Table 1). Solid fractions were tested for weight loss, cellulose and organic carbon content, which are presented in Table 1. The weight loss of pulp for all isolates was increased during degradation. The weight loss by

Table 1 — Degradation of pulp, and production of Cellulolytic, Xylanolytic and Lignolytic enzymes by different fungal isolates in 120 (h) incubation period.

Parameters	Isolated fungal strains								
	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9
% weight loss	49.1	43.2	60.2	37.2	40.2	44.6	34.6	32.1	50.4
% cellulose loss	46.3	36.2	49.5	32.4	37.3	36.8	32.8	30.4	56.4
% organic carbon	34.3	30.2	48.8	33.2	38.2	41.4	34.4	31.2	42.3
Xylanases activity (IU/ml)	0.50	0.59	0.85	0.49	0.52	0.48	0.39	0.38	0.65
CMCase (IU/ml) activity	0.32	0.23	0.49	0.17	0.18	0.23	0.37	0.29	0.39
FPase activity (IU/ml)	0.51	0.42	1.2	0.41	0.48	0.31	0.23	0.29	0.68
Lignin peroxidase activity (IU/ml)	2.5	2.3	3.3	2.3	2.1	2.5	2.8	3.0	3.1
Reducing sugar (mg/ml)	2.1	2.2	2.8	1.2	0.93	0.99	2.4	2.1	2.6
Total protein (mg/ml)	1.2	0.67	2.1	0.62	1.23	1.4	1.5	1.3	1.8

isolate F3 was 5.8% at 12 h, reached to 60.2% at 120 h followed by isolate F9, F1, F6, F5, F2, F4, F7, and F8. Similarly cellulose loss by isolate 3 was 5.8% at 12 h that reached to 49.5% at 120 h. Initially, the carbon content in the degrading material was 15.2%. The loss in carbon content was increased gradually as the degradation progressed and attained a maximum of 48.4% in case of isolate F2 but was 42.5% in *Phanerochaete chrysosporium*. Stroller *et al.* and Rana observed 41.2% weight loss with *Humicola grisea var. thermoide*.<sup>7-8</sup> The production and changes in hemicellulolytic enzyme pattern together with protein and reducing sugar during degradation of pulp by all nine isolates presented in Table 1. Both, the cellulolytic (FPase, CMCase), xylanolytic, lignolytic enzyme production proceeded and reached a maximum level after 96 h in all the strains. Decline in growth started thereafter but protein and reducing sugar content increased up to 120h. Increase in protein and sugar was expected because increase in microbial biomass would add to total protein availability and also production of hemicellulolytic and lignolytic enzymes. A moderate increase in sugar content upto 48h and later steep increase in sugar content was found in case of *Paecilomyces* that was similar to *Humicola grisea var thermoide*.<sup>9</sup> This could be due to utilization of glucose by fungal isolates. During course of investigation all the isolates showed increase in the production of protein. Nagarathnamma reported increase in protein content by *Ceribromyces subermispora* in pulp<sup>10</sup>. This could be due to availability and accessibility of substrates. Among the eight isolates, isolate F3, *Paecilomyces* sp., was quite effective in producing more protein and sugar. In case of F9 *Phanerochaete chrysosporium* sugar and protein formation was less

as compared to *Paecilomyces* sp. Very little decline in formation of sugar and protein after 96 h reflects in most of the substrate available in the medium was utilized by fungi. The available data suggested much greater production of lignin peroxidase than xylanases and cellulolytic enzymes by isolate F3. FPase activity was more in isolate F3 and *P. chrysosporium*. Carboxymethyl cellulase activity was shown maximum by isolate F3, F1 and *P. chrysosporium* than the other isolates. Xylanases and lignin peroxidase activity was more in isolate F3 than *P. chrysosporium*. Among all the isolates, isolate F3 was found to be more significant, and further studies were carried out using isolate F3 i.e. *Paecilomyces* sp. Biswas *et al.* reported the production of xylanases and  $\beta$ -xylosidases by *Aspergillus ochraceus* under solid and liquid state fermentation, under solid-state fermentation enzyme production was much higher than liquid state fermentation<sup>11</sup>. Culture filtrate of all the strains were dialyzed and subjected to Polyacrylamide gel electrophoresis for presence of protein. After the gel electrophoresis different molecular weight proteins were observed in all the strains. Culture filtrate was subjected to ammonium sulphate precipitation and best results were obtained at 80% saturation. It resulted in 1.23 fold increase in purity of lignin peroxidase. However, this precipitation step was extremely efficient<sup>12</sup>. Precipitation was dissolved in Tris buffer (pH 7.8, 1M). The crude enzyme preparation was subjected to dialysis using Tris buffer, it showed 6.3-fold increase in purity of lignin peroxidase and 8.5% yield. Dialyzing with Tris buffer desalted crude protein in this study was deep brown in colour and resemble in proteins isolated from other fungi as *Phanerochaete chrysosporium*. The pigmented protein retarded by

adsorption, was eluted near bed volume. Desalted protein was fractionated by ion exchange column and gave five different protein fractions showing lignin peroxidase activity (Table 2). All the fractions showed the activity of FPase, CMcase and Xylanases. Two major protein peaks were obtained after gel filtration chromatography and showed maximum lignin peroxidase activity, As presented in Table 2, fold purification of lignin peroxidase by fraction I, II was achieved, respectively. The molecular weight of the native enzyme was estimated by SDS-PAGE. Kav value estimated for active and purified proteins was 0.528, 0.538 corresponding to molecular weight of 38000 and 40000 Da. The purity of each fraction checked by PAGE indicated single band of each gel fraction after staining with Commassie Brilliant Blue R (CBB R). The molecular weight was also determined by SDS-PAGE. SDS-PAGE profile of the partially purified protein exhibited a single band on the gel (Figure 1). The band was calibrated with known molecular size protein. SDS-PAGE of purified lignin peroxidase revealed a single protein of 38000 and 40000 Da. These results are similar to the ion exchange observation by Tuisel *et al.* in *P. chrysosporium*. In fraction III yield higher than fraction II, III, IV and V. Fujian *et al.* reported 2600U/C-enzyme activity in solid-state fermentation<sup>13</sup>. The effect of temperature on lignin peroxidase is shown in Figure 2a. Enzyme assay was performed in the range of 10<sup>0</sup> to 50 °C. Lignin peroxidase fraction II showed optimum activity at 30°C, however, optimum temperature of lignin peroxidase activity of fraction I was 20°C. No activity of lignin peroxidases of the two fractions I, II above 50 °C was found (Figure 2a). Optimum temperature for enzyme

activity depends upon activation energy at given temperature and rate of thermal denaturation after a specific temperature enzyme become in active. The effect of pH on the two-lignin peroxidases is shown in Figure 2b. The enzyme activity at pH 1, 2, 3, 4, 5, 6 was measured. Fraction I was found active at optimum pH 3. Fraction II showed optimum activity at pH 2 influencing the acidic nature of enzyme. At pH 3, fraction II and I showed 51% and 80% relative activity respectively. The effect of substrate concentration on the rate of oxidation of veratryl alcohol with all two fractions of lignin peroxidase was studied in the range of 1.5 mm to 48 mm (Figure 2c). The maximum activity of enzyme was shown in the range of 6 mm to 48 mm. The effect of H<sub>2</sub>O<sub>2</sub>

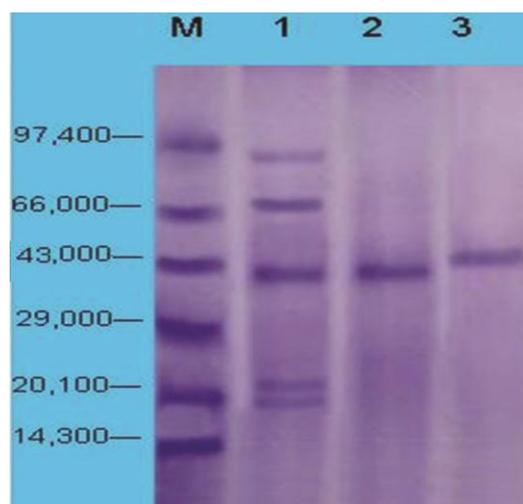


Fig. 1 — SDS-PAGE profile of partially purified lignin peroxidase enzyme extracted from fungal strain F<sub>3</sub> (*Paecilomyces* sp.). In figure lane M is molecular marker; lane 1, Crude extract, lane 2, enzyme fraction I and 3, fraction II.

Table 2 — Characterization of lignin peroxidase of *Paecilomyces* sp. in different steps of the purification of enzyme

Sl. No.	Purification step	Total volume	Protein (mg/ml)	Total protein (mg/ml)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield%
1.	Crude culture filtrate	850	2.1	1785	2.70	1700	0.95	1.00	100
2.	Precipitation with ammonium sulphate	50	2.8	140	3.28	164	1.17	1.23	9.64
3.	Desalting (Dialysing)	500	0.437	218.5	2.88	1440	659	6.93	85
4.	Sephadex G-100								
	Fraction I	5	0.372	1.660	4.92	19.68	11.8	12.42	1.16
	Fraction II	5	0.268	1.340	1.72	8.60	6.41	6.74	0.51

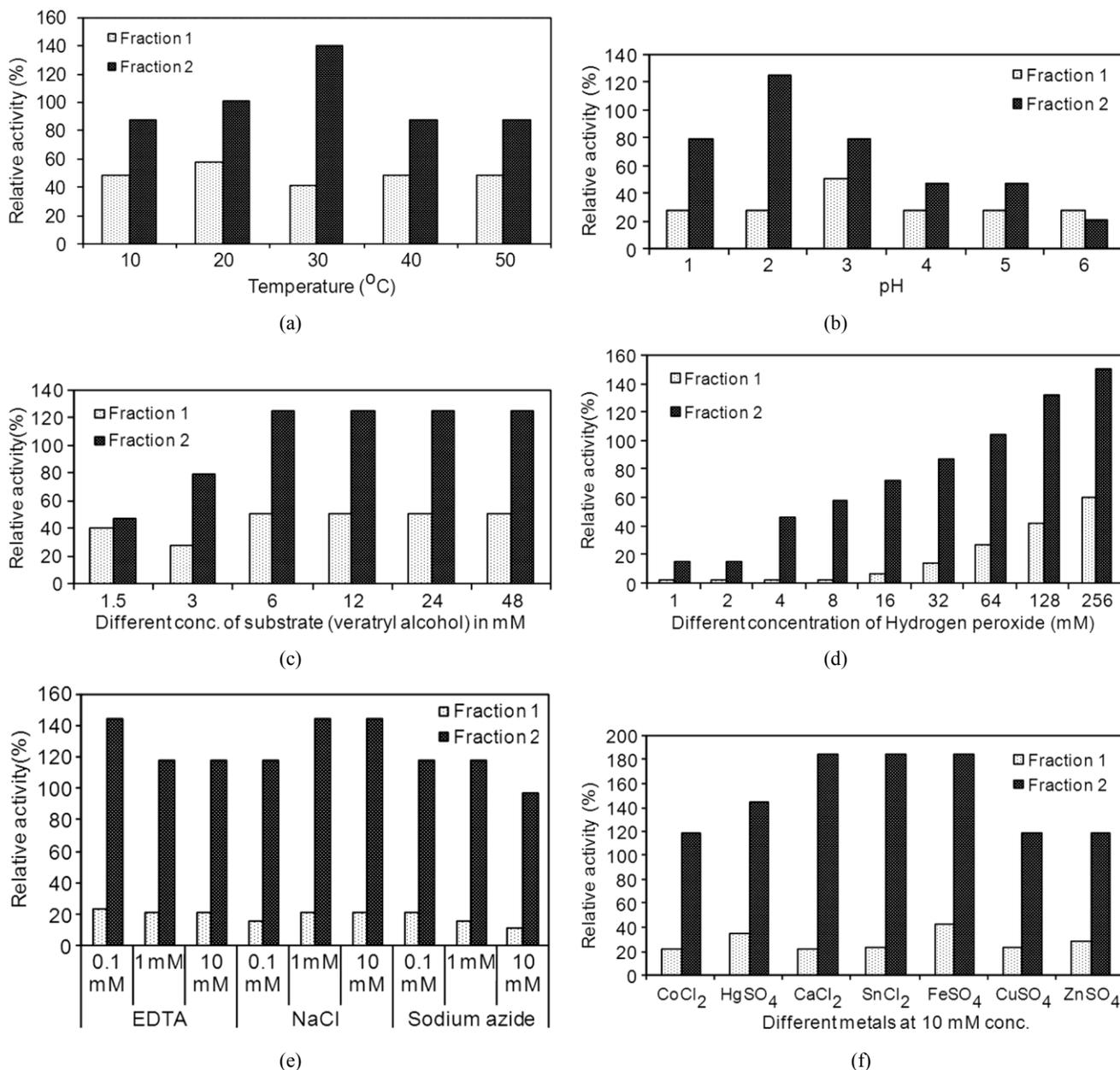


Fig. 2 — Relative activity of enzyme lignin peroxidase (fraction 1 and II) by fungal strain, *Paecilomyces* sp (a) at different temperature range (b) at different pH (c) at different concentration of substrate (Veratryl alcohol) (d) at different concentration of H<sub>2</sub>O<sub>2</sub>(e) at different conc. of EDTA, NaCl and sodium azide (f) at different conc. of heavy metals(10 mM)

concentration was studied in the range of 1 mM to 256 mM. Maximum activity was found at 256 mM H<sub>2</sub>O<sub>2</sub> in both fractions I and II (Figure 2d). Lignin peroxidase activity was not detected at pH above 6 (Figure 2b). In *P. chrysosporium* the effect of substrate concentration on lignin peroxidase activity was studied using veratryl alcohol<sup>14</sup>. The response of various chemicals on enzyme activity is given in Figure 6 and 7. Lignin peroxidase activity of fraction I and II was inhibited by sodium azide, and EDTA

and stimulated in presence of NaCl (Figure 2e). Fraction I and II showed stimulating effect in presence of CoCl<sub>2</sub>, HgSO<sub>4</sub>, CaCl<sub>2</sub>, SnCl<sub>2</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub> and ZnSO<sub>4</sub> and inhibited in fraction I (Figure 2f). Fraction I was found quite sensitive, and their activity was inhibited in presence of certain chemicals as EDTA, Sodium Azide, CoCl<sub>2</sub>, HgSO<sub>4</sub>, CaCl<sub>2</sub>, SnCl<sub>2</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub>, ZnSO<sub>4</sub>. Fraction II was stable in presence of these chemicals and showed stimulating effect with NaCl (10 mM). These results

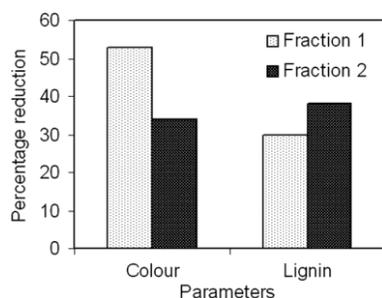


Fig. 3 — Effect of lignin peroxidase enzyme activity (0.429 U/mg) on decolourisation of pulp and paper mill effluent after 2 hrs by *Paecilomyces*

resemble with lignin peroxidase of several other fungi. Inhibition of lignin peroxidase activity by metal ions could be due to the formation of ionic bonds with active site amino acid residues. The complexity, structural and functional importance of crude lignin peroxidase was studied by carrying out decolourisation of pulp and paper mill effluent. Enzymatic decolourisation was carried out with fractionated enzymes separately. Lignin peroxidase fraction I and II resulted in 53% and 34% removal of colour and 30% and 38% lignin removal (Figure 3). More reduction in lignin was observed by fraction I, might be due to breaking the bonds between complex lignolytic materials. Fraction I and II also showed reduction in pH due to conversion of complex organic compounds into simple organic acid. Significant finding of the study was that the two fractions showed appreciable efficiency towards removal of colouring materials. So these enzymes help in decolourization of pulp and paper mill effluent. Several microorganisms have been reported to produce lignolytic enzymes that decolourize the pulp and paper mill effluent without requirement of cosubstrate<sup>15-16</sup>. It seems that this enzyme oxidizes the chromophores and removes the colour from bleaching wastewater<sup>17</sup>.

### Conclusion

Results of our study suggested that enzymes like lignase, cellulase, peroxidase etc. have potential to remove colour from pulp and paper mill effluent. Present study concluded that lignin peroxidase is the most efficient amongst the tested enzymes and it has capability to degrade toxic compound present in pulp and paper mill effluent. Conditions of this study were very much comparable with the field conditions and results were better than the previous studies reported. Hence the enzyme produced by native isolates of this study i.e. lignin peroxidase can be effectively applicable to the field scale.

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