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ENZYMATIC COLOUR REMOVAL OF PULP AND PAPER MILL EFFLUENT BY DIFFERENT FUNGAL STRAINS

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ABSTRACT

Pulp and paper mill effluent is posing a serious threat to the water and soil components of the environment due to its brown colour and high level of toxicity. Fungal treatment is one of the efficient methods for treating pulp and paper mill effluent. In the present study, for the treatment of from pulp and paper industry, nine efficient fungal strains including *Phanerochaete chrysosporium*, dedicated for the selective degradation of lignin, have been isolated. Enzyme activities (Fpase, xylanases, lignin peroxidases and carboxy methyl cellulase) required for the degradation of lignocellulosic waste, protein and reducing sugar content were determined. Among all the strains, F_3 (*Paecilomyces* sp.) strains showed highest activity of lignin peroxidase (3.3 $IUml^{-1}$), Fpase (1.2 $IUml^{-1}$), xylanase (0.85 $IUml^{-1}$), CMcase (0.49 $IUml^{-1}$), protein (2.8 $mgml^{-1}$), and sugar content (2.1 $mgml^{-1}$) at 120 h incubation period. Enzyme extracts of these fungal isolates were applied for the treatment of pulp and paper mill effluent. Data indicated that among all the strains, *Paecilomyces* sp. (F_3) showed highest removal of colour (80 per cent) and other pollution parameters (73 per cent lignin, 78 per cent COD and 76 per cent AOX) in pulp and paper mill effluent, on day 3 at 3.3 international unit per ml concentration of crude enzymes.

KEYWORDS: Lignin peroxidase, pulp and paper mill, effluent treatment

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INTRODUCTION

The pulp and paper industry categorized as wood/forest, agro and waste paper waste, accounting for 43 per cent, 28 per cent, and 29 per cent respectively, of the total installed capacity in India and releases a huge amount of waste material into the environment together with air, water and soil pollutant. The effluent has high biological and chemical oxygen demands (BOD and COD), lignin compounds and their derivatives. The dark brown colour of the effluent is due to the formation of lignin degrading products during the processing of lignocellulose from paper and pulp manufacture. Major contaminants of pulp and paper mill are ligno sulphonic acid, chlorinated resin acid, chlorinated phenols, dioxins and chlorinated hydrocarbons[1]. Several methods have been attempted for the removal of colour from the pulp and paper mill effluents. These can be classified into physical, chemical and biological methods. Physical and chemical processes are quite expensive and remove high molecular weight chlorinated lignins, colour, toxicity, suspended solids and chemical oxygen demand. But BOD and low molecular weight compounds are not removed efficiently. Flocculation study between dual polymer on pulp and paper mill waste water is reported by Razali *et al*[2]. The biological colour removal process in particularly attractive since in addition to colour and COD it also reduces BOD and low molecular weight chlorlignins[3]. Aerobic treatment including fungi and bacteria secreted degrading enzymes and helps in removing large amount of colour, COD, lignin and phenol. Various microorganisms including bacteria, actinomycetes, fungi or yeast and algae have been reported for production of hydrolytic enzymes, which are responsible for lignocellulose degradation[4-7]. A broad range of fungi has been described, that degrade lignin in a varying degree. They include soil fungi (*Fusarium* sp.), soft rot fungi (*Populaspora, chaetomium* sp.). Pseudo soft rot fungi (*Hyoxylen, xylaria*), lignin degrading fungi (*Callybia, Mycena*), white rot fungi (*Trametes, Phanerochaete*), brown rot fungi (Gleophyllium, Poria) and certain others. These fungi can also degrade modified lignin and other derivatives found in pulp and paper mill effluent[8]. However, most of the approaches to degrade high molecular weight chlorlignins are based on the use of white rot fungi, the only known microorganisms to efficiently degrade lignin and decolorize industrial effluents[9]. The white rot fungi, a limited group of basidiomycetes are unusual among microorganisms and possess an active lignolytic system which is able to degrade protolignin as well as heavily modified lignin such as kraft lignin and chlorinated lignin[10]. Therefore, their degradation in essentially a secondary metabolic process not required for the main growth[11]. Lignin degradation by white rot fungi has been extensively studied and results revealed that three kind of extracellular phenol oxidases, namely, lignin peroxidase (LiP), Manganese Peroxidase (Mnp) and Laccase (Lac) are responsible for initiating the depolymerization of lignin. The expression pattern of these enzymes depends on the organisms; some secrete LiP and MnP, whereas other secretes MnP and Lac (No Lip). In addition to lignin, these enzymes secreted by fungi are also able to degrade a variety of environmentally persistent pollutants such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbon, various dyes and synthesis high polymers[12]. Dutta *et al*[13] reported removal of organic halides in kraft mill effluent with residual enzymes. Therefore, the present study is undertaken to estimate different enzyme activities (CMcase, xylanases, Fpase, Lignin peroxidase), protein, and sugar content in different fungal strains growing on minimal salt medium containing pulp as a sole carbon source and their application in decolorisation of pulp and paper mill effluent.

MATERIALS AND METHODS

(i) Sampling sites and microorganisms

A sediment core together with degraded wood components in the effluent was collected for isolation of fungi from the drainage passing through the village of Bindukhatta outside the Century Pulp and

Paper Mill (Ghanshyamdham, Lalkuan, Nainital, Uttaranchal, India) premises. But for the purpose of treatment, the effluent was collected from the main canal, which receives the effluent of kraft pulping, recovery plant and bleaching located inside the industry. The effluent collected in clean plastic containers were brought to the laboratory and immediately stored in a refrigerator at 4°C until used for further analysis. Sediment core and decomposed woody material were mixed properly and filtered through muslin cloth. The filtrate was serially diluted 10-fold, and 0.1 ml of the diluted sample was spread on the potato dextrose agar plate and nutrient agar plates, incubated at 30°C for 4 days. The microbial colonies (fungi) appeared on potato dextrose agar plates and bacterial isolates on nutrient agar, were isolated and purified. Fungi were identified based on microscopic and morphological structures as colour, texture, mycelium and spore formation and attachment into the filaments. For the preparation of fungal inoculum, for treatment of kraft effluent, in the form of pellet, the fungal isolates were individually grown and cultured on potato dextrose agar plates. Plates were incubated at 30°C for 4 days. Fungal mycelium discs (six numbers) of about 1 cm diameter were cut from the zone of active growth and inoculated in to Erlenmeyer flasks containing potato dextrose broth and streptopenicillin (100 ppm). The flasks were then incubated at 30°C for 4 days in shake condition in orbital shaker. The culture content was centrifuged at 10,000 rpm for 15 min, and pellets of approximately 1.5–2 mm size were removed with the help of a sterile spatula. The above pellets were suspended in the pulp and paper mill effluent for treatment.

(ii) Microorganism growth medium and culture conditions

Fungal strains were grown in minimal salt medium containing (g/l): $Na_2HPO_4 \cdot 2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; Fe $(CH_3COO)_3NH4$, 0.01; Ca $(NO_3)_2 \cdot 4H_2O$, 0.05, and pH adjusted to 5.5 for fungi. Erlenmeyer flask with individual microbial isolates and incubated at 30°C in a rotary shaker for 7 days.

(iii) Growth and Enzyme assay

Culture filtrate was withdrawn from the flasks and filtered through G-2 sintered glass. The residue of the pulp was dried at 80°C for 48 h and used to estimate the loss in dry weight during degradation[14]. Cellulose content of the pulp before and after degradation was determined according to Updegraff[15] using microgranular cellulose as a positive control. Protein content was estimated according to the method of Lowry *et al*[16] using bovine serum albumin as standard. Reducing sugar was estimated according to the method of Somagyi[17] using glucose as standard.

Xylanase was determined by monitoring the release of reducing sugar from oat, spelt xylem (Sigma) by the dinitrosalicylic acid method[18]. CMCase was assayed as recommended by the Commission on Biotechnology, IUPAC[19] using 2% CMC in sodium citrate buffer (pH 4.8, 0.05 M) as substrate. FPase, activity was determined according to IUPAC[19]. Lignin peroxidase activity of the culture supernatant was assayed according to the method Yee *et al* [20] using 2,4-dichlorophenol assay which is based on reaction of A Li P-P_3 oxidized 2,4-dichlorophenol with 4 aminoantipyrene that strongly absorbed at 510nm.

(iv) Effect of different enzymes on decolourisation

Effect of enzyme on decolourisation of pulp and paper mill effluent was monitored. 100-unit/mg enzymes were mixed with effluent having pH and kept on rotatory shaker at 150 rpm. Colour, lignin, change in pH and AOX were determined at 2 h, 4 h, 6 h, 10 h, 12 h, and 1-day interval.

(v) Analytical methods

Reduction in colour was measured using a spectrophotometer using the method of Bajpai *et al*[21]. The sample was centrifuged at 10,000 rpm for 30 min to remove all 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[22] (1990). In these methods, the sample was centrifuged at 10,000 rpm for 30 min to remove all the

suspended matter. The pH of the supernatant was then adjusted to 7.6 with 2 M NaOH. The sample (50 ml) was mixed with 1 ml CH_3COOH (10per cent) and 1 ml $NaNO_2$ (10per cent). After 15 min, 2 ml of NH_4OH was added. The mixture was left for 5 min and absorbance was measured at 430 nm. For blank, 1 ml CH_3COOH (10per cent) was added in 50 ml distilled water and 2 ml NH_4OH. After 15 min, 1 ml of $NaNO_2$ (10per cent) was added. After 5 min, OD was taken at 430 nm. The absorbance value was transformed into lignin content (ppm). Adsorbable organic halogen was analyzed by IDC multi X-2000 AOX Analyzer. Hundred millilitres samples were drawn from the batch study experiment conducted with fungus and bacterium at an interval of 0, 1, 3, and 7 days interval. Similarly the same amount of the sample was drawn from the sequential treatment consisting of three sets, viz. treatment with F_3 (Set I), treatment with F_3 and bacteria (Set II) and treatment with only bacteria (Set III) for the same period. The pH of these samples was adjusted to 2 with the help of HNO_3 (15 per cent). Initially the constituent was concentrated by adsorption onto activated carbon. In the case of AOX, interfering inorganic substances must be removed from the loaded carbon by rinsing with $NaNO_3$ solution. In order to convert the halogenated hydrocarbons (HHC) into an analyzable state, the content was combusted in an oxygen stream at about 950 °C, whereby hydrogen halide, CO_2 and water were formed. After drying of the pyrolic gases, the halide is determined by microcolorimetry. The silver ions needed for halide precipitation were generated electrolytically at a silver anode. After quantitative conversion of the halide, the concentration of silver ions in the electrolyte increases. This moment is the end point of titration, which can be recognized by means of a polarized pair of indicator electrodes. The halide quantity was computed by means of Faraday's law from the amount of charge consumed until analytic conversion was complete. COD was determined by a dichromate reflux method[23]. In this method, the sample is refluxed with potassium dichromate and sulphuric acid and titrated with ferrous ammonium sulphate. The percent change in the size of the inoculum was measured in the culture filtrate. In this process culture filtrate was placed in pre-weighed centrifuge tubes and centrifuged at 10,000 rpm for 30 min. After discarding the supernatant, the centrifuged tube was kept for drying at 80°C overnight in an oven. Then the centrifuged tube with the cell mass was weighed. The samples for different analysis subjected to removal of colour and lignin was used for the changes in biomass represented the cell growth of the fungal and bacterial cells. The biomass measurement was made for each and every sample in triplicate

RESULTS

Eight different types of colonies of the fungi were isolated from pulp mill effluent and identified with the help of IMTECH, Chandigarh. They were further purified on potato dextrose agar plates. These purified fungal isolates were identified based on morphological characteristics and microscopic observations (Table 1). The nine strains were grown in liquid medium on pulp as substrate produced cellulolytic, hemicellulolytic and lignolytic enzymes (Table 2). The solid fractions were tested for weight loss, cellulose and organic carbon content, which are presented in Table 2. The weight loss of pulp for all isolated was increased during degradation. Isolate 3 (*Paecilomyces* sp.) resulted weight loss of 5.8 per cent at 12 h, reached up to 60.2 at 120 h followed by strains 9 (*Phanerochaete chrysosporium*), 1(*Phoma sp*.), 6 (*Torula theromophila*), 5 (*Torula herbarum*), 2 (*Paecilomyces varioti*), 4 (*Aspergillus fumigatus*), 7 (*Paecilomyces varioti*), 8 (*Paecilomyces varioti)*. Similarly cellulose loss at 12 h of isolate 3 (*Paecilomyces* sp.*)* was 5.8 per cent, which reached to 49.5 per cent at 120 h. Initially, the carbon content in the degrading material was 15.2 per cent. The loss in carbon content was increased gradually as the degradation progressed and attained a maximum of 48.4 per cent in case of *Paecilomyces* sp. but was 42.5 per cent in *Phanerochaete chrysosporium*.

Table 1
Morphological characterization and identification of different fungal strains (F_1-F_9)

Isolates	Characteristics	Growth	Characteristics
F_1 (*Phomas* sp.)	Salty dark colour appearance	Slow growth	▪ Septate hyphae ▪ Sporangiospore erect
F_2 (*Paecilomyces varioti*)	Yellowish white or muddy colour mycelia with greenish pigment	Fast growth	▪ Slender hyphae ▪ Spores attached at 45° on branch hyphae
F_3 (*Paecilomyces* species)	White snowy cottony mycelia with yellowish pigmentation	Medium growth	▪ White snowy mycelium ▪ Spores globose attached laterally
F_4 (*Aspergillus fumigatus*)	Whitish sticky mycelia	Slow growth	▪ Slender unbranched hyphae ▪ Spores attached directly or in branched hyphae
F_5 (*Torula herbarum*)	Black spotted cottony mycelia	Slow growth	▪ Existence of chlamydospore ▪ Globose spore found
F_6 (*Torula thermophila*)	White in colour and sticky mycelium	Slow growth	▪ Unbranched hyphae ▪ Bulbous tips
F_7 (*Paecilomyces varioti)*	TCR growth, with yellow dark	Slow growth	▪ Septate hyphae ▪ Bulbous spores attached laterally
F_8 (*Paecilomyces varioti*)	It is like F_2 but more muddy in appearance	Slow growth	▪ Septate hypahe ▪ Typical sporulation with lateral swelling at right angle
F_9 (*Phanerochaete chrysosporium*)	White in colour	Medium growth	▪ Chamydospore ▪ Spore globose

The production and changes in hemicellulolytic enzyme pattern together with protein and reducing sugar contents during degradation of pulp by all eight isolates and *Phanerochaete chrysosporium* are presented in Table 2.

Table 2
Degradation of pulp and production of Cellulolytic, Xylolytic 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
per cent weight loss	49.1	43.2	60.2	37.2	40.2	44.6	34.6	32.1	50.4
per cent cellulose loss	46.3	36.2	49.5	32.4	37.3	36.8	32.8	30.4	56.4
per cent 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
CM Case (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

Both, the cellulolytic (Fpase, CMCase), xylanalytic, lignolytic enzyme production proceeded and reached a maximum level often 120 h in all the strains. Decline in growth started thereafter. Protein and reducing sugar content increased upto 120 h. The available data suggested much greater production of lignin peroxidase than xylanases and cellulolytic enzymes in *Paecilomyces* sp. Fpase activity was more in *Paecilomyces* sp. and *P. chrysosporium*.Carboxymethylcellulase activity was found maximum by isolate 3 (*Paecilomyces* sp.), 1(*Phoma* sp.) and *P. chrysosporium* than other isolates. Xylanases and lignin peroxidase activity was more in *Paecilomyces* sp. than *P. chrysosporium*. Among all the isolates, isolate 3 was found to be more significant, and further studies were carried out using isolate 3 i.e. *Paecilomyces* sp. The dialyzed culture filtrate of all the fungal strains containing different concentration of crude protein was tested for decolourisation and bioremediation of pulp and paper mill effluent (Figure 1 to 5).

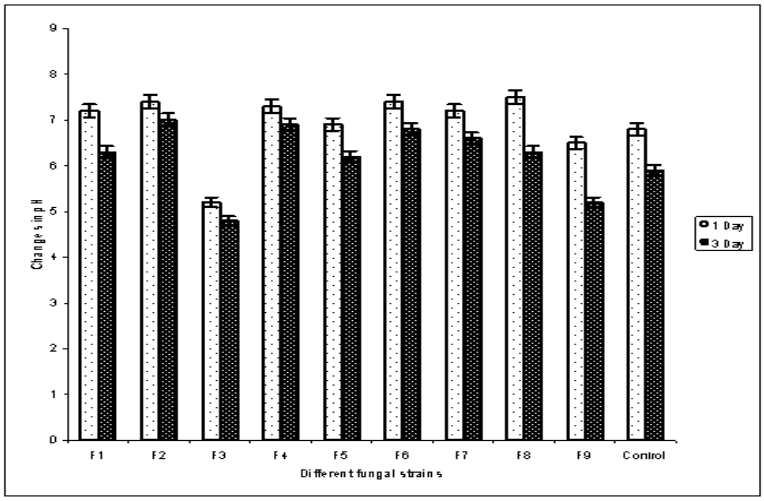

Figure 1
Changes in pH of pulp and paper mill effluent with the help of enzyme extracted (5 ml) from different fungal strains (F_1-F_9) during different time interval (0, 1 and 3 day).

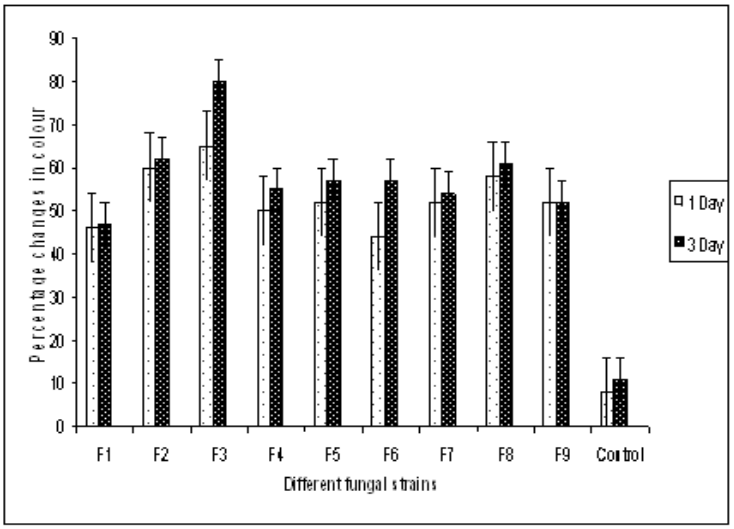

Figure 2
Changes in colour (CU) of pulp and paper mill effluent with the help of enzyme extracted (5 ml) from different fungal strains (F_1-F_9) during different time interval (0, 1 and 3 day)

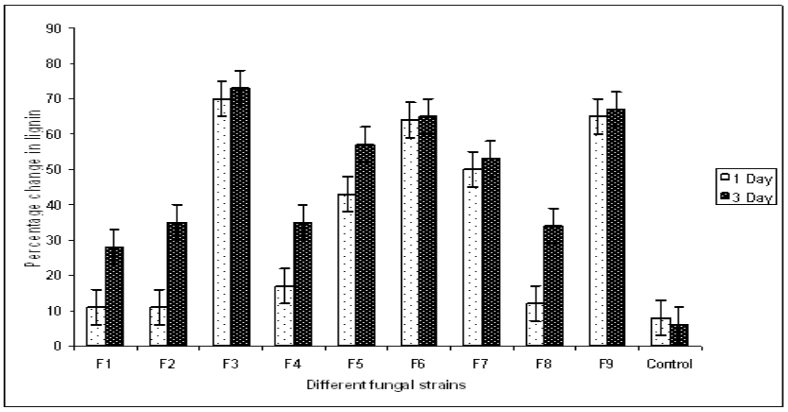

Figure 3
Changes in lignin content (mg l^{-1}) of pulp and paper mill effluent with the help of enzyme extracted (5 ml) from different fungal strains (F_1-F_9) during different time interval (0, 1 and 3 day).

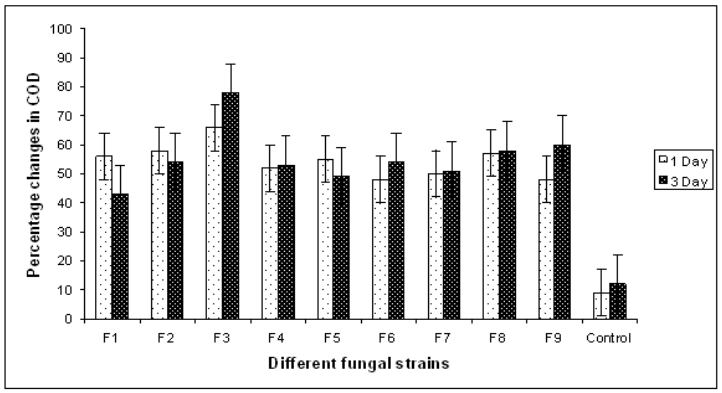

Figure 4
Changes in COD (mg l^{-1}) of pulp and paper mill effluent with the help of enzyme extracted (5 ml) from different fungal strains (F_1-F_9) during different time interval (0, 1 and 3 day).

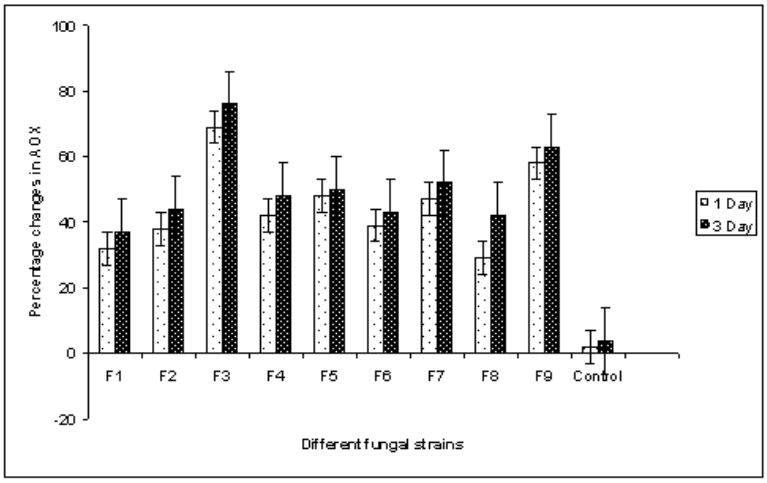

Figure 5
Changes in AOX (mg l^{-1}) of pulp and paper mill effluent with the help of enzyme extracted (5 ml) from different fungal strains (F_1-F_9) during different time interval (0, 1 and 3 day)

Among all the fungal strains, maximum significant reduction in colour (80 per cent), lignin (73 per cent), COD (78 per cent and AOX (76 per cent) were observed in pulp and paper mill effluent, on day 3 at 3.3 international unit per ml concentration of enzyme extracted from *Paecilomyces* sp. (Figure. 2 to 5). Significant change in pH was also observed during enzymatic treatment by different fungal strains (Figure.1).

DISCUSSION

Eight fungal isolates were recovered after 48 h incubation at 30°C. They are *Phoma sp*. (F-1), 3 strains of *Paecilomyces varioti* (F2, F7, F8), *Paecilomyces sp.* (F-3), *Aspergillus fumigatus* (F-4), *Torula herbarum* (F-5), *Torula theromophila* (F6) and *Phanerochaete chrysosporium* (F9) is taken as control. Distribution and occurrence of various white rot fungi have been reported earlier from pulp and paper mill effluent irrigated soil which includes *Coriolus versicolor* showing lignin degrading activity through various enzymes[24]. The efficiency of the degradation of pulp by the eight isolates and *Phanerochaete chrysosporium* was studied in this work to understand degradation of cellulose carbon and weight loss. In case of *Paecilomyces* sp. weight loss after degradation of pulp at 120 h was 60.2%, Stroller *et al*[25]and Rana *et al*[26]observed 41.2% weight loss with *Humicola grisea var. thermoidea.* The increase in carbon content is similar to the results of Jain *et al* [14], which is 48.8%, and cellulose loss was 49.5%. Hatakka[27] reported that *Sporotichum* sp. vigorously utilize hemicellulase. Degradation efficiency of other isolates was also found to be in agreement with other reports. The degradation capability was further monitored by studying changes in protein and sugar content. The increase in protein and sugar was expected because the 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 48 h, and later steep increase in sugar content was found in case of *Paecilomyces* sp was similar to earlier studied in *Humicola grisea* var thermoidea[26]. This could be due to utilization of glucose by fungal isolates. During the course of the investigation all the isolates showed an increase in the production of protein. Nagarathnamma *et al*[28] also reported increase in protein content by *Ceriporiopsis submersispora* in pulp. This could be due to accessibility of substrates. Langer and Bakshi[29] reported that an increase in biomass would add to the total protein availability and greater degradation of substrate in the liquid medium. Among the eight isolates, *Paecilomyces* sp. isolate 3 was quite effective in producing more protein and sugar. In case of *Phanerochaete chrysosporium* sugar and protein formation was less as compared to *Paecilomyces* sp. Very little decline in formation about sugar and protein after 96 h reflected that most of the substrate available in the medium was utilized by fungi[30]. All the eight fungal isolates with *P. chrysosporium* were studied for their degradation capability by production of CMCase, FPase xylanases and lignin peroxidase. Our results indicated production of all four enzymes by all eight isolates along with *P. chrysosporium.* But maximum xylanases, Fpase, CMCase and lignin peroxidase were produced by *Paecilomyces* sp. (isolate 3). Gaikwad *et al* [31] reported release of β-glucosidase and cellulase from culture of *Sporotrichum thermophile***.** Biswass *et al*[32] reported production of xylanases and β-xylosidases by *Aspergillus ochocaceus* under solid and liquid state fermentation. Under solid state fermentation enzyme production was much higher than liquid state fermentation. Tuisel *et al*[33] reported lignin peroxidase activity in *Phanerochaete chrysosporium* and found that in agitated nutrient nitrogen limiting culture, *P.chrysosporium* produces two lignin peroxidases in about equal proportion. Have *et al*[34] reported that the N-unregulated white rot fungus *Bjerkandera sp.* strain B0555 was cultured in 1 litre of peptone yeast extract medium to produce lignin peroxidase (LiP). Ek and Eriksson[35] purified an extra cellular lignin degrading enzyme from the basidiomycetes by ion exchange chromatography. From the results obtained with eight isolates along with *P. chrysosporium*, (isolate 3) *Paecilomyces* sp. was found significant as it showed maximum

degrading capability and more xylanases, lignin peroxidase, CMCase and FPase activity. Result of the study indicated that *Paecilomyces* sp, most efficient strain among all the strains, showed maximum removal of colour, lignin, COD and AOX. It may be due to the presence of some enzymes having potential to remove colour and AOX from pulp and paper mill effluents. Peroxidase, laccase etc are the most important among them. The use of microbial or enzyme based treatment offers some distinct advantages over physical and chemical AOX precipitation methods, in that only catalytic and not stoichiometric amounts of the reagent are needed, and the low organic concentrations and large volumes typical of bleaching effluents are, therefore, less of a problem. Also, both complete microbial systems and isolated enzymes such as peroxidases and laccase have been shown to reduce the acute toxicity by polymerizing and thereby rendering less soluble many of the low molecular mass non chlorinated and polychlorinated phenolics[2]. It has been reported that lignin peroxidases secreted by white rot fungi are involved in the degradation of a whole range of organic pollutants[36]. Lyr[37] reported lignin peroxidase of *T. versicolor* partially dechlorinates AOX. Hammel *et al*[38] and Kumar Swamy *et al*[3] reported that peroxidase from *P. chrysosporium* can partially dechlorinate chlorinated phenols in kraft mill effluent. Arcand and Archibald[39] carried out a systematic study on the direct dechlorination of chlorophenolic compounds in pulp and paper mill effluents by laccase from *T. versicolor*. It was found that most of lignin peroxidase secreted by *T. versicolor* could partially dechlorinate a variety of chlorophenolics. The rate and extent of chlorine release was substantially affected by substrate and enzyme concentration and the presence of multiple lignin peroxidase substrates. The dechlorination was accompanied by extensive polymerization of the substrate[3].

CONCLUSION

Results of enzymatic decolorisation also supported the batch culture treatment of different fungal isolates. Enzymatic colour removal has potential advantage over microbial treatment due to producing less sludge during treatment. It suggests the use of crude enzyme extracted from *Paecilomyces* sp. (F-3) strain in the large-scale bioreactor treatment of pulp and paper mill effluent.

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