# POTENTIAL FUNGI FOR BIOREMEDIATION OF INDUSTRIAL EFFLUENTS

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Two fungi (unidentified) were isolated from soil and marine environments. These isolates were used for bioremediation of pulp and paper mill effluent at the laboratory scale. The treatment resulted in the reduction of color, lignin, and COD of the effluent in the order of 78.6%, 79.0%, and 89.4% in 21 days. A major part of reductions in these parameters occurred within 5 days of the treatment, which was also characterized by a steep decline in the pH of the effluent. The enzyme activity of these fungi was also tested, and the clearance zone was obtained in the plate assay.

*Keywords: Marine fungus; Soil fungus; Paper mill effluent; Bioremediation; Enzyme assay*

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## INTRODUCTION

The production, use, and recycling of paper can have a number of adverse effects on the environment, i.e. pollution. Pulp mills can emit various wasteproducts to the air, water, and land. Discarded paper is a major component of many landfill sites, accounting for about 35% by weight of municipal solid waste before recycling (US EPA 2005). Even recycling of paper can be a source of pollution due to the sludge produced during deinking.

Pulp and paper production is a major industry in India, with a total capacity of over 3 million tonnes per annum (CPCB 2001). Large mills, with a capacity of over 10,000 TPA, produce just over half of this amount. The rest is produced by smaller mills, of which there are some 300 around the country. In the papermaking process, raw materials such as wood, bamboo, reeds, grasses, straw, and bagasse are mechanically and chemically pulped by cooking in a mixture of water, caustic soda, sodium sulphate, and sodium carbonate. The pulp is then bleached using chlorine or similar oxidising agents. The bleached pulp is then blown onto rotary driers, after which the paper is rolled out and cut to size.

Wastewaters from the pulping process, known as "black liquors", will usually contain high concentrations of lignin, as well as sodium hydroxide. Most paper mills with large capacities will have facilities for collecting and recovering black liquor; however, leaks, spills, and operational failures may occur and release the wastewaters into the effluent channel. Wastewaters from the bleaching process will contain sodium hydroxides, lignin and chlorinated lignin, and other chlorinated compounds. The wastewaters are not recycled and are released as effluent. From the point of view of

toxicity towards living organisms, the following are of concern: mercury, chlorinated lignins and their derivatives, chlorides, and suspended solids, all of which can originate from paper and pulp mill effluents.

Although physical and chemical methods are available for treatment of pulp and paper mill effluent, they are less desirable than biological treatment because of cost-ineffectiveness and residual effects. Biological treatment is known to be effective in reducing the organic load and toxic effects of pulp and paper mill effluent (Blair and Davis 1980; Chupal et al. 2005). Significant work has been reported on the problem of color removal from pulp and paper mill wastes at a global level (Abhay Raj et al. 2009; Manzanares et al. 1995). Processes such as ion flotation, chemical oxidation, ion exchange, soil percolation, electrochemical process, radiation and ultra-filtration are more of academic concern (Sharma 1983). Coagulation with alum, lime, and ferric salts is one of the treatment options available for color removal, but the treatment is quite expensive and also gives rise to problems due to the production of large volumes of sludge. A large number of adsorbent materials, viz., activated carbon, silica, wood, saw dust, peat, fuller's earth, fly ash, etc. have also been tried for removal of pollutants (Tan et al. 1985; Lee and Low 1989), but the problems of cost and handling still remain tagged with these adsorbents.

There have been several attempts to use biological methods to decontaminate effluent from Kraft mills because of their ability to degrade lignin by several microorganisms (Abadulla et al. 2000; Sukumar et al. 2006). Mycoremediation is an economically and environmentally sound alternative to extracting, transporting, and storing toxic waste. One of the primary roles of fungi in the ecosystem is decomposition, which is performed by the mycelium. White-rot fungi have been widely studied because of their efficient lignin degradation mechanism and possible applications in the pulp and paper industry (Akhtar et al. 1997; Hatakka 2001; Scott and Akhtar 2001). The ligninolytic enzymes of white-rot fungi are unspecific, and thus, these fungi are considered to be potential microorganisms for bioremediation of polluted soils (Orth et al. 1994; Paszczynski and Crawford 1995; Hatakka 2001; Pointing 2001). Indeed, the capability of white-rot fungi to degrade organic pollutants has been confirmed in many studies (e.g. reviewed recently by Pointing 2001). Determination of the maximal lignin-degradation activity of white-rot fungi in soil indicates the capability of fungi to degrade pollutants. The prime objective of the present study is to evaluate effluent degradation potential (with special reference to lignin) of fungi isolated from effluent contaminated soils and marine water.

## EXPERIMENTAL

### Materials

The study was conducted on the effluent released from the Andhra Pradesh Mills Ltd., situated on the banks of Holy River Godavari near Rajahmundry. This industry came into operation in June 1984. The industry uses eucalyptus wood as a raw material. The effluent was collected from inside the premises near the Paper Unit Laboratory and stored in refrigerator at 40 $^{o}$C.

The collected samples were initially subjected to the physico-chemical analyses listed in Table 1 as per the standard methods given by APHA (1998):

**Table 1.** List of Analysis Methods Performed

| Parameter | Measurement | Unit |
|---|---|---|
| Colour | Spectrophotometer | Pt-Co Scale |
| pH | pH Meter | - |
| Carbon dioxide release | As per method given by Gaur et al. 1971 | mg/l |
| Biological Oxygen Demand | Wrinkler's Method | mg/l |
| Chemical Oxygen Demand | Closed Reflux Method | mg/l |
| Lignin | Spectrophotometer | mg/l |
| Cellulose | Spectrophotometer | mg/l |
| Phenol | Spectrophotometer | mg/l |

## Methods

Fungi from the effluent contaminated soil and Marine water were isolated. *Phanerochaete chrysosporium* (MTCC 787) and *Trametes hirsuta* (MTCC 136) were procured from Microbial Type Culture Collection, Chandigarh, India. All the four species were maintained and sub cultured at $27^{0} \pm 1^{0}$C on Potato Dextrose Agar slants (Fig. 1). The initial analysis of the physico-chemical parameters of the effluent was followed by an experimental set up for evaluating the degradation of the effluent.

*Degradation studies*

The cultures of all the four fungal species were aseptically inoculated into 250ml conical flasks (reactors) containing 100ml effluent samples (50 and 30% dilutions) along with a control (blank without fungal strain). The flasks were incubated at a temperature of 27 $\pm$ 1$^{o}$C. The degradation studies were carried for 21 days, and the post analysis of the physico-chemical characteristics were performed periodically at alternate days (Abubacker *et al*. 2001).

*Production and Extraction of extra cellular enzymes*

Cultures of one-week-old colonies of fungi grown at 27$^{o}$C on PDA plates were inoculated in conical flasks (250ml) containing 100ml mineral medium and incubated at 27 $\pm$1 $^{o}$C. After 14 days of cultivation, culture aliquots were centrifuged at 5000xg to remove solids. The supernatants were assayed for their enzymatic activity (Peciulyte 2007).

*Enzyme assay*

Enzyme assays were done by the plate assay method, which allowed rapid determination of the presence of enzyme in the extracellular fluid. Potato Dextrose Agar Plates were prepared with lignin model compound – lignin sulphonate with a concentration of 5g/l each as per the method given by Kizhekkedathu *et al.* (2005). A well was made in the center of the medium with the help of a sterile well borer. The enzyme at a concentration of 1µl was added into the well and could be visualized by means of a decolorization zone.

## RESULTS AND DISCUSSION

The physico-chemical analysis of the paper and pulp mill effluent is given in Table 1 (see Appendix), which shows a higher amount of total solids, with higher COD and BOD. There were variations in their physical, chemical, and biochemical characteristics. The high pH of samples may be attributed to the nature of chemicals used for the pulping. Pulp and paper mill waste is a dark black colored liquid known as black liquor. It has characteristically high BOD, COD, suspended solids, and color (Sastry and Kamatchiammal 1988). Discharge of paper mill effluent into water courses results in oxygen depletion, unsightly appearance, and toxicity to aquatic life. The most noticeable and apparent characteristic of the effluent from such industries is color. Colors not only cause bad aesthetical effect, but also reduce the self-purification capacity of rivers by inhibiting photosynthetic production of oxygen and direct destruction of aquatic communities. The very high value of COD and BOD suggests that it is highly biodegradable and thus suitable for degradation by fungi.

The results of the study, indicating percentage degradation of paper and pulp mill effluents by different fungi on different day's incubation, are given in Table 2. Industrial wastes are usually discarded into water, with or without processing. When waste substances reach such a concentration that they exert measurable effects upon ecosystems, they are said to be pollutants. Physical and chemical methods, available to treat effluents, are expensive and do not provide satisfactory results. Biologically, decolorization can be achieved by the use of naturally occurring microorganisms such as bacteria and fungi. In recent years attention has been directed towards fungal decolorization systems (Moreira et al. 2000).

Table 2 shows the effect of time (days) on biodegradation of effluents. Seven days aged inoculum with 30 percent concentration to the working volume was selected for this purpose. To assess the potential of the fungi, the pulp and paper mill effluent was treated with four different fungal species, which are fungi isolated from marine sources (MF), and from soil (SF) polluted by paper and pulp mill effluents. Results were compared with the standard species *Phanerochaete chrysosporium*. Furthermore, colour, BOD, COD, lignin, cellulose, and phenols in the effluent are regarded as important factors to evaluate the water quality. Therefore, those parameters in a paper and pulp effluent were measured with the four different fungal species.

The measurement of carbon dioxide release during the biodegradation process may be used as an index of decomposition. The $CO_2$ release trends in all the four species showed continuous increase, and it was observed to be maximum (51%) on the 15th day in all the four species and then showed a decreasing trend. The results agree with the study conducted by Moore and Landecker (1972).

The BOD, COD, and color showed slow degradation rates until the 5th day in all the four species, whereas after the 5th day fast degradation rates were observed. This incubation period i.e. 5 days, is required for the full growth and adaptation by the fungi to the ligninolytic system. Avoidance of a lag phase before the start of the decrease in BOD and COD reflects depletion of nutrient nitrogen. This depletion triggers the development of ligninolytic activity in Phanerochaete chrysosporium and is therefore necessary for bleaching (Masud Hossain and Das 2002).

Adaptation significantly affects the specific growth rate, length of lag phase, and overall consumption of nutrients. However, this process is dependent on the production of extracellular lignin peroxidase enzyme. It was observed that optimum digestion time was 17days. A maximum of 73.54, 79.6, 66.4, and 47.6% of COD reduction in *Phanerochaete chrysosporium*, *Trametes hirsuta*, and the isolated MF and SF respectively was observed on 17th day (Fig. 2). The BOD reduction was observed to be 65.33%, 51.38%, 100%, and 100% in *Phanerochaete chrysosporium*, *Trametes hirsuta*, and the isolated MF and SF, respectively, as observed on 17th day (Fig. 1).

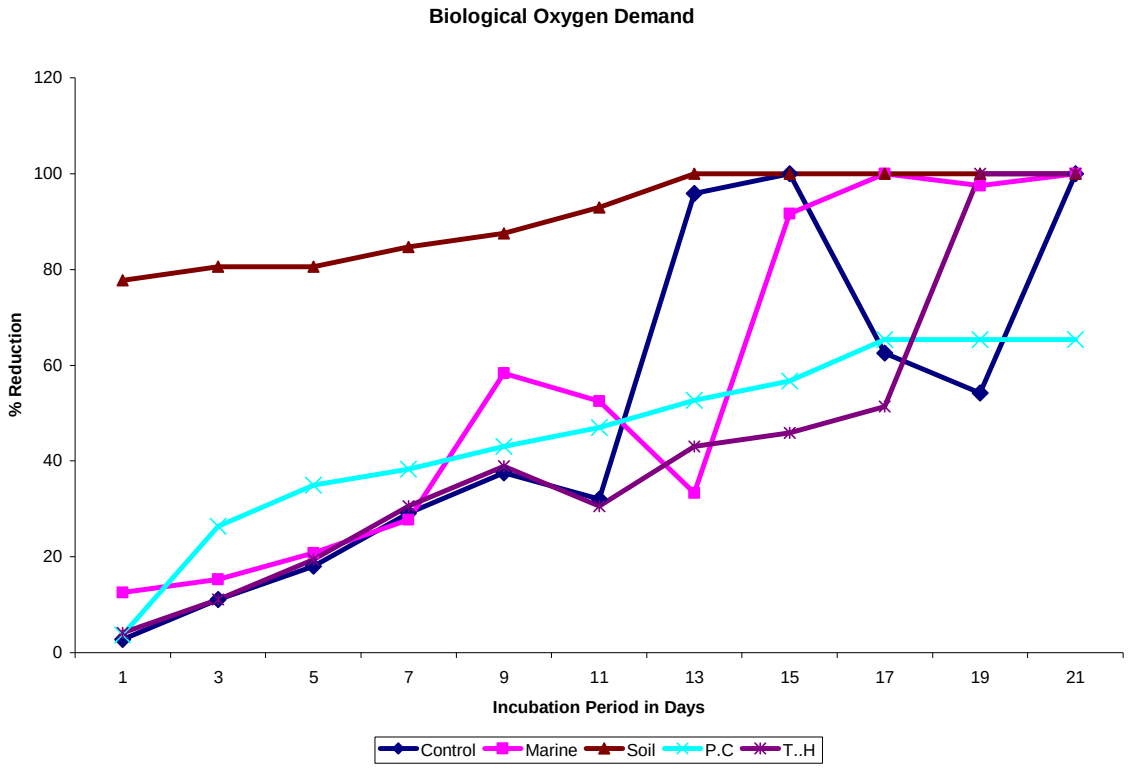


**Fig. 1.** Percent degradation based on Biological Oxygen Demand

After 17 days of digestion, the reduction of COD, BOD and colour was not significant and it became constant (Table 2). Similar results were observed in the studies performed by Masud Hossain and Das (2001).

There was 75.8%, 89%, 77.5%, and 69% phenol degradation of pulp and paper mill effluent by *P. chrysosporium*, *T. hirsuta*, MF, and SF at the end of 21st day, respectively. The extra cellular enzyme produced by the fungi is believed to be responsible for the phenol degradation. Similar results were reported by Peralta-Zamova et al., who observed the decolorization and pollutant degradation of pulp mill effluents with immobilized lignin and MnP from *P. chrysosporium* (Fig 4).

The colour data showed a remarkable trend along with the degradation of lignin. The most significant decolorization (60% on 19th day) was observed for all the species. The overall decolorization was 67.78%, 55.81%, 56.97%, and 58.37% in *P. chrysosporium*, T. hirsuta, MF, and SF, respectively (Fig. 3).

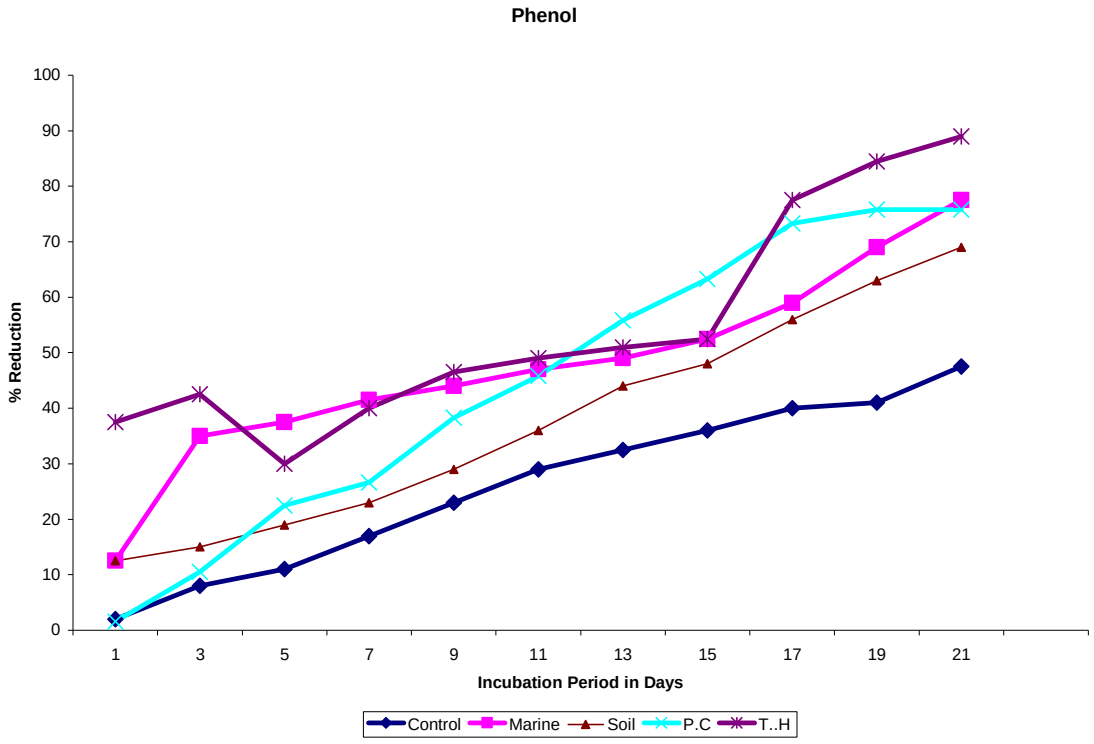


**Fig. 2.** Percent degradation based on Chemical Oxygen Demand.

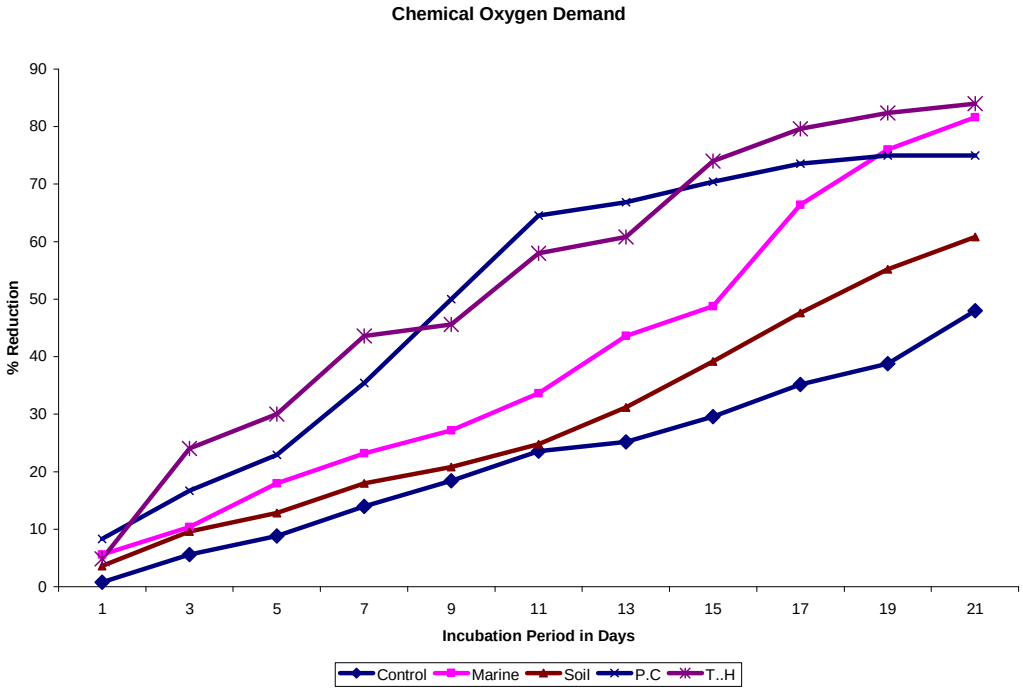


**Fig. 3.** Percent degradation based on Colour

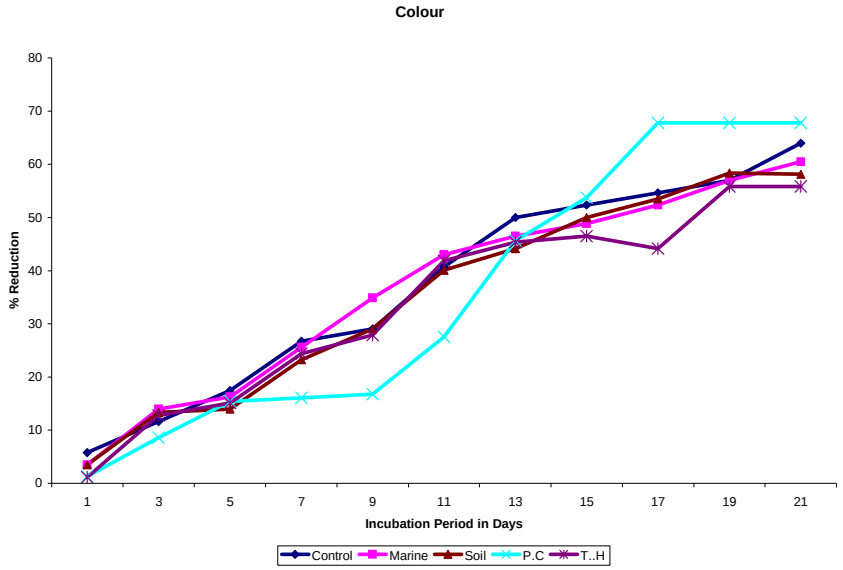


**Fig. 4.** Percent degradation of Phenol

The effective degradation of lignin was observed *by P. chrysosporium*, *T. hirsuta*, and MF, whereas SF showed the least efficiency in the degradation of lignin. In all the treatments, the greatest loss of lignin occurred during the exponential phase of growth. The most significant loss of lignin (78.4% on the 21st day) was observed by all the species. The overall lignin degradation was 63.8%, 78.4%, 63.4%, and 61.5% in *P. chrysosporium*, *T. hirsuta*, MF, and SF, respectively (Fig. 5). The results are in accordance with those obtained with alkaline paper-mill effluent used by Hernandez et al. (1994).

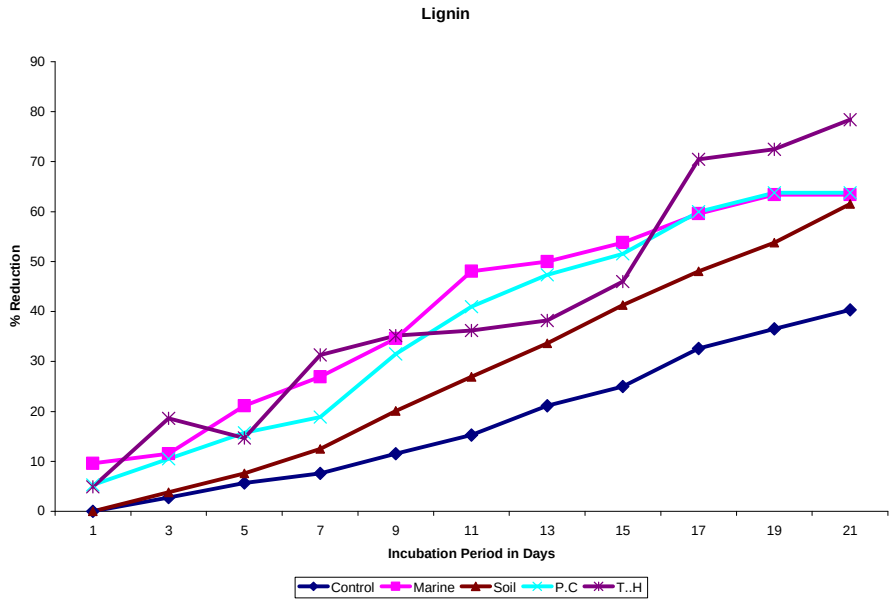


**Fig. 5.** Percent degradation of Lignin.

The degradation of cellulose was observed throughout the study until the 15th day in both the treatments, after which the degradation was stabilized. The efficiency of cellulose degradation varied as 52.38%, 68.14%, 80%, and 72.22% in *P. chrysosporium*, *T. hirsuta*, MF, and SF respectively (Fig. 6). The result correlates with the study of Abdullah et a1. (2006). This may be related to the utilization of cellulose by the fungi as a source of energy for the production of hydrolytic enzymes.

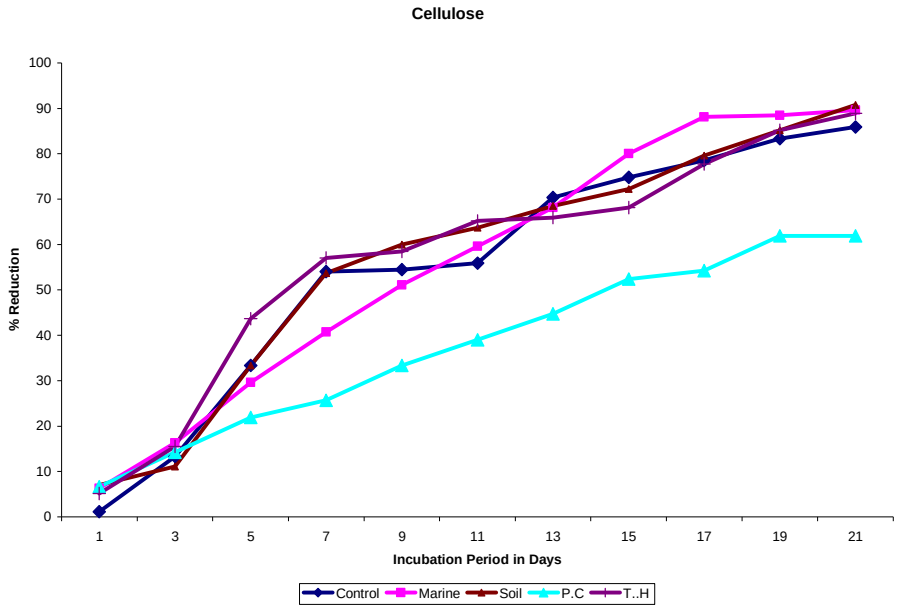


**Fig. 6.** Percent degradation of cellulose

The enzyme assay showed clearance zones for all the four fungal species (Plates 1, 2, 3, 4).

## CONCLUSIONS

1. The isolated fungal strains were capable of decomposing the lignin – cellulose complex from paper and pulp mill effluents.
2. Soil fungus was found to be able to mineralize synthetic lignin to a moderate extent. Marine fungus proved to be the most active strain in the investigation and degraded lignin to the maximum extent in with the potential equal to the standard species *P. chrysosporium* and *T. hirsuta*.
3. This study indicates that the treatment of the paper and pulp mill effluent by Marine Fungus and Soil Fungus is a competent and cost-effective method.
4. Lignin is the single most important activity in the biological cycle of carbon. The multitude of inter-unit bonds and functional groups and the heterogeneity of the polymer is the main reason for the resistance of lignin to microbiological attack and it is in fact one of the most recalcitrant naturally occurring biological materials (Prabhu and Udaya Soorian 2005). Biological treatment of pulp and paper mill effluent using

these fungi to decolorize effluent has yielded results similar to some of the best decolorizing activities reported in the literature.

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**Table 2.** Degradation of Paper and Pulp Mill Effluents by Various Fungal Species

| Parameter | Species | D-l | D-3 | D-5 | D-7 | D-9 | D-11 | D-13 | D-15 | D-17 | D-19 | D - 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | Control | 7.48 | 6.52 | 7.03 | 7.39 | 8.41 | 7.21 | 6.45 | 6.87 | 7.23 | 7.44 | 6.89 |
| | Phanerochaete chrysosporium | 8.6 | 8.4 | 8.5 | 8.44 | 8.39 | 8.36 | 8.32 | 8.3 | 7.6 | 7.6 | 7.6 |
| | Trametes hirsuta | 7 | 6 | 9 | 9 | 8 | 9 | 7 | 10 | 9 | 9 | 8 |
| | Marine Fungus | 7.51 | 7.35 | 6.58 | 6.52 | 7.13 | 7.1 | 6.6 | 7 | 6.8 | 7.2 | 6.7 |
| | Soil Fungus | 6.09 | 7.08 | 7.84 | 8.09 | 7.74 | 7.33 | 7.25 | 6.57 | 7.31 | 7.14 | 7.21 |
| | | | | | | | | | | | | |
| $CO_2$ Release | Control | 5.4 | 5.72 | 7.04 | 4.4 | 23.54 | 38.5 | 38.94 | 38.06 | 39.92 | 36.63 | 39.16 |
| | Phanerochaete chrysosporium | 8.2 | 14.8 | 13.6 | 24.7 | 31.9 | 44.4 | 46.9 | 51.6 | 52.0 | 49.8 | 47.9 |
| | Trametes hirsuta | 13.45 | 5.72 | 8.04 | 4.6 | 33.54 | 39.5 | 40.94 | 38.06 | 40 | 34.63 | 37.16 |
| | Marine Fungus | 4.96 | 9.14 | 12.48 | 23.7 | 32.34 | 43.56 | 45.54 | 44 | 38.5 | 38.72 | 40.7 |
| | Soil Fungus | 6.7 | 8.7 | 12.92 | 16.48 | 27.28 | 37.4 | 39.82 | 40.48 | 38.06 | 32.34 | 40.26 |

Table 2 Continued....

| Parameter | Species | D -1 | D-3 | D-5 | D-7 | D-9 | D-11 | D-13 | D-15 | D-17 | D-19 | D - 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BOD | Control | 700 | 640 | 590 | 510 | 450 | 489 | 30 | 0 | 270 | 330 | 0 |
| | Phanerochaete chrysosporium | 3.66 | 26.33 | 35 | 38.33 | 43 | 47 | 52.66 | 56.66 | 65.33 | 65.33 | 65.33 |
| | Trametes hirsuta | 690 | 640 | 580 | 500 | 440 | 500 | 410 | 390 | 350 | 0 | 0 |
| | Marine Fungus | 690 | 640 | 580 | 500 | 440 | 500 | 410 | 390 | 350 | 0 | 0 |
| | Soil Fungus | 160 | 140 | 140 | 110 | 90 | 51 | 0 | 0 | 0 | 0.3 | 0.2 |
| | | | | | | | | | | | | |
| COD | Control | 0.8 | 5.6 | 8.8 | 14 | 18.4 | 23.6 | 25.2 | 29.6 | 35.2 | 38.8 | 48 |
| | Phanerochaete chrysosporium | 8.33 | 16.67 | 22.91 | 35.41 | 50 | 64.58 | 66.87 | 70.41 | 73.54 | 73.54 | 73.54 |
| | Trametes hirsuta | 4.8 | 24 | 30 | 43.6 | 45.6 | 58 | 60.8 | 74 | 79.6 | 82.4 | 84 |
| | Marine Fungus | 5.6 | 10.4 | 18 | 23.2 | 27.2 | 33.6 | 43.6 | 48.8 | 66.4 | 76 | 81.6 |
| | Soil Fungus | 3.6 | 9.6 | 12.8 | 18 | 20.8 | 24.8 | 31.2 | 39.2 | 47.6 | 55.2 | 60.8 |

Table 2 Continued....

| Parameter | Species | D-1 | D-3 | D-5 | D-7 | D-9 | D-11 | D-13 | D-15 | D-17 | D-19 | D -21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Colour | Control | 5.81 | 11.62 | 17.44 | 26.74 | 29.06 | 40.69 | 50 | 52.32 | 54.65 | 56.97 | 63.95 |
| | Phanerochaete chrysosporium | 1.34 | 8.59 | 15.43 | 16.10 | 16.77 | 27.51 | 45.63 | 53.69 | 67.78 | 67.48 | 67.78 |
| | Trametes hirsuta | 1.16 | 12.79 | 15.11 | 24.41 | 27.90 | 41.86 | 45.34 | 46.51 | 44.18 | 55.81 | 55.81 |
| | Marine Fungus | 3.48 | 13.95 | 16.27 | 25.58 | 34.88 | 43.02 | 46.51 | 48.83 | 52.32 | 56.97 | 60.46 |
| | Soil Fungus | 3.48 | 13.37 | 13.95 | 23.25 | 29.06 | 40.11 | 44.18 | 50 | 53.48 | 58.37 | 58.13 |
| | | | | | | | | | | | | |
| Lignin | Control | 0 | 2.8 | 5.76 | 7.69 | 11.53 | 15.38 | 21.15 | 25 | 32.69 | 36.53 | 40.38 |
| | Phanerochaete chrysosporium | 5.26 | 10.52 | 15.78 | 18.94 | 31.57 | 41.05 | 47.36 | 51.57 | 60 | 60 | 60 |
| | Trametes hirsuta | 4.91 | 18.65 | 14.70 | 31.37 | 35.29 | 36.27 | 38.23 | 46.07 | 70.58 | 72.54 | 78.43 |
| | Marine Fungus | 9.61 | 11.53 | 21.15 | 26.92 | 34.61 | 48.07 | 50 | 53.84 | 59.61 | 63.46 | 63.46 |
| | Soil Fungus | 0 | 3.84 | 7.69 | 12.5 | 20.19 | 26.92 | 33.65 | 41.34 | 48.07 | 53.84 | 61.56 |

Table 2 Continued....

| Parameter | Species | D -1 | D-3 | D-5 | D-7 | D-9 | D-11 | D-13 | D-15 | D-17 | D-19 | D -21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cellulose | Control | 1.11 | 13.33 | 33.33 | 54.07 | 54.44 | 55.92 | 70.37 | 74.81 | 78.51 | 83.33 | 85.92 |
| | *Phanerochaete chrysosporium* | 6.66 | 14.28 | 21.9 | 25.71 | 33.33 | 39.04 | 44.76 | 52.38 | 54.28 | 61.90 | 61.90 |
| | *Trametes hirsuta* | 5.18 | 15.55 | 43.70 | 57.03 | 58.51 | 65.18 | 65.92 | 68.14 | 77.77 | 85.18 | 88.88 |
| | Marine Fungus | 6.29 | 16.29 | 29.623 | 40.74 | 51.11 | 59.62 | 68.14 | 80 | 88.14 | 88.51 | 89.62 |
| | Soil Fungus | 7.03 | 11.11 | 33.33 | 53.77 | 60 | 63.70 | 68.51 | 72.22 | 79.62 | 85.18 | 90.74 |
| | | | | | | | | | | | | |
| Phenol | Control | 2 | 8 | 11 | 17 | 23 | 29 | 32.5 | 36 | 40 | 41 | 47.5 |
| | *Phanerochaete chrysosporium* | 1.66 | 1.66 | 22.5 | 26.66 | 38.33 | 45.83 | 55.83 | 63.33 | 73.33 | 73.33 | 73.33 |
| | *Trametes hirsuta* | 37.5 | 42.5 | 30 | 40 | 46.5 | 49 | 51 | 52.5 | 77.5 | 84.5 | 89 |
| | Marine Fungus | 12.5 | 35 | 37.5 | 41.5 | 44 | 47 | 49 | 52.5 | 59 | 69 | 77.5 |
| | Soil Fungus | 12.5 | 15 | 19 | 23 | 29 | 36 | 44 | 48 | 56 | 63 | 69 |

**Enzyme Assay**

Plate 1: Marine Fungus

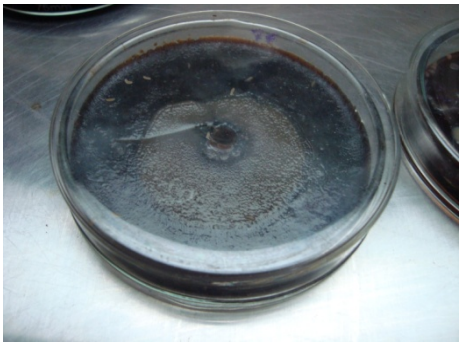

Plate 2: *Trametes hirsuta*

Plate 3: *Phanerochaete chrysosporium*

Plate 4: Soil Fungus