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Research Article

Isolation of fungi for delignification of Pulp and Paper mill effluent

Lokeshwari.N[a*], G.Srinikethan[b], S.G.Joshi[a], Shasikala. Inamdar[d] , Srikanth. B[c], Bashirahmed[a] and Sushma.L[a]

[a]Department of Chemical Engineering, SDM College of Engineering and Technology -Dharwad. 580 002 Karnataka. India
[b]Department of Chemical Engineering, National Institute of Technology Karnataka -Surathkal. 575 025 Karnataka. India
[c]Department of Bio Chemistry, Karnataka University Dharwad -Dharwad 580 008 Karnataka. India

Abstract

Biodegradation of industrial Lignin by a fungal isolate identified as Aspergillus flavus, white rot fungi which was isolated from Pulp and Paper effluent was studied in batch flask system with industrial effluent. The flasks were operated at temperature 32°C at 200 rpm for eight days in continuous mode. The average overall pH, Temperature, DO, C.O.D, T.D.S, T.S.S, Lignin, were up to 4.56, 32^oC, 4.2mg/l, 104mg/l, 6000 mg/l, 4000mg/l, 575.5mg/l, respectively after treatment. The Aspergillus flavus sp was the most effective in the biodegradation of Lignin of pulp industry for 94% at 480nm and of chemical oxygen demand levels for 45% after 8 days of incubation. The optimal conditions found were 4 pH and 32^oC temperature for lignin degradation

Keywords: *Lignin, Aspergillus flavus, Screening, Optimal conditions*

1. Introduction

Waste water from production of bleached kraft pulp contains organic acids, carbohydrates, resin acids, lignin transformation products and variety of chlorinated derivatives (L. Sunito *et al*, 1988). The paper mill wastewater characteristically contains colour, very high level of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) ,due to presence of lignin and its derivatives from the raw cellulosic materials , chlorinated compounds, suspended solids (mainly fibres), fatty acids, tannins, resin acids, sulphur and sulphur compounds, etc (M. Ali *et al*, 2001). These effluents contain many organic compounds, derived from lignin, which are responsible for their brown colour and also for increasing water temperature and decreasing photosynthesis rate of the phytoplanktonic community. The content in low molecular weight chloro-organics of residues, generated by pulp and paper industry, is the major contributors to mutagenicity and bioaccumulation, due to their hydrophobicity and ability to penetrate cell membranes (A.M. Pedroza *et al*, 2007). Most of these industries discharged their insufficiently treated waste into the rivers or streams, environmental impact of black liquor results not only from its chemical nature, but also from its dark coloration that reduces oxygen availability and negatively affects aquatic fauna and flora (H.R. Jones, 1973). All these organic compounds are toxic to aquatic organisms and resistant to microbial degradation, resulting in a decrease of the ecological value of natural systems surrounding the pulp mill (E. Esposito *e t al*, 1991).

Conventional procedures to treat these effluents involve physical and biological techniques with no complete degradation of the recalcitrant organic matter (M.C. Yeber, 1999). Therefore it is required to find out alternative treatment mechanisms, which enable the release of effluent to the environment or the reuse of effluent at some point along the same process. Biological processes using microbial systems provide an alternative to the existing physical/ chemical technologies (expensive and commercially unattractive) because they are more cost-effective, simple in design (I.M. Banat *et al*, 1996), environment friendly and do not produce large quantities of sludge (H. Kariminiaae-Hamedaani *et al*, 2007). Biodegradation is used to describe the complete mineralization of the starting compound to simpler ones like CO_2, H_2O, NO_3 and other inorganic compounds (R.M. Atlas and R. Bartha, 1998). There is a real need to treat the wastewater with biological systems. In biological methods, microorganisms having ligninolytic properties have been used for degradation of major contaminants of industrial effluent. Several fungal strains have proved effective in decolourization, and decontamination of effluent.

Black colour of the effluent is due to lignin and its derivatives which may increase water temperature and leads to decrease concentration of dissolved oxygen. Based on these problems, it is required to degrade lignin and colour from pulp and paper industry (R. Nagarathnama *et al*, 1999). Bacteria fail to degrade high molecular weight chlorolignin, the reason may be due to

*Corresponding author: **Lokeshwari.N**

the fact that bacteria can produce intracellular enzyme capable of degrading lignin like structures, and the high molecular weight chlorolignin cannot penetrate the bacterial cell membrane (S.Pallerla *et al*, 1996). Few microorganisms especially fungus *P.chrysosporium, Trametes, Phlebia, Aspergillus* sps, *Cladosporium* sps are commonly used for lignin degradation (M. Tuomela *et al*, 2000). The comparison of decolourization by different organisms show that white rot fungi *P.chrysosporium* and *C. versicolor* were suitable for efficient degradation of the recalcitrant chromophoric material in bleach plant effluents (P. Bajpai and P. K. Bajpai, 1994).

The wood degrading white rot fungi are effective in significant reduction of color, lignin and COD, however, there are a few fungi to reduce chlorinated aromatic compound. White-rot fungi are primarily responsible for the initial decomposition of lignin in wood, which occurs via an oxidative and relatively nonspecific process (A. Hatakka 1994; K.E. Hammel *et al*, 2002). *Phanerochaete chrysosporium*, a white-rot-wood decaying basidiomycete, produces a potent lignin degrading system that oxidizes lignin completely to CO_2 (A. Breen and F.L.Singleton 1999). The utilization of *P. chrysosporium* in waste water treatment is gaining importance in paper industries, because of their ability to degrade lignin in wood (D. Eaton *et al*, 1980). Attempts have been made to remove the colour of the effluent from a Kraft mill by using *P. chrysosporium* (Thomas *et al*, 1981) and from the pulp waste by *Tinctoria* sp (T. Fukuzumi 1980) and *Aspergillus* sp (S.A. Dutta *et al*, 1985).

Recent developments in new technologies and improvement of existing ones for the treatment of effluents from the pulp and paper industries include the use of the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* (A. Mehna et al., 1995). Very limited experience is available on the possibility of direct degradation of highly contaminant black liquors by fungi. Hence the study reports on effective biodegradation of Industrial Lignin with optimum process parameters by white rot fungi which was isolated and identified as *Aspergillus flavus.*

Materials and Methodology

2.1 Sampling and analysis of waste water

Wastewater of pulping process from the Pulp and Paper Company in Karnataka state was used in this study. The samples were collected in the plastic container and were brought to the laboratory and immediately stored in refrigerator at 4^oC until used for further analysis. The colour unit of the effluent was by taking Absorbance readings at 465 nm of both the original and the decolorized effluents were converted to color units (CU) by the equation: CU = 500 X Am/ 0.132 where 0.132 is the absorbance of 500 CD platinum-cobalt standard solution (S. Davis *et al*, 1990). The pH of the effluent was measured with the help of electronic pH meter. COD, BOD, Total dissolved and suspended solid were analyzed according to the standard method for the Examination of Water and Wastewater, APHA, 1992. Lignin concentration was analyzed by UV-Visible spectrophotometer (U- 3010) with detection wavelength of 300-700 nm (D. Eaton *et al*, 2005).

2.2 Isolation of the fungus

Waste water samples were collected from near by Paper and Pulp industries. The standard method, called drop spore or shoot spore technique was used for fungal spore isolation. The technique used was aimed to obtain samples onto Potato Dextrose agar (PDA; pH 5-6) containing antibiotics (0.3 g/L penicillin and 1.3 g/L streptomycin). The agar plates containing the waste water samples were then incubated at 30 C for 24 hours. The resulting spores were observed as groups which would be afterwards isolated using Nichrome loop wire in Laminar flow chamber and placed onto PDA agar containing antibiotics. The growing fungal mycelium was sub cultured until the purified fungal strains were obtained and pure culture obtained was stored at 4 C.

2.3 Screening of the potential Fungus

Screening of the fungus was done by growing the fungal strains on broth media containing 1% lignin source at 30^oC for 5 days by providing nitrogen source (KNO_3). In the present study, black liquor was used as lignin source. The strains that were capable of degrading lignin in the wastewater from pulp and paper industries were theoretically able to survive and grow well on this agar. Plates were observed for growth and color change from colourless to green around the culture growth, which indicate the ligninolytic nature of the culture. The reduction in lignin content was determined by using the formula of absorbance using UV-spectrophotometer.

2.4 Identification and Characterization of the potential Fungus

The selected strain was identified according to their basic morphology at mycology department. Morphological examination was performed using a light microscope equipped with a micrometer eyepiece with 400x magnifications. The strain is identified as Fungi *Aspergillus flavus*. It grows by producing thread like branching filaments known as hyphae. Filamentous fungi such as *A. flavus* are sometimes called molds. A network of hyphae known as the mycelium secretes enzymes that break down complex food sources. The resulting small molecules are absorbed by the myceilium to fuel additional fungal growth. When young, the conidia of *A. flavus* appear yellow green in color. As the fungus ages the spores turn a darker green. Colonies variable ranging from rapidly growing 6-7 cm in diameter slowly growing 3-4 cm in diameter, consisting of submerged mycelium commonly plane but occasionally radially followed are wrinkled colony pattern. Young conidial heads in yellow shades to yellow-green becoming deep green in age reverse uncolored. Conidial heads typically radiate

splitting into several poorly defined columns smaller heads 62.7-69.3 micron. Conidia subglobose to globose 33 to 36.3 micron. Foot cell is of 49.5*6.6 – 9.9 micron.

2.4 Process Optimization parameters

The optimum conditions of temperature and pH were determined for percentage degradation of Lignin by isolated fungi. The Shake flask experiments were carried out using broth media in 250 ml conical flasks inoculated with fungi and incubated for 48 hours for different temperatures (15^{o}C, 32^{o}C and 37^{o}C) and pH (4, 6 and 8).

2.5 *Degradation of Lignin*

The shaker flask with BR media, lignin solution and with optimum process parameters was autoclaved and inoculated with the fungi. The BR media used was KH_2PO_4-70ml, KCL-15ml, $ZnSO_4$-8ml, $MgSO_4$-20ml, $FeSO_4$-20ml, $MnSO_4$-6ml, NH_4NO_3/KNO_3-1g. The inoculated flask was subjected to shaker with the speed of 200rpm for 2hrs. Then the flask was kept undisturbed for 48hrs for period of 8days and the degradation in Lignin was observed.

2.6 Lignin analysis

The Lignin concentrations in the samples were analysed using UV-Vis Spectrophotometer (U- 3010). Lignin absorbance was measured between the wavelength range of 280 nm 480 nm. Lignin concentration was measured by the calibration chart as shown in Fig 2.7.

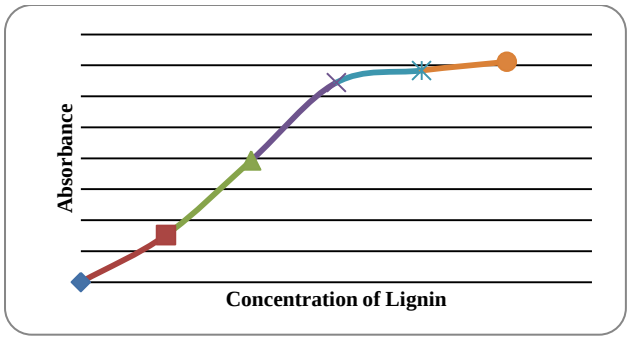


Fig 2.7: Calibration Curve of Lignin

Results and Discussion

3.1 Analysis of Waste water quality

The effluent of the samples was obtained from local Kraft Pulp and Paper mill. The characteristics of waste water analyzed, are given in Table 3.1.

3.2 Isolation, Screening and Identification of Fungi

The isolate was isolated from Pulp effluent on PDA agar medium. The PDA medium was prepared, autoclaved and inoculated. Serially diluted samples were inoculated into the petri-dishes containing solidified PDA media in a sterilized environment. The inoculation of the sample is done by adding the 1 ml of sample into the petri-dish containing PDA media. The inoculated petri-dishes were kept undisturbed for 48 hours for the spore formation. By

Table 3.1: Characteristics of Waste Water form Pulping Process.

Parameter	**Values**
pH	6.5
Colour	Dark Brownish
COD (mg/l)	289.33
DO (mg/l)	7.9
TDS (mg/l)	12000
TSS (mg/l)	8000
Lignin Concentration (mg/l)	9590.64

using the single spore isolation method, the spore of the fungus was isolated and transferred to the test-tubes containing the solidified PDA media. The culturing of the isolated fungus occurs in PDA media. After 2 days, the growth of the fungus was observed. The maintenance was done by sub-culturing of the isolated fungus onto the PDA slants and incubating at room temperature for 2 days. The sub-culturing helps in obtaining the pure culture of the isolated fungus. The sub-cultured fungus was stored at 4^{o}C. Screening was done for Lignin quantification. Based on the microscopic studies, the fungal isolate was found to be *Aspergillus Flavus* as shown in Fig 3.21. A network of hyphae known as the mycelium secretes enzymes that break down complex food sources.

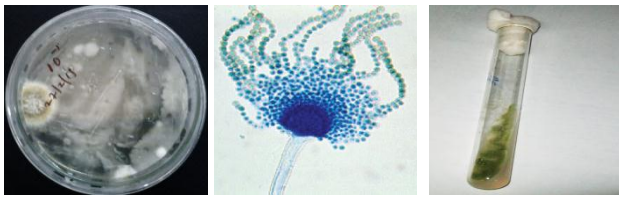


Fig 3.2.1: Isolated strain on Petridish, *Aspergillus Flavus*, Pure Culture of fungi

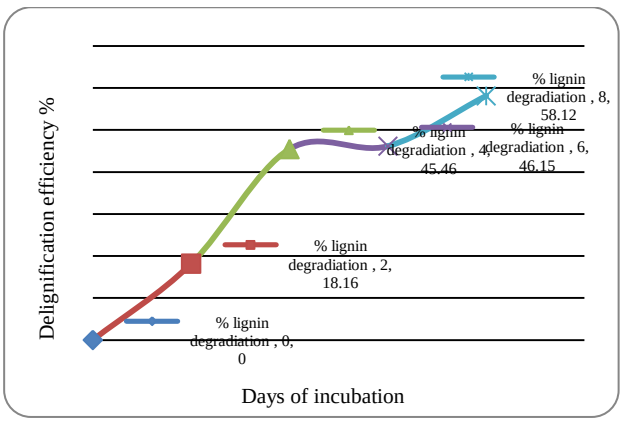


Fig 3.2.2: Lignin Quantification

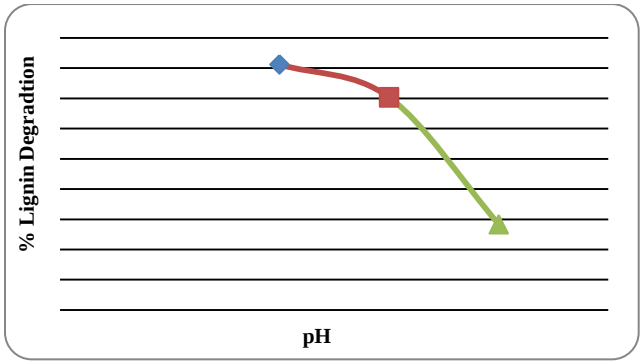
Isolation of *Aspergillus flavus* from the wastewater of pulp and paper industries and the examination of their ability to biodegrade lignin could be of great advantage. Several factors, such as temperature, pH, oxygen concentration and the microorganism influence the degradation of lignin (M.A. Tiirola *et al*, 2002). Hence, in this section, lignin quantification was carried out to determine whether *Aspergillus flavus* feeds on lignin as shown in Fig 3.22. In lignin quantification, the lignin was reduced upto 58% in 8 days. The degradation percentage of lignin was low, it may be because *Aspergillus flavus* mainly fed on the broth media.

3.3 Optimization studies

The optimum temperature was found to be 32^{o}C. White-rot fungus which can grow in a wide range of temperature has no growth at any temperature below 10^{o}C and no significant change in growth rate occurs between 30^{o}C and 39^{o}C. And also, according to literature, the optimal growth of white-rot fungus occurs at 39^{o}C but unlike most fungi, *Aspergillus flavus* readily grows between the temperatures of 25-32^{o}C. Lignin degradation was adversely affected with increase in temperature, but in contrast M. Tuomela *et al*., 2000 showed optimum temperature for lignin degradation as 40 to 50°C by fungal consortia.

The studies (M.A. Lara *et al*, 2003; B.K. Belsare *et al*, 1988) reported that 4.0 to 8.0 ranges are best suitable for the treatment for pulp and paper effluent. The optimum pH was found to be 4 as the optimal growth of *Aspergillus flavus* occur between the range of 4.0 to 4.5, and at high oxygen content. In contrast a previous report (D.K. Sahoo *et al.*, 2005; V.B. Damiano *et al*., 2003) showed alkaline pH as best suitable for lignin degradation by *Aspergillus fumigatus* and *Bacillus licheniformis* respectively. Fungi are recognized for their great ability to produce a large variety of extra cellular proteins, organic acids and other metabolites (C. Palma et al., 1999), being this process highly dependent from the substrates used by the fungi, which in turn influences the pH of extracellular environment.

The *Aspergillus flavus* is capable of degrading the lignin producing vanilic acid & methanol.The degradation at 32^{o}C and 4 pH was maximum from experiments conducted as shown in Fig 3.3. The delignification efficiency was found to be 60% for optimum temperature and 81% for optimum pH in broth medium.



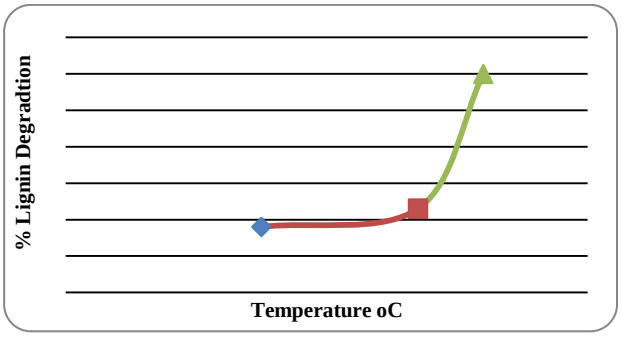


Fig 3.3: Temperature and pH effect on Degradation of Lignin.

3.4 Degradation of Lignin

Lignin biodegradation by white-rot fungi is an oxidative process probably involving enzymes such as lignin peroxidases (LiP), manganese peroxidases (MnP) and laccascs (T.K. Kirk *et al*, 1987). After treating the effluent of paper industry with *Aspergillus flavus*, the lignin was reduced upto 94% from 0 to 8 days of Incubation as shown in Fig 3.4. After 10 days of incubation the decreasing trend in absorbance was reversed which could be related to secondary metabolic compounds produced by these species (A.C. Freitas *et al*, 2009). This observation suggested that greater time of incubation was not a positive factor for higher degradation rates of organic compounds present in the final effluent. The pH of the effluent was reduced after treating it with *Aspergillus flavus*. The decrease in pH (acidic) may be due to conversion of complex organic compounds in simple inorganic acids (Pratibha Singh, 2005 and P. Sehanat *et al*, 2009). The COD of the effluent also decreased due to removal of lignin. The reduction in COD was also supported by Sehanat *et al*. (2009). The COD of the sample was reduced by 45 %.

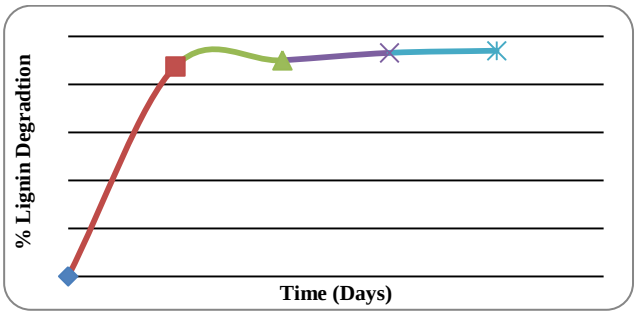


Fig 3.4: Degradtion of Lignin by *Aspergillus flavus*

Conclusion

The samples were collected from Paper mills and were subjected to isolated fungus for delignification. The single fungus used was *Aspergillus flavus* and it was isolated from the same samples. The optimum temperature and pH for the biodegradation of lignin were found to be 32^{o}C and 4. On treating the waste water samples with this fungus at

the optimum conditions, we were able to notice degradation of lignin upto 94%. The study concluded that the isolated fungus form Pulp effluent was efficient in degradation of lignin (colour causing compound) and reduction in level of COD. The decrease in the pH value of treated samples was due to the metabolism of fungus & metabolic production of acid by *Aspergillus flavus.* The decrease in suspended solids in sample is due to the presence of fibers.

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