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Decolourization and Biological Treatment of Pulp and Paper Mill Effluent by Lignin-Degrading Fungus *Aspergillus flavus* Strain F10

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ABSTRACT

Keywords

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A potential lignin-degrading fungal strain, identified as *Aspergillus flavus* strain F10 have been studied for the treatment of pulp and paper mill effluent. The results of this study revealed that *A. flavus* effectively reduced the colour and lignin content (in the term of total phenolic content). It reduces colourity up to 31–51 % and lignin content 39–61% in ten days of incubation when used as a free form, while in immobilized condition it reduces more colourity and lignin content within 6 day of treatment. A significant reduction in color and lignin content by this fungus strain was observed after four days of incubation, indicating that fungus subsequently utilized chromophoric compounds thereby reducing lignin content and color. The toxicity of paper and pulp effluent was measured in the term of phytotoxicity. A high germination index of treated samples indicate that the treatment have reduced pollution load in effluent to low levels which are not toxic to plants.

Introduction

Pulp and paper milling are major industrial sectors using a huge amount of lignocellulosic raw materials and water during manufacturing processes, and releasing non-biodegradable organic materials, adsorbable organic halogens (AOX), chlorinated lignosulphonic acid, chlorinated resin, chlorinated phenol and chlorinated hydrocarbon in the effluent (Abhay Raj *et al.*, 2007; Thompson *et al.*, 2001; Buyukkamaci *et al.*, 2010). In India, pulp and paper manufacturing is one of the eldest and leading industry, which produced

about three million metric tons per annum finished products (Malaviya *et al.*, 2007). Pulp and paper mill wastewater is produced from wood preparation, pulping process, pulp washing, screening, washing, bleaching, and paper machine and coating operations (Pokhrel *et al.*, 2004). The generation of wastewater and the characteristics of pulp and paper mill effluent depend upon the type of manufacturing process adopted and the extent of recycling of water employed in the plant (Abhay Raj *et al.*, 2007). 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 (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. 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 (Clesceri *et al.*, 1999; Ali *et al.*, 2001). The raw wastewater from paper and board mills can be, potentially, very polluting. Indeed, a recent internal survey within the industry has shown that chemical oxygen demand (COD) values. Thus, wastewater from the industry needs to be treated to reduce any possible impacts on the aquatic environment (Thompson *et al.*, 2001; Buyukkamaci *et al.*, 2010).

The conventional treatment methods, such as aerated lagoons and activated sludge plants are ineffective in removing colour and phenolics. In most cases, this effluent (raw or treated) is discharged into the rivers, stream or other water bodies; resulting in high BOD, COD and also causing problems to community and environment. In many developing countries farmers are irrigating their crop plants with water bodies which might be severely exposed to industrial effluents. This leads to risks of bioaccumulation of toxicants as we move-up the food chain. Thus, it is important to treat the industrial effluents before their final discharge (Yang *et al.*, 2008). Despite the

fact that, wide range of physical and chemical treatment methods (electrocoagulation, ozonation, adsorption, advanced oxidation, and ultrafiltration) or combination of different methods in series are available for the treatment of effluent, but they are more energy intensive and suffer from residual effects. Thus, there is still a need for energy efficient, affordable and environment friendly technologies (Yang *et al.*, 2008; Raj *et al.*, 2014). In recent years the biotechnological approaches based biological treatment came in to scenario in present treatment systems, in which wide variety of microorganisms including fungi, actinomycetes, and bacteria as well as enzymes have been implicated but the recent research has been focused on with ligninolytic fungi (white rot and brown rot) due to their powerful lignin-degrading enzyme system (Malaviya *et al.*, 2007; Kumar *et al.*, 2012; Jha *et al.*, 2013).

In the present study isolated ligninolytic fungus producers and studied their suitability for decolourisation of pulp and paper mill effluent. The immobilization of fungus also have an alternative mode of decolourisation of paper pulp effluent in contrast to long term use and storage.

Materials and Methods

Chemicals

All reagent used in experiments were of analytical grade and were purchased from Merck Pvt. Ltd. and Hi-Media Mumbai (India). Millipore Deionized Mili-Q water (Aliks, Millipore, Mumbai, India) were used for biochemical assay.

Screening and Isolation of Fungi

For the isolation of ligninolytic fungus soil samples were collected from Guru Ghasidas

University campus, Bilaspur, C.G. Isolation of fungus was done in PDA medium with conventional dilution method. To screen the potent ligninolytic fungi, 32 isolated purified fungal strains were subjected to Bavendam's test (Bavendam, 1928) for initial screening and among them, a potent fungus F10 was considered for the further studies. Isolated fungus was purified and grown on optimized malt extract agar (MEA) medium and stored at 4°C.

Identification of Fungus Strain

The isolated potent lignin biodegrading fungus were identified by polyphasic approach (based on their microscopic, morphological characteristics (Holt *et al.*, 1994) and by sequencing of their 18S rRNA). Partial gene sequencing (including ITS1, 5S rRNA, ITS2 and partial 28S rRNA) of both fungus was performed by the commercial service provider, Chromous Biotech Pvt. Ltd., Bangalore, India. Strain identification was carried out by amplifying partial 18S rRNA using PCR with two primers ITS1 (5'TCCGTAGGTGAA CCTGCGG3') and ITS4 (3'TCCTCCGCT TATTGATATGC5').

The sequences of the partial 18S rRNA were compared with the 18S rRNA sequence available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using their World Wide Web site (<http://www.ncbi.nlm.nih.gov>) to identify the approximate species. Multiple alignments of the sequences in this work and all reference sequences were performed using CLUSTAL W. The phylogenetic trees were constructed based on a neighbor-joining algorithm in the MEGA 4.0 software program. The confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1000

interactions (Tamura *et al.*, 2007).

Decolourisation of the Paper Pulp Effluent

Effluent Origin and Physicochemical Characterization

Two types of paper mill discharge effluents were obtained from Amlai paper plant (M.P., India). Sample A, was produced through chemical alkaline pulping process of hardwood (soda cook liquor, without any treatment) and sample B, was the soda cooked liquor with slight aerobic treatments. These effluents were collected in air tight 5L bottle and stored at 4°C till use. The physico-chemical characterization [pH, TS (Total solid), TSS (total suspended solid), BOD (biological oxygen demand), COD (chemical oxygen demand)] were done according to standard method.

Analytical Methods for Physic-chemical Characterization of Pulp Mill Discharge Effluent

Colourity Determination

The colourity of cultures was determined according to the CPPA standard method (1974). According to this method the pH of effluent sample was adjusted to 7.6 with the help of 2 M NaOH and centrifuge at 10,000 g. The obtained clear supernatant was used for taking the absorbance at 465 nm against distilled water. The A_{465} values were transformed into colour units (CU) according to the formula

where A_1 is the absorbance of 500 CU platinum-cobalt standard solution ($A_{465}=0.132$) and A_2 is the absorbance of the wastewater sample (CPPA, 1974).

Dissolved oxygen (DO)

The DO was determined by the standard dilution technique according to method No. 5210 of APHA methods (Clesceri *et al.*, 1999). The method consists of filling an airtight bottle of the specified size with sample to overflowing and incubating it at the specified temperature (20 °C) for 5 days. Dissolved oxygen concentration (DO) was measured before and after incubation. The final DO was computed from the difference between initial and final DO value.

Chemical Oxygen Demand (COD)

The COD of the effluent sample was determined by method No. 5220 of APHA methods (Clesceri *et al.*, 1999). The effluent sample is refluxed in strongly acid solution with a known excess of $K_2Cr_2O_7$. Oxygen consumed was measured against standards at 600 nm with a UV-visible spectrophotometer (UV-1800, Shimadzu, Japan).

Solids (Total Solids and Total Suspended Solids)

Total solids (TS) and total suspended solids (TSS) were estimated by method Nos. 2540-B and 2540-D respectively, of APHA methods (Clesceri *et al.*, 1999). The sample was evaporated in a pre-weighted dish and dried to gain a constant weight in an oven at 103-105°C. The increase in weight over that of an empty dish represents TS. For estimation of TSS, the effluent sample was filtered through glass fibre filter and the residue retained on filter was dried up to a constant weight at 103-105°C.

Other Biochemical Analysis

Total carbohydrate (by Anthron), reducing sugar (by DNS method) and total phenolic content (by Folin-Denis method) content were also quantified before and after

Decolourisation experiments (Hedge *et al.*, 1962; Miller, 1959; Barapatre *et al.*, 2015).

Decolourisation Conditions

A total of 100 mL mineral salt medium were taken in a 250 mL flask and 3 bores of 7 day old fungal culture were inoculated in it. The 100 mL mineral salt medium consist of 80% (v/v) total pulp effluent and 20% of mineral salt medium (KH_2PO_4 0.2%; $MgSO_4 \cdot 7H_2O$ 0.05%; $CaCl_2 \cdot 2H_2O$ 0.01% (w/v); mineral salt solution 1 mL/L) supplemented with a carbon source (D-glucose) at a concentration of 0.5% (w/v) and a nitrogen source (0.3% w/v, ammonium nitrate). The flasks were inoculated at 28 °C in static and shaking condition (120 rpm). Uninoculated media serve as a control. The 5 mL sample was withdrawn at every 2 day interval up to 10 days to determine the decolouration.

Microbial Decolourisation of Paper Pulp Effluent by Immobilized Fungus

Aspergillus flavus F10 was entrapped in Calcium-alginate polymer beads using a method based on that described by Enayatizamir *et al.* (2010). Five millilitres of mycelia suspension was mixed with 100 mL of sodium alginate 2.1% (w/v) under shaking. The final concentration of alginate in the beads was 2%. The mixture was dropped by means of a syringe into a $CaCl_2$ solution (3% w/v) under shaking. To minimize cellular leaking, a method of gel re-coating was used. After 30 min, the beads were collected from the solution and washed with distilled water and placed into a solution of sodium alginate 0.5% (w/v) for 5-10 min (this makes it possible for the diffusion of the Ca^{+2} from the beads to produce the gelification on the bead surface of a second alginate cover). After that, the beads were washed with sterile distilled water and left to harden for 30 min into a 3% (w/v) solution of $CaCl_2$. Finally, the beads were washed with 0.7 % (w/v) NaCl.

Alginate beads were produced under sterile conditions and hence, all solutions involved were autoclaved until used.

Phytotoxicity Evaluation by Seed Germination Tests

The phytotoxicity study was carried out to check the toxicity the treated effluent. Experiment was done at room temperature in relation to germination of *Phaseolus mungo* (15 seeds). Seeds were sterilized with 10% (v/v) sodium hypochlorite for 20 min to prevent any microbial contamination. Germination of seeds were done by watering separately 5 mL treated and untreated pulp effluent per day. Control set was carried out using distilled water at the same time. All results were recorded in terms of % germination, length of plumule (shoot) and radicle (root) was recorded after 7 days and the germination index (GI) was calculated.

The germination index (GI) was calculated as follows:

$$GI = \frac{100 \times (G \times L)}{(G_c \times L_c)}$$

where G and L are the germination and radicle growth of the seeds germinated in the tested solution, respectively, and G_c and L_c the germination and radicle growth of the seeds in the control (distilled water), respectively. According to Zucconi *et al.*, (1981), GI values below 50% are considered to be indicative of a high toxicity, values between 50-80% represent a slight toxicity, and when the GI is higher than 80%, no phytotoxicity is considered.

Result and Discussion

Isolation of Test Strain

All of the 32 morphologically different fungal isolates exhibited a different ability to degrade lignin when tested on tannic acid amended media. Eighteen fungal strains

shows positive reactions in which fungus, named *Aspergillus flavus* strain F10, the potent fungus (form highest diameter of dark zone) was selected for the decolouration of pulp and paper mill effluent studies. Bavendam medium were used for the selection of the ligninolytic organism. In early several authors revealed that the incorporation of tannic acid in the culture medium provide preliminary test to detect the production and presence of ligninolytic enzymes (Thormann *et al.*, 2002; Kausar *et al.*, 2010). Polyphenol oxidases includes a group of the enzymes (laccase, lignin peroxidase, manganese dependent and independent peroxidase and versatile peroxidases) which catalyzed the reaction between polyphenol and molecular oxygen, and form the dark brown quinone complexes, a positive reaction of the degradation of the phenolic compound like lignin, gallic acid and tannic acid. The polyphenol oxidase is mainly produced by the ligninolytic fungus produce a dark colour zone around the mycelia growth in the Bavendam medium (Thormann *et al.*, 2002).

Microscopic Observation of Fungal Strain F10 and Strain Identification by 18S rRNA Sequencing

Microscopic observations of fungal appearance: mycelium composed of hyaline, branched, septate, smooth-walled hyphae. Conidiophores are smooth-walled and brittle, hyaline to pale brown; conidial heads are loosely radiate; conidiogenous cells biseriate; vesicles spatulate to subclavate; metulaes and phialides covering from 60% to almost the entire vesicle; conidia are globose, blue green, more or less rough-walled. The result of partial 18S rRNA sequence alignment based on BLAST analysis revealed that the isolated fungus strain F10 were shows high homology with (> 98%) *Aspergillus flavus*. The sequence

were deposited in the NCBI Genomic Bank nucleotide database and have been assigned accession numbers KC911631.1 (F10). Phylogenetic tree analysis were shown in Fig. 1.

Characterization of Waste Effluent

The paper pulp mill effluent used in the decolourisation experiment had a dark

brown colour and turbid colloidal appearance with a large amount of solid particulate matter. The analysis of effluent were done by different parameters showing high TS, TSS, pH, COD, and colourity. The observed values were presented in Table 1. From the observation table it was clearly seen that the sample B (biologically treated) having low pH, TS, TSS, COD, DO and CU than sample A (non-treated).

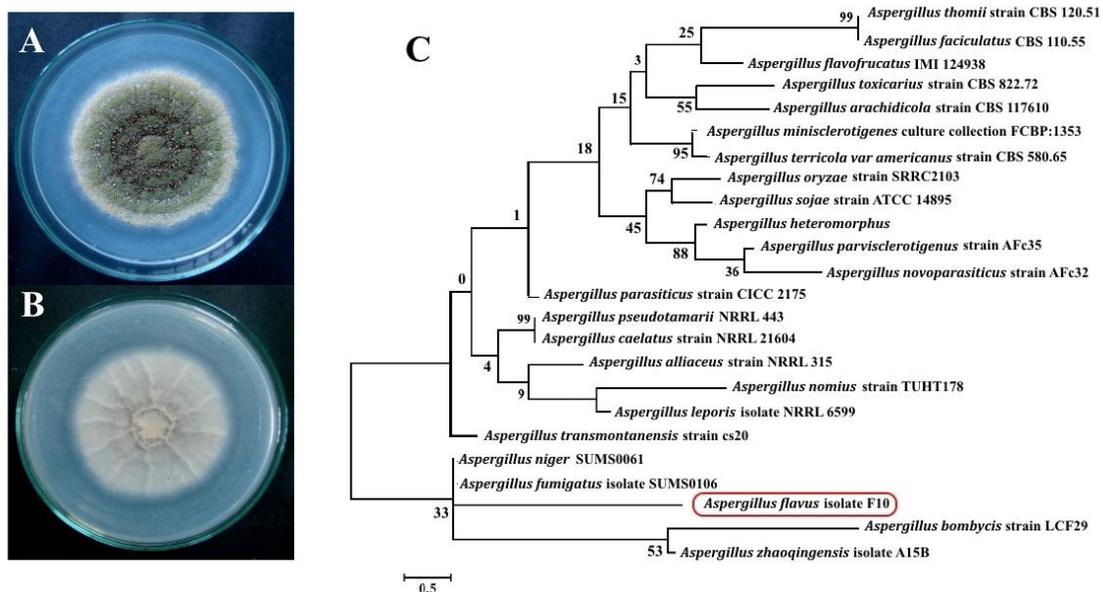


Fig.1 A & B Front and back view of selected fungus *Aspergillus flavus* (F10). (C) Phylogenetic tree of *A. flavus* F10

Table 1: Characteristic of paper pulp effluent

Parameter	Sample A	Sample B
Ph	10.28	7.44
TS (mg/L)	6410.00	1860.00
TSS (mg/L)	910.00	130.00
COD (mg/L)	253.20	174.40
DO (mg/L)	28.00	32.00
CU	5029.00 U	1853.50 U

Decolourisation of Paper Pulp Effluent by *A. flavus* F10

The white and brown rot fungi are unique among eukaryotes for having capability to degradation of lignin; and the lignin degradation is a secondary metabolic process in which they do not use lignin as a primary carbon source for their growth (Murugesan, 2003). Lignin is one of the major component which was the main cause of the colourity of pulp mill effluent. The decolourization of both effluent were done by *A. flavus* F10 and percent colour

decolourisation were shown in Table 2. Results depicted that there was a change in the reducing in colour unit in both type of treatment conditions (static and shaking), which confirm that the decolourisation was also influenced by culture condition. It was found that the maximum decolourisation occurred in sample A was 33.68% in the shaking condition as compared to 29.39% in static condition. The decolourisation in the effluent was also high for in shaking condition for sample B (66.32%) in comparison to static condition which is 61.91%.

Table.2 Percent (%) colour removal of paper pulp effluent treated by *A.flavus* F10

Day	Percent (%) colour removal			
	Sample A static	Sample A shaking	Sample B static	Sample B shaking
0	0	0	0	0
2	5.46	5.09	10.32	13.48
4	11.35	13.69	33.33	35.75
6	17.45	20.38	40.90	46.15
8	27.74	30.76	54.06	58.60
10	29.39	33.68	61.91	66.32

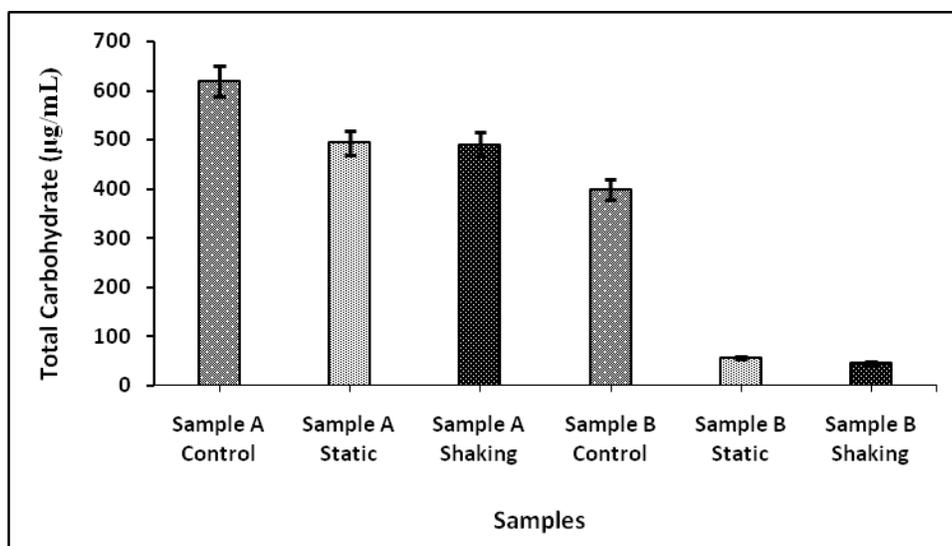


Fig.2 Total carbohydrate content of control and treated paper and pulp effluent

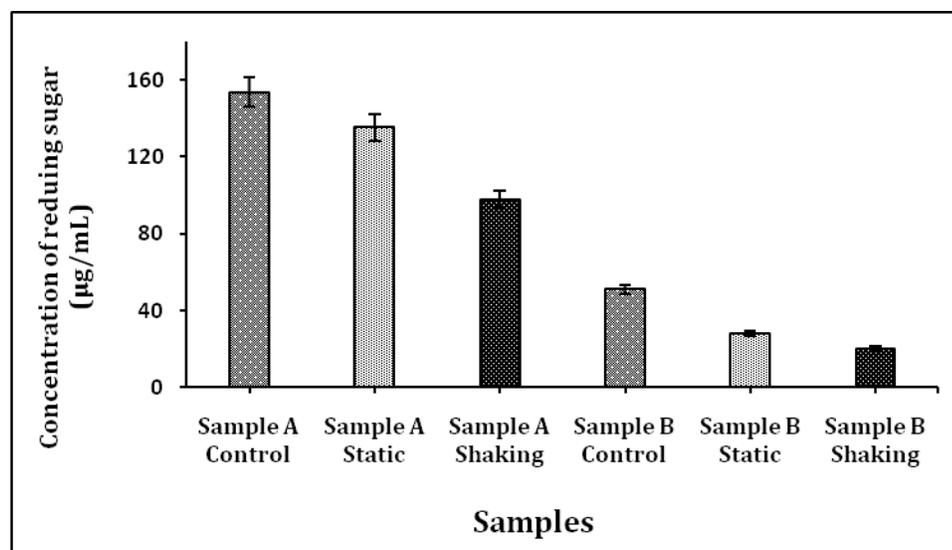


Fig.3 Reducing sugar content of control and treated paper and pulp effluent

Biochemical Analysis of Control and Treated Paper and Pulp Effluent

Quantification of Total Sugar through Anthrone test

After treatment (at day 10) by *A.flavus* F10, the total sugar content was decreased in treated sample as compare to their control (Fig. 2). The decrease in total sugar content was more in sample B as comparison to sample A. The incubation conditions did not affect the utilization of the carbohydrate in treated samples.

Glucose was added during the biological treatment of effluent, which was used as a supportive carbon as well as energy source for rapid growth of fungus and degrade the phenolic or aromatic compounds present in the effluent. The study shows that the fungus utilized the added glucose as a primary carbon source. The efficient decolourization of paper pulp effluent in the presence of glucose was also reported in several previous studies (Ragunathan *et al.*, 2004; Prasongsuk *et al.*, 2009; Singhal *et al.*, 2009).

Quantification of Reducing Sugar

Result were presented in Fig. 3 indicated that both types of treated samples (shaking and static) the amount of final reducing sugar was significantly low. Among treated samples, the utilization of reducing sugar were high in shaking condition as compare to static.

Total Polyphenol

Result presented in Fig. 4 indicate that the test fungus reduce the polyphenolic content in both type of treatment condition (shaking and static). The amount of final polyphenolic was significantly low in treated sample as compared to both control samples. The reduction in polyphenolic content is the clear sign that the fungus utilize polyphenol efficiently. Fungus ligninolytic enzymatic system reduced these polyphenol into small monomeric fractions, which were ultimately consumed by the fungus as a carbon source (Barapatre *et al.*, 2015). In the static condition the % reduction of lignin and carbohydrate content was high as compare to shaking condition,

which might be due to the production of high amount of ligninolytic enzymes. According to the Górska *et al.*, (2014) a higher production of the ligninolytic enzymes under agitation conditions may be the result of varied resistance of fungal hyphae to mechanical damage caused by shaking the culture as well as different requirements regarding oxygen and CO₂ concentration in the mycelium growth phase.

Decolourisation of Paper Pulp Effluent by Immobilized *A.flavus* F10 into Alginate Bead

The experiment result showed the decrease in the colour unit of effluent samples after treatment with the fungus. The percent decolourisation is shown in Table 3. It was found that the maximum decolourisation of 49.58% occurred in sample A treated by immobilized fungus whereas in sample B it was 93.11%. The percent decolourisation

was high in immobilized fungus sample in comparison to free fungus cells. The immobilization of microbial cell on the solid support or entrapment play an important role in the treatment of industrial waste water. The alginate immobilized fungus were also used for the decolourisation of paper and pulp effluent (Jha *et al.*, 2002; Ortega-Clemente *et al.*, 2009). Calcium-alginate beads with and without fungus were prepared by the liquid curing method in the presence of Ca²⁺ ions. Ca-alginate was used as a support material for the immobilization of the *A. flavus* (F10). The advantage of immobilized fungus was the repetitive batch decolourisation and long term preservation. Data of the study indicated that more efficient treatment was achieved by the immobilized fungi as compare to free cells. Jha *et al.*, (2002) and Ortega-Clemente *et al.*, (2009) found that immobilized fungus efficiently remove/degrade the lignin and reduce the colourity of pulp effluent.

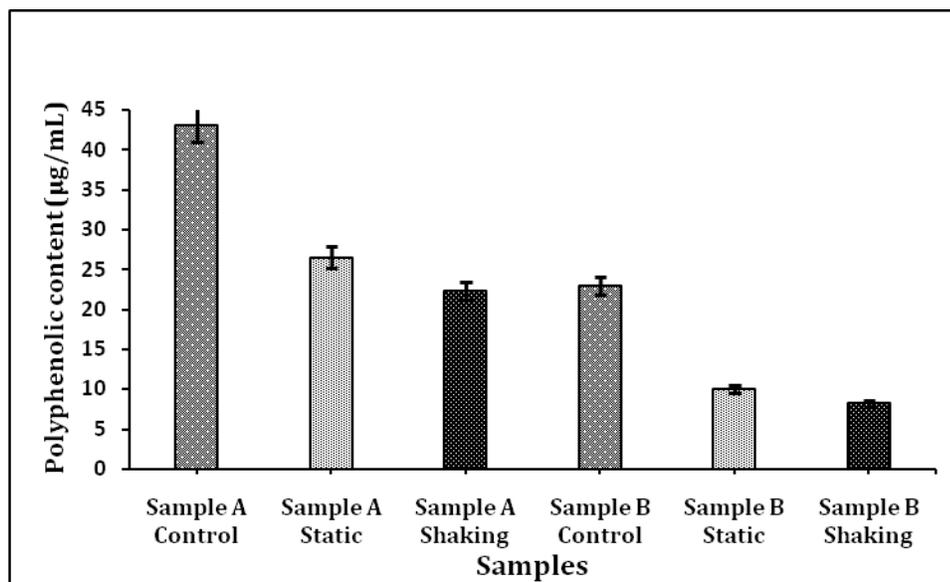


Fig.4 Total polyphenol content of control and treated paper and pulp effluent

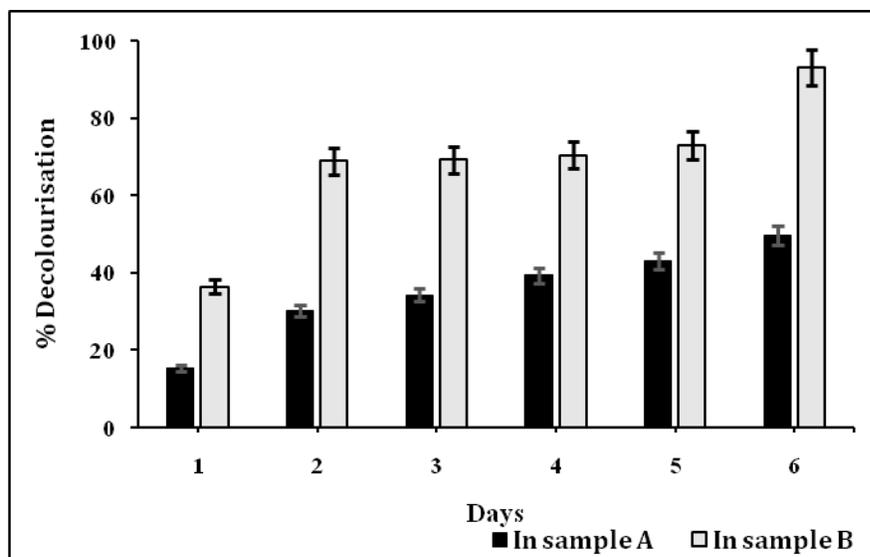


Fig.5 Colour decolorization of paper pulp effluent using Immobilized *A.flavus* F10 into alginate bead

Table.3 Phytotoxicity analysis

Sample	Length of shoot (cm)	Length of root (cm)	Germination %	Root: shoot ratio	GI index (%)
Control	14.50	8.60	100	1 : 1.69	-
Sample A untreated	3.80	2.30	26.66	1 : 1.65	7.04
Sample A treated static	10.66	7.20	73.66	1 : 1.48	61.67
Sample A treated shaking	10.75	6.7	86.66	1 : 1.6	67.51
Sample B untreated	10.68	6.0	80.00	1 : 1.78	55.81
Sample B static treated	12.22	5.80	100.00	1 : 2.11	67.44
Sample B shaking treated	13.67	7.70	100.00	1 : 1.77	89.53

Seed Germination Test

The result of phytotoxicity test of treated and non-treated paper and pulp mill effluent was verified on *Phaseolus mungo* seed germination test on different parameter like the percentage of seed germination, root-shoot length and root shoot ratio and presented in Table 3. It was observed that *Phaseolus mungo* when grown on

distilled water (control) and treated effluent sample showed a high percentage of germination, and a high shoot and root growth. On the other hand, the *Phaseolus mungo* seeds grown on untreated effluent showed low germination percentage, high growth inhibition and less root, shoot length as compared to the seeds grown on control. As expected, GI was low for the untreated

sample and the sample A was most toxic for the growth of *Phaseolus mungo* in present study. The sample which treated in shaking condition show high GI (67.51 and 89.53%) index in comparison to sample treated in static condition (61.67 and 67.44%). The poor germination and growth of seeds on raw effluent were due to high toxicity and high pollution load associated with the effluent, which was the one of the reason due to which untreated sample A have lowest GI index. Whereas sample B shaking

having highest GI index ($\approx 90\%$) indicate that the sample having no phytotoxicity, while all other samples having GI index (50-90%) indicates low phytotoxicity. These results indicate that the biological treatment have reduced pollution load in effluent and reduce the possible toxicant to low levels. The reduction of the toxicity of pulp effluent by biological treatment was also successfully reported (Malaviya *et al.*, 2007, Raj *et al.*, 2014).

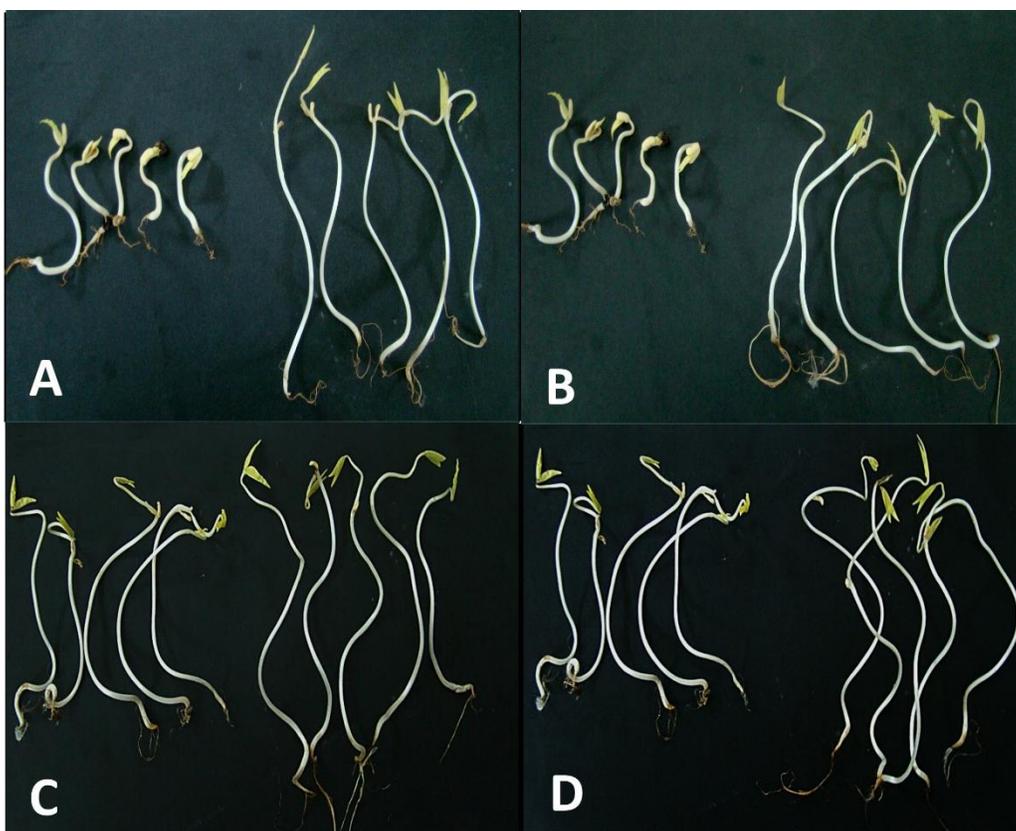


Fig. 6: (A) Untreated Sample A (left) with treated sample A (right) in static condition. (B) Untreated Sample A (left) with treated sample A (right) in shaking condition. (C) Untreated Sample B (left) with treated sample B (right) in static condition. (D) Untreated Sample B (left) with treated sample B (right) in shaking condition.

Pulp and paper industry is one of the most water-dependent industries, which utilize about 50 m^3 water to produce a ton of paper (Buyukkamaci *et al.*, 2010). Effluents of the pulp and paper industry contain a number of

toxic compounds and may cause deleterious environmental impacts upon direct discharge to receiving waters (Catalkaya *et al.*, 2007). Paper and pulp effluent comprises an offensive colour due to the presence of

lignin and its derivatives and also distort the water quality which is not only aesthetically unacceptable, but also inhibit the photosynthesis process in the stream due to absorbance of sunlight. This reduction in the sunlight amount will leads to a great adverse effects on the aquatic ecosystem, and as well as in the growth of primary consumers, and these will ultimately affect the secondary and tertiary consumers (Murugesan, 2003).

In conclusion, the results indicate that the utilization of *Aspergillus flavus* F10 is suitable for the Decolourisation and polyphenolic groups removal from soda pulping effluents in the presence of a carbon source. The overall removal efficiencies of *Aspergillus flavus* F10 for reduction in the colour of effluent were observed after 10th and 6th day of incubation in free and immobilized form. Further, the toxicity of treated effluent was significantly reduced and the treated effluent was able to support the growth of *Phaseolus mungo* even in undiluted form suggesting improved properties of biologically treated effluent.

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