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Mycoremedial Approach for Biodegradation of Textile Dyes

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ABSTRACT

Mycoremediation, a form of bioremediation facilitated by fungi, has been employed for the biodegradation of azo dyes, including congo red, acid red, and basic blue. In this study, four fungal isolates, namely *Aspergillus niger, Aspergillus piperis, Penicillium oxalicum, and Penicillium chrysogenum,* obtained from dye effluent were used. Four biodegradation methods were selected, including solid media dye accumulation, liquid media methods under stationary and shaking conditions at 28°C, and biosorption using dead biomass. For the first time, we report the involvement of the isolated fungal species *A. piperis*, derived from textile effluent, in the biodegradation of textile dyes. Comparative spectrophotometric analysis revealed more than 90% of dye decolourization in the fungus-inoculated medium supplemented with dyes, compared to control. Higher percentage of decolorization were obtained under shaking conditions compared to the stationary method. Dead fungal biomass exhibited effective dye absorption, resulting in a noticeable color change during dye degradation. The seed germination bioassay revealed that treated dye solutions promoted germination; however, untreated inhibited it. Further, untreated dye effluent hindered microbial growth. The excellent performance of *A. piperis* in the biodegradation of textile azo dyes with diverse chemical structures highlights and reinforces the bioremediation potential of these fungi for environmental decontamination.

Keywords: *Aspergillus piperis*, Biosorption, Bioremediation, Decolorization, Dyes, Mycoremediation

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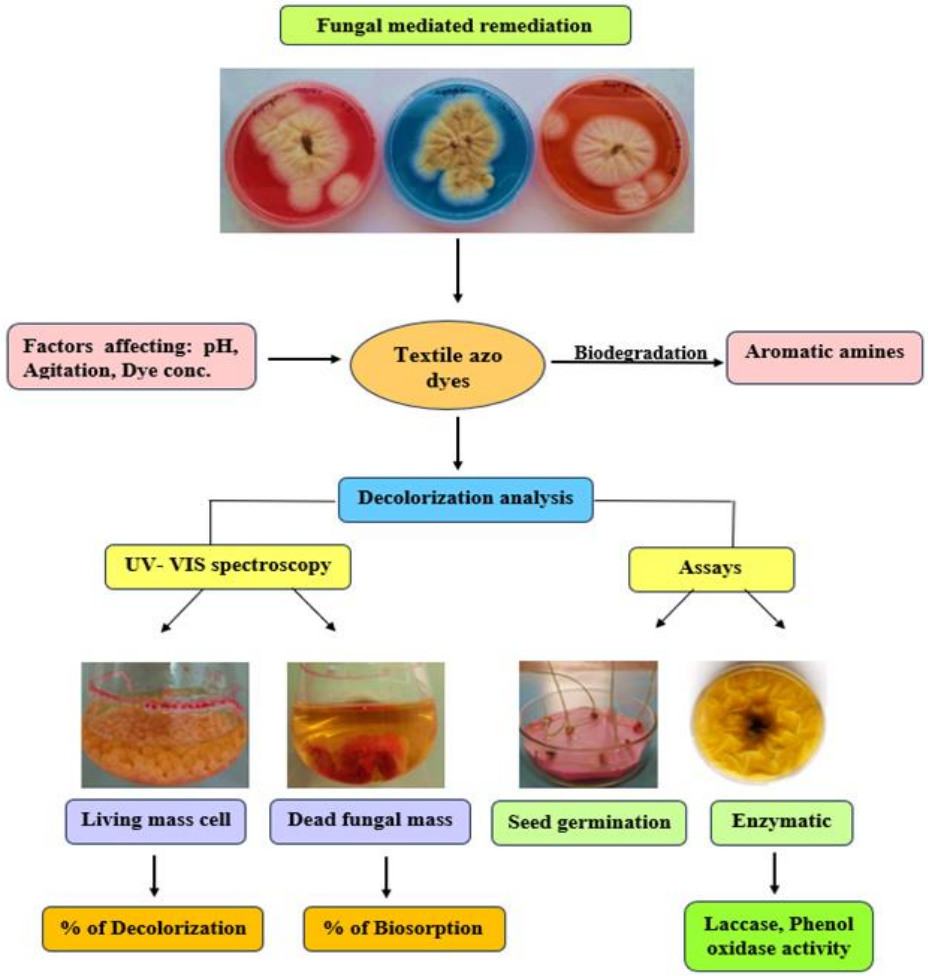

I. INTRODUCTION

A significant amount of chemicals, including dyes, are being manufactured and used in daily life as a result of rising industrialization and urbanization. Worldwide, textile wastewater is one of the most hazardous pollutants , with detrimental effects on the environment and human health [13, 28]. About 100 000 commercial dyes are processed such as acidic, basic, reactive, azo, diazo, anthraquinone based metal complex dyes [5] in food, cosmetics, paper, plastic and textile industries. But, most of them are potential toxins and carcinogen for living organisms. As per reports, 15–50% of these dyes end up in wastewater, which is frequently used for irrigation in underdeveloped nations [35]. The azo dyes have an impact on nitrogen and carbon cycles in the environment and cause pollution in the soils. Azo dyes, which are widely used in the textile industry, contain electron-withdrawing groups that cause electron depletion and lead to resistance of degradation [11, 27]. The by-products of azo dyes degradation are primarily aromatic amines with complex configurations, and they can potentially be cancerous. Toxic compounds from the dye effluents pass through the food chain and ultimately reach humans causing various physiological disorders, like hypertension, sporadic fever, renal damage and cramps due to biomagnification. Some azo dyes have been linked to human bladder cancer, splenic sarcomas,

hepatocarcinoma and nuclear anomalies in experimental animals and chromosomal aberrations in mammalian cells [34]. Further, studies have shown that azo dyes at concentrations ranging from 5 to 50 mM impacts zebrafish development [34] such as hatch difficulties, genetic defects including heart-swelling, reduced heart rate, placenta swelling, and spinal deformities including spine bending and tail deformation [34].

A very minimum amount of dye in water (10–50 mgL-1) is highly visible and reduces light penetration in aquatic systems, which causes a negative effect on photosynthesis [10, 23]. Agricultural lands with textile wastewater severely affect soil fertility, altering its chemical and biological status and decreases the plant productivity. Effluents make plants susceptible to many pathogens. Some of the textile dyes are even carcinogenic and mutagenic, and effluents reduce the rate of seed germination and plant growth. It has been reported that there is a decrease in the chlorophyll content and endogenous cytokinin, while there is an increase in endogenous abscisic acid [15, 29] and proline concentration [29]. Directly or indirectly these toxic effluents are being settled in water bodies that finally contaminate the water and soil. It has the various adverse impacts on the water quality in terms of total organic carbon (TOC), biological oxygen demand (BOD), chemical oxygen demand (COD), color, pH and presence of recalcitrant synthetic compounds, such as azo dyes and heavy metals [1, 4].

The dye effluents are mutagenic, carcinogenic and toxic [7, 34]. The major structural element that is responsible for absorption of light and imparts color of the dye is the chromatophore group [2], while the electron withdrawing or donating substituents that cause or intensify the color of the chromophores are the auxochromes. The synthetic origin and complex aromatic structures of dyes make them stable and difficult to degrade. The color fastness, stability and resistance of dyes to degradation have made color removal from textile wastewaters difficult, as they are not readily degraded. The common treatment methods applied for color removal from dye effluents consist of integrated processes involving biological, physical and chemical decolorization methods [3, 16, 36]. Physico-chemical treatment processes, such as coagulation, precipitation, filtration, adsorption, photolysis and oxidation with hydrogen peroxide or ozone, can produce a large volume of sludge and usually need the addition of other environmental hazardous chemical additives [25, 26, 33, 41]. For treatment of the dye wastewater some methods like chemical and physical methods are not widely applied to textile industries because of exorbitant costs and disposal problems. Methods like green technologies can be used to deal with such problem include adsorption of dyestuffs on bacterial and fungal biomass [14, 22, 42] or low-cost non-conventional adsorbents [9, 12].

Further, chemical treatments can create an additional chemical load in water bodies and end up with sludge disposal issues. The alternatives to the traditional physicochemical methods are biological treatment technologies as they are low-cost, environmentally friendly and act selective to provide a complete degradation of organic pollutants without destruction of flora or fauna on the sites [1, 8, 20]. Due to this, as better alternatives, biological processes are getting more and more attention since they do not produce harmful by-products [22].

One of the most effective cleaning techniques for the removal of toxicants from polluted environments is bioremediation [24, 39]. The microbial degradation of textile dyes has been reported using different microorganisms including bacteria, yeasts and filamentous fungi [8, 36, 38]. Among them, mycoremediation based on the exploitation of fungal strains for dye degradation have been quite preferable, as fungi due to their large surface area and ease of solid-liquid separation provide an effective system and multiple mechanisms for the degradation of organic and inorganic contaminants [39]. Most of mycoremediation studies utilizing fungi *Phanerochaete chrysosporium,*

Bjerkandera adusta, Trametes versicolor, Phlebia radiata and *Pleurotus* spp. have also focused on production of enzyme laccase that was found to be related to lignin and dyes degradation [19, 39]. These fungi can remove dyes and pigments either in living or dead form using lignin peroxidase, manganese peroxidase, manganese independent peroxidase and laccase through bio-sorption, bio-degradation, bioaccumulation and enzymatic mineralization [17, 40].

Our study explored the role these potential fungal species for bioremediation; inhibition activity and ligninolytic enzymatic activity; and the percentage of achieved dye decolorization in both semi-solid and liquid media; and assessment of the impact of mycoremediated and untreated dye solutions through assays on seed germination and microbial growth.

II. MATERIAL AND METHODS

A. Dyes used

Azo dyes are artificial compounds that include intramolecular N– –N bonds. It provides vivid, high-intensity colors and its main benefit is, their low cost of production. The following azo dyes were used for the present study: Congo Red, Basic Blue (7) and Acid Red (1).

B. Sources of fungal strains

The dye effluent was collected from Textile Mill near Sector 23, Mathura Road, Faridabad, Haryana. The fungal strains used in the study were isolated from the samples from industrial effluent and maintained on Sabouraud Dextrose Agar (SDA) medium. The fungal strains were isolated from the sample and grown as pure cultures [18].

C. Molecular identification of fungal strains

Molecular identification of these fungal strains was undertaken. DNA was isolated as described in Cetyl Trimethyl Ammonium Bromide (CTAB) protocol [31]. PCR was performed using the ITS 4 and 5 primers [21]. For sequencing the amplicon was outsourced to Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka. The sequence was analysed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

D. Assay for the measurement of percentage of inhibition in solid media

The fungal mycelial agar plugs (~5 mm) were cut approximately from the colony margin and inoculated on SDA plates. The optimum growth was evaluated by measuring growth diameters on solid culture media. Percentage inhibition of fungal growth during degradation was measured and calculated using the following formula:

$$I=C-T/C \times 100$$

Where, I = Percentage of inhibition in fungal growth, C= growth in terms of colony diameter in control and T=Growth in terms of colony diameter in the sample.

E. Assay for the dye decolorization activities of fungi in liquid media

100 mL of Erlenmeyer flask containing 70 mL of liquid culture medium and the dye (0.2 gL-1) was used for the assay. One fungal agar disc of 1 cm diameter was added. The culture media were incubated for 10 days (~28°C and humidity 75%) either under static conditions and shaking conditions. The culture filtrates were collected using centrifugation at 10000 rpm for 20 min and subjected to spectrophotometric analysis. For the congo red, basic blue and acid red, the absorbance reading was done at 497 nm, 630 nm and 494 nm wavelengths respectively. The extent of percentage of dye decolorization was calculated using the formula below:

$$\text{Percent dye decolorization (PDD\%)} = \text{Abs. (C)} - \text{Abs. (I)} \times 100 / \text{Abs. (C)}$$

Where, C = Absorbance of control and I = Absorbance of inoculant

F. Assay for the effect of dead fungal mass on biosorption

The fungal cultures were grown in 1000 mL Erlenmeyer flasks using spore suspensions in 700 mL of SDA liquid medium and incubated in an orbital shaker at 150 rpm. After 5 days, the biomass was collected by vacuum filtration, and dried. The mycelium was inactivated by autoclaving. About 2 g of inactivated mycelium was taken to 50 mL of culture media in 100 mL Erlenmeyer flasks containing dyes. The pH of the medium was adjusted to 8 by using 1 N NaOH before autoclaving, and the cultures were incubated in an orbital shaker (150 rpm at 28°C). The kinetics of decolorization was studied by collecting 2 mL of aliquots at 24, 48 and 72 h. The absorbance reading was done at 497 nm, 630 nm and 494 nm wavelengths for congo red, basic blue and acid red respectively. Control experiments were prepared without the mycelium. Percentage of dye biosorption was calculated using the following formula:

Percent dye biosorption (%) = Abs. (C) –Abs. (I) ×100 /Abs. (C)

Where, C = Absorbance of control and I = Absorbance of inoculant

G. Seed germination bioassay

Effect of bioremediated and untreated dye solution were observed on wheat seed germination. The wheat seeds were sterilized using 0.1% $HgCl_2$ solution for 50 sec, washed 5–6 times with sterile distilled water to remove traces of $HgCl_2$. In sterile petri plates sterile filter paper was kept soaked in bioremediated, untreated dye solution and with sterile distilled water-soaked filter paper as control, respectively. 10 wheat seeds were kept in each petri plate. Observation on seed germination was taken for four consecutive days. The experiment was conducted at room temperature of 25°C.

H. Ligninolytic enzymatic activity on solid medium

2.8.1 Laccase activity: The solid culture SDA medium contained 0.01% (w/v) of guaiacol (orthomethoxy phenol) was prepared and autoclaved (at 121°C for 20 min). 5 mm^2 of agar containing the fungus was placed in the petri dish. After a few days, at ~28°C a red zone of coloration appeared around the colony, indicating the presence of laccase [6, 30].

2.8.2 Phenol oxidase activity: A piece of 5 mm^2 agar with the fungus inoculated in a petri dish containing the solid culture SDA medium with 0.5% of gallic acid. The appearance of a brown area around the colony after 3 days at ~28°C indicates the presence of polyphenol oxidases [6, 32].

III. RESULTS AND DISCUSSION

The present study has been carried out to examine the degradation of textile dyes such as congo red, acid red and basic blue from the isolated fungi. The isolated fungi were identified by ITS gene sequencing as *Aspergillus niger, Aspergillus piperis, Penicillium oxalicum* and *Penicillium chrysogenum*. We first time reported the involvement of an isolated fungal species *A. piperis* obtained from the textile effluent in biodegradation of textile dyes.

These colonies were first observed for the macroscopic growth on the plate. The *A. niger* formed white sparse colonies which turned to black colonies with conidial production. *A. piperis* colonies were creamy white to yellowish color and later had the wrinkled mycelial growth which turned to brown-black conidia production. Initially, *P. oxalicum* colonies were white in color and later turned to bluish- greenish color giving it a fuzzy texture. *P. chrysogenum* had the fluffy blue color mycelium (Fig.1).

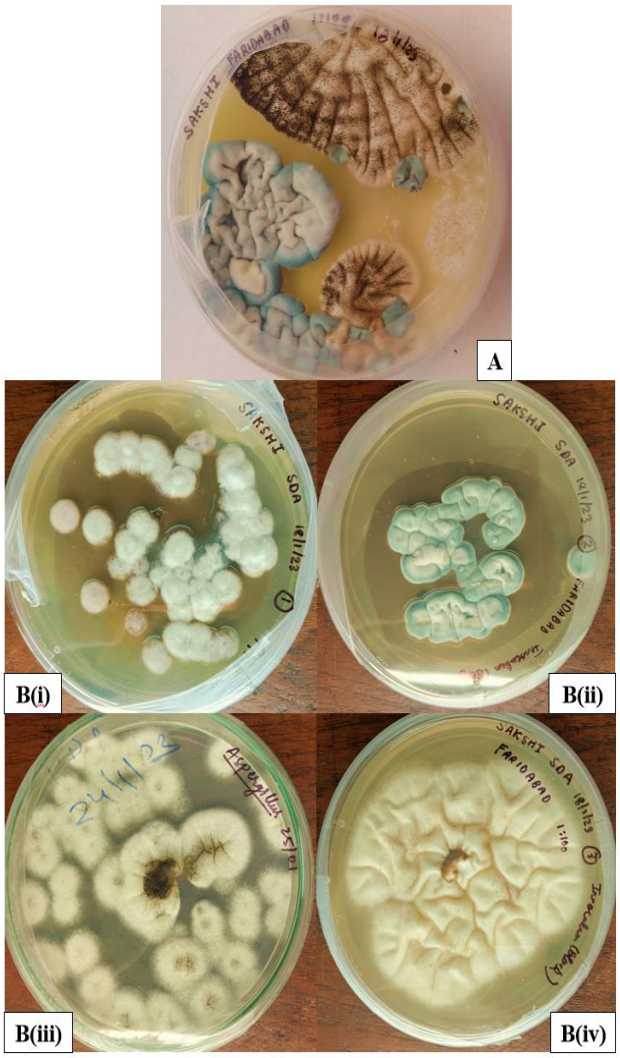

Fig. 1 Isolation of fungal strains from textile effluents

A) Mixed culture plate after the inoculation of textile effluents

B) Individually isolated pure colonies:

i) *Penicillum oxalicum* ii) *Penicillium chrysogenum* iii) *Aspergillus niger* iv) *Aspergillus piperis*

The initial screening for dye decolorization by fungal strains were done using semi-solid media (Fig.2).

Fig. 2 Growth of *A. niger*

In Congo Red, Basic Blue and Acid Red after inoculation A) On 1st day B) On 4th day C) On 7th day

The degradation was assessed either by disappearance of color from the culture medium or accumulation of color by the fungal colonies during their growth (Fig.3).

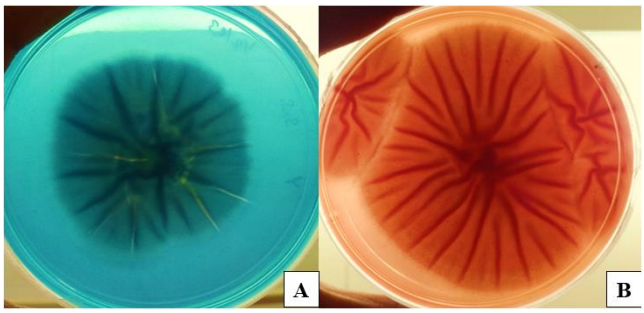

Fig. 3 Accumulation of dyes

A) Basic Blue dye by *P. oxalicum* B) Acid Red dye by *A. piperis*

Decolorization of dye was seen in all four fungal strains. Moreover, the dye accumulation was also observed in all four fungal species but to varying degrees.

P. oxalicum and *P. chrysogenum* exhibited more inhibition of its growth in the media containing all types of dye. Out of these dyes, acid red has been found to show maximum growth inhibitory effect in *P. chrysogenum* (52%). On the other hand, *A. niger* (30.68%) showed the minimum inhibitory growth. The maximum accumulation of dyes was also observed in acid red and basic blue by *A. piperis* and *P. oxalicum* respectively which might be due to the production of extracellular enzymes by the respective fungus, during the biodegradation of tested dyes.

We also studied the dye decolorization under stationary and shaking conditions; we got encouraging results after 5 days, but maximum decolorization of all the dyes were obtained after 10 days. After 15 days of growth in liquid medium, approximately 95% decolorizations was observed for the all dyes. Under stationary conditions, highest decolorization was observed by *P. oxalicum* in congo red (97.1%), followed by *A. piperis* in basic blue (94.8%). In acid red dye the fungus showed relatively less decolorization as the rate of decolorization were moderately slowed down (Fig.4).

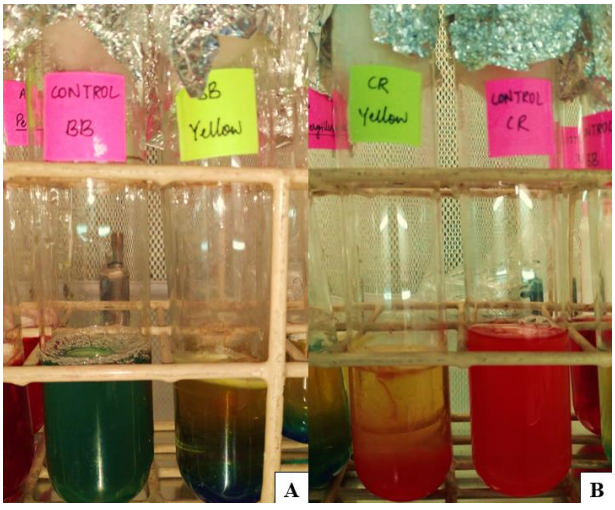

Fig. 4 Degradation of dyes under static conditions

A) Basic Blue dye degradation by *A. piperis*
Left tube: Control; Right tube: Test

B) Congo Red dye degradation by *P. oxalicum*
Left tube: Test; Right tube: Control

Similarly, under shaking conditions showed, in congo red highest decolorization was observed by *P. oxalicum* (99.5%) (Fig.5).

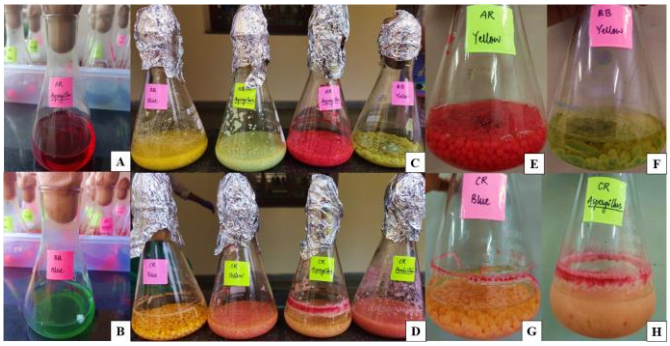

Fig. 5 Decolorization of dyes by living fungal cell under shaking conditions

A and B shows the initial dye concentration **C and D** shows decolorization of Acid Red and Congo Red dyes by all isolated fungal species, **E and F** shows decolorization of Acid Red and Basic Blue dye by *A. piperis* under shaking conditions, **G and H** shows decolorization of Congo Red by *P. chrysogenum* and *A. niger* respectively

In acid red highest decolorization was observed by the fungus *P. chrysogenum* (93.4%), followed by *A. niger* (92.9%), *P. oxalicum* (92.2%) and *A. piperis* (91.8%). In basic blue the maximum decolourization was observed by *A. niger* (99.1%), followed by *A. piperis* (97.1%). All the fungal isolate from the effluent showed the ability to degrade the dyes. On collecting the filtrates of the culture containing the dyes after definite time intervals a gradual decrease in absorbance was seen. These colour changes resulted in changes of UV-VIS absorption values during this period and these absorption values may have been due to a reaction of the dyes with enzymes secreted by the fungal mycelia. Under constant shaking conditions the overall dye biodegradation was better compared to stationary state (Fig.6 &7).

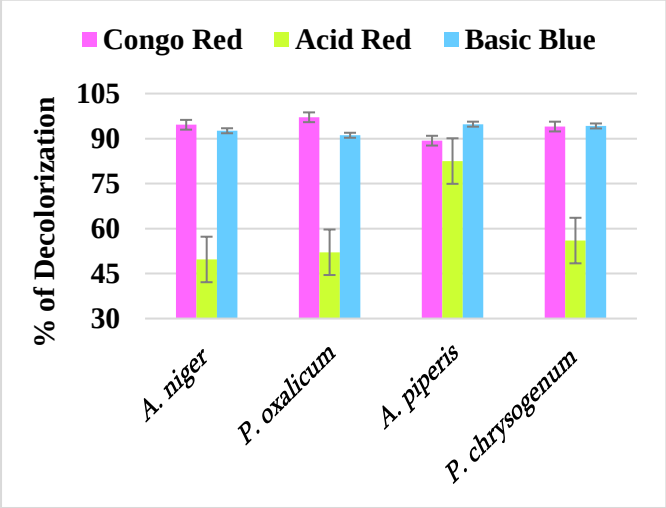

Fig. 6 Percentage of dye decolorization in liquid media under static conditions

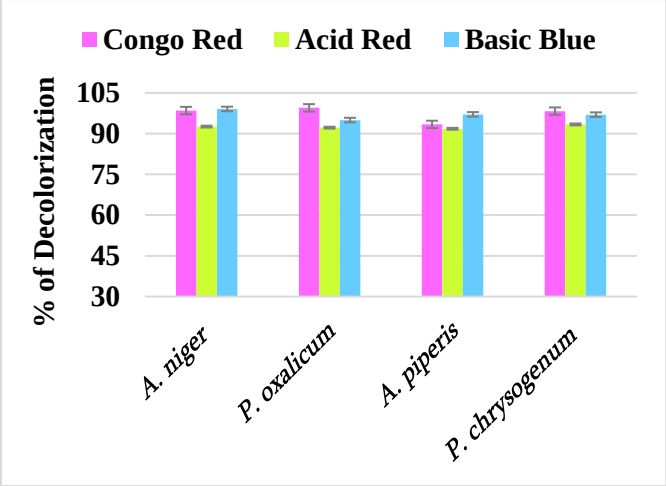

Fig. 7 Percentage of dye decolorization in liquid media under shaking condition

Agitated conditions offer many advantages over static conditions for development of practical processes as because of high decolorization yield (Fig.7). Under shaking conditions, agitation increases mass and oxygen transfer between cells and the medium. In addition, enzyme activity too could have depended on the presence of oxygen [37]. In degradative decolorization for optimal activity of dye degradative enzymes, optimum temperature and oxygen transfer is necessary.

Our results showed that dead fungal biomass used in this study could effectively remove textile dyes. It was observed that within the 4 days of incubation, the media containing the dyes gradually become colourless or less coloured (Fig.8).

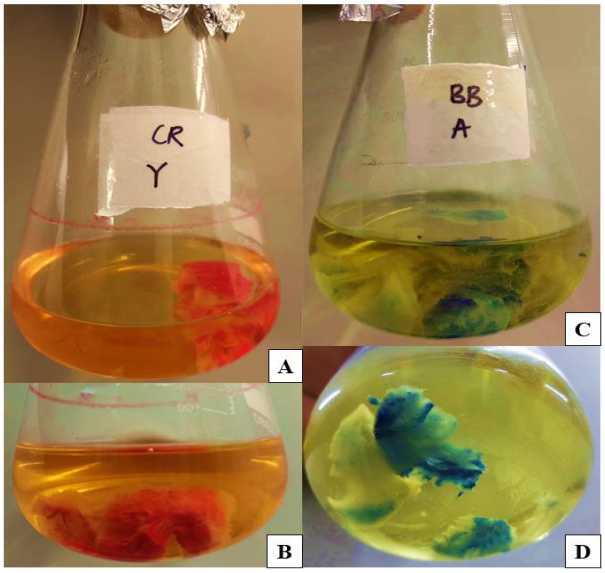

Fig. 8 Biosorption of dyes by dead fungal mass under shaking conditions

A and B shows the absorption of Congo Red dye by *A. piperis,* **C and D** shows absorption of Basic Blue dye by *A. niger*

All the fungal species showed the percentage of biosorption between 90-99.4% except in fungi *P. chrysogenum* for the dye acid red (83.5%). *P. oxalicum* showed the highest biosorption rate in congo red (99.4%) as well as in acid red (96.3%). In basic blue, *A. niger* (99.1%) showed the highest rate, followed by *P. chrysogenum* (98.4%). It was noticed that the effect of dead fungal mass was higher than living cell, in the rate of decolorization (Fig.9).

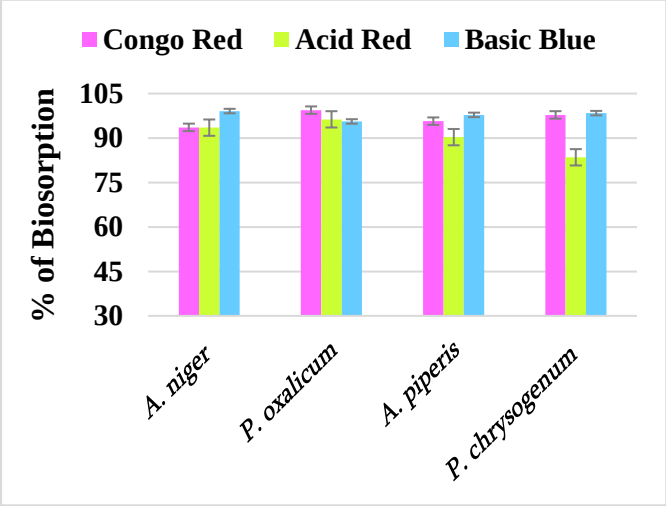

Fig. 9 Percentage of biosorption by dead fungal mass in liquid media under shaking conditions

The drastic colour change was observed in all the dyes degradation. The dead fungal biomass showed effective dye absorption. It was observed that after 3 to 4 days of incubation, the media containing dyes gradually become less coloured. The reason might be that the powdered form of fungi had smaller particle size apparently resulting in higher as well as faster adsorption. This change can be due to increase in adsorbent surface area and availability of more adsorption sites. The biosorption of dyes is due to the cell wall composition of fungi that is made up of heteropolysaccharide and lipid components, which contain different charged functional groups causing strong attractive forces between the azo dye and the cell wall [43].

Ligninolytic enzymatic activity on solid medium was analysed by the presence of a red zone of coloration around the colony, indicating the presence of laccase. Laccase activity was observed in *A. niger* using guaiacol as a substrate. After 5–6 days of growth, a reddish-brown coloration around the colony, indicating activity of laccase. This coloration might be due to polymerization by oxidation of guaiacol in the presence of laccase. The maximum red zone was created by *A. niger.* The appearance of a brown area around the colony indicates the presence of polyphenol oxidases. All the fungus shows the phenol oxidase activity. The appearance of the brown area was arranged in increased order *P. chrysogenum* < *A. niger*< *A. piperis*< *P. oxalicum.*

Detoxification of all the dyes was finally approved and tested by the wheat seed germination bioassay. The untreated dyes or textile effluent (control) inhibited the wheat seeds germination after 3–4 days of incubation, while in treated dyes treatments the seed germination was observed after 48 h (Fig. 10).

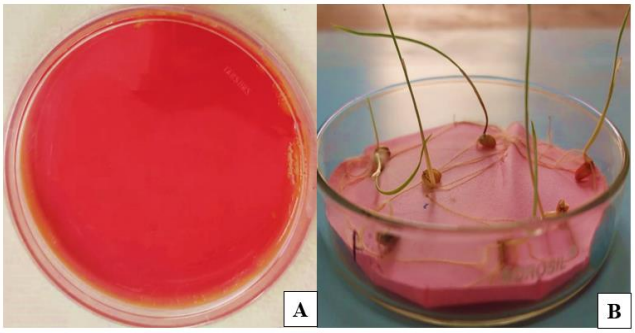

Fig. 10 Germination assay

A) Control (Untreated dye) **B)** Germination of wheat seeds in treated dye

From the above result it shows that the strain of *A. piperis* has a strong ability to degrade these dyes studied i.e., in static and shaking conditions. Even in dead fungal mass it shows the positive result, it can degrade the dyes up to 90%. It was probably due to the action of the enzyme laccases in that strain.

IV. CONCLUSION

The degradation results obtained from fungal utilization in dye decolourization are mainly from the laboratory tests. Therefore, essential work on this topic is still in the laboratories and less at commercial level to solve the problem through mycoremediation. In this study we have observed higher decolorization under shaking conditions, which could be due to better oxygenation of the fungus and regular contact of secreted enzymes with dye molecules to decolorize it, moreover agitation also helps the fungus to grow better.

For development of practical processes agitated conditions offer many advantages over static conditions. Further, operating conditions like dye concentration, pH and temperature may negatively affect the decolorization potential of growing cells but, compared with living cells, dead cells may be stored or used for extended periods and regeneration is simple. Dead biomass can be obtained easily and can be used as cheap and effective biosorbents. Being environmentally friendly, bioremediation has been characterized as an attractive and alternative soft technology. A well-planned strategy is needed to substantially exploit fungal strains for bioremediation. One of the most important factors, which have a great impact on the setting of a proper bioremediation plant for textile wastewater is the identification and research of new fungal strains with the aid of molecular techniques that may improve applicability in mycoremediation processes. It is anticipated that fungal remediation will be soon a reliable and competitive dye remediation technology. Further investigations on isolation and purification of enzymes involved in the biodegradation of hazardous dyes are required for the proper exploitation of this mycoremedial approach.

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