International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 159-169
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Degradation of Azo Dyes Using Mycoremediation

E. Pundalik[1], A. Salagare[2] and G.D. Tandon[3]
[1-3]Dr. D Y Patil Biotechnology, Bioinformatics Institute, Dr. D Y Patil Vidyapeeth, Pune, Tathawade-411033 (India)

Received: 15 Mar 2019 / Accepted: 19 Apr 2019 / Published online: 1 Jul 2019
***Corresponding Author Email:** g.tandon@dpu.edu.in

Abstract
Environmental pollution from human activities is a major challenge for civilization today. Synthetic dyes such as azo dyes are hazardous and toxic for humans, animals as well as aquatic life at the concentration at which they are being discharged in natural waters. Microbial degradation of dyes has received much attention for treating industrial wastewater containing dyes in an environment friendly way. Among biological treatment, a potential method is the fungal bioremediation. Current study aims to investigate the potential of certain fungi for decolourization of textile dyes. In the present study, the bio-chemical and morphological characterization of fungal species was done according to the conventional methods. The fungal isolates used were *Aspergillus fumigatus, Aspergillus niger, Penicillium purpurogenum, Acremonium strictum and Fusarium moniliforme*. The decolourization of selected azo dyes viz. Acid Orange 7, Acid Violet 49, Basic Blue 3 and Basic Yellow 3 was carried out using these fungal isolates at different time intervals under different conditions of pH and temperature. The isolated fungi showed varied degrees of decolourization with different dyes. During the decolourization process the bio-sorption by fungal mycelia played the major role compared to the enzymatic degradation of the dyes. The maximum adsorption was observed in Basic Blue 3 by *Penicillium purpurogenum*, which adsorbed 96% of the dye.

Keywords
Azo dyes, Acid Orange 7, Acid Violet 49, Basic Blue 3 and Basic Yellow 3, Decolourization and Fungal Bioremediation

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1. INTRODUCTION
Two of the most hazardous aquatic pollutants that are affecting aquatic ecosystem include heavy metals and dyes. Dyes are the chemical compounds that impart colour by attaching themselves to fabrics. These are used in different industries like cosmetics, plastics and textile, pharmaceutical, food and paper industries. Textile dyes constitute a major source of pollution. They are chemically diverse in nature and can be divided into azo, reactive, triphenylmethane, heterocyclic and polymeric dyes [5]. Most commonly used are azo dyes, accounting for 70% of the total dye production [1]. Reactive azo dyes are highly recalcitrant to conventional wastewater treatment processes, with as much as 90% of reactive dyes remaining unchanged after activated sludge treatment [10]. Wet processing in textile industry generates a large amount of wastewater, whose pollution load arises not only from the removal of impurities from the raw materials themselves but also from the residual chemical reagents used for processing. Colour is one of the major problems of

these types of wastewaters. Most synthetic dyes are not degraded by conventional physical and chemical processes and therefore microbial degradation of dyes has received much attention from the viewpoint of treating industrial wastewater containing dyes.

Similar results have been reported from bacteria isolated from textile effluents discharging sites [3, 9, 13-17]. Some researchers have isolated effluent adapted bacteria such as *Bacillus firmus* and *Halomonas sp*. [25] that have the potential of reducing textile azo dyes. Other dye decolorizing bacteria, such as *Pseudomonas aeruginosa* [15] and *Comamonas sp.* [13] were also reported from waste contaminated sites. This study aims to investigate the potential of certain fungal cultures isolated from similar wastewater samples for decolourization of textile dyes such as Basic Blue 3, Acid Orange 7, Basic Yellow 3 and Acid Violet 49.

2.MATERIALS AND METHODS

2.1 Isolation of Fungal Strains

The fungal cultures were isolated from various soil and water samples from Pune, Maharashtra, India. The cultures were re-isolated by growing on Sabouraud Dextrose Agar (SDA) media. The fungal isolates were subcultured on different agar media for their growth studies and identification. Their growth was observed on Potato Dextrose Agar (PDA), SDA and Czapek Dox Agar plates after 5 days of incubation at 28°C. The fungal stock cultures were maintained on Sabouraud Agar slants stored at 4°C with sub culturing after 30 days.

2.2 Selection of Dyes

Four dyes were selected from the commonly used textile dyes which were found to be fairly soluble in water. They were used to explore the potential of fungal isolates towards the decolourization of textile dyes **(Table-1**). Spectrum scan of the dye was obtained using Spectronic 20 spectrophotometer by finding the wavelength (λ) at which absorbance of light by a solution of coloring agent has a maximum value (λ_{max}).

Table 1: Different Textile Dyes Used

Dyes	Molecular Formula	Molecular Weight
Basic Blue 3	$C_{20}H_{26}ClN_3O$	359.89g/mol
Acid Orange 7	$C_{16}H_{11}N_2NaO_4S$	350.32g/mol
Basic Yellow 3	$C_{17}H_{22}ClN_3$	303.83g/mol
Acid Violet 49	$C_{39}H_{40}N_3NaO_6S_2$	733.87g/mol

2.3 Identification of Fungal Isolates

A fungal mycelium was taken on a clean grease free slide containing a drop of cotton blue, the mycelium was teased using a sterile needle. The slide was observed under light microscope (40X/100X) to study their morphological characteristics with size measurements. The identification of fungal isolates was carried out based on microscopic morphological characteristics and colony characteristics on PDA, SDA and Czapek Dox agar plates and comparing them with the description given in the taxonomic guide [26, 27 and 28]. The identified fungal strains were preserved on SDA slants at 4°C in refrigerator and were served as stock cultures.

2.4 Screening of Isolates

For screening the dye decolourizing potential of fungal strains, the fungal colonies from all the isolates were grown on SDA medium for 5 days at 28°C. A circular piece of grown culture was cut with a cork borer with 8 mm diameter and placed on dye containing agar medium plates which was not supplemented with nutrients, so as to prevent the growth of fungi and for distinct observation of the zone of clearance due to the absorption of the dye. The dye agar plates without fungal discs were also incubated to serve as control. Triplicates were created and stored at 28°C for 7 days. The plates were observed for the appearance of zone of clearance. The size of the zone of clearance was measured In millimeters. The fungal strains which showed the zone of clearance on plates were selected for further dye remediation study in submerged culture.

2.5 Assay for the Dye Decolourization Activity of Fungi in Liquid Medium

The dye decolourization studies were carried out in submerged culture and all the experiments were performed in triplicates for statistical analysis. The selected strains of fungi were inoculated in 100ml Conical flasks containing 50ml of SD broth medium. The flasks were incubated on a rotary shaker at 28°C at 150rpm for 24hrs to allow the fungal growth and then supplemented with 0.5ml concentrated dye solution (2.0g/l) every 2hrs to make the strength to 0.5mg/50ml from each type of selected dyes.

The inoculated flasks after supplementation of dyes were continued to run on the rotary shaker for 6 days, the amount of dye decolourized was observed on each day. The uninoculated flask with only media supplemented with dye was maintained as control to determine the rate of dye absorbance. Every day, 2ml sample was drawn out from different dye flasks containing different fungal strains, centrifuged at 5000rpm for 10mins and the supernatant was collected to observe their optical density. The decolourization assay was carried out by measuring the optical density at specified wavelength [19]. The absorbance was taken at their respective wavelengths using spectrophotometer (Basic Blue 3 – 650nm, Acid Orange 7 – 480nm, Basic Yellow 3 – 430nm and Acid Violet 49 - 520nm).

After optical density measurement, percent decolourization was calculated using the following equation:

$$\mathbf{PDD} = \frac{\boldsymbol{A}\mathbf{bsorbance\ C} - \mathbf{Absorbance\ F}}{\mathbf{Absorbance\ C}} \boldsymbol{X}\ \mathbf{100}$$

PDD = Percentage of dye decolourization
Absorbance C = Absorbance of the control
Absorbance F = Absorbance of the inoculated flask

2.6 Effect of pH and Temperature on Growth and Decolourization Activity

Decolourization studies were also carried out for specific fungal cultures under a variable range of pH (4-9) and temperature (10- 45°C) to determine the most optimum conditions for the maximum decolourization activity of each fungal strain.

2.7 Determination of Rate of Dye Adsorption by Fungi

A loopful of culture of selected fungal isolate was inoculated in 250ml flask containing 50ml of SD broth medium and was incubated on a shaker incubator at 28°C at 150rpm for 24hrs. After a good growth was observed in the flask, the mycelium was filtered and taken out aseptically on a pre-weighed Whatman paper number 1, dried in hot air oven at 70°C for 4hrs and reweighed to get the weight of mycelia. The process was followed from the flasks incubated after the addition of individual dyes to different fungal cultures. The rate of bio-sorption of different dyes with various fungal strains was determined using the following equation:

The % of dye adsorbed by mycelia = Wt. of mycelia without dye / Wt. of mycelia with dye X 100

2.8 Determination of Enzyme Activity on Decolourization

The fungal culture was grown using 50ml Sabouraud Dextrose Broth and kept on 150rpm at 28°C for 24hrs. 2ml of dye solution was added to the broth and kept for 24hrs. The broth with dye adsorbed by mycelia was filtered out and 500µl of dye was again added and incubated in a shaker at 28°C for 4hrs. Optical density at required wavelength was measured every hour to determine the effect of extracellular enzymatic activity of the fungal isolate on the dye solution.

3. RESULTS AND DISCUSSIONS

3.1 Microscopic Studies for the Identification of Fungi

The fungal isolates were grown on SD Agar by incubating at 25^{0}C for four days. They were identified by microscopic examination using cotton blue stain. The colony colour and morphology, microscopic observations and other culture characteristics were used for the identification of the fungi **(Table 2)**. Five species of fungi were identified as *Acremonium strictum, Fusarium moniliforme, Aspergillus fumigatus, Aspergillus niger* and *Penicillium pupurogenum.* They were maintained on SD agar slants for further studies.

Table 2 - The Morphological and Microscopic Characteristics of Different Fungal Isolates

ISOLATE	MICROSCOPIC CHARACTERISTICS	IDENTIFICATION
F-01	Colonies on Czapek Dox agar are typically blue green with a suede-like surface consisting of a dense felt of conidiophores. Conidial heads are typically columnar (up to 400x50µm but often much shorter and smaller) and uniseriate. Conidiophore stipes are short, smooth-walled and have conical-shaped terminal vesicles which support a single row of phialides on the upper two thirds of the vesicle. Conidia are produced in basipetal succession forming long chains and are globose to sub-globose (2.5-3.0µm in diameter), green and finely roughened [27].	***Aspergillus fumigatus***
F-02	Colonies are usually slow-growing, often compact and moist at first, becoming powdery, and suede-like or floccose with	***Acremonium strictum***

	age, and may be white, grey, pink, rose or orange in colour. Hyphae are fine and hyaline and produce mostly simple awl-shaped erect phialides with inconspicuous collarettes. Conidia are usually one-celled, hyaline or rarely pigmented, globose to cylindrical, and mostly aggregated in slimy heads at the apex of each phialide. Chlamydospores present. [26].	
F-03	Rapid growth on Czapek Agar. Colonies initially white becoming tinged with lavender with a colourless to dark purple reverse. Hyphae are septate and hyaline. Conidiophores are medium simple or branched. Conidiogenous cells are monophialides. Macro conidia are sparse, very slightly sickle-shaped to nearly straight, i.e., "string bean-like", 5-septate, measuring 31-58 x 2.7-3.6µm. Micro conidia abundant, 0 to 1-septate, oval to clavate, measure 7-10 x 2.5-3.2µm, and occur in both false heads (a collection of conidia at the tip of the phialide) and chains [26].	***Fusarium moniliforme***
F-04	On Czapek Dox agar, colonies consist of a compact white or yellow basal felt covered by a dense layer of dark brown to black conidial heads. Conidial heads are large (up to 3µm by 15 to 20µm in diameter), globose, dark brown, becoming radiate and tending to split into several loose columns with age. Conidiophore stipes are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae twice as long as the phialides. Conidia are globose to subglobose (3.5-5µm in diameter), dark brown to black and rough-walled [27].	***Aspergillus niger***
F-05	Colonies on Czapek Agar 1530mm diameter, plane or radially sulcate, dense, velutinous, mycelium bright yellow or red due to encrusted hyphae; Conidiogenesis moderate to heavy, dark green, exudates orange to red; Soluble pigment vivd red, reverse dark red or purple. Conidiophores borne from surface or aerial mycelium, stripes 70-300µm long, smooth walled, bearing terminal biverticillate penicilli; Penicilli narrow, metulae and phialides appressed, each 10-14µm long; Conidia ellipsoidal, sometimes becoming sub-spheroidal at maturity, 2-3µm in diameter [28].	***Penicillium purpurogenum***

Table 3 – Screening of Fungal Isolates on Different Dyes in Solid Media

FUNGI SPECIES	BASIC BLUE 3	ACID ORANGE 7	BASIC YELLOW 3	ACID VIOLET 49
A.strictum	-ve	-ve	-ve	-ve
F.moniliforme	**+ve**	**+ve**	**+ve**	**+ve**
A.niger	**+ve**	**+ve**	**+ve**	**+ve**
P.purpurogenum	**+ve**	**+ve**	**+ve**	**+ve**
A.fumigatus	**+ve**	**+ve**	**+ve**	**+ve**

Where, +ve = zone of clearance observed. –ve = No zone of clearance observed

3.2Screening of Decolourization of Dye in Solid Media

From all the five fungal isolates, four were selected after comprehensive screening for decolourization of textile dyes for further studies. In the dye agar plate technique, decolourization zone was found in *A.fumigatus*, *F. moniliforme, A.niger* and *P.pupurogenum* while *A.strictum* did not exhibit any decolourization activity **(Table 3).**

3.3Decolourization in Submerged Batch Culture

3.3.1Fusarium moniliforme

Fusarium moniliforme showed a moderate and steady growth over the span of a week which is reflected in the periodic growth and increase in dye adsorption rate**.** The optical density was measured at fixed intervals to find the percentage of decolourization and recorded on the 2nd, 4th and 6th day. The rate of adsorption was directly proportional to the time duration and growth of mycelia. For all the four dyes, the highest percentage of decolourization was observed on the 6th day. The highest decolourization rate was observed in case of Basic Blue 3 and Basic Yellow 3, which was 85% each, followed by Acid Orange 7 (80%) and Acid Violet 49 (76%), as shown in **Figure 1**.

3.3.2Aspergillus fumigatus

The rate of growth of *Aspergillus fumigatus* was high as compared to *Fusarium moniliforme.* The bio-sorption of the dyes was very rapid on the first two days of the growth which gradually continued till the sixth day of incubation. The highest percentage of absorption was observed in case of Basic Blue 3 (94%), followed by Acid Violet 49 (90%), Basic Yellow 3 (80%) and Acid Orange 7 (75%), as shown in **Figure 2**. The fungal degradation of various azo dyes has already been reported using *Aspergillus flavus* (74%), *Aspergillus niger* (89%), *Candida albicans* (67%), *Polyporus borealis* (52%), *Pholiota squarrosa* (65%), *Omphalotus olearius* (42%), as observed by T. Marimuthu et.al [20]. In the present study the rate of colour removal of the dyes by the fungal isolates increased with the increase in the incubation period. This was also in confirmation with the earlier finding of Nehra et.al, Mahbub et.al and Spadaro.et.al [21, 22, and 23].

3.3.3Aspergillus niger

Aspergillus niger showed a steady growth over a period of time. The decolourization percentage was higher than *A.fumigatus* and *F.moniliforme*. The best results were obtained in Basic Yellow, where the decolourization was above 90% **(Figure 3).** It showed similar results in Basic Blue (90%). The efficiency was slightly less in Acid Violet 49 and Acid Orange 7. Acid Violet 49 showed 85% decolourization, while in Acid Orange 7 it reached up to 83%.

3.3.4Penicillum purpurogenum

Penicillium purpurogenum was equally effective in all the dyes. While the decolourization was up to 90% in Basic Blue 3, Acid Violet 49 and Acid Orange 7, it was slightly lower in Basic Yellow 3 (82%), as shown in **Figure 4**. Overall, it was the most efficient of all the fungi, with the maximum decolourization reaching 93% in Acid Orange 7.

3.4 Effect of pH on Decolourization Activity

Dye decolourization was analysed at different pH scale **(Figure 5 a-d).** The pH optima varied (pH 4-9) with fungal isolates as well as with the type of dye used. For Basic Blue 3, the maximum decolourization of 88% was obtained at pH 4, by *A. fumigatus*. For Acid Orange 7, maximum decolourization of 90% was achieved by *A. fumigatus* at pH 7 while *F.moniliforme* and *A.niger* showed 79% and 78% decolourization, respectively at pH 7. In Basic Yellow 3, the maximum decolourization of 90% by *A. fumigatus* was observed at pH 7. In the case of Acid Violet 49, the entire range of pH from 4.0 to 9.0 was found to be suitable for all the fungal isolates. The maximum decolourization of 90% was achieved at pH 7 by *A. fumigatus,* closely followed by *P. purpurogenum* at pH 9.

P. purpurogenum performing decolourization at alkaline pH range has practical importance to develop industrial wastewater treatment that has alkaline nature. Since textile industries use different salts and sodium hydroxide before dyeing steps, the effluents are characterized by high salinity and alkaline medium [32]. Results of this study are consistent with previous findings [33, 34]. Chen et al. [33] has also reported that the most suitable pH for colour removal was between pH 5.5 and 9.0 under anoxic conditions.

3.5Effect of Temperature on Decolourization Activity

The fungal activity was observed under a wide range of temperature varying from 10℃ to 45℃ **(Figure 6 a-d)**. With all the dyes, the most optimum temperature turned out to be 25℃ where maximum decrease in optical density was observed. It was reported that lower and higher temperature values significantly inhibited the growth of organism and the activity of the enzymes that were responsible for decolourization. In this study, the effect of temperature was investigated by considering a wide range of temperature values (15°C to 45°C) and the decolourization results differed significantly. Strains showed enhanced decolourization when the temperature was increased from 10°C to 25°C, reached the plateau between 25°C and 37°C, and the

decolorizing activity was suppressed when the temperature further increased to 45°C. This might be due to the loss of cell viability or the deactivation of the enzymes responsible for decolourization [30]. The optimum decolourization efficiency (80 to 85%) of all the strains was found at 25°C to 37°C, a range which appeared to be favourable for the growth of the fungi.

3.6 Mechanism of Mycoremediation by Fungal Isolates

3.6.1 Adsorption Experiment

The fungal isolates were studied to find out the mode of decolourization. As mentioned earlier the bio-sorption of dye by fungal mycelia was determined by difference in dry weight of mycelia from different fungal strains on all the dyes used. The rate of adsorption was determined and was found to be in the range of 50-96% **(Figure 7)**. The kinetics of dye adsorption was calculated as shown in **Table 4**. It was evident that in all dyes, the maximum rate of adsorption was after 2 days. However, within 4 days, the adsorption slows down and attains saturation. The best results were obtained with Basic Blue 3, by *P.purpurogenum* adsorbing 95% of the dye **(Figure 8).** The weight of dye adsorbed per gram of mycelia is shown in **Table 5**.

Table 4 – Rate of Dye Adsorption by Mycelia in mg/hr

Fungi	Dyes			
	Basic Blue 3`	Acid Orange 7	Basic Yellow 3	Acid Violet 49
A.fumigatus	1.3mg/hr	0.56mg/hr	0.58mg/hr	0.68mg/hr
F.moniliforme	0.84mg/hr	0.49mg/hr	0.66mg/hr	0.66mg/hr
A.niger	1.1mg/hr	0.80mg/hr	0.65mg/hr	0.70mg/hr
P.purpurogenum	0.83mg/hr	0.63mg/hr	1.02mg/hr	1.24mg/hr

Table 5 – Weight of Dye Adsorbed per Gram of Mycelia

Fungi	Dyes			
	Basic Blue 3`	Acid Orange 7	Basic Yellow 3	Acid Violet 49
A.fumigatus	0.044g	0.034g	0.039g	0.035g
F.moniliforme	0.046g	0.050g	0.054g	0.04g
A.niger	0.052g	0.038g	0.422g	0.036g
P.purpurogenum	0.59g	0.465g	0.040g	0.044g

3.6.2 Enzymatic Dye Degradation Activity

The enzymatic activity for dye decolourization was observed for *A.fumigatus*, *F.moniliforme, A.niger and P.purpurogenum* **(Figure 9)**. It was observed that the enzymatic activity (intracellular and extracellular) showed negligible effect on dye remediation. The reason likely being, that the enzymes are highly specific for temperature and pH. Any change from the optimum condition results in decline of their activity. The other justification is that maximum dyes have been adsorbed by fungal mycelia by active transportation along with the nutrients and are retained by the fungal cells as it has been confirmed by microscopic observation fungal of the fungal mycelia. The maximum decolourization happened because of bio-sorption.

The present data is in parallel with previous report by Acemioglu B. et.al. [24], which showed the use of *Aspergillus wentii* for bio-sorption of methylene blue from aqueous solution. The adsorption of dyes by fungal isolates is an effective and eco-friendly method [31]. It was concluded that some fungal isolates used in the present study have great potential for decolourization of textile dyes so they can be further explored for bioremediation of textile dyes. Still this was a primary study and more detailed study is needed to see the actual mechanism of dye decolourization. There is need for further investigation on enzymes and the mechanism involved in dye decolourization.

Figure 1 - Percent Decolourization by *F.moniliforme* at Different Time Intervals

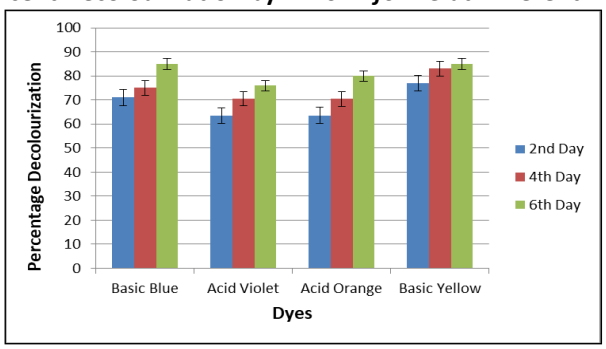


Figure 2 - Percent Decolourization by *A.fumigatus* at Different Time Intervals

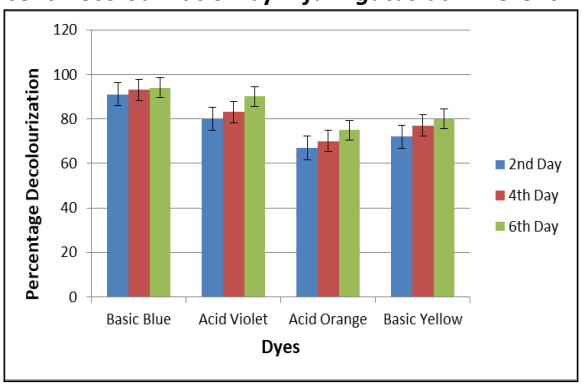


Figure 3 - Percent Decolourization by *A.niger* at Different Time Intervals

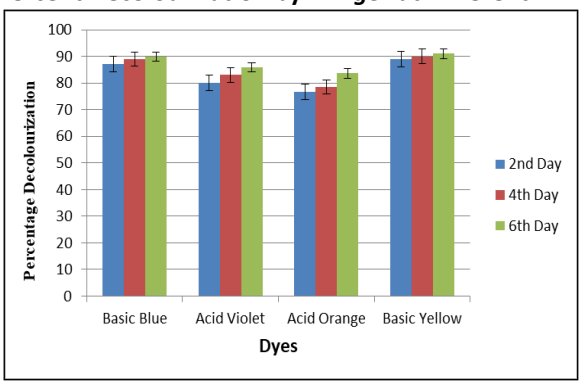


Figure 4 - Percent Decolourization by *P.purpurogenum* at Different Time Intervals

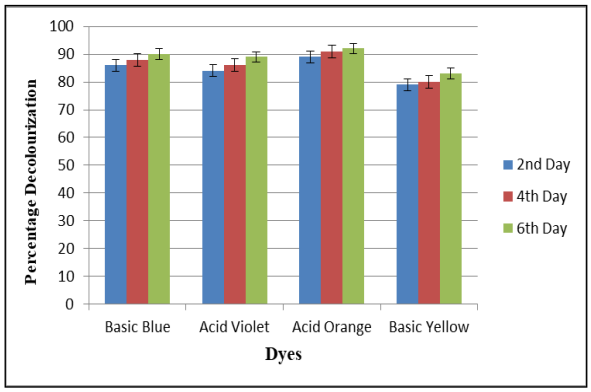
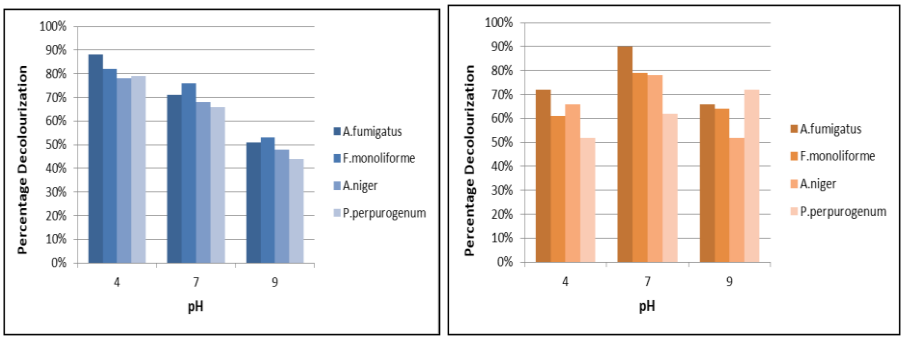
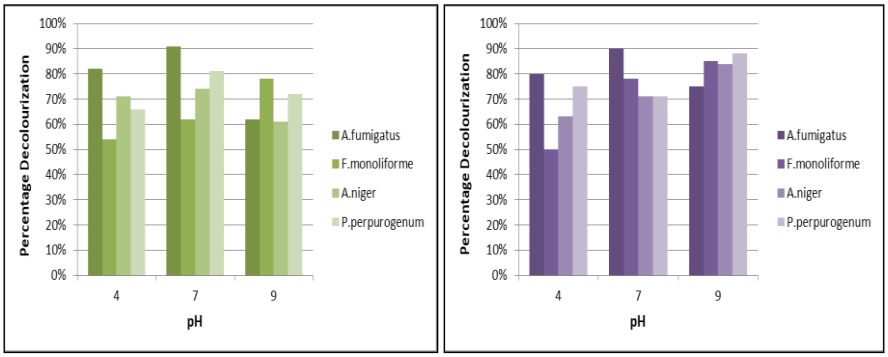


Figure 5 - Effect of pH on Fungal Decolourization of Dyes

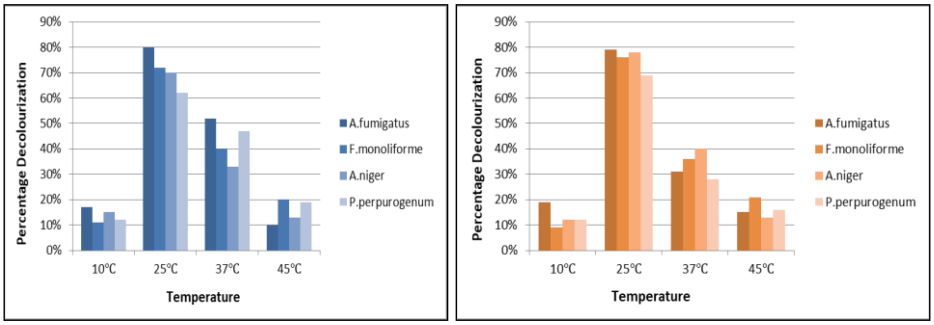


a. Basic Blue 3 b. Acid Orange 7

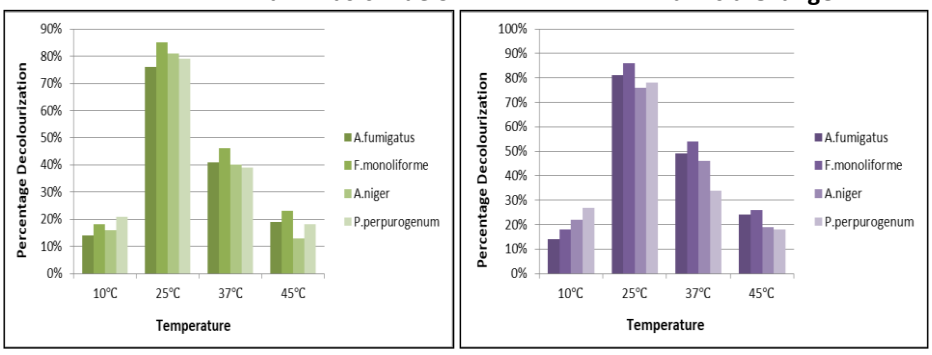


c. Basic Yellow 3 d. Acid Violet 49

Figure 6 - Effect of Temperature on Fungal Decolourization of Dyes



a. Basic Blue 3 b. Acid Orange 7



c. Basic Yellow 3 d. Acid Violet 49

Figure 7 – Percentage of Dyes Adsorbed by Fungal Isolates

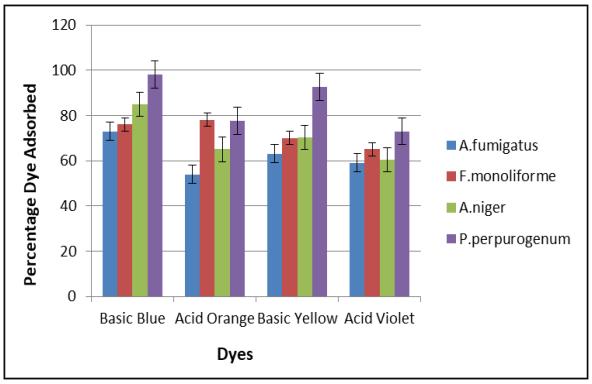
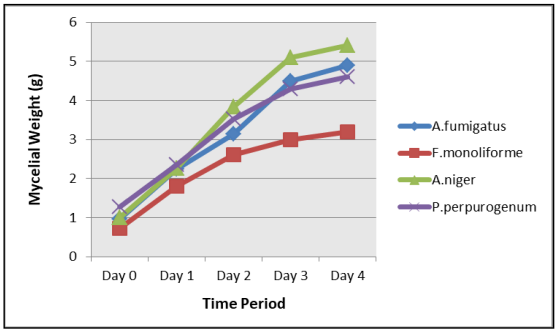
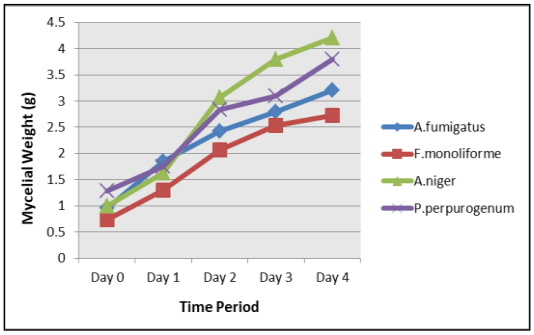


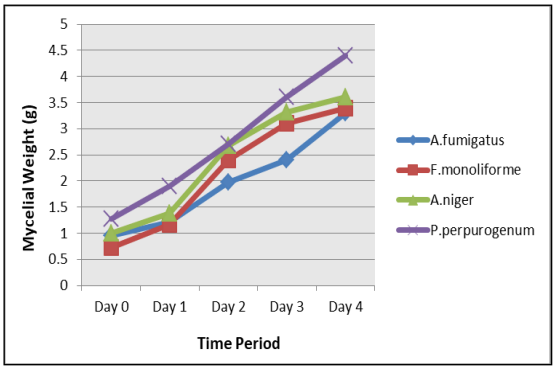
Figure 8- Rate of Adsorption of Dyes at Different Days of Incubation of Fungal Isolates



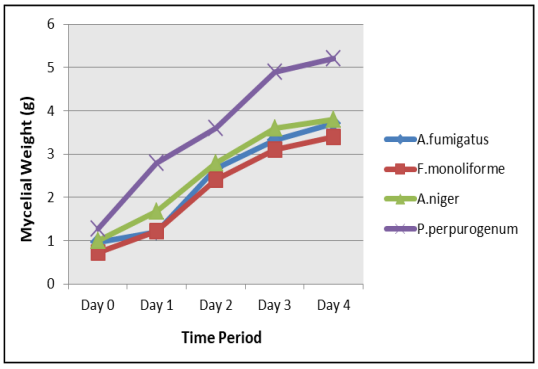
a. Basic Blue



b. Acid Orange

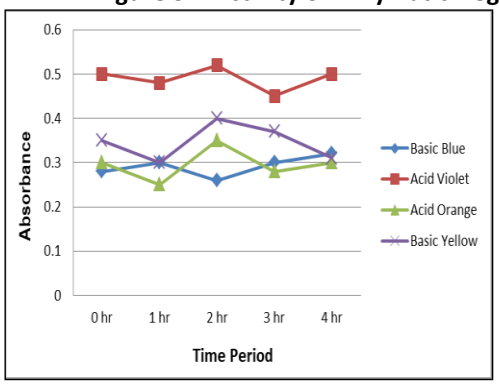


c. Basic Yellow

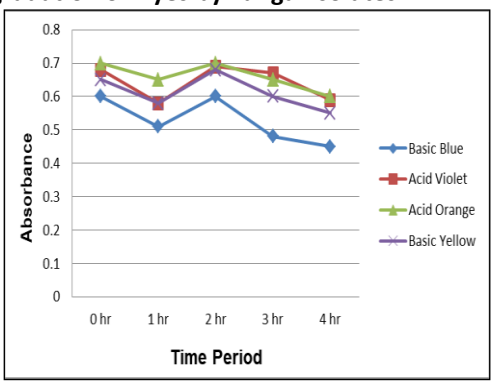


d. Acid Violet

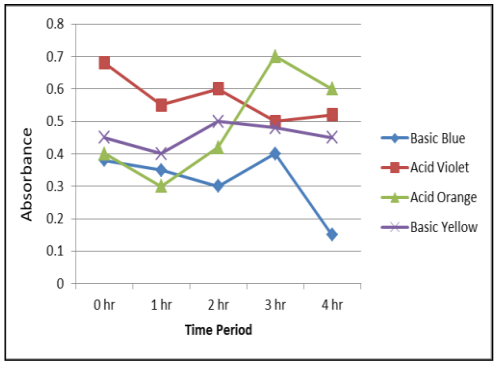
Figure 9 – Activity of Enzymatic Degradation of Dyes by Fungal Isolates



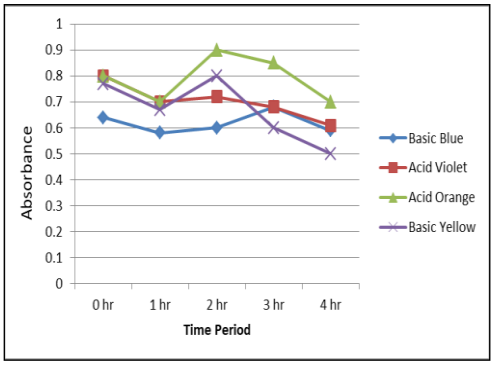
a. *A.fumigatus*



b. *F.moniliforme*



c. ***A.niger***



d. ***P.pupurogenum***

4. CONCLUSION

Several fungal isolates were exploited to examine their growth associated dye decolourization potential in this study. The decolourization of dyes was studied on dye agar plates as well as under shaking conditions; encouraging results were obtained after 3 days, but maximum decolourization of all the dyes were obtained after 6 days. Higher decolourization under shaking conditions could be due to better oxygenation of the fungus, increased growth rate and increased mass transfer rate of dyes along with the available nutrients. Disappearance of dye color could be due to biodegradation of chromophore in dye molecule because of production of intracellular and extracellular enzymes but it was found to be mainly due to the absorption and adsorption of dye by the growing fungal mycelia. It also demonstrated that these fungi have a good capacity to treat textile dye wastewaters in an ecofriendly manner. These results also indicated the availability of newer fungal isolates with still higher dye removing or bioremediation potentials.

Due to the environment friendly techniques, these bioremediations have been characterized as a soft technology. Its cost-effectiveness and the little disturbance to the environment render this technology a very attractive and alternative method of choice. The identification and research of new fungal strains with the aid of molecular techniques will further improve practical applications of fungi and it is anticipated that fungal remediation will soon be a reliable and competitive dye remediation technology.

ACKNOWLEDGEMENT

Authors express their gratitude towards Dr. D Y Patil Biotechnology and Bioinformatics Institute, Dr. D Y Patil Vidyapeeth, Pune for providing the research facilities.

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