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Isolation and process optimization of textile dye degrading fungi.

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Abstract

The aim of the present study was to screen and isolate a potent fungus capable of degrading and supporting the decolourisation of an azo dye Trypan Blue and to optimize the medium conditions and factors for maximum dye decolourisation. The decolourisation/removal of various industrial dyes like trypan Blue using isolated fungal strain has been an important area of research and the present work is focused on this specific aspect. The effect of independent variables such as time, temperature, pH, agitation on decolourisation efficiency is discussed. Biodegradation of Trypan Blue was demonstrated by decolourisation of culture medium, the extent of which was determined by monitoring the decrease in absorbance at or near the maximum wavelength of this dye. The biodegradation of the dye was related to its decolourisation during growth of fungi. There was increase in decolourisation % along with increase in incubation time. While performing the process optimization studies for the decolourisation of the dye, maximum decolourisation was observed at temperatures 28°C and 37°C, pH 6 and under static conditions. It was found that the isolated fungal strain was considered to be well adapted, resistant and highly acclimatized to dye contaminated soils showing the decolourisation of trypan blue dye. Mycoremediation is a great technology and can be exploited for the bioremediation of dye contaminated soils and also to reclaim wastewater. Isolated fungal strain in the present study is found to be capable of degrading Trypan Blue under its optimized medium and growth conditions and it seems to be best candidate for textile effluent decolourisation, so this strain can be used for bioremediation of environs polluted with textile effluents.

Keywords: trypan blue; mycoremediation; decolorization; recalcitrant compounds

Running title: Mycoremediation of trypan blue by a soil fungal isolate

Introduction

Dyes and dyestuffs are used in a wide variety of industries *viz.* textile, leather, cosmetics, paper, printing, plastic, food and pharmaceutical industries to color their products. Effluents of these industries are very important source of chemicals entering into aquatic ecosystem, deteriorating the water quality, affecting the sunlight penetration and thereby affecting the flora and fauna. Major classes of synthetic dyes include the azo, anthraquinone and triphenylmethamne dyes. Dyes are difficult to degrade biologically, so that degradation of dyes has received considerable attention. About 10-15% of all dyes are directly lost to wastewater in the dyeing process. (Kalme *et al.*, 2006). Thus, the wastewater must be treated before releasing into the natural environment.

The removal of dyes from the textile effluents is one of the most significant environmental problems. The elimination of colored substances in wastewater is based on mainly physical and chemical methods.

Due to mutagenicity, carcinogenicity and complicated molecular structures of dyes, make dye wastewater difficult to be treated by conventional biological and physico-chemical process. These methods have several disadvantages like high cost, incomplete removal, low selectivity and high energy consumption due to low biodegradability of dyes. (Olukanni *et al*., 2006). Therefore, innovative treatment technologies need to be investigated. Bioremediation is an expanding area of environmental biotechnology to decontaminate polluted water and soil, and may be defined as use of living systems especially micro-organisms to catalyze the degradation of wastes without disruption of the environment. At present there is no satisfactory method to economically decolourize textile wastewater. Recent studies point out the potential of fungal wastewater treatment for textile industries .Fungal systems appear to be appropriate in the treatment of colored and metallic effluents (Ezeronye and Okerentugba, 1999). Several combined aerobic and anaerobic microbial treatments had been suggested to enhance the biodegradation of textile dyes. (Huag *et al*., 1991). In recent years, there has been an alternative research on fungal decolourization of dye present in wastewaters, and it is turning into a promising alternative to replace or supplement for present treatment processes (Ramya *et al*., 2007).

There are various methods to observe the dye removal like the decrease in COD, the reduction in absorbance of the dye at its λ_{max}. In the present study the decrease in the absorbance value was used to study the dye removal by the fungal strain because absorbance is the parameter that can be easily studied in the laboratory using spectrophotometer.

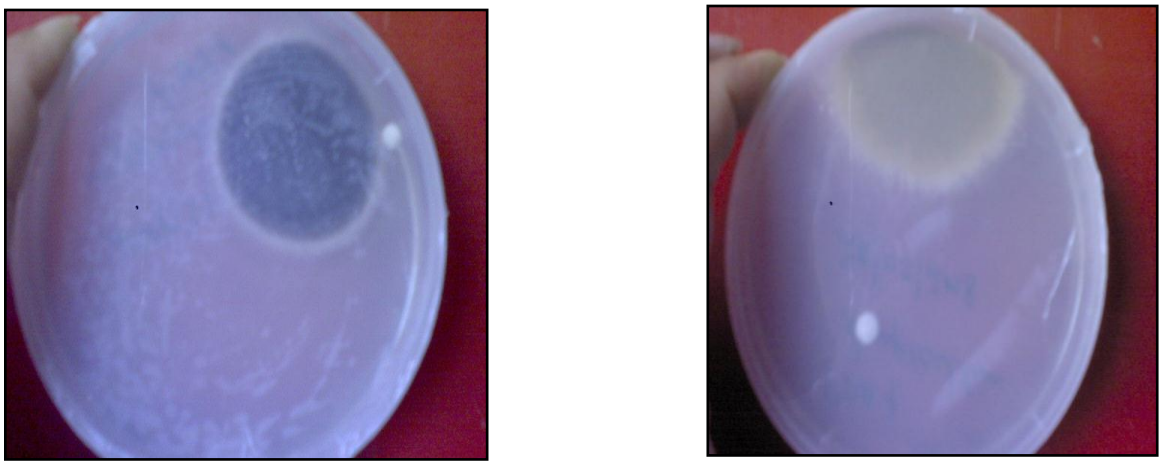
Among many classes of synthetic dyes used in the textile and dyeing industries, azo dyes such as trypan blue is the most versatile and play predominant role in almost every type of application. Trypan blue has been extensively used in human as a biological stain and it is also used in pharmaceutical ,cosmetic and textile dye industries. In the present study, fungi are being investigated for their potential to decolorize textile effluents and removal of toxic dyes from wastewaters. The present study was carried out with an aim to isolate the fungal strain from the soil and optimization of conditions that support the maximum decolourisation of trypan Blue.

Materials and Methods

Collection of soil samples and isolation of fungal isolates

Soil samples, each measuring approximately 10 grams were taken randomly to a depth of 5 cm from the topsoil using sterile spatula and stored in sterile screw-capped polycarbonate tubes. The soil samples were taken from several locations in Chandigarh, India near textile or dye-based industries. The samples were immediately placed on ice until further examinations. Approximately one gram of each soil sample was serially diluted ten times in sterile double distilled water and plated over PDA medium (Potato Dextrose Agar) (HiMedia Pvt. Ltd.) amended with 60µg/ml Ampicillin {(Ranbaxy Laboratories, Sunderabad (India)}. The plates were incubated at 37°C inverted in incubator for 5 days. The isolated fungal strains (Fig.1) were further purified by subsequent subculturing and maintained in slant culture at 4°C.

Fungi from soil samples were tested for their abilities to decolorize azo dye trypan blue. The dye was procured from HiMedia Pvt. Ltd. (Mumbai, India.)



(A) **(B)**

Fig.1. Petriplate showing growth of **(A)** strain 9 and **(B)** strain 1 on PDA.

Morphological identification of isolated fungal strains

The morphological identification of isolated fungal strains was performed using lactophenol cotton blue stain (Sukumar *et al.*, 2007). Clean glass slide was taken and a drop of lactophenol cotton blue stain was mounted on centre of glass slide. A portion of mycelial mat from the colony was transferred into the drop of stain with the help of flamed needle. With the help of two needles the propagules were gently spread, so that the mycelial were mixed with stain. Excess stain washed out with distilled water and observed under compound microscope at 10X and 40X and the types of conidia, hyphae and their arrangement noted (Harigan and Mccane, 1966).

Standardization of absorbance characteristics of trypan Blue:

Stock solution of trypan blue dye was prepared (100 µg/ml). Dye was scanned in UV- Visible spectrophotometer to ascertain the maximum wavelength and maximum absorbance. The working concentration range was determined by scanning different concentrations (0.1-1 µg/ml) of the dye in spectrophotometer and absorbance was recorded. A plot of absorbance v/s concentration was plotted for the dye (Fig.2). From the linear working range, the higher concentrations were selected because at the lower concentrations, decolorization of the dye by fungal strain could not be visualized.

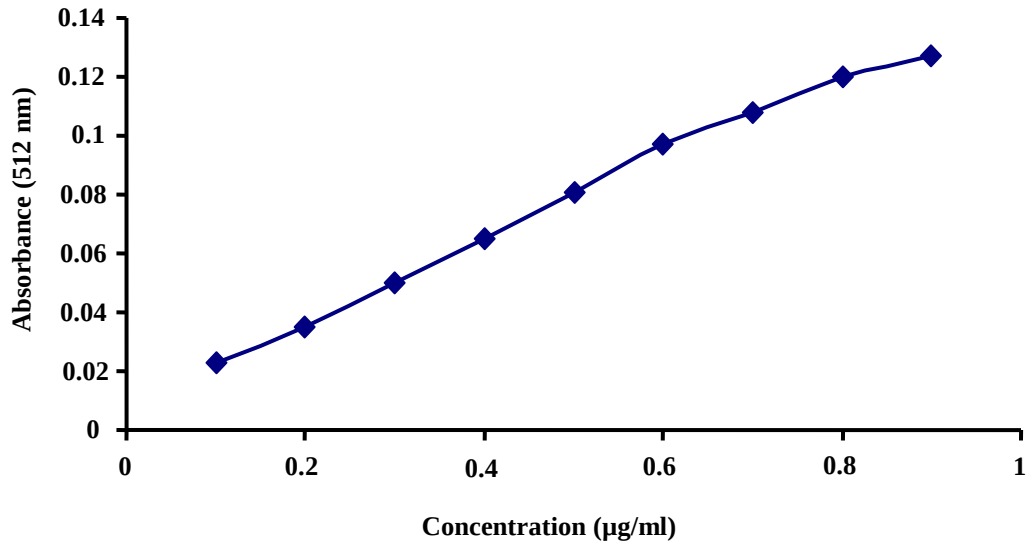


Fig.2. Standard absorbance curve for trypan blue

From graph it was interpreted that linear working range for the dye should be 0-1µg/ml

Assessment of dye degradation based on decolourisation:

1 µg/ml and 0.1 µg/ml concentrations of dye was prepared and streaked with isolated fungal strain (Fig.3) on PDA petriplates amended with 60 µg/ml ampicillin. Petriplates were incubated for 5-6 days to observe decolourisation.

(A)

(B)

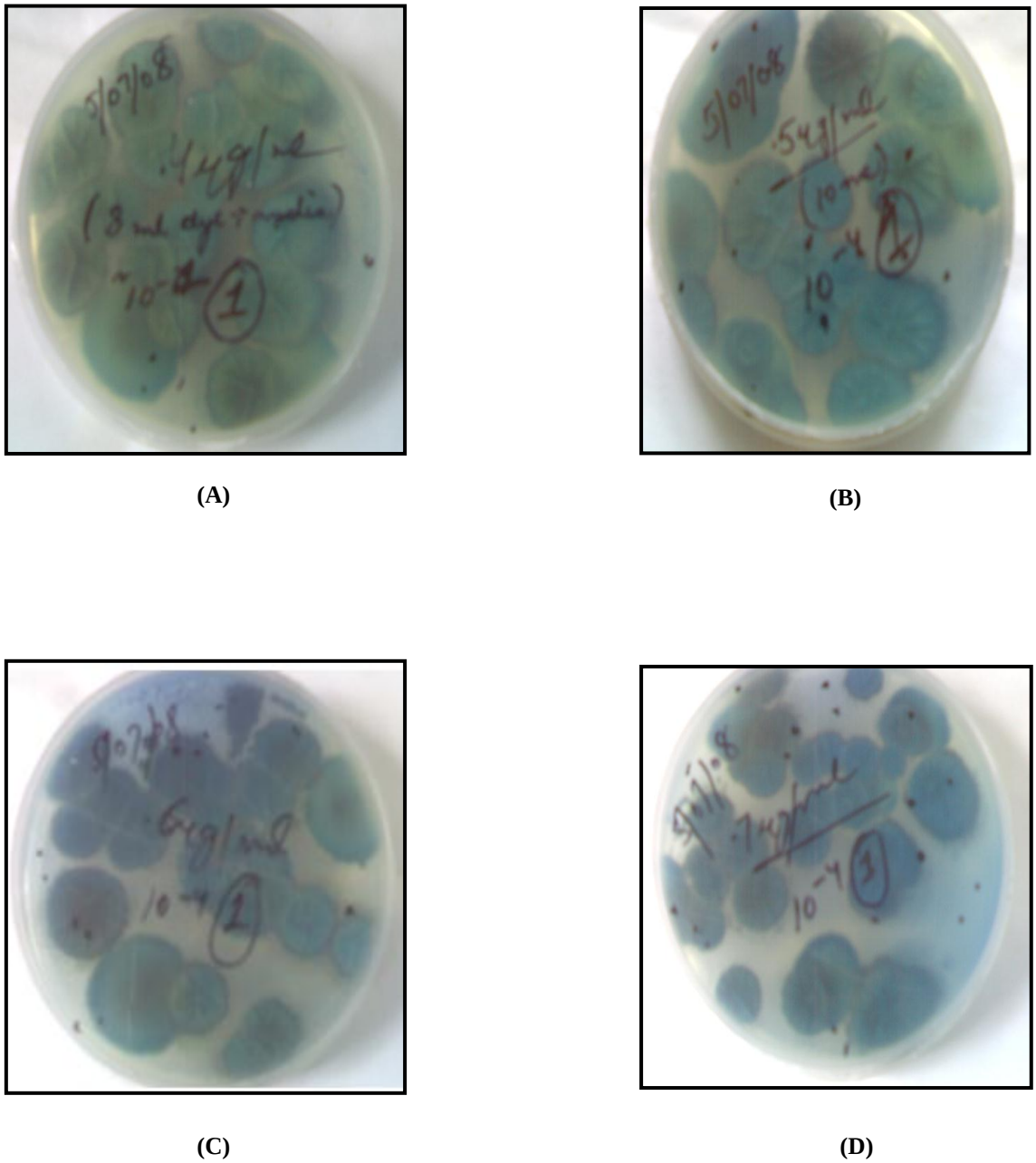
(C)

(D)

Fig.3. Media containing trypan blue dye (1 µg/ml) **(A)** before fungal growth **(B)** after fungal growth and trypan blue dye (0.1 µg/ml) **(C)** before fungal growth **(D)** after fungal growth.

Study of effect of dye on fungal growth:

Trypan blue dye was selected and experimental range of dye considered was 0.4 µg/ml-0.7 µg/ml (Fig.4) because graph plotted between absorbance vs. concentration came out to be linear in this range.



(A) **(B)**

(C) **(D)**

Fig.4. Petriplate showing fungal growth on PDA having trypan blue **(A)** 0.4 µg/ml **(B)** 0.5 µg/ml **(C)** 0.6 µg/ml **(D)** 0.7 µg/ml

Serial dilution and spread plating:
Loopful inoculum from slant culture of isolated fungal strain was taken and serially diluted up to 10^{-6} times using sterile double distilled water. 100 µl of 10^{-4} dilution of strain was spread plated on petriplates of prepared concentrations (0.4 µg/ml-0.7 µg/ml).

Dye degradation by isolated fungal strain in nutrient broth:
Two flasks containing nutrient broth having concentration of 2 µg/ml and 4 µg/ml of trypan blue were inoculated with fungal strain using disc method (Sukumar *et al.*, 2007). The flasks were incubated at 37°C in incubator. Absorbance was recorded after 6-7 days at its maximum wavelength (512nm).

Factors influencing growth of fungi and dye degradation:
Effect of time: Two flasks having 100 ml of nutrient broth in each (pH 5) with 2 µg/ml and 4 µg/ml concentrations of trypan Blue were inoculated by four discs of isolated fungal strain by disc method [disc taken from periphery i.e. mycelial mat (to avoid spores)] All flasks were incubated at 37°C in incubator. Absorbance was monitored for 6-7 days at 512 nm.

Effect of temperature: Four flasks having 100 ml **n**utrient broth (pH 5) in each were inoculated by four discs of isolated fungal strain by disc method and incubated at different temperatures (4°C, 28°C, 37°C, 50°C) for six days under static conditions. 5 ml samples drawn from the inoculated treatments and centrifuged at 17,000 rpm for 10 min. The maximum absorbance was observed for dye and rate of decolourisation was monitored at 512 nm.

Effect of pH: Five flasks having 100 ml of nutrient broth in each of pH adjusted to 5, 6, 7, 8 and 9 were inoculated by four discs each of isolated fungal strain by Disc method and incubated for six days under static conditions. 5 ml samples drawn from the inoculated treatments and centrifuged at 17,000 rpm for 10 min. The maximum absorbance was observed for dye and rate of decolourisation monitored at 512 nm.

Effect of agitation: Two flasks having 100 ml of nutrient broth (pH 5) in each were inoculated by four discs of isolated fungal strain by disc method and flasks were incubated separately at static and at 60 rpm to observe effect of agitation for 6 days. 5 ml samples were drawn from the inoculated treatments and centrifuged at 17,000 rpm for 10 min. The maximum absorbance was observed for dye and rate of decolourisation monitored at its maximum wavelength (512nm).

Results and Discussion

Isolation of fungal strains from soil sample:
Total 11 fungal strains were isolated from soil for dye decolorization. Out of those strain 1 (Fig.1 b) was randomly selected for process optimization of trypan blue dye degradation.

Morphological identification of the fungal strains:

Strain 1:

Colonial morphology: Small, heaped colonies, greenish black and powdery colonies were observed.

Microscopic appearance: Spores (conidia) at the end of complex conidiophores arising from septate mycelium that was brownish in appearance were observed.

The above morphological features resemble those of the moulds suggesting that the fungal strain used may be the Molds.

Strain 9:

Colonial morphology: The fungus was observed to be rapidly growing, compact and there were moist colonies becoming cottony with aerial hyphae which were grey or rose colored.

Microscopic appearance: Single celled conical or elliptical spores (conidia) held together in clusters at the tips of conidiophores by a mucoid substance were observed. Erect, unbranched conidiophores arising from a septate mycelium were also seen. The above morphological features closely resemble those of the Cephalosporium, indicating that that the fungal strain isolated may be Cephalosporium

Degradation of trypan blue dye by isolated fungal strain:
Degradation of trypan blue dye by fungal strain 1 was observed. It was concluded that fungal strain 1 has the capability to adsorb and decolorize trypan blue. Process optimization of trypan blue was carried out by investigating various factors that support its decolourisation.

From above results it is concluded that trypan blue dye was absorbed maximum by fungal strain in dye concentration 0.6-0.7µg/ml. The probable reason of less decolorization at the lower dye concentration may be that at such lower concentrations, the fungal strain being used is unable to detect the dye. These results suggest that the higher dye concentrations should be used for further experimentation.

Effect of time on trypan blue dye degradation by fungal strain:
In experiments done to see the time dependence of fungal growth, we observed that upto day 4-5, absorbance decreased, possibly due to capability of isolated fungal strain under study to absorb and assimilate dye from the medium (Fig.5). This was indicated by decrease in the dye color in the medium.

But after day 5, absorbance increased unexpectedly which might be due to media depletion and possible excretion of metabolites by fungus into the medium leading to an increase in absorbance.

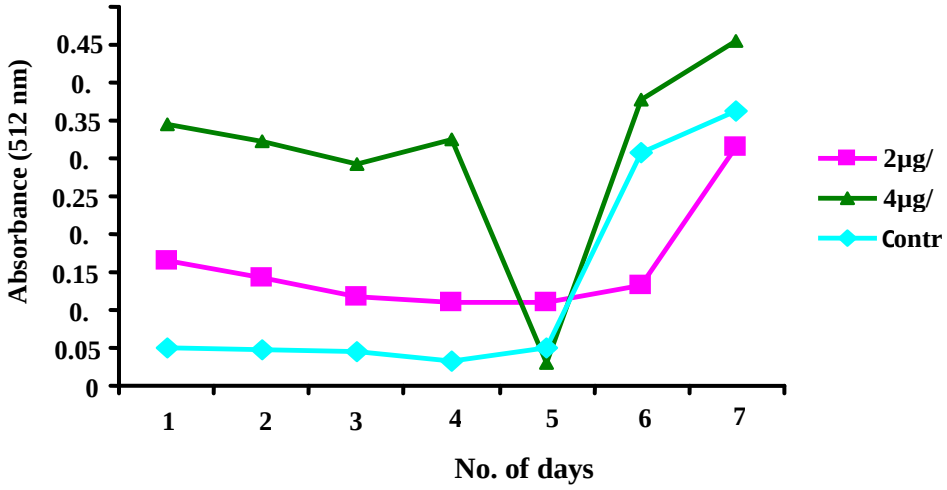


Fig.5.Effect of time on decolorization of trypan blue

Effect of temperature on growth of fungi and decolorization of trypan blue

At 4°C and 50°C: There was no visible growth at 4° C and 50 °C (Fig.6 A, D), hence dye decolorization had not been taken place as there is no major decrease in absorbance. The changes in absorbance may be due to other factors like media depletion, secretion of metabolites into media. At these temperatures, fungal strains recorded the lowest value.

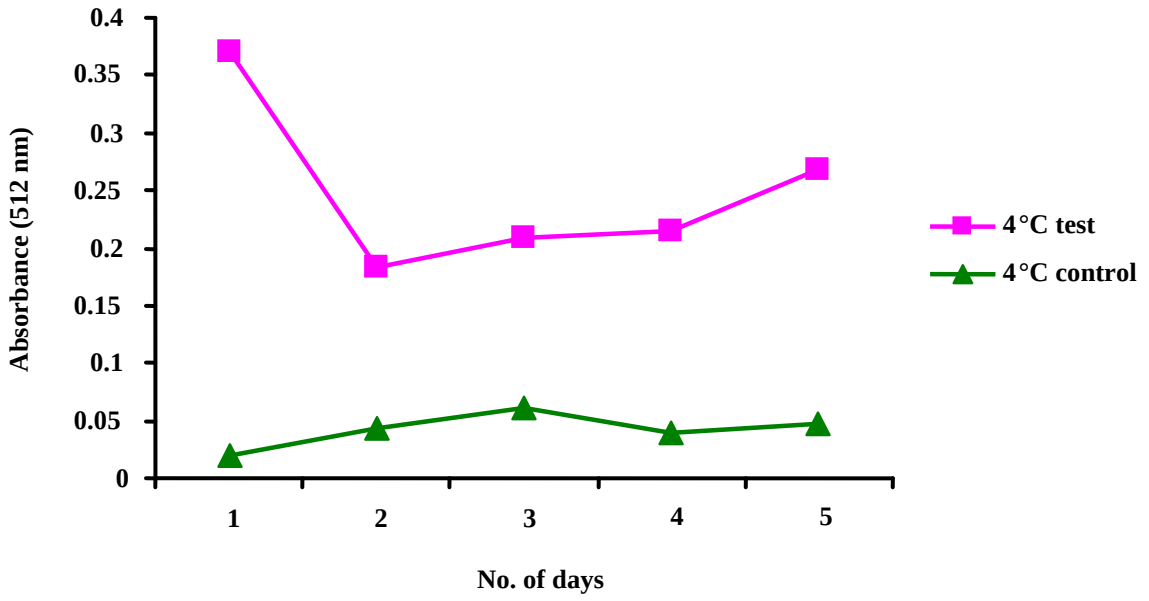
At 28°C and 37°C: There was visible growth at these temperatures (Fig.6 B, C) and hence dye degradation had taken place since there was decrease in intensity of dye color day by day. Absorbance decreased due to a possible consumption of media by fungal strain. Fungus was absorbing and assimilating dye in medium and there was qualitative decrease in dye color. In general 37° C appeared to be optimum for color reduction of trypan blue.

The optimum temperature for fungal growth is 30°C (Sukumar *et al.*, 2007). So we studied the effect of different temperatures i.e. 4°C, 28°C, 37°C and 50°C on fungal growth and hence dye removal. pH was set at 5 (the optimum pH for fungal growth) and under static conditions. So the fungal strain was inoculated in the dye containing nutrient broth (pH=5) and incubated at the above mentioned temperatures for 5-6 days and the O.D. read at its λ_{max}.

As indicated by the absorbance values [Fig. 6 (A) and (D)], at these temperatures the dye decolorization was observed to be less than at other temperatures. And also no visible fungal growth was observed at these temperatures.

As indicated by absorbance values [Fig.6 (B) and (C)] there was qualitative decrease in dye color. So, average temperature of 37°C could be an optimum temperature for color reduction of trypan blue like industrial dyes.

(A)



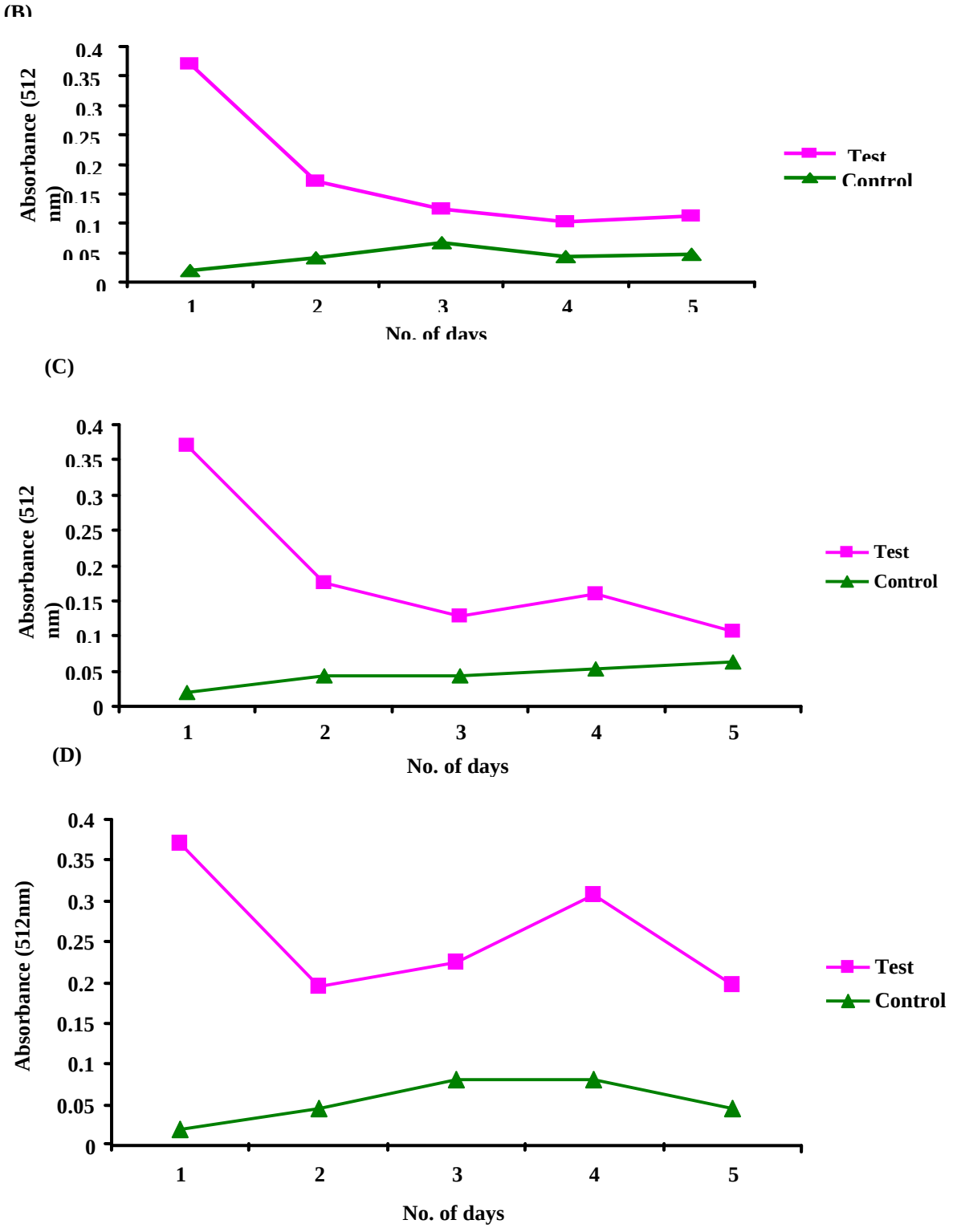
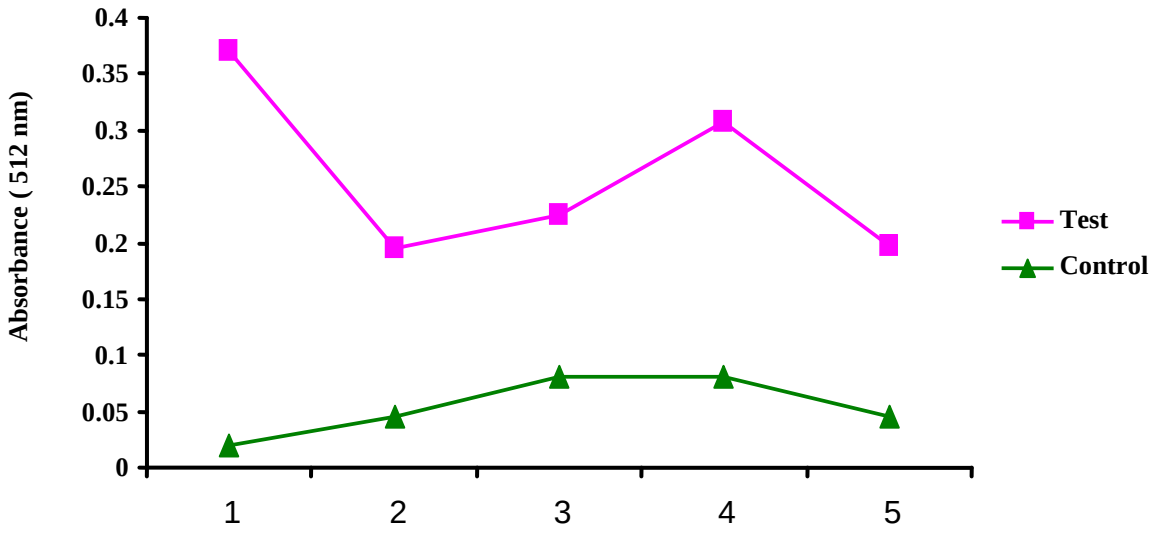


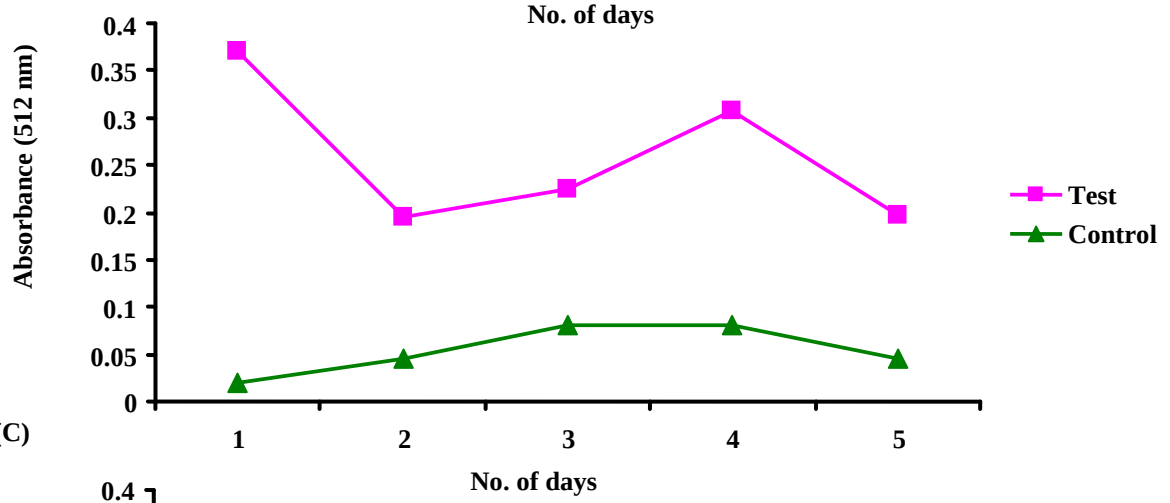
Fig.6. Decolorization of trypan blue at (A) 4 °C (B) 28 °C (C) 37°C (D) 50 °C

Effect of pH on growth of fungi and decolorization of trypan blue:

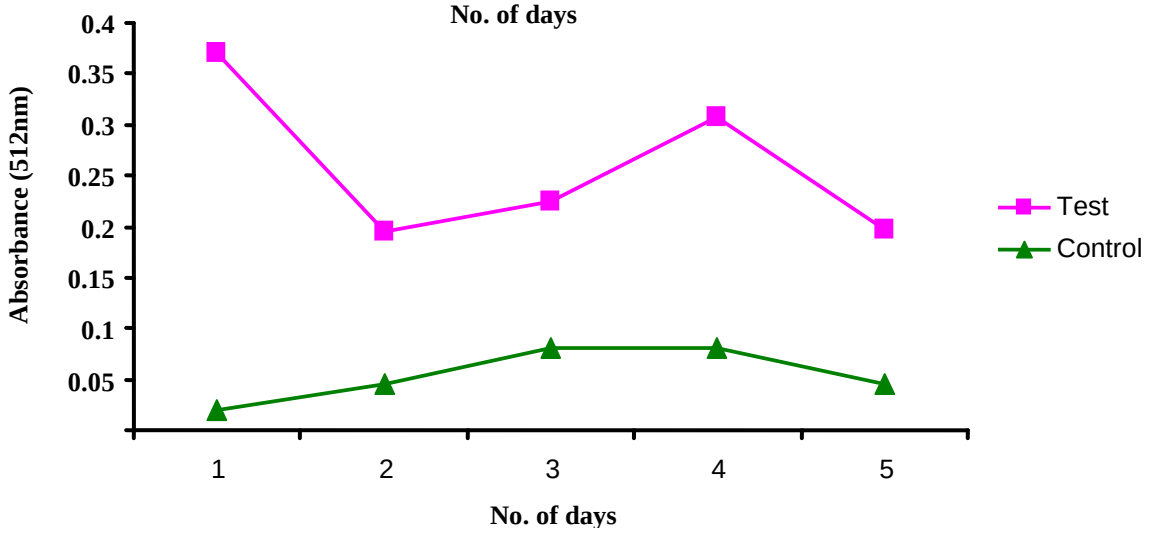
(A)



(B)



(C)



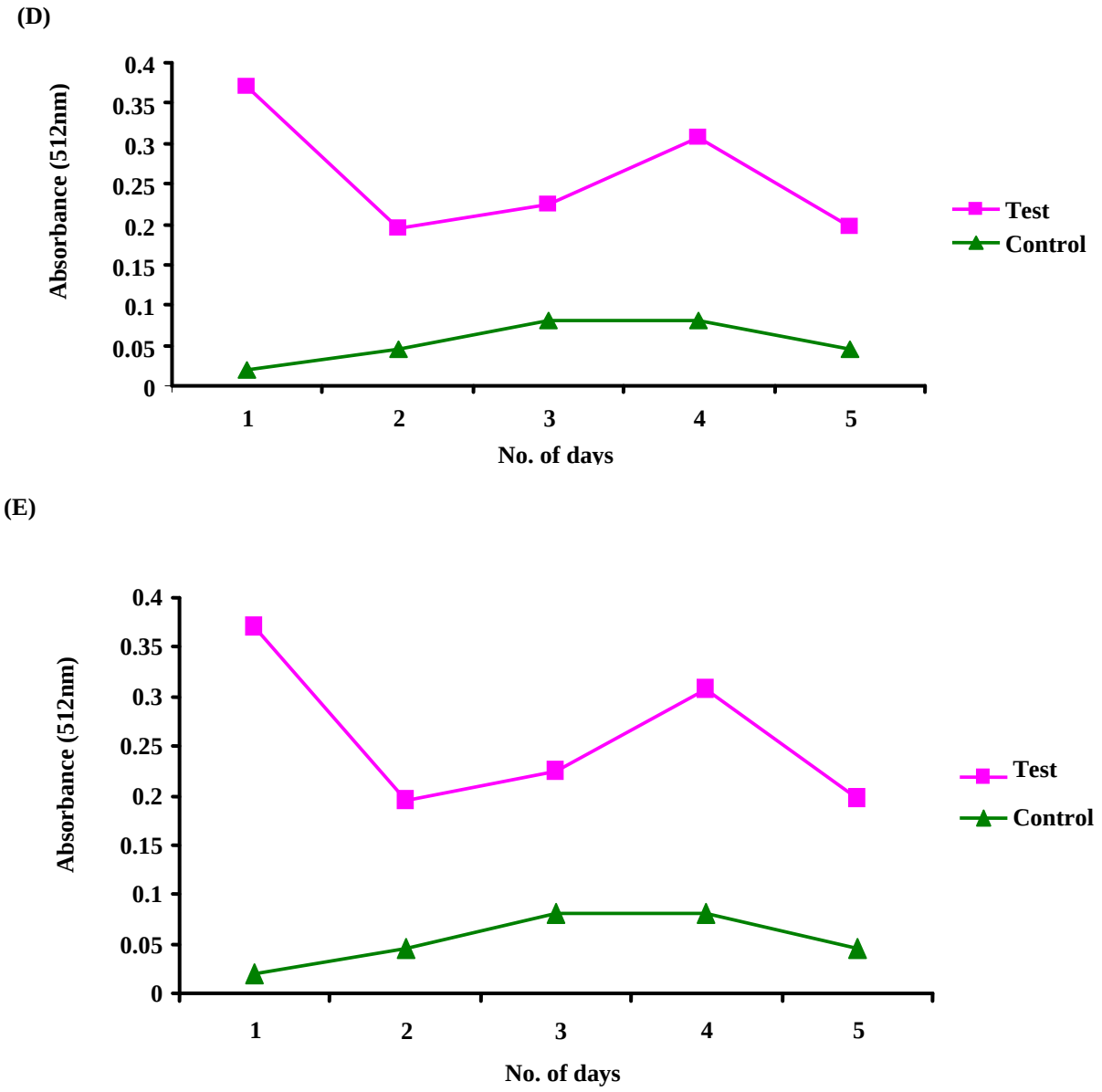


Fig.7. Decolorization of trypan blue at **(A)** pH 5 **(B)** pH 6 **(C)** pH 7 **(D)** pH 8 **(E)** pH 9

Media of pH 6 supported higher color reduction. At pH 9, the color reduction recorded the lowest value.

Fig.7. (A-E) showed the effect of initial pH on the efficiency of fungi to reduce the color intensity of the dye containing broth in the terms of decrease in absorbance values within the range of pH 5-9. It was clear that the maximum decolourisation was observed at pH-6. And the least decolourisation was observed at pH-9. These results clearly support the work done by Husseiny, 2008 that better fungal growth usually occurs at low pH values. They have used reactive and

direct dyes and supported that the maximum percentage of decolorization was 93.73% when *Aspergillus niger* was used at pH 4.5 and 78.4% removal of color at pH 4.5 in case of *Penicillium* spp.

Effect of Agitation:
The fungi recorded highest color reduction under static conditions for trypan blue. Increase in agitation tended to decrease the color reduction.

To compare the efficiency of static and shaking conditions (60 rpm) for activity of the fungal strain used on decolourisation of the dye was done. It was found that the static conditions are more efficient than the shaking for the dye decolorization. These results are similar to those obtained by (Daneshvar *et al.*, 2007) using microalgae *Cosmarium sp*, for decolorization of malachite green and can be discussed in terms of the high rate of the agitation that decreases the fungal growth and the activities of some biological substances such as enzymes which play important role in decolorization of dye (Husseiny., 2008). It has been reported earlier that the static conditions are more efficient than the shaking for both *Aspergillus niger* and *Penicillium* spp. for the decolorization of both reactive and direct dyes which is corroborative evidence in support of our study.

Discussion (Advantages)
Textile industries have been using synthetic dyes intensively because of their ease and cost effectiveness. The textile dyes are highly reactive and therefore during processing difficult to treat. During the past decade the use of microbial degradation methods have been under active development in textile and dyestuff industry (Knapp *et al.*, 1995). As can be seen from the literature *Phanerochaete chrysosporium* Basidiomycetes fungi is a choice organism reported to be potent decolorizer of the effluent (Wasenberg *et al.*, 2003). In the present work, an attempt has been made to study the common soil inhabiting fungi isolated indigenously from soil for the decolorization of trypan Blue. Trypan Blue (Laboratory dye) is selected for experimental studies because it is an azo dye and very closely resembles that of commonly used textile dyes in textile industries. The decolorization of trypan Blue by isolated fungal strain is discussed and effect of independent variables such as pH, temperature, time, agitation on the decolorization efficiency was observed to study the factors for the optimization of fungal growth conditions for decolorization of trypan blue. Decrease in absorbance is considered as indicator of dye removal and decolorization. The isolated fungal strain (Fig.1 B) has the potential to decolorize dye effluents. Isolated fungal strain showed maximum decolorization of dye at temperatures 28 °C and 37 °C, pH 6 and under static conditions. The color reduction increased linearly with increase in incubation time. Some other physical parameters in pH, temperature, concentration of xenobiotic/dyes in the wastewaters thus might have implications in the dye removal efficiency of most fungi. The study concluded that isolated fungal strain (Fig. 1 B) on its own can offer cost effective, easily applicable and an environmentally sound solution to dye effluents.

The present study was carried out to examine the microbial degradation of a hazardous dye, taking a fungus as the experimental organism and trypan Blue as a testing dye. The applied fungus has shown positive results for dye degradation/decolourization, as was

indicated by the change and disappearance of color of the dye from the dye-containing media of the petri plates as well as decrease in absorbance value at maximum wavelength of the dye. Microbial degradation of Congo red by *Gliocladium virens* (Singh, 2008a),various hazardous dyes likes, Congo red, Acid red, Basic blue and Bromophenol blue, Direct green by the fungus *Trichoderma harzianum* (Singh and Singh, 2010) by using different fungal strains has been investigated earlier. Cripps *et al*. (1990) also reported the biodegradation of three azo dyes (Congo red, Orange II and Tropaeolin O) by the fungus *Phaenerocheate chrysosporium*. In the present study dye might be degraded by the adsorption of dye by the mycelium of isolated fungal strain (Fig.1.B) during its growth in the dye-containing medium.

Adsorption of dyes to the microbial cell surface is the primary mechanism of decolourization (Knapp *et al*., 1995). In our study, the adsorption of dyes by the fungal mycelium was also observed, as it was confirmed by the change in the color of fungal mycelium in tested dye.

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