Microbial Decolorization of Synthetic Dyes by the fungus *Trichoderma longibrachiatum*

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Abstract
Synthetic colorants used in textile industries can enter our water systems and cause pollution if they are not treated before disposal. The present study focused on evaluating the decolorization of the textile synthetic dyes by Trichoderma longibrachiatum. The fungus was isolated from the bark of decaying tamarind plant. It was identified by morphological characterization and sequence analysis of the internal transcribed spacer (ITS) regions.

The fungal isolate showed the decolorization percentage of 90.16 %, 80.81 %, 76.4 %, 72.51 %, and 70.34 % of congo red, acid orange, bromocresol purple, crystal violet, and bromothymol blue respectively after 7 days treatment. Two azo dyes were only selected for the continuous study. The optimum conditions for decolorizing activity by T. longibrachiatum were alkaline pH (10) and 35 °C. After optimizing, the decolorization on congo red and acid orange reached a maximum of 95.61 % and 85.91 %.

Keywords: Synthetic dyes, decolorization percentage, optimum conditions.

Introduction

Contamination of soil, water, groundwater, and air with toxic chemicals is one of today's world's major environmental issues. Through rapid industrialization and widespread use of pesticides in different fields of agriculture and chemicals, environmental pollution through organic compounds has become a serious threat. Nonetheless, the aquatic system and soil contamination are global problems that can result in the accumulation of toxic chemicals in food chains, and also affect the flora and fauna. Environmental pollution of hazardous waste containing recalcitrant chemicals, mostly xenobiotic compounds, has, therefore, become one of the main ecological problems with increasing awareness worldwide[1].

Physico-chemical methods for treating effluents that are rich in synthetic dyes are typically techniques focused on the chemical interaction of the synthetic coloring with various solid surfaces or gel-based matrices under variable pH conditions, temperature, the available surface area for reaction and reaction time[2]. Synthetic dyes are artificial, colored substances with various industrial and biotechnological uses. Such colors vary from natural colors that are typically derived from living organisms and have been in use since 32-11 kyr B.P. (Thousand years to the present time)[2,3].

Various methods for remediating recalcitrant xenobiotics found in wastewaters and effluents released from various industries have been explored[4,5]. The approaches used to cure recalcitrant synthetic dyes are usually classified as physical methods, chemical methods, or physicochemical methods depending on the dye's chemistry with different physical substances or polymers, and biotic source methods. The main factor is the high cost and expenditure involved in the use of these techniques in the industry together with the infrastructure necessary to ensure continuous operation with minimal efficiency loss. Except for a handful, most of these methods have failed to completely remove the color and achieve good water quality for recycling and reuse[4,6].

Nowadays, biological processes are very popular for xenobiotics degradation. Among them, fungal ones have been extensively investigated being eco-friendly and cost-effective, because most basidiomycetes are more tolerant of high pollutant concentrations. Fungal bioremediation is a promising technology that exploits its metabolic ability to eliminate or reduce xenobiotics[7]. Whole fungal organisms or parts of fungal filaments also demonstrated potential in the bioremediation of synthetic dyes[6].

The fungal systems provide an environmentally friendly and less costly alternative to traditional color remediation, as do bacterial cells, but the drawbacks include the long-term viability of these systems in the decoloration of synthetic color and generally less efficient in the synthesis and secretion of sufficient quantities of enzymes necessary for decoloration and mining[8].

Trichoderma is an omnipresent, free-living genus of fungi found in a wide variety of environments at all latitudes from fallow land to orchards, from agricultural fields to forest lands and from industrial gardens to polluted sites[9,10]. Trichoderma species have also recently been considered potential bioremediation agents because they are capable of breaking down and converting organic contaminants such as polycyclic aromatic hydrocarbons (PAHs)[11,12] and diesel fuel residues[13] and extracting heavy metals[14,15]. A fungal strain's ability to decolorate aromatic colors and oxidize gallic acid is known to predict its capacity to degrade recalcitrant organopollutants[16].

Trichoderma atroviride[17], *T. longibrachiatum*[18], *T. harzianum*[17] are some of the *Trichoderma* sp. studied as

laccase sources. The purpose of this study was to isolate *T. longibrachiatum* from the decayed plant source and to evaluate its decolorizing activity on textile dyes.

Material and Methods

Sample collection and isolation of fungi: The samples were collected from the bark of the dead and decaying tamarind tree in Kyaukse at Mandalay Region. The collected samples were rinsed thoroughly with tap water and then put into the plastic boxes containing moist tissue papers. The samples were incubated at 25°C for two weeks.

The sample was periodically checked under a stereo-microscope for a fungal sporulating structure and labeled. Under a stereo-microscope, the fungal sporulating structure was picked up with a sterile needle and transferred onto a glass slide containing one drop of sterile distilled water. The conidial suspension was checked under a compound microscope to ensure the identity. Then, the conidial suspension was picked up with a sterile wire-loop and streaked on a medium plate (Corn Meal Agar with 0.5mg/L streptomycin sulfate).

Plates with fungal conidia were incubated at 25ºC for 24-48 h and periodically checked under a stereo-microscope for germination. When the germination occurred successfully, a small plug of agar with the germinated conidia was picked up and transferred onto the PDA medium plate. At least 5-10 isolates of each species were selected and transferred to ensure that these isolates were identical. The pure isolate was finally transferred to PDA (Potato Dextrose Agar) medium plate and incubated at 25°C for 3days. After incubation, the plates were maintained at 4°C for further study.

Morphological analysis: The sample or plate was checked under a stereo-microscope for a fungal sporulating structure. Under a stereo-microscope, the fungal sporulating structure was picked up with a sterile needle and transferred onto a glass slide containing a drop of lactophenol on a glass slide and then covered with a coverslip. Slides were examined under a compound microscope and photographed.

Molecular characterization of the isolated strain: The isolated fungal strain was sent to Biomed sequencing company, Beijing for DNA sequencing. The isolate's ITS1 and ITS4 gene sequences were then analyzed using BLAST tools. Those sequences with the highest identity were selected and aligned in the multiple-sequence alignment program, Clustal X[19] and MEGA v6.0 was used to construct the phylogenetic tree following the neighbor-joining method[20]. To evaluate the tree's topology, its neighbor-joined data were bootstrapped using 1000 randomized data sets[21].

Dyes: Synthetic dyes used in this study are acid orange 7, bromothymol blue, bromocresol purple, congo red and crystal violet. Their detail structure and maximum absorption – λ_{max} (nm) are shown in table 1. Acid orange was kindly provided by the textile industry and others are analytical grade dyes purchased from the chemical company.

Screening of the decolorizing activity of *Trichoderma longibrachiatum*: Decolorization of the isolated fungi on synthetic dyes was qualitatively measured on malt extract agar plate containing 0.01 % of each representative dye. To describe briefly, the 5 mm fungal plug of freshly prepared *T. longibrachiatum* was placed on the center of the plates containing the respective dye and the plate without any fungus was also prepared as a control. The plates were incubated at 25 °C for seven days and the decolorizing activity was recorded by comparing it with the control plate.

Decolorization assay: Decolorization assay was evaluated in the terms of percentage decolorization using UV-vis Spectrophotometer. A fresh overnight culture broth of *T. longibrachiatum* was first prepared using Potato Dextrose Broth (PDB). About 10 % of the overnight inoculum was added into 100 mL conical flasks containing 45 mL of malt extract broth medium amended separately with 0.01 % of each of the previously mentioned dyes. Each flask was incubated at 150 rpm on a rotary shaker at 25 °C for seven days and its decolorization was performed at 2, 4, 8, 24, 48, 72, 120, and 168 h respectively.

1 mL of each inoculated flask was taken and centrifuged at 6000 rpm for 10 min. Decolorization was assessed by measuring the OD value of the supernatant with the help of a UV-vis Spectrophotometer at wavelength maxima (λm) of the respective dye. Un-inoculated malt extract medium supplemented with 0.01 % of the respective dye served as control. The percentage of decolorization was calculated using the following equation:

Percentage of decolorization = Initial OD – Final OD/Initial OD x 100 [22]

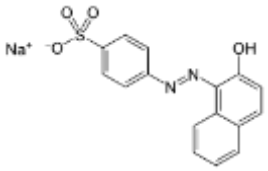
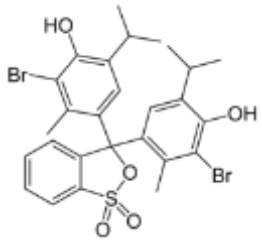
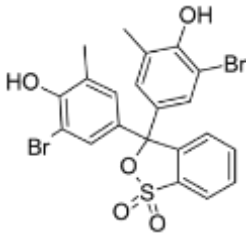
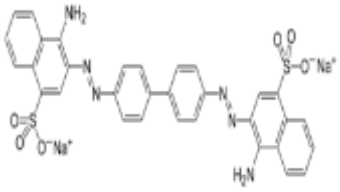
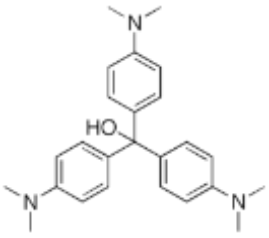
Optimum factors affecting the decolorization of the synthetic dyes by *T. longibrachiatum*

Effect of different dye concentrations on the decolorization: This assay was investigated using malt extract liquid medium supplemented with different concentrations of two types of dyes (100, 150, 200, 250 ppm); acid orange and congo red. As previously mentioned, 5 mL of the overnight fresh liquid culture of the fungal isolate was added into the MEA medium containing the respective dyes.

After seven days of incubation at 150 rpm on a rotary shaker at 25 °C, the culture was centrifuged at 6000 rpm for 10 min. The decolorization assay was performed as stated before.

Effect of pH on the decolorizing activity of *T. longibrachiatum*: Effect of pH on dye decolorization by *T. longibrachiatum* was investigated by adjusting the pH of the medium using hydrochloric acid (HCl) and sodium hydroxide (NaOH).

Table 1
Structures of the synthetic dyes used in the present study and the wavelength of maximum absorption – λ_{max} (nm).

Dye	Structure	λ_{max} (nm)
Acid orange 7		485
Bromothymol blue (BTB)		434
Bromocresol purple		437
Congo red		520
Crystal violet		580

The pH levels performed in this study were 3, 4, 5, 6, 7, 8, 9, 10, and 11. Dye decolorization experiments were carried out in 100 mL conical flasks containing 50 mL of the malt extract medium amended with 0.01 % of the respective dye. Decolorization assay was performed by a UV-vis spectrophotometer.

Effect of temperature on the decolorizing activity of *T. longibrachiatum*: The fungal isolate was incubated at various temperatures in the range of between 20 and 45°C with an interval difference of 5°C. *T. longibrachiatum* was incubated using malt extract medium amended with 0.01 % of the respective synthetic dyes at different temperatures for seven days. Each inoculum was centrifuged at 6000 rpm for 10 min and decolorization assay was evaluated by using the method previously described.

Analysis of Variance (ANOVA): Data analysis was evaluated by One-way ANOVA followed by the least significant difference (LSD) tests at p <0.05 using the Minitab 17.0 statistical software package (State College, PA 2010).

Results

Morphological analysis: Trichoderma colonies grew fast on PDA and matured within 5 days. The colonies were wooly at 25 ° C and on potato dextrose agar and become compact in time. The color was white from the front and, scattered patches of blue-green or yellow-green became visible as the conidia formed. Often, these patches could form concentrated rings. This species could grow over a wide temperature range; however, the optimum temperature for growth is about 35°C.

Under a stereomicroscope, septate hyaline hyphae, conidiophores, phialides and conidia were observed. Chlamydospores were also produced by *T. longibrachiatum*. Conidiophores were hyaline, branched and may display a pyramidal arrangement on occasion. Phialides were flask-shaped, hyaline and inflated at the base. They were attached to the conidiophores at right angles. The phialides were solitary or arranged in clusters. Conidia (3 µm in diameter, average) were one-celled and round or ellipsoidal. They were smooth-or rough-walled and grouped in sticky heads at the tips of the phialides (Figure 1).

Molecular characterization of the isolated strain: The isolated fungal strain was sent to Biomed sequencing company, Beijing for DNA sequencing. The isolates ITS 1 and ITS 4 gene sequences were then analyzed using BLAST tools. Those sequences with the highest identity were selected and aligned in the multiple-sequence alignment program, Clustal X[19] and MEGA v6.0 was used to construct the phylogenetic tree following the neighbor-joining method[20]. To evaluate the tree's topology, its neighbor-joined data were bootstrapped using 1000 randomized data sets[21]. Phylogenetic analysis of ITS region sequences with other Trichoderma species was illustrated in figure 2.

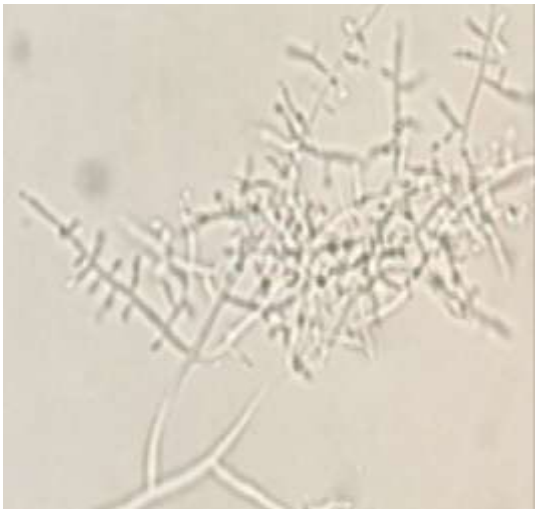


Figure 1: Morphology of *Trichoderma longibrachiatum* under a stereo microscope.

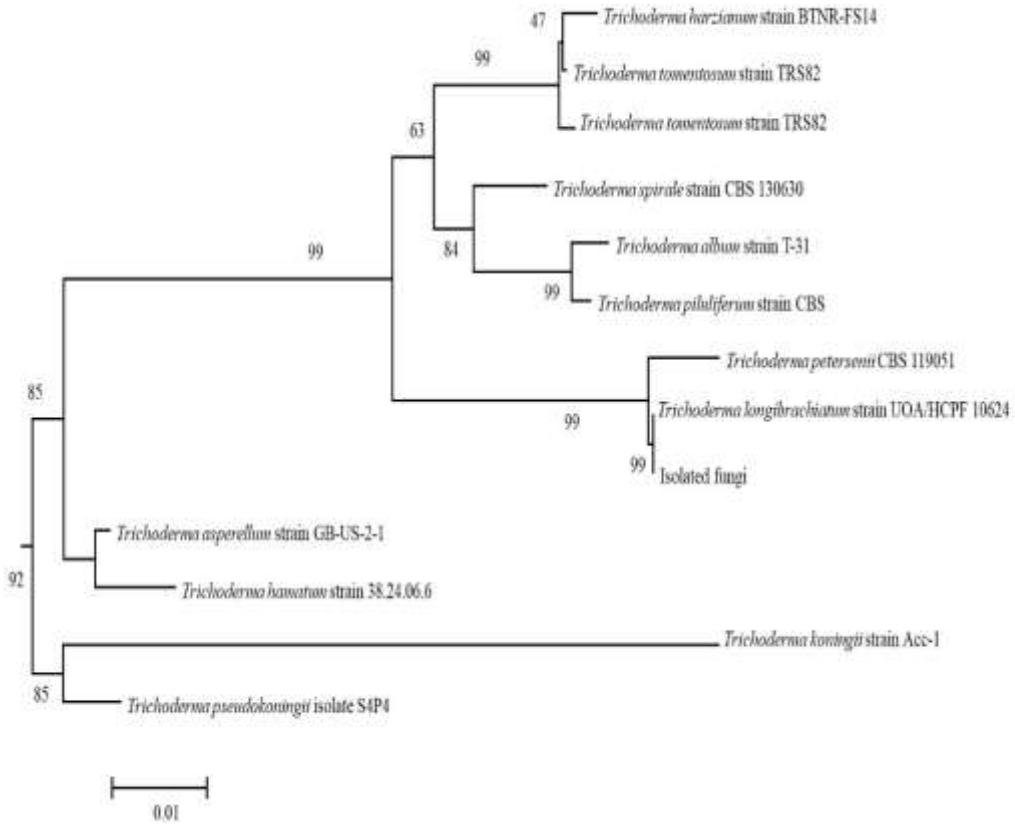


Figure 2: Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method[33]. The optimal tree with the sum of branch length = 0.21659941 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches[34,35]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[36] and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 507 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[20].

Preliminary screening on decolorizing activity by *Trichoderma longibrachiatum*: The decolorization of the dye in the medium was measured by the visual loss of color from the Petri plates inoculated. After seven days period, the radial growth and change in color intensity were calculated. For the visual absence of color from the background of the semisolid media of plates, the dye-containing plates were examined. The decolorization of dye in the culture medium was found compared to the control plates without inoculum. However, results indicated low fungal degradation efficiency in agar-based dye decolorization assay which may be due to higher concentrations of dyes used (100 ppm) or because the fungus needs longer incubation periods to extract the dye completely. Therefore, in liquid culture systems, where dye is more susceptible to fungal enzymes, further evaluation of dye decolorization was performed.

Quantitative measurement of the decolorizing activity of *Trichoderma longibrachiatum*: From the quantitative evaluation of the decolorization, *T. longibrachiatum* reduced the color intensity of the synthetic dyes used in this experiment. The percentage of decolorization was the best after 168 h incubation. *T. longibrachiatum* decolorized congo red the best and bromothymol blue was the dye that decolorized the least by the fungal isolate.

At the final sampling time (168 h), the decolorization of congo red by *T. longibrachiatum* reached a maximum of about 90.16 % followed by 80.81 % of decolorization on acid orange while the fungal isolate reduced only 70.34 % of the color intensity of bromothymol blue (Figure 3).

From this experiment, congo red and acid orange dyes were selected for next study because not only the fungal isolate decolorized these two dyes strongly but also these dyes were azo dyes which are mostly used in textile industries.

Effect of different dye concentrations on the decolorization: Dye concentration in the culture medium exerted greatly on decolorization by fungi. The dye decolorization was maximum (51.82 and 79.27 %) in 100 ppm of acid orange and 100 ppm of congo red after 14 days of incubation time. *T. longibrachiatum* showed the slower decolorization on more concentrations of dye, which was only about 23.1 % decolorization of acid orange and 65.56 % of congo red even after the fourteen incubation days (Figure 4 and 5).

Effect of pH on the decolorizing activity of *T. longibrachiatum*: pH is one of the most important factors controlling the adsorption of a dye onto suspended particles. The acidic or alkaline condition of the medium highly affected the dye removal by the microbes. A spontaneous pH change in the solution may occur during the decolorization process which affects the decolorization of the dye. In this experiment, *T. longibrachiatum* showed the decolorizing activity at the alkaline condition of the culture medium. It reached a maximum (87.63 and 83.09 %) reduction of acid orange and congo red at pH 10. At acidic conditions (pH 3), there was a significant decrease in color removal and the decolorization was only 48.57 % and 62.995 % of acid orange and congo red. In this study, alkaline conditions strongly favored the decolorizing ability of *T. longibrachiatum* (Figure 6).

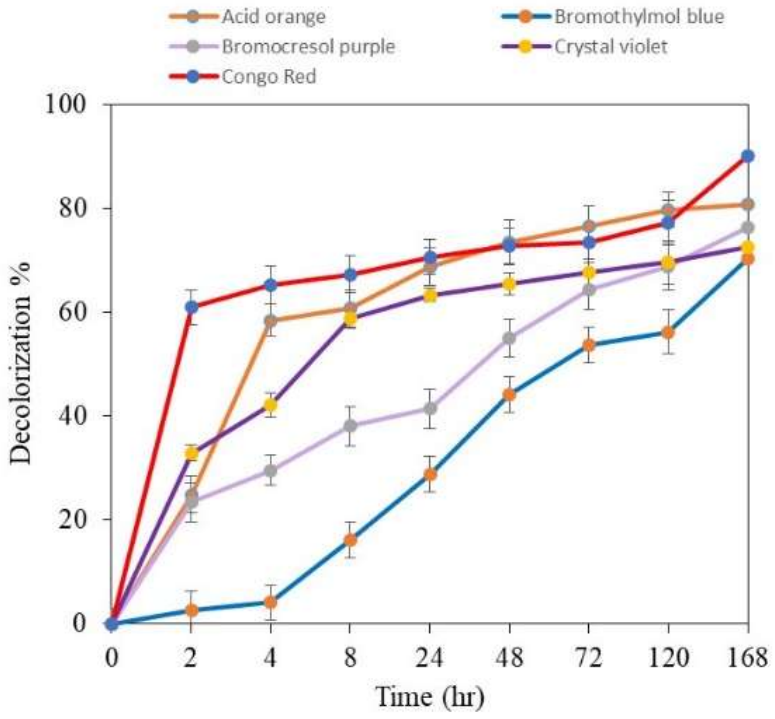


Figure 3: Decolorizing activity of *T. longibrachiatum* on the synthetic dyes at different incubation periods.

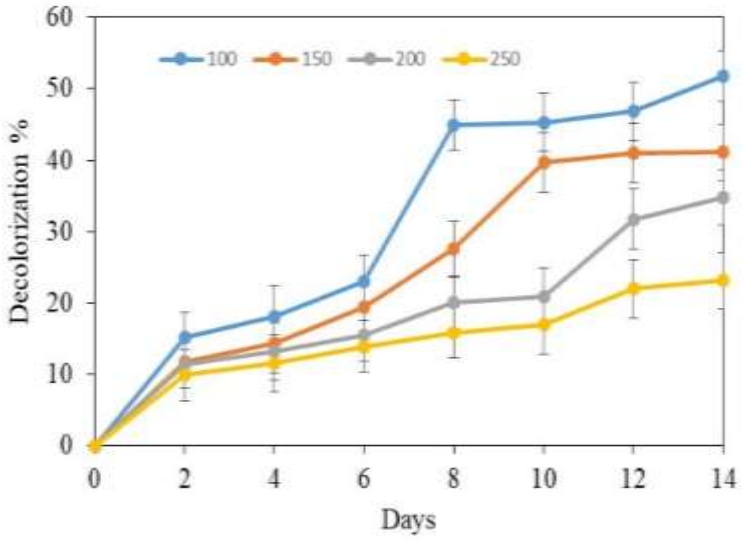


Figure 4: Effect of dye concentration on color removal of acid orange dye.

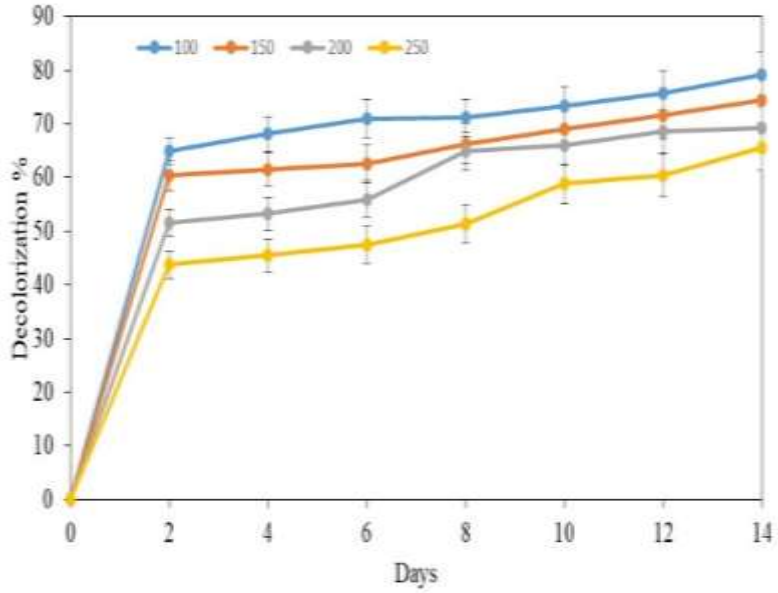


Figure 5: Effect of dye concentration on color removal of Congo red dye.

Effect of temperature on the decolorizing activity of *T. longibrachiatum*: Temperature greatly affected on the decolorizing activity of the microbes. The highest decolorization of 86.016 % of acid orange and 73.033 % of Congo red was achieved by incubating *T. longibrachiatum* at 35°C and the activity slowly decreased at higher temperatures.

It declined to 40.34 % decolorization of acid orange and 39.7 % reduction of congo red at 45°C. The decolorizing activity was not also efficient at lower temperatures. The fungal isolate showed the decolorization of 44.39 % of acid orange and 51.383 % of congo red at 20 °C (Figure 7).

Discussion

The present study was conducted to investigate the microbial decolorization of synthetic dyes, taking a fungus *Trichoderma longibrachiatum* strain as the experimental organism and the synthetic dyes; acid orange 7, bromothymol blue, bromocresol purple, congo red and crystal violet as the test coloring.

In the preliminary screening test, the isolate had grown well on the special solid media according to Pointing[23] substituted with various dyes and stains (0.01 %). Hyphal features such as the colors of these colors and stains were found to adhere to the fungal hyphae.

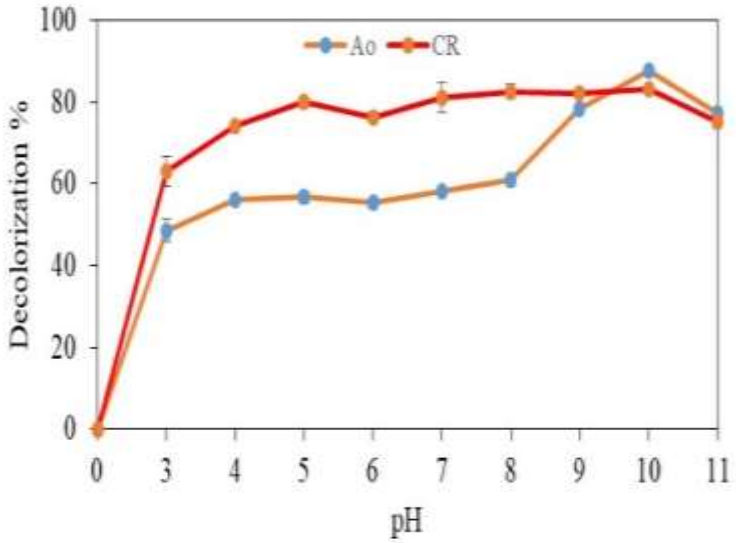


Figure 6: Effect of pH on color removal of two azo dyes.

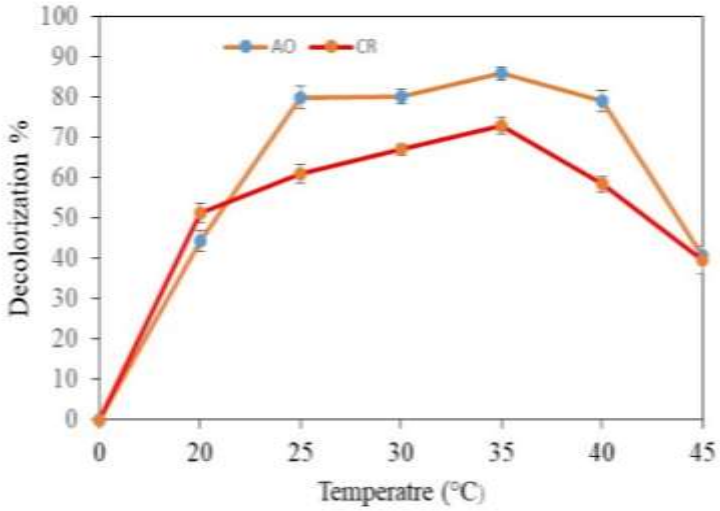


Figure 7: Effect of temperature on color removal of two azo dyes.

The effect of dye concentration on microbial growth is an important consideration for its field application, as the higher dye concentration inhibits the biosynthesis of nucleic acid and cell growth[24]. To study the effect of varying dye concentrations, the experiment was performed at a fixed concentration of fungal inoculum (10 percent) at various dye concentrations (100, 150, 200 and 250 ppm). The rate of color removal decreased to a significant extent with an increase in the initial dye concentration. *T. longibrachiatum* decolorized about 51.82 % of acid orange (100 ppm) and 79.27 % of Congo red dye (100 ppm) while it showed the color reduction of 23.1 % of acid orange (250 ppm) and 65.56 % of Congo red dye (250 ppm).

A literature survey suggests that increasing the concentration of dye slowly decreases the rate of decolorization possibly due to the toxic effect of dyes and/or insufficient concentration of biomass (or inappropriate ratio of cell to dye), as well as blocking the active sites of azoreductase by dye molecules with different structures[25-27].

Changing the pH of the culture medium appears to positively affect the decolorizing activity of *T. longibrachiatum*. The pH has a profound effect on dye decolorization efficiency. In earlier studies, the optimum pH for color removal was often at a neutral pH or a slightly alkaline pH. Color removal levels were only higher at optimum pH but continued to decrease rapidly at a strongly acidic or strongly alkaline pH. Color removal was the highest in the pH range of 6.0-10.0 as recorded by several researchers for different colors studied[28,29]. Our results were consistent with these reports since the fungal isolate showed the maximum decolorizing activity on the synthetic dyes at alkaline pH (6-10).

Incubation temperature is an important process parameter that varies from one microorganism to another. A slight change in the incubation temperature will influence the microorganisms' growth and enzyme activities. Thus, temperature is a paramount factor for all processes associated with microbial vitality, including water and soil remediation. Several experiments have been conducted about the activation energy of microbial decolorization of azo dyes[30]. Narrow temperature ranges were established as required for the decolorization of azo dyes by extremely complex microorganisms inhabiting active sludge. It has also been documented that changes in temperature in microbial physiology lead to a sudden change in the activation energy. Previous literature indicated that the rate of color removal raised with increasing temperature to a certain limit; subsequently, the decolorization frequency is slightly reduced. For optimal growth of the microorganisms, the temperature range of 30-45°C is generally required and this also corresponds to maximum removal of the color[31]. *T. longibrachiatum* reached a maximum color removal at 35°C and a further increase in temperature above 35ºC resulted in a decrease in decolorizing activity.

This result was in agreement with the report discovered by[32] that the optimal temperature for growth of *T. longibrachiatum* is 35°C. Decolorization rate of azo dyes increases to the optimum temperature, and consequently the decolorization frequency is marginally reduced. This decline may be due to the loss of cell viability or the denaturation of an azo reductase enzyme at higher temperatures[26].

Conclusion

Thus, by the present study, we concluded that the fungus *Trichoderma longibrachiatum* could be effectively used as a good microbial source for the wastewater treatment system. The efficiency of color removal was found to depend on some test parameters such as incubation time, dye concentration, pH and temperature of the culture medium.

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