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Original Article

Decolorization of Synthetic Dyes by *Aspergillus flavus* Strain EF-3 Isolated from Textile Dyeing Sludge

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Treatment of textile and dyeing wastewater using indigenous dye-degrading microorganisms is considered to be a sustainable bioremediation strategy. In the present study, a dye-decolorizing fungal strain was isolated from dyeing sludge and identified as *Aspergillus flavus* strain EF-3 on the basis of cultural, morphological and internal transcribed spacer (ITS) region sequencing. The fungal strain was tested to decolorize five commercially available textile reactive dyes and dye mixture as well. The decolorization efficiencies were ranged from 62% to 100% for 100 mg/L concentration of each dye in Czapek Dox broth within 6 days of incubation at 27°C, and 120 rpm batch-culture conditions. The dyes Novacron Brilliant Blue FN-R, Bezema Yellow S8-G and dye mixture were completely decolorized within the stipulated time period. The nature of decolorization was found to be co-metabolic, i.e., dyes were not used as the sole source of energy for fungal growth and required an external co-substrate, sucrose for induction of decolorization process. The dye removal mechanism involves initial biosorption of the dye in fungal mycelium followed by subsequent biodegradation to colorless end-product. The findings of the present study provide valuable insights to desgin effective biological treatment for dyeing wastewater using the fungus *Aspergillus flavus.*

Keywords *Aspergillus flavus*; Biosorption; Reactive dye; Decolorization; Biological Treatment.

Introduction

Synthetic dyes are widely used in the textile, pharmaceutical, cosmetic, paper, printing, drug, and food processing industries[1]. These dyes fall into various categories based on their mode of application viz., reactive, basic, acid, fluorescent, direct, sulphurous, or vat dyes, based on the staining requirements of different fiber types such as cellulose (cotton, rayon, linen etc.), protein (wool, angora, mohair, cashmere and silk etc.) and synthetic fibers (polyester, nylon, spandex, acetate, acrylic etc.)[2]. However, in the textile industries, reactive dyes are widely used due to their wide variety of color shades, high wet fastness, ease of application and minimal energy consumption[3].

In typical textile wastewater, the average concentration of dye is 300 mg/L; although above 1 mg/L are naturally visible in water[4]. During textile processing such as dyeing, washing, de-sizing and finishing processes, a significant amount of water is consumed which consequently produces a huge volume of dyeing wastewater[5]. Improper treatment of this dyeing wastewater leads to the pollution of soil and natural water bodies due to the presence of toxic and hazardous aromatic compounds, heavy metals, chlorides, etc.[6]. Discharge of dyeing wastewater into nearby receiving waters bodies hinder sunlight penetration, causes eutrophication, and thereby, threatens the ecology and biodiversity [7, 8]. In addition, such noxious dyeing effluent can adversely affect water quality for agricultural purposes and are reported to change the microbial community and soil enzyme activities[9, 10]. Untreated dyeing wastewater can be a big public health concern due to their mutagenic and carcinogenic properties[11,12].

The treatment of synthetic dyes is an arduous task due to their synthetic nature and complex aromatic structure. Despite this, various physico-chemical methods such as adsorption, coagulation, flocculation, ozonation, electrochemical treatment and advanced oxidation methods such as Fenton, photo Fenton, and photocatalytic processes and their combinations have been used to treat dyeing wastewater[13]. Though, these processes are relatively efficient in the treatment of various dyes from industrial wastewaters; however, their application is not cost-effective and formation of toxic by-products[14].

Microbial decolorization and degradation is an environmentally friendly and cost-effective alternative to conventional physico-chemical treatment processes[16, 17]. Biosorption and biodegradation are the two major decolorization mechanisms of dyeing wastewater by microorganisms[18]. The former process is mediated by inactivated biomass, commonly known as 'biosorbent' whereas both mechanisms can act together in living cells[19]. Over the past few decades, bioremediation approaches using the principle of biodegradation and biosorption in aerobic,

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anaerobic or combined aerobic/anaerobic treatment processes have been investigated[20, 21]. Biological processes are advantageous due to the conversion of organic compounds to non-toxic products[22]. In contrast to bacteria, the fungal decolorization using an oxidative mechanism has the advantage of giving products that are less toxic than the parent dye and also serve as a promising biosorbent due to their high biomass content and unique cell surface properties[13]. In order to develop efficient bioremediation technology, it is important to understand the microbial decolorization and degradation processes to search for broad-spectrum and highly efficient dye mineralizing microorganisms.

In the way of employment-intensive industrialization, textile and dyeing industries are playing an important role in the economic development of Bangladesh. But still, the treatment of industrial wastewater in Bangladesh isn't up to the mark. For this reason, it is an important issue to develop novel environment-friendly and cost-effective technologies to solve these environmental problems.

In the present work, a fungal strain, *Aspergillus flavus* strain EF-3, isolated from textile dyeing sludge for decolorization was used for the decolorization of five commonly used textile reactive dyes.

Material and methods

Dyes

Dyes used throughout the present study were commercially available as reactive textile dyes *viz.* Novacron Orange FN-R, Novacron Brilliant Blue FN-R, Novacron Super Black G, Bezema Yellow S8-G and Bezema Red S2-B which were collected from a local textile dyeing industry of Chattagram, Bangladesh. Absorption Maxima (λ_{max}) of each dye was determined by scanning aqueous solution of each dye within visible range (400-700nm) using a double beam UV-VIS Spectrophotometer (Optima sp 3000 nano, Japan) as described in the previous study[23].The stock solution of the experimental dyes was prepared by dissolving 1 g dye powder into 100 mL sterile distilled water, filtered with a 0.22 µm membrane filter and stored in the brown bottle at room temperature. A mixed solution of all dyes was prepared by adding a 1:1 portion of each dye solution. From the stock, working solutions were prepared with a final concentration of 100 mg/L. According to the scientific literature, dye concentration in a typical dye-house effluent is in the range of 10 to 50 mg/L[24], and for this reason, a final dye concentration was selected as 100 mg/L for the present study.

Mycological Media and Chemicals

Complex nutrient-rich medium such as Potato Dextrose Agar (PDA) was used for the isolation and preservation of fungi from collected samples. Defined minimal salt medium such as Czapek Dox agar (CDA) was used for screening of dye decolorizer. PDA, CDA, and Sabouraud Dextrose Agar were used for phenotypic characterization of the selected fungal isolate. Liquid Czapek Dox medium was used for the dye-decolorization assay. All media ingredients and reagents were of analytical grade (Hi-Media, India; Merck, India; and Sigma-Aldrich Ltd., USA).

Isolation and Screening of dye-decolorizing fungi

Textile dyeing sludge samples were collected from the effluent treatment plant of a local textile dyeing industry of Chattagram, Bangladesh, and transported in sterile plastic bags to the laboratory and kept at 4°C before and after analysis. In order to isolate dye-decolorizing fungi, serial 10-fold dilutions (10^{-1} to 10^{-7}) of the collected sludge samples were carried out. Briefly, 1 g sludge sample was suspended in 10 ml sterile distilled water (10^{-1}) and vortex well to get uniform sludge suspension. Likewise, the sample was serially diluted by transferring 1 mL sample from each dilution to the next 9 mL of sterile distilled water to yield the corresponding dilution, i.e. 10^{-2} to 10^{-7}. Finally, 1 mL aliquot of each dilution was added to a sterile PDA plate and inoculated by the spread plate technique. To avoid bacterial contamination, 200 gm/L of streptomycin was added with the medium. After inoculation, plates were incubated at 27°C for 6 days till the appearance of visible growth. Based on colony characteristics, different colonies were isolated and purified by repeated streaking. The purified isolates were maintained in PDA slants and preserved as stock culture in the refrigerator at 4°C for further study.

The screening of potent dye-decolorizing fungi was carried out by the dye-agar plate assay method. Briefly, freshly prepared fungal disks (9 mm) were inoculated in the center of CDA medium supplemented with 100 mg/L of experimental dye mixer solution and incubated at 27°C for 6 days. Inoculated plates were observed periodically.

Fungal Characterization and Identification

Phenotypic Characterization

The selected dye-decolorizing fungal isolate EF-3 was characterized based on the cultural and morphological characteristics and provisionally identified up to the genus by following the taxonomic guidelines[25].

Genotypic Characterization

The genomic DNA of the fungal isolate EF-3 was extracted by using Plant/Fungi DNA Isolation Kit (Sigma-Aldrich, USA) in accordance with the manufacturer's instruction. The extracted genomic DNA was quantified using a nano-drop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, USA). Polymerase chain reaction (PCR) of the isolated fungal DNA was carried out in Veriti™ thermal cycler (Applied Biosystems, USA). The internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rDNA regions (600 bp) was amplified using universal primers of ITS-1 (5Ê-TCCGTAGGTGAACCTGCGG-3Ê) and ITS-2 (5Ê-TCCTCCGCTTATTGATATGC-3Ê)[26]. PCR reaction was performed in a final volume of 25 µL containing 12.5 µL 2×GoTaq® G2 Green Master Mix (Promega, USA), 2 µL of each

forward and reverse primer (10 pmol), 2 µL DNA template and 6.5 µL nuclease-free water. The PCR conditions were set as follows: initial denaturation at 94ºC for 5 min; 35 cycles of (30 s at 94ºC for denaturation, 30 s at 54ºC for annealing and 1 min at 72ºC for extension), followed by a final extension step of 5 min at 72ºC and 4°C hold. The PCR product was separated by electrophoresis on 1% agarose gel containing ethidium bromide (0.5 mg/mL) and visualized under UV light in an Axygen™ Gel documentation system (Fischer Scientific, USA). Finally, the approximately 600 bp PCR product was purified with Pure Link™ PCR Purification Kit (Invitrogen, USA) and sequenced with 3130 ABI genetic analyzer using Big Dye Terminator v3.1 Cycle Sequencing Kit following the manufacturer instructions (Applied Biosystems, USA) at National Institute of Biotechnology, Dhaka-1349, Bangladesh.

The contig sequence was generated from the raw sequences of forward and reverse primer using DNA baser sequence assembly software v5.15. The contig sequence was searched for similarities in the NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search program and multiple sequence alignment of the highly similar sequences was done by using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) online platform. The phylogenetic analysis was conducted using the Molecular Evolution Genetic Analysis Version X (MEGA X) software[27]. The evolutionary history was inferred using the Neighbor-Joining method[28] wheras the evolutionary distances were computed using the Tamura 3-parameter method[29]. The pairwise deletion option was used to remove all ambiguous positions from each sequence pair. There were a total of 968 positions in the final dataset.

Dye-decolorization assay in batch-culture

The mycelial disks (8 mm diameter) were cut from the actively growing region of the fresh fungal culture and inoculated to 250 mL Erlenmeyer flasks containing 50 mL of sterile CDB (pH 6.5) amended with each of the filter-sterilized experimental dye solutions to a final concentration of 100 mg/L. CDB broth without co-substrate i.e., sucrose, was also inoculated to assess the fungal utilization of dyes as the sole source of energy. Control was maintained without inoculation of the fungal culture. Incubation was carried out at 27°C and 120 rpm shaking condition for a period of 6 days. After 2, 4 and 6 days of interval, the culture broth was withdrawn, filtered through Whatman® No. 1 filter paper followed by centrifugation at 10,000 rpm for 15 min by a centrifuge (Kubota 6930, Japan). The absorbance of the supernatant was recorded at the corresponding λ_{max} of each dye by a double-beam UV– visible spectrophotometer. The decolorization (%) was calculated from the standard curve of each dye using the following formula:

Dye decolorization (%) = [(Initial dye concentration - Final dye concentration) / Initial dye concentration] × 100

NCBI Gene Bank Deposition

The nucleotide sequence of the identified fungal strain *Aspergillus flavus* EF-3 was deposited to NCBI gene bank database under the accession Number MN447393.

Statistical analysis

All experiments were conducted in triplicate. Variation within a set of data was analyzed by using GraphPad Prism Software (v.6 for Windows) and mean ± standard deviation values were expressed.

Results

Isolation and screening

A total of five morphologically distinct fungal colonies were isolated from the collected samples. For screening of potent dye decolorizing fungi, all the isolates were grown in CDA medium supplemented with each of the dye solution (100 mg/L). The apparent dye decolorization efficiency and growth of different fungal isolates were monitored for seven days and the observation was recorded on the basis of mycelial growth on dye supplemented CDA medium and dye uptake into/onto the hyphal mass macroscopically. Among the fungal isolates, isolate EF-3, was screened out based on its ability to grow on the dye supplemented medium, as evident by the formation of zone of decolorization around the colonies and reverse coloring of the fungal mat as that of the dye color.

Phenotypic and genotypic characterization

The selected fungal isolate EF-3 was identified on the basis of cultural, morphological and molecular characteristics. The cultural and microscopic characteristics of the isolate EF-3 are outlined in Table 1. By following the taxonomic guidelines of

Table 1: *Cultural and morphological characteristics of the dye decolorizing fungal isolate EF-3.*

Parameters	Observations
Cultural Characteristics	Growth pattern followed the order as SDA>CDA>PDA. Young colonies were wooly and become olive to lime green with cream colored reverse background.
Microscopic characteristics	Hyphae were septate and hyaline. Conidiophores (200.85-265.41µm × 11.41-17.31 µm) were long, hyaline, smooth, un-branched, coarsely roughened with down thick walled foot-cell. Conidiophores enlarging upward and terminated at globose to subglobose vesicle (10.44-16.65 µm). Sterigmata (5.50-6.34 µm) borne in one series bearing conidial chains. Conidia light green in color and globose to subglobose (2.60-4.96 µm).

the 'A Manual of Soil Fungi' by Gillman[25], the isolate EF-3 was provisionally identified as *Aspergillus sp.* (Figure 1). The taxonomic status of the isolate EF-3 was further determined by the sequencing of approximately 600 bp ITS genes (ITS-1 and ITS-2). The result of the sequence analysis revealed that the fungal isolate EF-3 was clustered with *Aspergillus flavus* strain M68 (accession no. MH746007.1) with 98.31% identity and the isolate was finally identified as *Aspergillus flavus* strain EF-3 (Figure 2).

Dye decolorization extent

The extent of decolorization of five reactive textile dyes by the fungal strain EF-3 was investigated in Czapek-Dox broth medium in the presence and absence of co-substrate, sucrose. To understand the ability of the fungal strain to treat actual dyeing effluent containing a complex mixture of dyes, we also tested the decolorization extent of all five dye mixture as well. After 2, 4 and 6 days of the incubation period, the satisfactory result of dye decolorization was recorded as shown in Figure 3 and Figure 4. Almost all of the experimental dye solution, as well as dye mixture, was completely decolorized after 6 days of incubation, except Bezema Red S2-B and Novacron Super Black-G. Decolorization percentage of Bezema Red S2-B was found as 77.0% whereas that of Novacron Super Black-G was 62.0% after the complete incubation cycle. Initially, partial decolorization was recorded for almost all of the dyes after 2 days of the incubation period, but surprisingly, in case of Bezema Yellow S8-G and dye mixture almost complete decolorization was observed within that time period.

Nature and mechanism of dye decolorization

During decolorization assay, however, no visual decolorization was observed in broth medium without the presence of external co-substrate, i.e. sucrose and fungal growth in this medium was also suppressed. Whereas, significant fungal growth, as well as decolorization, was noticeable in the broth medium containing the co-substrate, sucrose. In addition to this observation, a preliminary assessment was made to investigate the dye decolorization mechanism. The mycelium of the fungal strain turned colored due to the adsorption of dye which further degraded and became colorless. The result indicated the initial biosorption of dyes followed by subsequent degradation to colorless end-products.

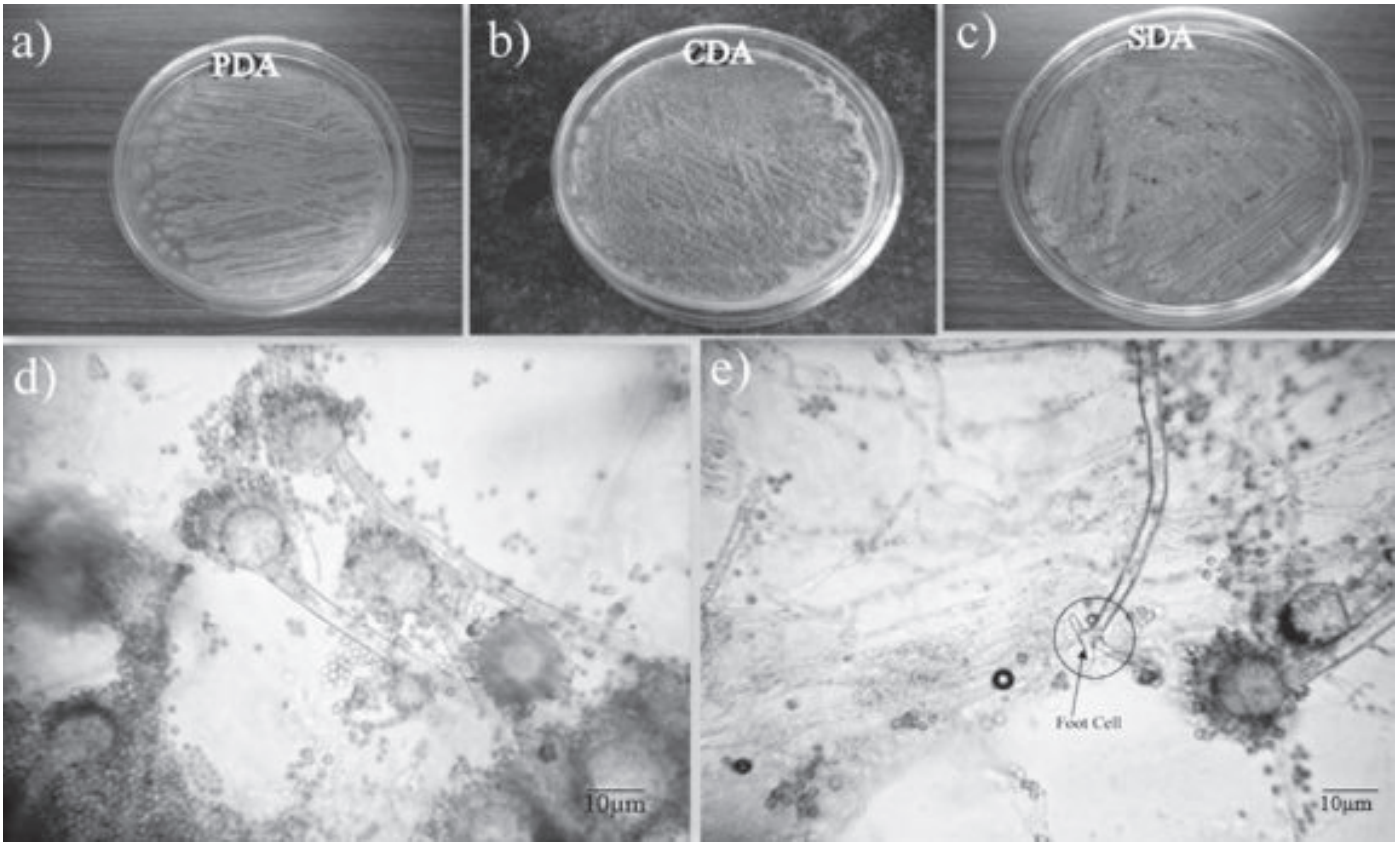

Figure 1. *Cultural and morphological characteristics of the fungal isolate EF-3. Growth pattern on a) Potato dextrose agar (PDA), b) Czapek dox agar (CDA) and c) Sabouraud dextrose agar (SDA). Photomicrograph at 40× magnifications showing cell-morphology (d) and foot-cell (e).*

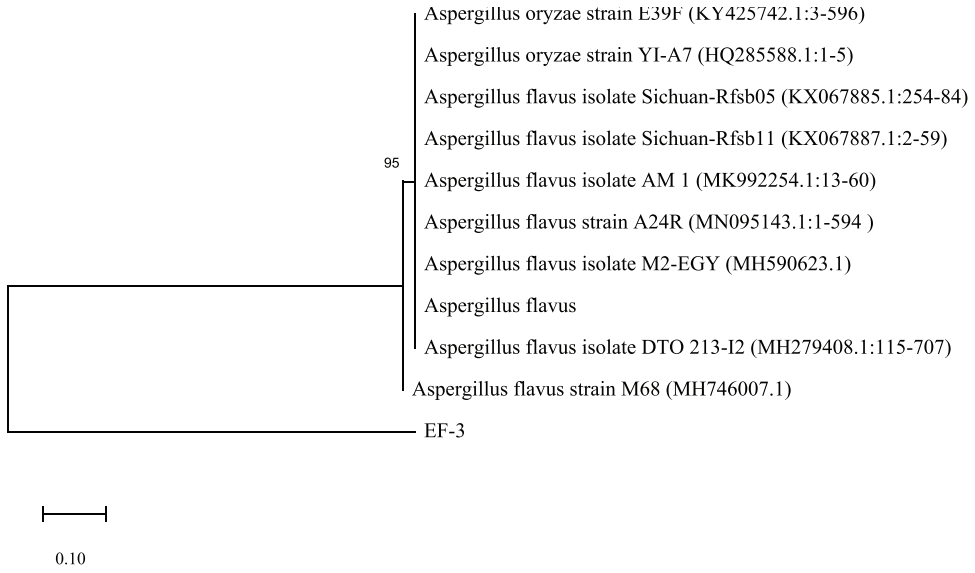

Figure 2. *Neighbor-Joining phylogenetic tree of the fungal isolate EF-3 based on ITS gene sequencing. The optimal tree with the sum of branch length = 1.29957418 is shown in Figure. The bootstrap test values (1000 replicates) are shown next to the branches. The scale bar represents sequence divergence. Database accession numbers are indicated in parentheses.*

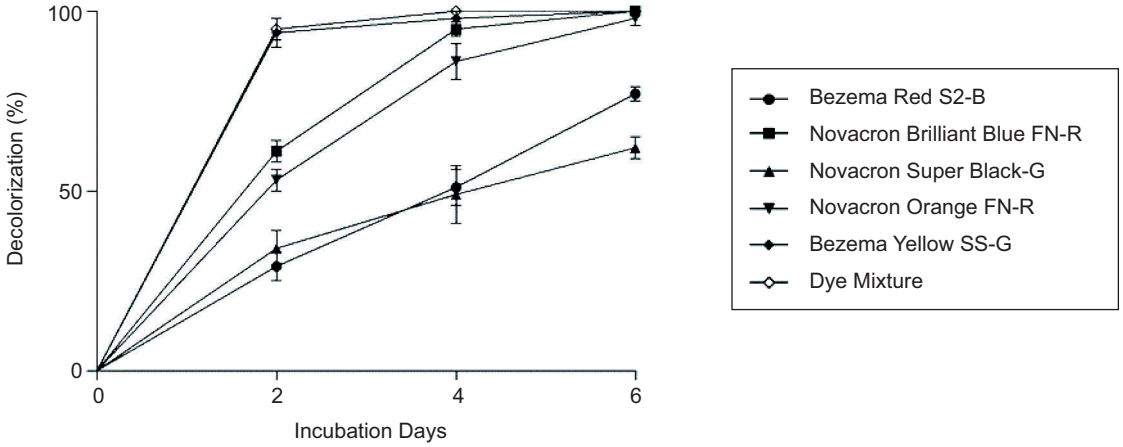

Figure 3. *Decolorization percentage (%) of the experimental dyes by Aspergillus flavus EF-3 after 6 days of incubation. The assay was performed in Czapek Dox broth containing 100 mg/L of each experimental dyes followed by incubation at 27°C and 120 rpm for 6 days. Samples were withdrawn at 2 days interval and decolorization percentage was calculated from the standard curve of each dye. The data is represented as mean ± SD of triplicate samples.*

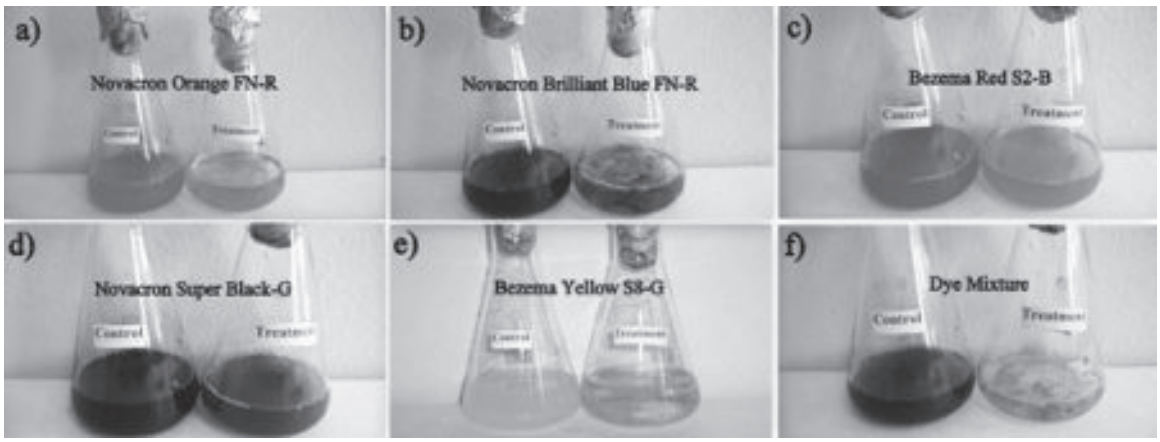

Figure 4. *Photograph of the decolorization of experimental dyes by Aspergillus flavus EF-3 in Czapek Dox broth after 6 days of incubation*

Discussion

Indiscriminate discharge of industrial effluent containing synthetic dyes and their toxic derivatives is a serious environmental threat in Bangladesh. As still there are no viable options to remediate this menace, biological treatment approaches involving biosorption and biodegradation by utilizing naturally adapted microorganisms seem to be promising in this regard. Hence, this study highlights some preliminary outcomes related to microbial remediation of synthetic dyes that might aid in the bioremediation process design.

In this study, a potent dye decolorizing fungus, designated as EF-3 was isolated and screened from local textile dyeing sludge. During the screening process, the fungal mycelium showed characteristic color similar to that of the tested dyes which presumptively suggests biosorption of the experimental dyes. According to Chen et al.[30] in the case of biosorption, the fungal mat becomes colored as that of the dye color while retaining the original mat color indicates biodegradation. Phenotypically the isolate was assigned to *Aspergillus sp.* which further identified as *Aspergillus flavus* strain EF-3 by ITS gene sequencing. The ITS region is the most widely sequenced DNA region in the molecular ecology of fungi and has been recommended as the universal fungal barcode sequence[31]. It has been observed that strain EF-3 shows good decolorization potential for almost all of the dyes compared to the decolorization activity of the bacterial isolates as described in our previous study[23]. The variation in decolorization efficiency and the time required for decolorization of various dyes may be due to the molecular complexity of the dyes, culture conditions and the enzyme system produced by the fungi[32]. A slower rate of decolorization can also be attributed to higher molecular weight and the presence of inhibitory groups like $–NO_2$ and $–SO_3$ groups in the dyes[6].

Aspergillus flavus are common inhabitants of synthetic dye contaminated sites and are recognized as active dye degrading fungus as evident from previous studies[33, 34]. The decolorization efficiencies of *Aspergillus flavus* strain A5p1 for 15 dyes were ranged from 61.7 to 100% at an initial concentration of 100 mg/L[35]. Another study showed 85.0% decolorization of Reactive Red 198 at a concentration of 50 ppm[36]. Andleeb et al. reported about 85.57% decolorization efficiency of 50 mg/L anthraquinone dye, drimarene blue K_2RL in lab-scale immobilized fluidized-bed bioreactor system using *Aspergillus flavus*.[37].

Media composition has greatly influenced the nature of decolorization and degradation of synthetic dyes by microorganisms. The present study has demonstrated that synthetic dyes are not used as a sole source of energy for fungal metabolism which may be due to their structural complexity and synthetic nature. The study, thus, highlights the obligate requirements of a labile organic nutrient source for fungal growth and decolorization. Previously, Abd-El Rahim *et al.*[38]. also revealed that enrichment of mineral salt medium with yeast extract significantly enhanced the decolorization extent of direct violet dye from 0.0% to 54.0% by *Aspergillus niger* whereas mineral salt medium devoid of yeast extract but with added inorganic nitrogen source, ammonium sulfate, lacked decolorization. This phenomenon was due to the co-metabolic nature of the fungus which is also evident from our previous study[19]. According to Gingell and Walker[39], the main mechanism of dye reduction is probably due to co-metabolic reaction, in which the reducing equivalents or reduced cofactors like NADH, NADPH, $FMNH_2$ and $FADH_2$ generated from the metabolism of organic co-substrates, act as a secondary electron donor for reductive cleavage of the azo bond. To date, very few researches were successful in isolating microorganisms capable of utilizing synthetic dyes as the sole source of carbon and energy[40]. Therefore, significant consideration should be given to the choice of microbiological media during microbial biodegradation as well as biotransformation assays; otherwise, it will create scientific ambiguity of the published literature.

A preliminary assessment by visual observation of the color of fungal mycelium indicates that the decolorization was probably due to initial biosorpion followed by subsequent enzymatic degradation to color-less end products. According to the literature, decolorization of dye involved adsorption of the dye compound at the initial stage followed by the decolorization through microbial metabolism[41, 42]. The reason behind the biosorption process is that reactive dyes typically contain azo based chromophores attached with different types of reactive groups that interact with the active sites (such as chitin, chitosan acidic polysaccharides, lipids, amino acid, and other cellular components), on the surface of fungi[43].

Conclusion

The present study demonstrated the potential of a fungal strain *Aspergillus flavus* EF-3 for decolorization of five textile reactive dyes and dye mixture. The strain was unable to utilize the dyes as the sole source of energy and require an obligate carbon source for fair growth and initiation of decolorization. The dye decolorization mechanism was preliminarily assessed and involved both biosorption and biodegradation. The studied strain should be viewed as a promising candidate for the biotechnological removal of dyes from industrial dyeing effluent. Although certain points need to be clearly addressed before its potential application, such as toxicity assessment of biodegraded products and the presence of mycotoxin in the treated effluent for ensuring environmental health and safety. Further research works might include the study of dye-removal kinetics, bioreactor design, and operational factors influencing the biological treatment process.

Conflict of interest statement

The authors declered that they have no conflict of interest.

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