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Research Article
Factors Influencing Decolourization and Detoxification of Remazol Brilliant Blue R Dye by *Aspergillus flavus*

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Abstract

Background and Objectives: Anthraquinone synthetic dyes are widely used in textile, dyeing and paper painting. The discharge of these dyes into the environment causes detriment. The removal of physiochemical dyes is sometimes unsuccessful and expensive. Biological removal is inexpensive, eco-friendly and may break down organic contaminants. In the current work, a fungal technique was applied to decolorize and detoxify dye. **Materials and Methods:** Dye decolorizing fungi isolation, selection and identification of the most effective isolate and dye decolorization optimization based on carbon and nitrogen sources. In addition, the product's cytotoxicity and metabolites were tested. The enzyme activities were measured to determine dye decolorization. **Results:** Decolorization of reactive blue 19 dye by the most effective fungal strain isolate (5BF) isolated from industrial effluents were studied. This isolate was identified as *Aspergillus flavus* based on phenotypic characteristics and confirmed using 18S rRNA gene sequencing. Thin-layer chromatography indicated that this strain is aflatoxins free. Furthermore, metabolites produced from dye treatment with *A. flavus* were assessed using gas chromatography-mass spectrometry. Toxicity data revealed that *A. flavus* metabolites were not toxic to plants. Using a one-factor-at-a-time optimization technique, a maximum decolorization percentage (99%) was obtained after 72 hrs in the presence of mannitol and NH_4NO_3 or NH_4Cl as carbon and nitrogen sources. Two enzymes (laccase and manganese peroxidase) were shown to be active during dye decolorization by *A. flavus*. **Conclusion:** The *A. flavus* strain was shown to be safe when it came to removing dye from a synthetic medium with high efficiency and their metabolites had no negative influence on the environment. As a result, this strain will be used in the future for dye wastewater bioremediation.

Key words: *Aspergillus flavus*, biodegradation, decolorization, remazol brilliant blue R, 18S rDNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A dye is a compound that may absorb part of the visible spectrum (chromophore). Over 10,000 dyes are accessible in the global markets, with a total yearly production of more than $7x10^5$ tons of dyestuff, almost 10% of which is released in the environment in wastewaters. Dyes are applied to painting and colouring paper, skin and clothes in nearly all aspects of our everyday routine. Up to 50% of dyes are not attached to textile fibre but remain in the liquid phase as contaminants. A variety of organic dyes, based on chemical structure are classified as azo and nitro-dyes, phthalocyanines, di-arylmethane and anthraquinones. Reactive blue 19 or Remazol Brilliant Blue R (RBBR) is an anthraquinone dye that is applied to textiles because of its high fastness[1]. This dye was chosen because it is widely used in Egypt's textile industry.

There is increasing public awareness about discharging textile dyes into the environment. Textiles industries provide considerable employments with low specialized skills and play an important part in many countries' economies. A great deal of liquid waste is produced every day from these industries[2]. Physical and chemical techniques for detoxification of dyes, including flocculation, adsorption, photochemical oxidation, Fenton oxidation, ozonation and ultrasound have been used but their continual application is limiting because of high energies, time and cost are necessary and because they produce significant quantities of toxic byproducts and sludge. The biological practice was seen as a good solution to this hazard[3]. Microorganisms including bacteria, fungi and yeasts are being intensively studied regardless of physical and chemical options available. Fungal bioremediation was shown to be more acceptable than bacterial bioremediation giving the availability of more biomass at a lower cost[4]. The efficient biodegradation of textile dyes by these fungi suggests that these microorganisms could be used in the bioremediation process. Fungi of *Aspergillus niger* and *Phanerochaete chrysosporium* isolated from soil contaminated with dye effluents (malachite green, nigrosin and basic fuchsin) showed strong bioremediation properties [5]. Various fungi are effective in mineralizing hazardous chemicals with highly oxidative and non-specific ligninolytic enzymes that are also relevant for the decolorization and breakdown of numerous dyes[6].

The eco-friendly microbial decolorization and detoxification by fungi have the potentials to degrade these organic compounds into simpler fragments. Fungi are capable of decolorization and biodegradation of many dyes including *Aspergillus flavus*, *A. niger*, *A. terricola* and *Phanerochaete chrysosporium*[4].

The current work was carried out to obtain an efficient local fungal isolate capable of degrading synthetic dyes and assessing the toxicity of intermediate substances synthesized during the biodegradation process to plants.

MATERIALS AND METHODS

Study area: The study was carried out at Fermentation Lab., Microbial Inoculants Center, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Egypt from January, 2018-December, 2020.

Collection of synthetic dye and samples: Commercial reactive blue 19 (RB19) textile dye or (disodium,1-amino-9,10-dioxo-4-[3-(2-sulfonatooxyethylsulfonyl)anilino]anthracene-2-sulfonate). It was obtained from Nabil Tex Dyeing and Printing Company in Shubra El Kheima, Qalyubia governorate, Egypt. The chemical structure of this dye was presented in (Fig. 1).

Textile dye contaminated soil and effluent samples were obtained from the surroundings of the Atlas and Nabil Tex Dyeing and Printing Companies in Shubra El Kheima, Qalyubia governorate, Egypt. These samples were collected in sterile plastic bags or bottles and sent to the laboratory in an ice-box. All samples were kept refrigerated in the laboratory until isolation.

Broccoli (*Brassica oleracea* var. italic) and lettuce (*Lactuca sativa* var. *capitata*) seed samples were obtained from Fajr for Development and Project Management S.A.E and used in the phytotoxicity assay.

Media used: Bushnell and Haas Agar (BHA) medium[7] was used to isolate the RB19 decolorizing fungi. It had the following composition (g L^{-1}): K_2HPO_4, 1.0, KH_2PO_4, 1.0, $MgSO_4$, 0.2, $FeCl_3$, 0.05, NH_4NO_3, 1.0, agar, 20.0, adjusted pH to 5.0 and supplemented with 100 mg L^{-1} RB19 dye.

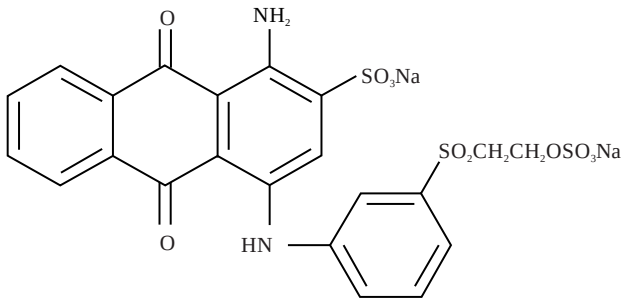


Fig. 1: Chemical structure of reactive blue[19]
Source: Fazli *et al.*[33]

Bushnell and Haas dextrose yeast extract (BDY) medium[8] was used for batch culture and optimization of dye decolorization. It consists of basal minerals of Bushnell and Haas (BH) medium as mentioned above, plus dextrose, 3.0 g L^{-1} and yeast extract, 2.5 g L^{-1}.

Malt agar medium[9] was used for fungal maintenance and preservation, which contained (g L^{-1}), malt extract, 30.0, agar, 20.0 and adjusted to pH 5.0.

Czapek's agar (CZA) medium[9]. It is used for fungal identification. It has the following composition (g L^{-1}): sucrose 30.0, $NaNO_3$, 3.0, K_2HPO_4, 1.0, KCl, 0.5, $MgSO_4.7H_2O$, 0.5, $FeSO_4.5H_2O$, 0.001 and agar 20.0 and adjusted pH at 5.5-6.0:

- The liquid media used in this study is the same as previously shown without adding agar
- All media were autoclaved at 121°C for 20 min at 1 atm

Isolation and screening of RB19 dye degrading fungi: Ten grams of dye-contaminated soil or 10 mL samples of industrial effluent were suspended in 90 mL of sterilized saline solution (0.85% NaCl) and agitated in a rotary shaker for 1 hr at 150 rpm. Serial dilution method[10] was used to isolate RB19 degrading fungi from collected samples, using BHA medium containing 100 mg L^{-1} RB19 dye as the sole carbon source and plates were incubated at 28°C for 7 days. Following the incubation period, colonies with clear halo-zones around them suggest dye decolorization. These colonies were selected and purified using the streak plate technique[11]. The pure fungal colonies were grown on malt agar slant, kept in a refrigerator at 5°C and sub-cultured at monthly intervals for continued study.

The second screening was done in broth medium for quantitative assessment of decolorization by the previously chosen fungal isolates. It was conducted out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL of Bushnell and Haas broth (BHB) medium supplemented with 100 mg L^{-1} RB19 dye, inoculated with 5 mL of the selected fungal culture and incubated on a rotary shaker at 150 rpm and 28°C for 7 days. At the end of the incubation period, the culture medium was filtrated through Whatman number 1 filter paper and then dye decolorization was measured in the supernatant as described later.

Phenotypic and molecular identification of the most dye decolorizing fungal isolate: The selected fungal isolate (5BF) was identified based on the microscopic shape and colour of conidia according to Barnett and Hunter[12] and confirmed identification based on molecular characterization (18S rRNA sequencing). The fungal isolate was grown on sterile Petri plates containing autoclaved CZA medium and incubated for 7 days at 28°C[13].

The culture was sent to the Molecular Biology Research Unit Assiut University for DNA extraction using a Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. Fungal DNA product was then sent to SolGent Company, Daejeon, South Korea for Polymerase Chain Reaction (PCR) and rRNA gene sequencing. The PCR of the fungal isolate was performed using ITS1 (forward) and ITS4 (reverse) primers which were incorporated in the reaction mixture. Primers have the following composition: ITS1: (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4: (5'-TCCTCCGCTTATTGATATGC-3'). The purified PCR product (amplicons) was sequenced with the same primers with the incorporation of ddNTPs in the reaction mixture[13]. The Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website was used to evaluate the sequences retrieved. MegAlign (DNA Star) software version 5.05 was used to perform a phylogenetic analysis of sequences.

Detection of aflatoxins: Following degradation, the supernatant sample was submitted to the Animal Health Research Institute, Agricultural Research Center, Giza, Egypt for analysis of aflatoxins formation by *Aspergillus flavus* as the method described by Wacoo *et al.*[14].

Inoculum preparation and dye decolorization batch culture: The *A. flavus* 5BF fungus inoculum was made by scratching the spores from cultivated slants in 100 mL plugged Erlenmeyer flask containing 50 mL malt broth medium and cultivated for 2 days on a rotary shaker at 150 rpm and 28°C. The number of fungal spores was counted by using haemocytometer slide[15].

The dye decolorization batch culture was performed as described above in the isolation and screening section which the flasks containing 100 mL of BDY medium were inoculated with 5% v/v (2×10^7 spores mL^{-1}) standard *A. flavus* 5BF inoculum. Samples (10 mL) of a batch culture were drawn every 12 hrs during 72 hrs incubation periods under aseptic conditions. The culture was filtrated through Whatman No. 1 filter paper (Whatman® qualitative filter paper, Grade 1, circles, diam. 45 mm, SigmaAldrich).

The cell dry weight was assayed using pellets after washing three times with distilled water, then dried in a hot air oven at 80°C[16]. Decolorization percentage, metabolites products, toxicity and enzyme activities were assayed in cell-free supernatant as described later.

The relationship was plotted between time (hrs) and dye decolorization (%). During the logarithmic phase, the specific dye decolorization rate (μ_d) was calculated using the following formula according to Maier and Pepper[17]:

$$\text{Specific decolourization rate } (\mu_d)\ (h^{-1}) = \frac{(X\text{-}X0)}{(t\text{-}t_0)}$$

where, X is the value of dye decolorization after t time (t) and X0 is the value of dye decolorization at the beginning time (t_0).

Dye decolorization determination: The dye decolorization was measured using a UV/Vis spectrophotometer (T60 UV-VIS spectrophotometer) at 610 nm in the supernatant (cell-free extracted). As a control, uninoculated flasks containing BHB medium plus 100 mg L^{-1} RB19 dye were used to measure the initial absorbance value. The dye decolorization percentage was calculated according to Cheriaa *et al.*[18] using the following Equation:

$$\text{Decolourization (\%)} = \frac{A0\text{-}A1}{A0} \times 100$$

where, A0 and A1 represent the initial (control) and final absorbance values of RB19 dye, respectively.

Gas chromatography-mass spectrometry (GC-MS) analysis of RB19 dye and decolorized products: The GC-MS analysis of metabolite products in *A. flavus* 5BF cell-free supernatant and comparison with control RB19 dye was performed at the Regional Center for Mycology and Biotechnology Laboratory, Al-Azhar University, Cairo, Egypt, according to Nashwa *et al.*[19].

Extraction of degradation products of RB19: The degradation products were extracted from the culture supernatant by an equal volume of ethyl acetate. The extract was evaporated under vacuum to 2 mL final volume.

GC/MS system: ISQ LT single quadrupole mass spectrometer connected to Thermo Scientific Trace 1310 gas chromatograph. Colum: DB5-MS, 30 m, 0.25 mm ID (J and W Scientific). Ionization mode: EI. Ionization voltage: 70 ev. Temperature program: 40°C (3 min)-280°C (5 min) at 5°C min^{-1}. -290°C (1 min) at 7.5°C min^{-1}. Detector temperature: 300°C. Injector temperature: 200°C. Carrier gas: Helium, Flow rate 1 mL min^{-1}. Searched library: Wiley and Nist Mass Spectral Data Base.

Phytotoxicity assay of RB19 dye and *A. flavus* 5BF cell-free supernatant: The phytotoxicity assays were performed by the method described by Nedra *et al.*[20] to assess the toxicity of RB19 dye before and after degradation. The experiment was conducted using broccoli (*Brassica oleracea* var. italic) and lettuce (*Lactuca sativa* var. *capitata*) seeds. Ten seeds were wetted (3 mL per day) with RB19 dye solution (100 mg L^{-1}) or dye decolorized products in separated Petri dishes. The control groups were treated with distilled water. All samples were cultured under the same ambient conditions three times. After 7 days the percentage of germination was recorded and calculated as follows:

$$\frac{\text{Total number of seeds germinated}}{\text{Total number of seeds sowed}} \times 100$$

Enhancement of RB19 dye decolorization by *A. flavus* 5BF using a classical method as one-factor at-a-time: To optimize the carbon sources, the carbon source in the medium (dextrose, 3 g L^{-1}) was replaced by a similar amount of nine carbon sources (sucrose, lactose, glycerol, mannitol, sugar cane molasses, sugar beet molasses, sugar beet wastes, olive cake and starch. As well as nitrogen sources, five organic nitrogen sources (yeast extract, beef extract, malt extract, peptone and tryptone) and four inorganic sources (NH_4NO_3, $(NH4)_2SO_4$, KNO_3, $NH_4Cl$and mixture of yeast extract + NH_4NO_3) containing equivalent nitrogen amount as that of the original medium was separately tested. The previous procedure of propagation and dye decolorization percentage was done as mentioned above.

Enzyme activities assay: Detection of laccase production by *A. flavus* 5BF was measured by using guaiacol agar plate assay as described by Fatemeh *et al.*[21].

Manganese peroxidase (MnP) activity was determined by measuring the purpurogallin formation rate at 420 nm, from the reaction between pyrogallol and hydrogen peroxide catalyzed by peroxidase. This was done using the extinction coefficient (ε) (2640 M^{-1} cm^{-1}). One unit of peroxidase is defined as the amount of enzyme that catalyzed the production of 1 mg of purpurogallin in 20 sec at 25°C and pH 6.0[1].

Statistical analysis: Data were statistically analyzed using IBM® SPSS® Statistics program version 19 based on Duncan's Multiple Range Test[22] at the level of 5%.

RESULTS AND DISCUSSION

Isolation and screening of dye degrading fungal isolates: Among 64 fungal isolates obtained from dye contaminated soil and effluent samples on BHA medium with 100 mg L^{-1} RB19 dye, only 9 isolates showed strong growth on solid medium, with halo-zones of decolorization around the fungal colonies. This denotes that, these isolates utilized the dye as their sole carbon source[23]. These isolates (1-9 BF) were chosen for quantitative estimation in BH broth medium as shown in (Fig. 2). The results showed that the efficacy of chosen isolates to remove the RB19 dye ranged from 55.0-91.1%. The 5BF isolate had the highest percentage of significant decolorization (91.1%). While isolate 7BF had the least amount of decolorization. Thus, 5BF isolate was chosen for further investigation. These results are following Lokendra and Ved[24] who reported that *A. flavus* is an active azo and anthraquinone dyes-degrading fungi and potent to grow and decolorize the azo and anthraquinone dyes in a liquid medium. Huiran *et al.*[25] also reported that *A. niger* and *A. flavus* showed the highest decolorization percentages of reactive blue and mordant red 3.

Identification of the most efficient dye decolorizing fungus isolate: According to the morphological properties (microscopic shape and colour of conidia) of fungal isolate 5BF, the preliminary identification indicates that it may belong to be the genus *Aspergillus* according to Campbell *et al.*[12]. This isolate displayed yellow-green colonies scattered throughout the agar plate. Microscopic fungal isolate examination revealed heavily walled and coarsely roughened conidiophores with elongated vesicles containing globular and occasionally elliptical globular to pyriform conidia as shown in Fig. 3a.

This isolate identification was confirmed by using 18S rRNA gene sequencing based on sequencing the ITS1 and ITS4 primers. The isolated genomic DNA was amplified and analyzed with the aim of sequence and evolution analysis using PCR amplification of the 18S rDNA gene. The results indicated the effectiveness of amplification. One set of DNA products was amplified by 580 base pairs. As shown in (Fig. 3b) the fungal strain *A. flavus* 5BF showed 99.48-99.83% identity with several strains of *A. flavus* including the type of strain ATCC 16883 (NR_111041). *A. fumigates* was included in the tree as an outgroup strain. Moreover, the ITS sequences of *A. flavus* 5BF showed 100% coverage when aligned with closely related strains. Therefore, the fungal 5BF isolate was classified as *Aspergillus flavus* strain 5BF.

Detection of aflatoxins: The supernatant after degradation samples was sent to Animal Health Research Institute, Agricultural Research Center for detection of aflatoxin production by *Aspergillus flavus* and the result is negative for aflatoxins (Fig. 4).

Decolorization of RB19 dye by *A. flavus* 5BF as a batch culture: The capacity of *A. flavus* 5BF to grow and decolorize RB19 dye in BDY medium after 72 hrs incubation periods reached 3.04 g L^{-1} of cell dry weight and 94.88% dye decolorization, respectively. The percentage of dye decolorization in the BDY medium was higher than in the BHB medium, because the BDY medium had sugar and

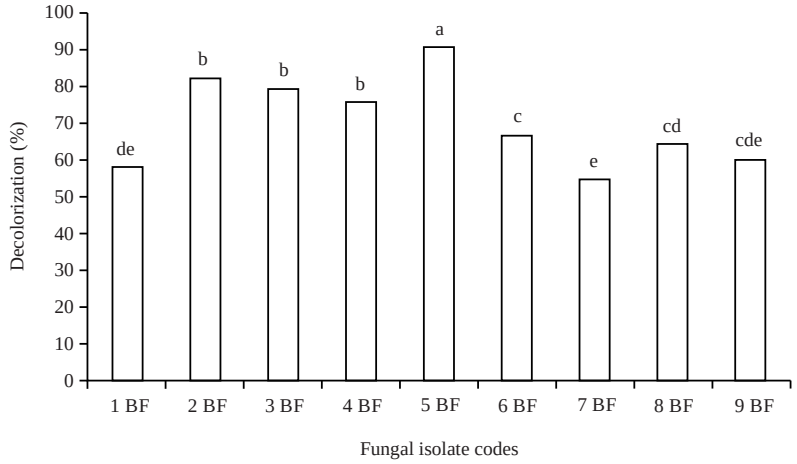


Fig. 2: Reactive Blue 19 dye degrading fungal isolates in broth medium at 28°C for 7 days

*a,bValues with small letters above column having different superscripts are the significant difference (at $p \leq 0.05$) between different isolates for dye decolorization

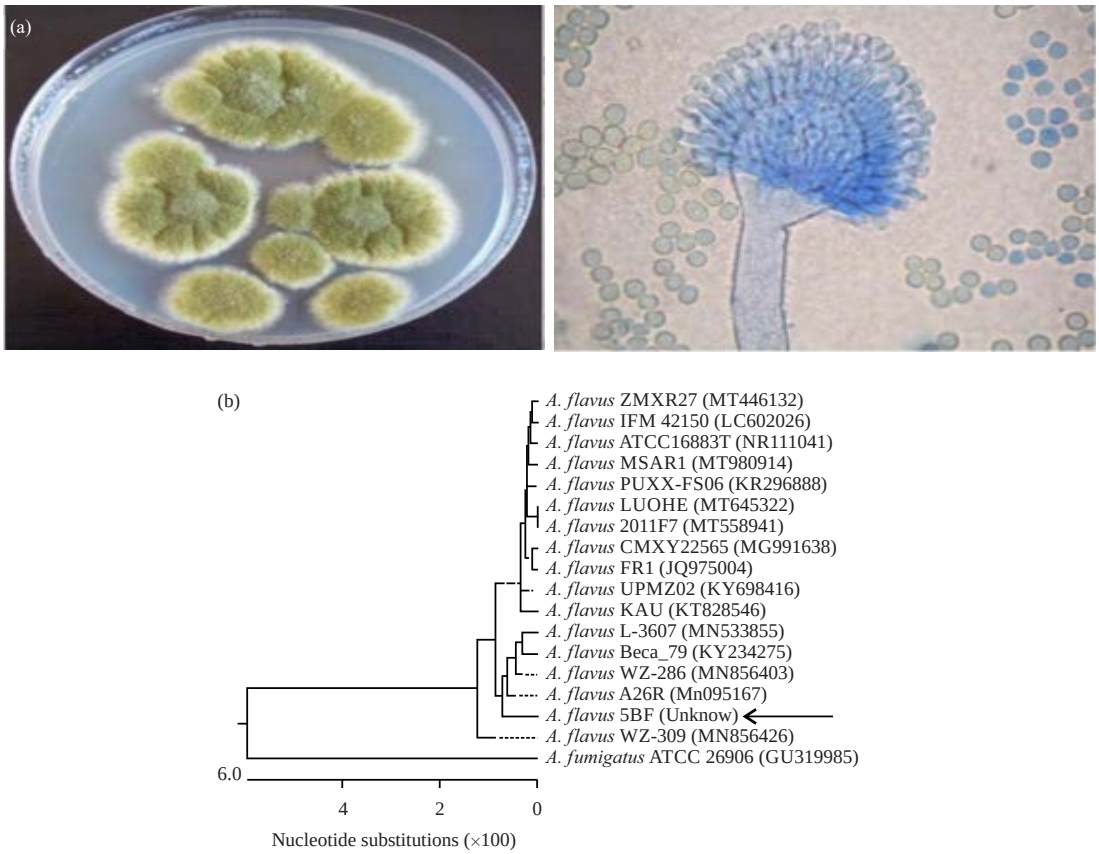


Fig. 3(a-b): Phenotypic and genotypic identification of the most dye decolorization isolate (5BF)

(a) Cultural examination of isolate 5BF under a light microscope (1000x) and on an agar plate and (b) Phylogenetic tree based on ITS sequences of 18S rDNA of the fungal strain isolated in the present study (*A. flavus* 5BF (Unknown), arrowed) aligned with closely related sequences accessed from the GenBank, (*A. aspergillus*)

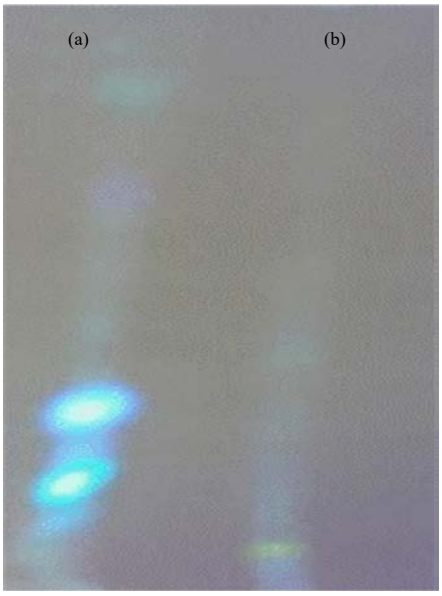


Fig. 4: A thin layer chromatography (TLC) of aflatoxin standard (lane A) and *Aspergillus flavus* 5BF strain (lane B)

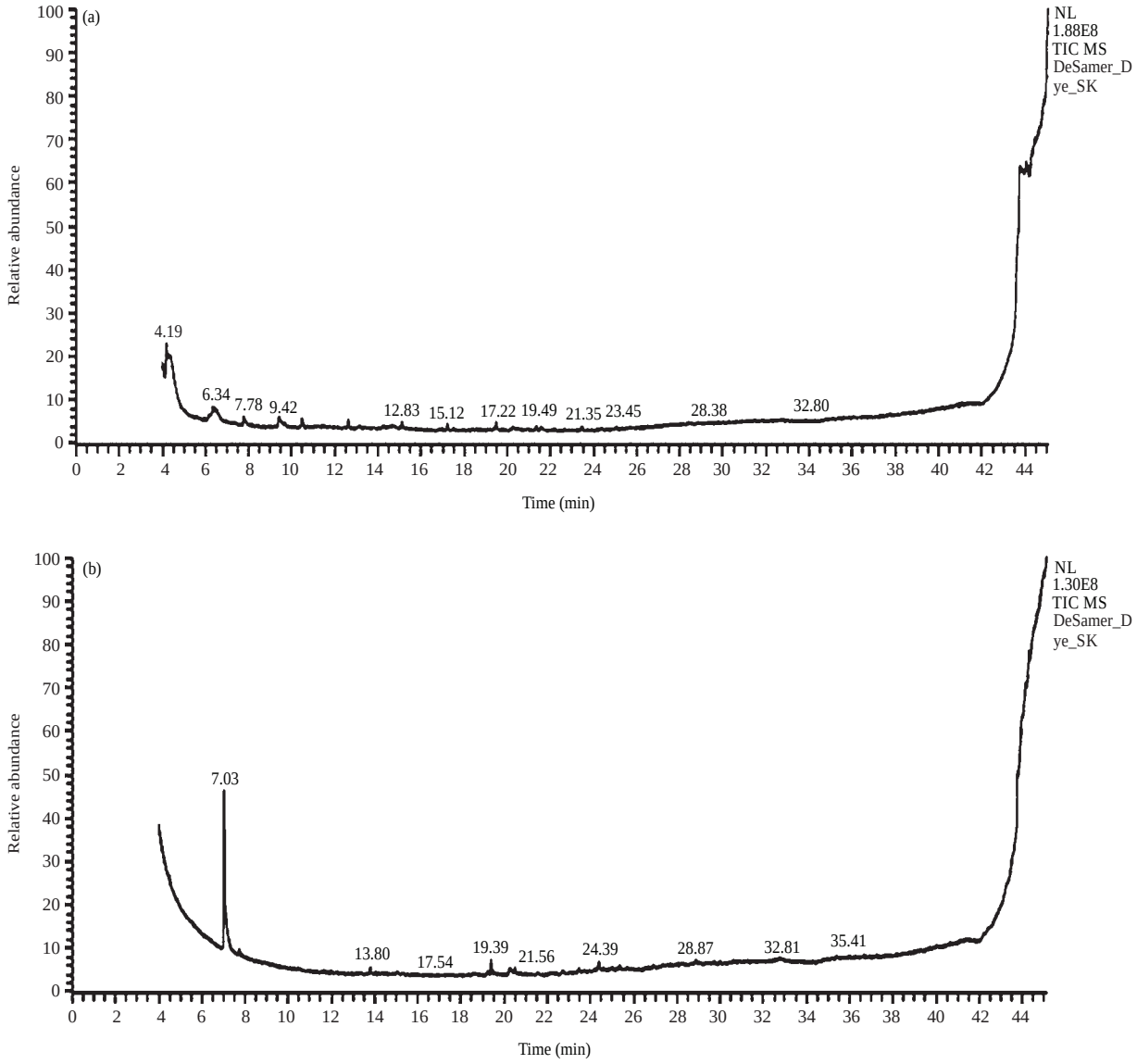


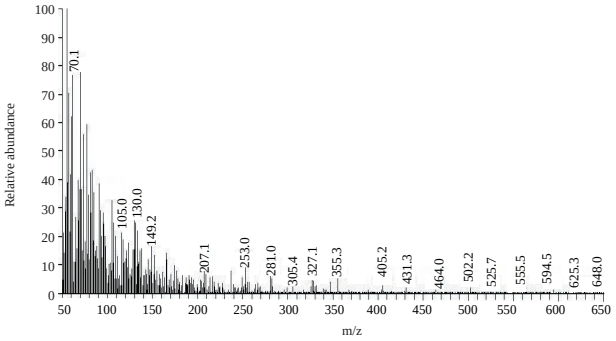
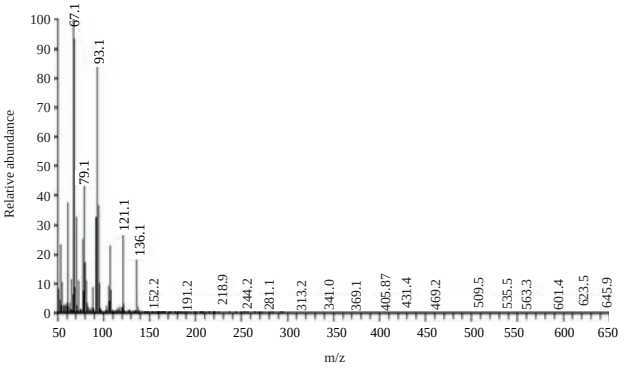
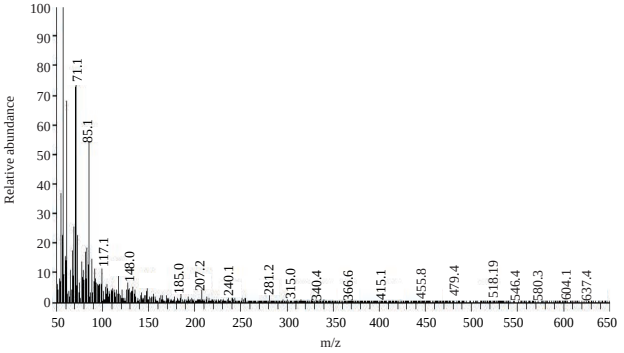
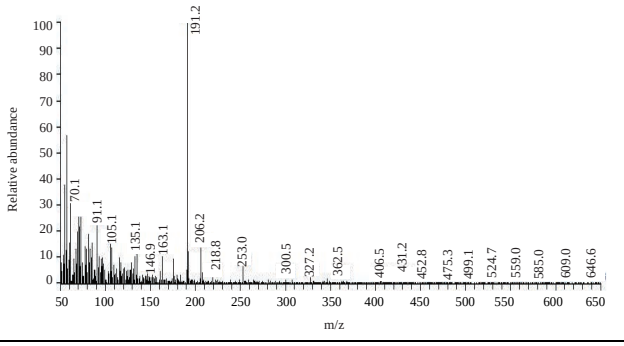
Fig. 5(a-b): (a) GC mass spectra of RB19 dye as control before decolorization and (b) Its metabolites obtained after decolorization by *Aspergillus flavus* 5BF

nitrogen supplies stimulating fungal growth and dye removal efficiency, whereas the BHB medium solely contained minerals. This might be related to the fact that the dye molecule is not suitable as a sole source of carbon and the addition of external co-substrate seemed important in improving dye decolorization as described by Gahlout *et al.*[26].

Analysis of RB19 dye and decolorized products using GC-MS: Data presented in (Fig. 5a and Table 1) clearly show the mass spectrum of 2,7-diphenyl-1-6-dioxopyridazino (4,5-2,3) pyrrolo (4,5,d)pyridazine (dye before degradation) with a mass peak of 355 at a retention time of 22.20 min. Data in (Fig. 5b) show the mass spectra of 3 different metabolites (dye after degradation). The product with a mass peak of 136 was concluded to be cyclohexene at a retention time of 7.03 min. Cyclohexene is one of the products biodegradation by *Aspergillus* as have been reported by Manisha *et al.*[27]. The m/z peak of 173 was identified as 1,3,5-triazine-2,4-diamine-6chloro-N-ethyl at a retention time of 13.80 min. The m/z peak of 206 was identified as 2,4-di-tert-butyl-phenol at a retention time of 20.26 min.

These products were found to be the degradation products of anthraquinone dye and these compounds leading to the generation of TCA cycle intermediates in organisms[28].

Table 1: GC mass spectra of reactive blue 19 before and after degradation and the retention time and molecular weight

Peak	RT (min)	MW (M)	Name of metabolite	Mass spectrum
Before	22.2	355	2,7-diphenyl-1-6-dioxopyridazino (4,52,3) pyrrolo (4,5,d) pyridazine	
After	7.03	136	Cyclohexene	
	13.8	173	1,3,5-triazine-2,4-diamine-6chloro-N-ethyl	
	20.26	206	2,4-di-tert-butyl phenol	

Toxicity studies

Phytotoxicity analysis: The effect of RB19 dye and its decolorized products on germination of broccoli (*Brassica oleracea* var. italic) and lettuce (*Lactuca sativa* var. *capitata*) seeds in comparison with distilled water (control) was shown in Fig. 6. Results indicated that 95 and 97% of broccoli and

lettuce seeds were germinated in tap water as well as high germination percentages appeared in both test seeds reaching 84 and 93% in the presence of decolorized products (with no significant difference with germination in control). Before degradation, RB19 dye solution (100 mg L^{-1}) significantly decreased lettuce and broccoli germination rates (76 and 73%, respectively). As a result, the decolorized products could be less toxic than the dye solution[29].

Enhancement of RB19 dye decolorization by *A. flavus* 5BF using one-factor at-a-time technique

Incubation periods: Results in Fig. 7a show that RB19 dye decolorization by *A. flavus* 5BF was started after a 12 hrs incubation period and the decolorization percentage was significantly increased (94.88%) with the increase of the incubation period up to 72 hrs. Results also indicated a high coefficient (R^2) between incubation period and dye decolorization was giving of 0.98. The results revealed that *A. flavus* 5BF dye decolorization during the first 12-48 hrs of incubation periods. The specific decolorization rate calculated during the logarithmic phase was 0.053 h^{-1}.

Carbon sources: The effect of different supplementary carbon sources (sucrose, lactose, glycerol, mannitol and starch) and agro-industrial residues (sugar cane molasses, sugar beet

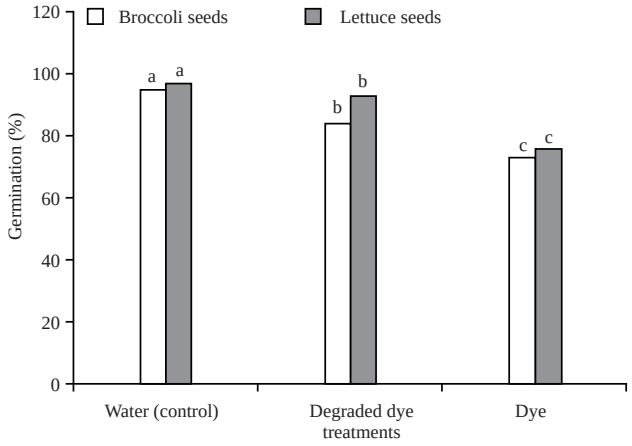


Fig. 6: Phytotoxicity assay of RB19 dye and *Aspergillus flavus* 5BF cell-free supernatant

*a,b,cValues with small letters above column having different superscripts are the significant difference at ($p \leq 0.05$) between different isolates for dye decolorization

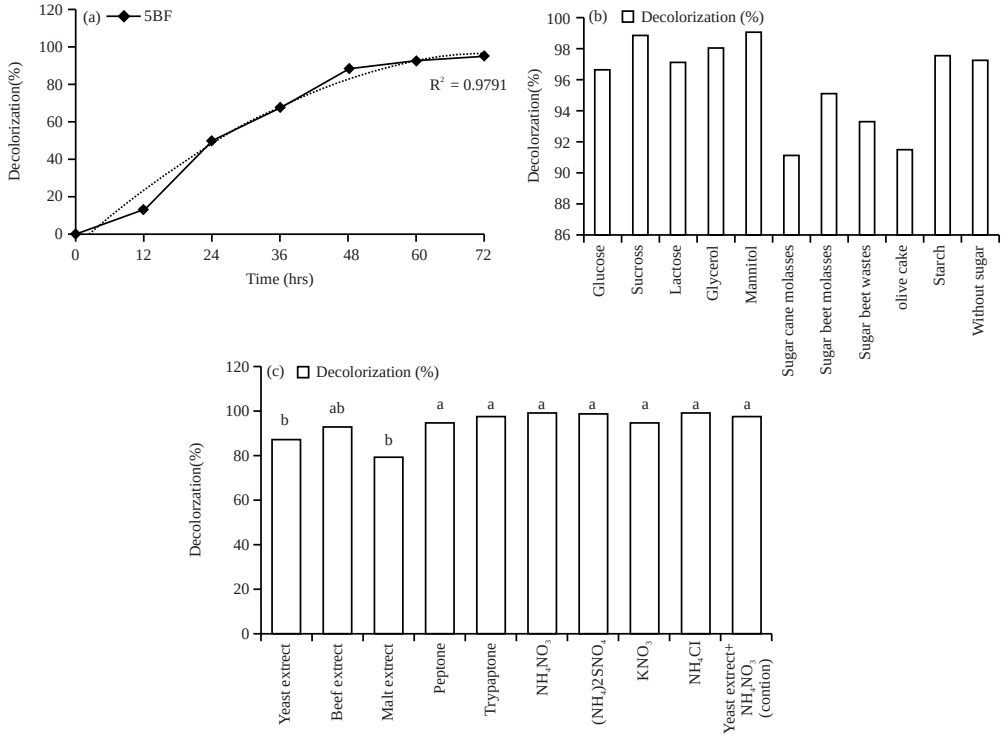


Fig. 7(a-c): (a) Influence of incubation period, (b) Carbon sources and (c) Nitrogen sources on the percentage of reactive blue 19 dye decolorization by *Aspergillus flavus* 5BF

*a,bValues with small letters above column or line having different superscripts are the significant difference at ($p \leq 0.05$) between different isolates for dye decolorization

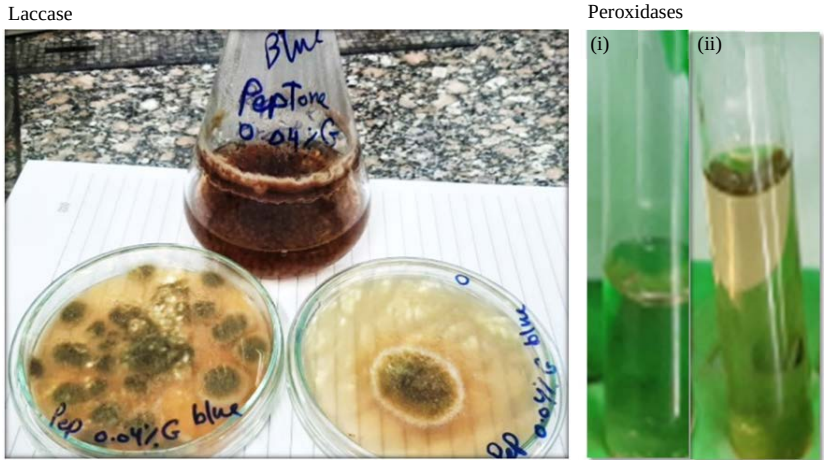


Fig. 8: Detection of some enzymes activity of laccase and manganese peroxidases produced from *Aspergillus flavus* 5BF strain for RB19 dye decolorization

Laccase detected on guaiacol agar plate assay and formed orange or brown colour halo-zones, (i) Blank, (ii) Treatment

molasses, sugar beet waste and olive cake waste) improved *A. flavus* 5BF dye decolorization as compared with the control (dextrose). According to the data in (Fig. 7b), mannitol and sucrose were the best carbon sources for dye decolorization (99.11 and 98.90%, respectively) and the level of decolorization in these treatments was enhanced by 2.5 and 2.3% as compared to the control, respectively. Furthermore, Ruijter *et al.*[30] showed that D-mannitol was the most abundant carbon-containing molecule and accounts for 10-15% of the dry weight of some species of *Aspergillus* conidiospores, suggesting that it plays a function in spore survival. Mannitol is rapidly metabolized during spore germination in *A. niger*. In addition, Mannitol is involved in conidial stress resistance in *A. niger*. The mannitol cycle might be used to regulate the coenzymes NADH and $NADP^+$, i.e., a method to create NADPH at the expense of NADH and ATP with each cycle turn. Additionally, Meena *et al.*[31] suggested that polyols play key roles in fungal physiology.

Also, the results revealed that mannitol was the best sugar, in increasing decolorization when compared to the control (with dextrose)[32].

Besides that, when sugar cane molasses and olive cake waste were utilized, the RB19 colour was removed by 91.13 and 91.52%, respectively. On the other hand, Fazli *et al.*[33] found that glycerol was the optimum carbon source for maximal decolorization (95.3%) of reactive blue dye by *Ganoderma* species on the fifth day of incubation.

Nitrogen sources: Batch cultures containing glucose was supplemented separately with sources of organic and inorganic nitrogen. Data in (Fig. 7c) show that the mineral nitrogen source seems to be important as it was necessary for better decolorization of RB19 dye. Among the nitrogen sources added to the BDY medium, NH_4Cl enhanced the dye decolorization reaching its maximum of about 99.41% followed by NH_4NO_3 (99.04%). This could be due to the presence of assimilatory enzymes for nitrate reduction in the organisms[34]. Furthermore, Böhm and Boos[35] indicated that inorganic nitrogen sources are quickly utilized. Furthermore, Abd El-Rahim *et al.*[1] found that inorganic nitrogen sources outperformed organic nitrogen sources in terms of improving decolorization (%) in several microorganisms.

Evaluation of degrading enzymes: Some enzymes presentence in microorganisms are responsible for degrading the dyes such as azo-reductase, manganese and lignin peroxidase and ascorbate oxidase indicating their prominent roles in dye degradation[36].

The role of laccase and peroxidase produced by *A. flavus* 5BF in the decolorizing RB19 dye was investigated. The result of Fig. 8 *A. flavus* 5BF showed the ability to produce laccase on guaiacol agar plates forming orange or brown colour halo zones around fungal growth. Moreover, the activity of manganese peroxidase was determined in culture. This enzyme produced purpurogallin in the fungal culture giving yellow colour in the medium.

Likewise, Hofrichter[37], Kaushika and Malik[38] mentioned that fungi could destroy textile dyes through their extracellular oxidoreductive, nonspecific and non-stereoselective enzyme system, which includes lignin peroxidase, tyrosinase,

manganese peroxidase and laccase. Laccases from the lignin-degrading basidiomycetes *Trametes versicolor*, *Polyporus pinisitus* and *Myceliophthora thermophila* were identified to decolorize synthetic dyes to varying degrees[39]. In specifically, Ahmed *et al.*[36] showed that the capacity of S8 isolate to create phenol red manganese peroxidase and break down reactive blue 19 and reactive yellow 4GL, with enzyme activity reaching 2.74 and 2.44 U mg^{-1} protein, respectively.

CONCLUSION

According to the results of this investigation, reactive blue 19 dye was decolorized by *A. flavus*. Incubation periods as well as carbon and nitrogen supplies, had a great influence on the decolorization process. Furthermore, laccase and manganese peroxidases were shown to play an essential role in the oxidative degradation of dye. As a result, this strain was safe as it did not produce aflatoxins and their decolorized products are nontoxic to plants, being environmentally friendly. It thus can be utilized in the future in the treatment of dye wastewater and the bioremediation of environmental contaminants.

SIGNIFICANCE STATEMENT

This study discovers the fungal technique was applied to decolorize and detoxify anthraquinone synthetic dye that can be beneficial for the discharge of these dyes into the environment causes detriment. This study will help the researcher to uncover the critical areas of removing dye with high efficiency and their metabolites had no negative influence on the environment that many researchers were not able to explore. Thus a new theory on dye wastewater bioremediation may be arrived at.

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