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Decolorization and detoxification of batik dye effluent containing Indigosol Blue-04B using fungi isolated from contaminated dye effluent

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ABSTRACT Fungi are capable of treating various synthetic dye effluents. Previously, we isolated seven strains of fungi from contaminated batik dye effluent at Banyumas, Central Java. The aims of this study were to screen the ability of these fungi to decolorize batik dye effluents containing Indigosol Blue-04B and to investigate the phytotoxicity effects of biodegraded effluent on the germination of corn seeds *Zea mays* L. and green bean seeds *Vigna radiata* (L.) Wilczek. In addition, the decolorized effluents were tested for toxic effect on the agriculturally important gram-positive and gram-negative soil bacteria *Bacillus cereus* and *Azotobacter* sp., *Staphylococcus aureus* and *Escherichia coli*, respectively. Study of decolorization showed that fungi were able to decolorize Indigosol Blue-04B batik dye effluents by 21.04% to 99.89% at room temperature after three days of incubation. The assay of phytotoxicity showed that both plumule and radicle length of *Z. mays* and *V. radiata* grown on the decolorized effluent was longer than on untreated effluent. The percentage of *Z. mays* and *V. radiata* seed germination in decolorized effluent was higher than in untreated effluent. There was no inhibition zone found around the decolorized effluent samples after incubating the bacteria for 48 hours. *Aspergillus* sp. 3 was the most effective for degradation and could be used for batik effluent mycoremediation processes.

KEYWORDS batik dye effluents; decolorization; Indigosol Blue-04B; microbial toxicity; phytotoxicity

1. Introduction

Vat dyes, such as indigo, are widely used in the batik industry. Many steps are involved in the batik industry, such as dyeing and the final washing process, in which large quantities of indigo are rinsed off and disposed of in waste water (Campos et al. 2001b; Sunarto 2008). The disposal of dye waste into water without proper effluent treatment can cause environmental pollution. Many types of dyes used in the batik industry are recalcitrant to decolorization and have toxic properties for aquatic living organisms including plants, animals, and microbes, hence causing serious long-term health effects (Tian et al. 2013).

Large amounts of effluent containing indigo dye must be treated before being discharged into the environment. Indigo dye is water-insoluble and considered as a recalcitrant substance that causes environmental concerns (Balan and Monteiro 2001), and such effluents must be effectively treated. Therefore, methods for removing synthetic dyes from effluents to reduce their impact on the environment have been developed using microbiological technologies. Microbiological remediation is considered a cost-effective and eco-friendly method for the treatment of recalcitrant dyes and effluents. Fungi are capable of treating various synthetic dye effluents. A number of dyes from various chemical groups have been found to be effective against fungal oxidation due to their lignin modification systems (Fu and Viraraghavan 2001; Arora and Chander 2004). A large number of research studies of fungal decolorization through biosorption or enzymatic mineralization (lignin peroxidase, manganese peroxidase, manganese-independent peroxidase, and laccases) have been reported (Wong and Yu 1999; Zheng et al. 1999; Ferreira et al. 2000; Wesenberg et al. 2003). Indigo dye and effluent containing indigo dye could be decolorized by fungal action (Abadulla et al. 2000; Balan and Monteiro 2001; Campos et al. 2001a). The authors of this study previously found that seven fungi were able to decolorize Indigosol Blue-04B dye.

The aims of this study were to assess the ability of these fungal isolates to decolorize Indigosol Blue-04B batik dye effluents and to investigate the microbial toxicity and phytotoxicity of the degraded products of Indigosol Blue-04B.

2. Materials and methods

2.1. Chemicals and effluents

Chemical compounds used in this research were obtained from the Laboratory of Mycology, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia. The Indigosol Blue-04B batik dye effluents were collected from local batik factories in Banyumas, Central Java.

2.2. Isolation of fungal strains and growth conditions

The seven ascomycetous fungi strains used in the present study were previously isolated from sludge, effluent and effected soil from the local environment of batik effluent storage ponds at several factories in Banyumas. The fungal isolates were preliminarily identified by taxonomic criteria: color, density and appearance of colonies and microscope observations with guidelines that facilitate the identification of fungi developed by Pitt and Hocking (2009) (Table 1). Potato dextrose agar (PDA) as a solid medium was used for pre-culture fungal growth and per liter contained 200 g of potato extract, 20 g of dextrose and 20 g of agar. The liquid cultivation medium for decolorization studies was potato dextrose broth (PDB) containing PDA without agar.

2.3. Decolorization assays

Five plugs (eight mm disks) of freshly grown mycelium and spores were inoculated into Erlenmeyer flasks (250 mL) containing 100 mL of liquid cultivation medium. These were then incubated under shaking (reciprocal shaker) conditions for 3 d. After fungal biomass had formed, the culture was washed from its medium and used in the next step. About 100 mL of effluent was used to culture fungal isolates without dilution or prior treatment. This was placed in the Erlenmeyer flasks, incubated under the same conditions and performed as triplicates of each culture flask. Decolorization assay was carried out by taking an aliquot of effluent. After three days of incubation, samples were taken, and supernatants were used to estimate the number of effluents by UV–Vis spectroscopic analysis. Percentage of decolorization was calculated as the initial absorbance substracted by the final absorbance divided by the initial absorbance times 100 (Kariyajjanavar et al. 2013). Mycelial biomass was then harvested and then a protocol in which clean glass microscopic slides were prepared and observed directly by microscopy was used to estimate the biosorption of dyes by hyphae (Rani et al. 2014).

2.4. Growth profile

The dry weight of mycelia was obtained by using the two-day mycelia culture dried in an oven at 50°C overnight. The mycelia were dried by filter paper to enable measurement of dry weight. Data was designed by averaging the value of the three cultures.

2.5. Microbial toxicity and phytotoxicity

The toxic effect of the decolorized effluent was tested on agriculturally important soil flora bacteria (*Bacillus cereus* and *Azotobacter* sp.) (Ilyas and Rehman 2013) and prevalent species of gram-positive and gram-negative bacteria *Staphylococcus aureus* and *Escherichia coli* (Rani et al. 2014). Minimal salt medium was used for the culture of *B. cereus* and *Azotobacter sp.*, and Luria-Bertani broth medium was used for *S. aureus* and *E. coli* (Rani et al. 2014). Microbial toxicity was developed using the agar diffusion method. This method was used for in vitro evaluation and to determine the inhibition of antimicrobial activity. Decolorized broth was inoculated with all of the bacteria, placed into plated agar medium and incubated for 48 h. Two hundred µL of prepared suspension bacteria was inoculated on the dried surface of PDA medium using spread plate method and an index of toxicity was indicated by the presence of an inhibition zone around the inoculation well (Kurniasih et al. 2018).

Phytotoxicity tests were performed in order to assess the toxicity of the treated decolorized effluent samples. The decolorized products were extracted and dissolved in 10 mL of distilled water for the tests. Solutions of untreated samples and treated decolorized compounds were prepared in distilled water and were tested on seeds of *Zea mays* and *Vigna radiata* for toxicity at room temperature. Approximately 20 seeds were sown in autoclaved sand. The study was carried out by watering the seeds with 5 mL of the samples. The control set was carried out at the same time using irrigation water. After 7 d, the percent-

TABLE 1 Morphological characterization of macroscopic and microscopic fungal isolates.

Isolate code	Colony color	Colony diameter (mm)	Color of mycelium	Specific character	Spore diameter (µm)	Preliminarily identification
E-LcmA	yellow/pale brown	40	white mycelium	vesicle, metulae & phialides, foot cell	2.5–3.5	*Aspergillus* sp. 1
J-LimT	yellow green	60	white mycelium	vesicle, metulae & phialides, foot cell	5.0	*Aspergillus* sp. 2
G-LnsP	golden yellow	45	white mycelium	vesicle, metulae & phialides, foot cell	2.5–3.0	*Aspergillus* sp. 3
M- TT	black	60	white mycelium	vesicle, metulae & phialides, foot cell	3.0–4.0	*Aspergillus* sp. 4
S-TR	black	60	white mycelium	vesicle, metulae & phialides, foot cell	3.0–4.0	*Aspergillus* sp. 5
T-TR	dark brown	50	white mycelium	vesicle, metulae & phialides, foot cell	3.0–4.0	*Aspergillus* sp. 6
AU-TSs	dark brown	55	white mycelium	vesicle, metulae & phialides, foot cell	3.0–4.0	*Aspergillus* sp. 7

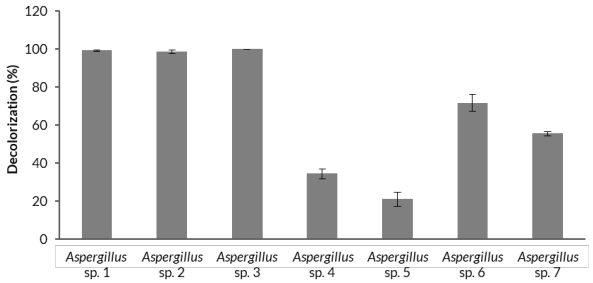

FIGURE 1 Decolorization of Indigosol Blue-04B batik dye effluents by seven isolated fungi.

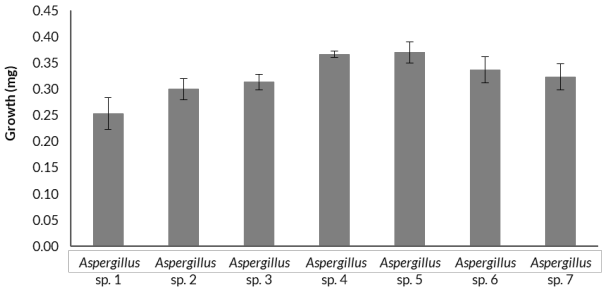

FIGURE 2 Mycelial dry weight of fungal isolate to decolorize Indigosol Blue-04B batik dye effluents.

age of seed germination was recorded and the plumule (shoot), and radicle (root) lengths were measured (Ilyas and Rehman 2013).

3. Results and discussion

3.1. Decolorization assays and growth profiles

Seven ascomycetes strains from a local batik environment were isolated and identified as *Aspergillus* species based upon their morphology. The *Aspergillus* species showed significant decolorizing ability for Indigosol Blue-04B batik dye effluents. Decolorization assays were carried out at room temperature after 3 d of incubation by placing freshly grown fungal pellets in 100 mL of the effluent. Study of decolorization showed that fungi were able to decolorize the effluents by between 21.04% and 99.89% (Figure 1). As mentioned earlier, Manu and Chaudhari (2003) reported the same results for efficient decolorization of effluent containing vat dye (C.I. vat blue 1: indigo) which was recalcitrant and difficult to degrade. Alternatively, indigo dye can be removed from effluent through degradation using pure cultures of fungi (Balan and Monteiro 2001; Campos et al. 2001a; Dewi et al. 2016).

Aspergillus sp. 1, 2, and 3 showed that decolorization percentages were higher (at 99.05, 98.50, and 99.90%, respectively) than that of the remaining four fungi, *Aspergillus* sp. 4, 5, 6, and 7 (at 34.30, 21.04, 71.60, and 55.45%, respectively). These results also showed a good mycelial growth of *Aspergillus* sp. 4 and 5, but the decolorization efficiency was low (Figure 2). Mycelial dry weights of fungal strains in decolorizing the effluent were respectively 0.25, 0.30, 0.31, 0.37, 0.37, 0.37, and 0.32 g. The same results were obtained by AI-Jawhari (2015), in that high levels of mycelial growth do not necessarily also produce good decolorization ability. *A. niger* was not able to effectively decolorize Methylene Blue dye although it had a good mycelial growth. It was grown on different dyes but did not show any considerable decolorization. Other species (*Aspergillus fumigates*, *Penicillium funigulosum* and *Fusarium solani*) had higher decolorization rates than *A. niger*.

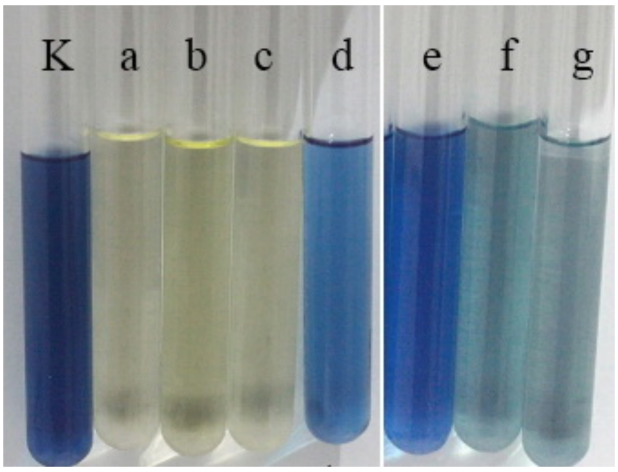

FIGURE 3 The appearance of Indigosol Blue-04B batik dye effluent decolorization. Removal of color from the effluent by isolated fungi. K: the Indigosol Blue-04B batik dye effluents as control; fungi isolates: a: *Aspergillus* sp. 1, b: *Aspergillus* sp. 2, c: *Aspergillus* sp. 3, d: *Aspergillus* sp. 4, e: *Aspergillus* sp. 5, f: *Aspergillus* sp. 6, g: *Aspergillus* sp. 7.

Aspergillus species 5, the poorest fungi isolate for decolorization, had colony and morphological features similar to *A. niger* (Table 1). *Aspergillus* species 4, 5, and 6 showed similar characteristics to *Aspergillus* species 5, so these isolates were also identified as *A. niger*. In the same study, (AI-Jawhari 2015) states that *A. niger* has a lower decolorizing ability than any other species. In a review by Kaushik and Malik (2009), the results of a research study conducted by Fu and Viraraghavan (2001) showed that *A. niger* was able to decolorize Congo Red dyes via a biosorption mechanism, but that other *Aspergillus* species could decolorize dyes by both biosorption and biodegradation (Parshetti et al. 2007). This result may be due to the biodegradation being an additional mechanism resulting from these fungi having unique systems of enzymes which break down complex organic structures into simple fragments, and because of stress on mycelial cells causing low growth (AI-Jawhari 2015).

Decolorization of the effluents was well over 98.5% by *Aspergillus* species 1, 2, and 3 isolates, seen by the removing of color from the effluent and thickening of the mycelium color after treatment (Figures 3 and 4). These fungal isolates change the color of the dye and this implies that they are changing its chemical form (Figure 3). The color of effluent was reduced when the color of the biomass changed (Figure 4) and the mycelium biomass was observed adsorbing the blue dye contained in the effluent.

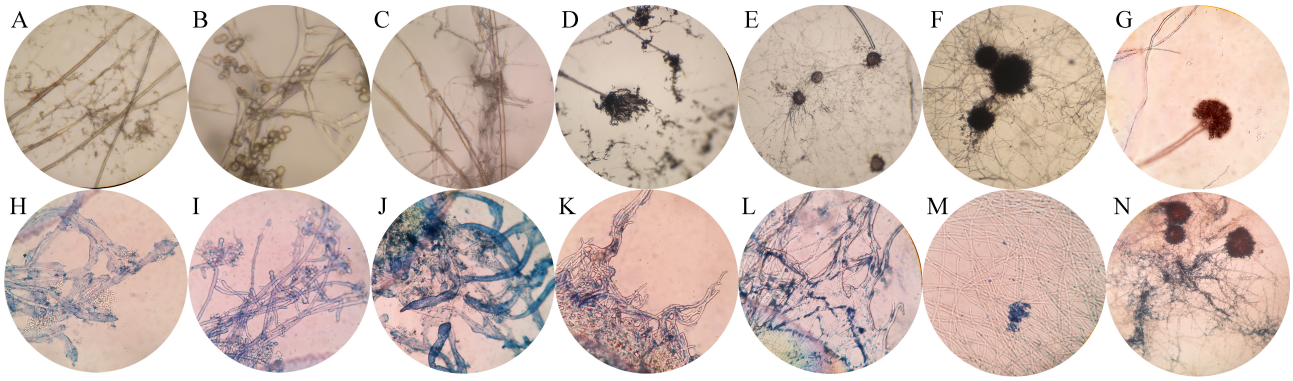

FIGURE 4 Color reduction before (A-G) and after treatment (H-N) by *Aspergillus* sp. 1 (A, H); *Aspergillus* sp. 2 (B, I); *Aspergillus* sp. 3 (C, J); *Aspergillus* sp. 4 (D, K); *Aspergillus* sp. 5 (E, L); *Aspergillus* sp. 6 (F, M); *Aspergillus* sp. 7 (G, N).

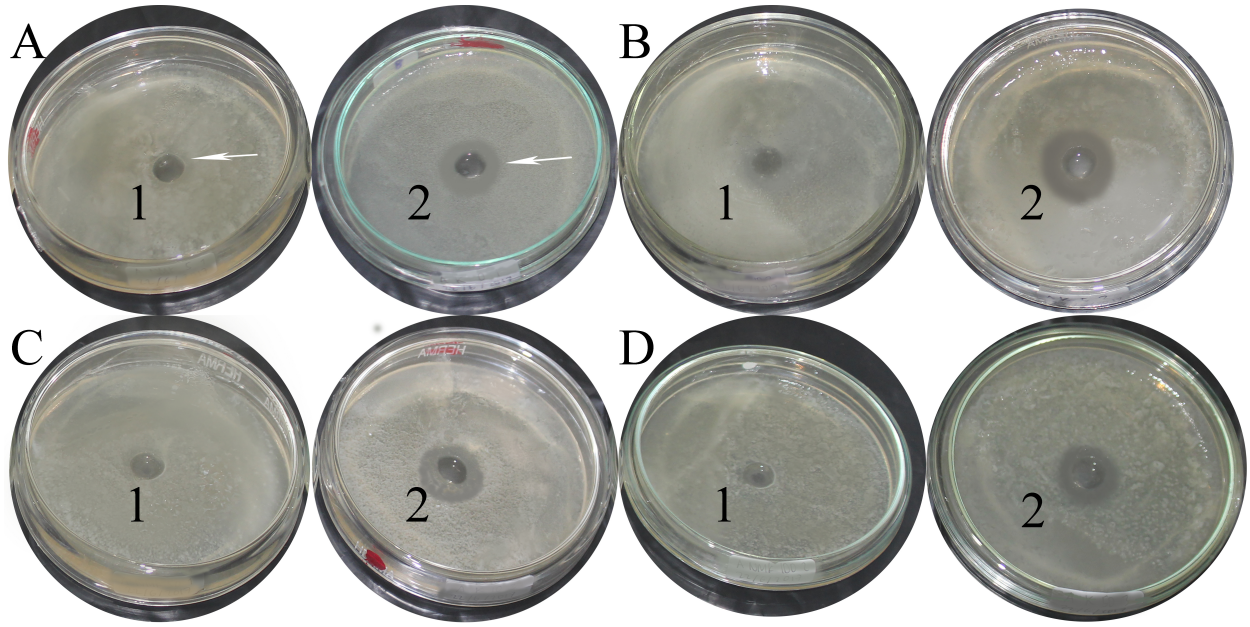

FIGURE 5 Antimicrobial activity of the effluent after fungal treatment (1) and control effluent (2). A: *Bacillus cereus*; B: *Azotobacter* sp.; C: *Staphylococcus aureus*; D: *Escherichia coli*. *Aspergillus* sp. 7 (G, N).

Aspergillus species 3 almost completely (99.9%) decolorized the effluent in the liquid medium after a three-day incubation. This result showed that *Aspergillus* species 3 was the best isolate for decolorizing the effluent. Degradation by *Aspergillus* species 3 as the most effective fungi is seen more clearly than in the other species (Figure 3). The mycelium which caused degradation using the *Aspergillus* species 3 fungus culture was seen to be absorbing more dye than the other cultures (Figure 4).

Color removal from effluent containing dyes appeared to be due to mechanisms of the fungal biomass, primarily biosorption/bioadsorption. After decolorization of dyes by the biomass mechanism and then subsequent formation, dyes were metabolically degraded by fungal strains into their derivative products (Naeem Ali 2009). Removal of color from effluent by bioabsorption/bioadsorption of fungal hyphae is shown in Figure 4. Other studies have mentioned that in fungal bioabsorption/bioadsorption (Knapp and Newby 1995; Sumathi and Manju 2000; Fu and Viraraghavan 2001; Naeem Ali 2009) as the primary dye removal method coupled with electrostatic pull between negatively charge dyes, the positively charged cell was the primary dye removal agent. It coupled with the electrostatic pull between negatively charge dyes and the positively charged cell wall (Aksu et al. 1999; Aksu and Tezer 2000). Apparently, besides their metabolizing properties, fungi were acting as cultural filtrates (extracts) containing enzymes of the dyes for effluent degradation. This study suggests application of fungal biomass rather than cell-free systems containing enzymes for the treatment of dye effluent, as carried out by previous studies (Braun and Vecht-Lifshitz 1991; Zhou and Zimmermann 1993; Aksu and Tezer 2000; Coulibaly et al. 2003).

3.2. Microbial toxicity

In the present investigation, toxicity decreased concurrently with the decrease of effluent containing Indigosol Blue-04B dye. The concentration of dye toxicity after the

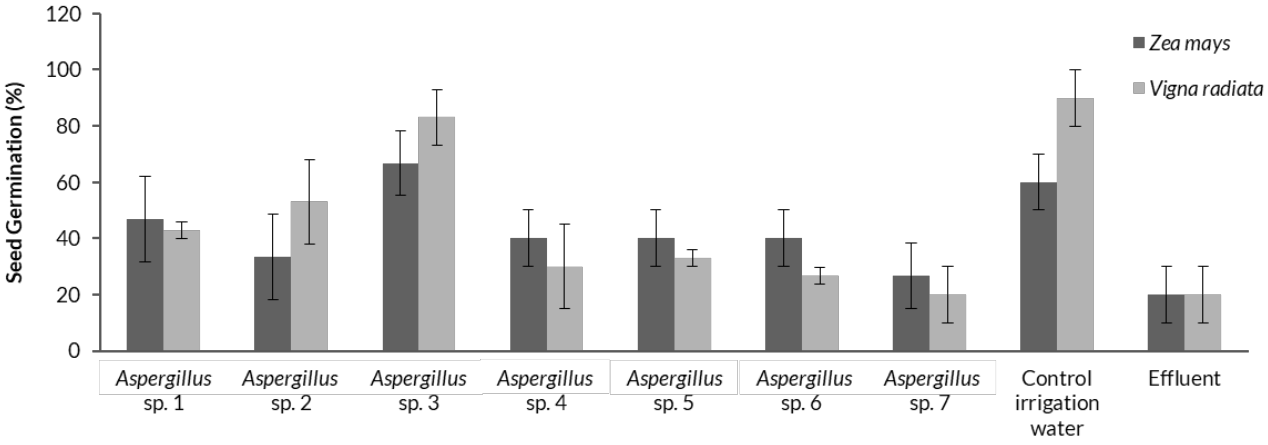

FIGURE 6 Percentage of seed germination of *Zea mays* and *Vigna radiata* in treated fungal isolate.

decolorizing in effluent was reduced compared to the initial condition. There was no inhibition zone found around the effluent samples treated by fungi after incubation of *B. cereus*, *Azotobacter* sp., *S. aureus*, and *E. coli* for 48 h (Figure 5). The total dye content of the effluent and its toxicity were reduced significantly during incubation. This study also suggests that fungi worked efficiently to decrease toxicity.

3.3. Phytotoxicity studies

Soil fertility depends on the characteristics of irrigation water. Decolorization of effluent produces various degradation products. It is important to learn the toxicity impact of those degradation products on plants. The seed germination of *Z. mays* was 26.67%–66.67% when treated by degradation products of effluent containing Indigosol Blue-04B dye, and this was higher than when treated with untreated effluent (20%). Similarly, the seed germination of *V. radiata* was 20%–83% higher than with untreated effluent (20%) (Figure 6). Table 2 shows that the plumule length and radicle length of *Z. mays* (5.2–10 cm and up to 4.4 cm) on the decolorized effluent were higher than in untreated effluent (2.4 and 2.1 cm) and similar results were achieved for *V. radiata* (5.3–12.4 cm and up to 3.5 cm in decolorized effluent rather than 3.6 and 1.8 cm in untreated effluent). These were similar results to some previous studies by Watharkar and Jadhav (2014) and Mahmood et al. (2015), that is, in terms of length of plumule and radicle of *Z. mays* when treated with decolorized effluent products of red, green, black, and yellow dyes. Plumule and radicle length was reduced when treated with untreated effluent treatment compared to decolorized effluent.

Figure 7 illustrates the growth of seeds after being treated using fungal isolates. All seven fungal treatments were growing as well as those in the water control treatment, while there were just a few growths in the dye effluent. This indicated that this fungal-treated effluent did not adversely affect plant growth.

4. Conclusions

In this study, seven fungi isolated from batik dye effluent were able to decolorize batik dye containing Indigosol

TABLE 2 Phytotoxicity test of different treated and untreated effluents containing Indigosol Blue-04B dye.

Fungi	Treatments	*Zea mays*		*Vigna radiata*	
		Plumule (cm)	Radicle (cm)	Plumule (cm)	Radicle (cm)
	Control (irrigation water)	10.10 ± 0.19^{a}	6.29 ± 0.25^{a}	16.50 ± 0.50^{a}	7.40 ± 0.20^{a}
	Effluent	2.40 ± 0.48^{c}	2.10 ± 0.02^{bc}	3.60 ± 0.10^{d}	1.80 ± 0.30^{bc}
Aspergillus sp. 1	Decolorized effluent	6.85 ± 1.15^{b}	3.10 ± 0.66^{bc}	5.30 ± 0.90^{cd}	1.50 ± 0.37^{c}
Aspergillus sp. 2	Decolorized effluent	6.75 ± 1.56^{b}	3.60 ± 1.04^{bc}	11.30 ± 0.70^{b}	2.10 ± 0.17^{bc}
Aspergillus sp. 3	Decolorized effluent	10.10 ± 0.60^{a}	4.40 ± 0.56^{ab}	12.40 ± 1.30^{b}	3.50 ± 0.50^{b}
Aspergillus sp. 4	Decolorized effluent	5.40 ± 1.14^{bc}	1.75 ± 1.09^{c}	11.30 ± 0.90^{b}	2.70 ± 1.30^{bc}
Aspergillus sp. 5	Decolorized effluent	5.20 ± 1.08^{bc}	2.30 ± 0.90^{bc}	7.50 ± 1.06^{c}	2.10 ± 0.68^{bc}
Aspergillus sp. 6	Decolorized effluent	7.90 ± 1.40^{ab}	3.30 ± 0.38^{bc}	11.70 ± 1.04^{b}	3.30 ± 0.57^{b}
Aspergillus sp. 7	Decolorized effluent	6.10 ± 0.99^{b}	2.45 ± 1.30^{bc}	6.40 ± 1.38^{c}	1.10 ± 0.54^{c}

The numbers followed by different letters in each column indicate that mean values are significantly different ($p \leq 0.05$) by Tukey test method.

FIGURE 7 The growth of *Zea mays* (A) and *Vigna radiata* (B) in control irrigation water (C), effluent (E), and dye effluent treated with *Aspergillus* sp. 1 (1), *Aspergillus* sp. 2 (2), *Aspergillus* sp.3 (3), *Aspergillus* sp. 4 (4), *Aspergillus* sp. 5 (5), *Aspergillus* sp. 6 (6), *Aspergillus* sp. 7 (7).*Vigna radiata* in treated fungal isolate.

Blue-04B. The fungal degradation products did not cause any toxicity in plants (*Z. mays* and *V. radiata*) as shown by plumule and radicle length of *Z. mays* and *V. radiata* grown on the decolorized effluent being longer than in the untreated effluent. The percentage of *Z. mays* and *V. radiata* seed germination on decolorized effluent was higher than in untreated effluent. There was no inhibition zone found around the decolorized effluent samples after incubating with bacteria (*B. cereus*, *Azotobacter* sp., *S. aureus*, and *E. coli*) for 48 h. *Aspergillus* sp. 3 showed the highest efficiency for degradation of effluent containing Indigosol Blue-04B and so could be used for batik effluent mycoremediation.

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Authors' contributions

RSD, RSK, EM, and YAP designed the study. RSD performed the field, laboratory works, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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