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Indigenous Fungi from Batik Wastewater Exhibit Decolorization Potential for Remazol Red Dye

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ABSTRACT: The study aimed to isolate and characterize indigenous fungi from batik waste-contaminated soil in Surakarta for their potential in decolorizing Remazol Red dye. Six fungal isolates (7 SPG, 10 SPJ, 12 SPL, 19 SPS, 41 SRP, and 99 RQX) were successfully isolated and screened for ligninolytic activity. The decolorization assay of Remazol Red at 250 ppm for 120 hours showed that the isolate with 41 SRP demonstrated the highest decolorization percentage of 87.72%. Morphological identification revealed the isolates (12 SPL, 19 SPS, 41 SRP, and 99 RQX) identified as *Trichoderma* sp., 7 SPG as *Fusarium* sp., and 10 SPJ as *Penicillium* sp.. These findings suggest the potential of indigenous fungi in bioremediating batik waste contamination

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INTRODUCTION

Textile dyes are the primary pollutants in the batik industry. The accumulation of dyes in the environment is carcinogenic and damages the environment. If the dye decomposes under anaerobic conditions, it will produce aromatic amine groups, which are also carcinogenic. Discharging these dyes into water bodies can cause allergic dermatitis and skin irritation (Lellis et al., 2019). Dyes without proper treatment can persist in the environment for long periods and can interfere with the photosynthesis process of aquatic plants and all living organisms. Water that has been polluted by batik waste dyes increases the levels of COD, BOD, salt, reactive dye residues, and organic matter in water bodies. This causes an imbalance in the water-holding capacity of the ecosystem and limits the amount of light penetration (Afiya et al., 2019).

Bioremediation is a branch of biotechnology that involves the use of biological agents, especially microorganisms such as fungi, bacteria, and algae, to clean up contaminated environments. Microflora in contaminated areas are capable of detoxification activities because, in the bioremediation process, these microorganisms can use some contaminants as nutrients or energy sources (Prasad, 2017).

According to Singh, (2006), fungi can degrade various substrates, known as biodegradation. Fungi can break down organic compounds by secreting various kinds of enzymes. One of the enzymes that plays a role in the process of organic degradation, such as dyes, is laccase (benzenediol-oxygen-oxidoreductase, EC 1.10.3.2). Laccase can oxidize various substrates such as ortho- and para-diphenols, methoxy-substituted phenols, aromatic amines, phenolic acids, and several other compounds by reducing oxygen molecules to water with an electron oxidation mechanism. Laccase's substrate specificity varies from one organism to another(Sadhasivam et al., 2008; Shanmugam et al., 2018).

The study aimed to improve batik waste treatment by isolating and characterizing fungi that can decolorize Remazol Red. Fungi from polluted environments, capable of utilizing existing carbon sources and adapting to contaminated environments, offer a promising approach for large-scale bioremediation.

MATERIALS AND METHODS

a. Sampling collection

Sampling was carried out using purposive sampling. Samples of soil polluted by batik waste were taken around the waste disposal pipe or reservoir. Soil samples were taken from 5 batik home industries in Surakarta using a shovel, approximately 5 g, at a depth of 15-20 cm from the soil surface. The soil samples were then put into a sterile closed container and filtered using a filter with 2 mm pores.

b. Fungi isolation

Isolation was carried out using serial dilution by adding 1 mL of sterile distilled water to a test tube. Serial dilution was done up to 10^{-4}. Take 500 µL of sample from serial dilution and spread on the surface of PDA medium that has been added with streptomycin at a concentration of 100 mg/L. Purification was carried out by taking mycelium at the end of the media using aseptic streaking on PDA media and then incubating it at room temperature for seven days. (Kumar et al., 2012) stated that pure cultures on slant agar media can be used as stock culture by storing at 4°C.

c. Screening ligninolytic activity

Screening of potential fungal isolates is carried out by inoculated one plug of fungal isolate (5 mm) into PDA medium (g/L) combined with 1% tannic acid. The culture medium was incubated for seven days at room temperature(Dewi et al., 2018). Discoloration of the medium was observed on the culture plates (Senthivelan et al., 2019). Table 1 shows the size of the brown zone and the classification of the color formed based on the ligninolytic ability (Lee, 2000).

Table 1. Classification of Ligninolytic activities

Scale	Description
-	No brown zones formed
+	Light brown colored zone, visible only on the underside of the colony
++	Light brown colored zone, the brown zone is slightly wider than the colony and can be seen at the top of the colony
+++	Light to dark brown colored zone, brown zone wider than colony
++++	Dark brown, large and wide brown zones can be seen on the upper and lower sides of the colony.

d. Decolorization Assay

The decolorization test was conducted by inoculating five plugs (5 mm) into 100 mL of PDB (24 g/100 mL) supplemented with Remazol Red at a concentration of 250 ppm. The samples were then incubated on a shaker at 150 rpm for 120 hours at room temperature (25 °C ± 2 °C) (El-rahim et al., 2017; Parmar, 2014). Decolorization activity was measured by taking out 10 mL aliquots of samples centrifuged at 6000 rpm for 5 minutes. (Singh and Singh 2012). The percentage of decolorization was measured using a UV-Vis spectrophotometer. Absorbance measurements were taken at a wavelength (λ) of 530 nm for both the untreated and treated samples by potential fungi. The percentage of decolorization was calculated using the following formula: % Decolorization= (initial absorbance-final absorbance)/initial absorbance x 100 (Dewi et al., 2018).

e. Morphological identification

Morphological identification was carried out by growing pure culture on PDA media to observe the characteristics of the colony surface, radial lines, concentric circles, growth rate, color, and edges. Microscopic observations were carried out using the culture slide technique with Lactophenol Cotton Blue dye to observe the microscopic morphology of fungi, such as hyphae type, conidia or spore type, conidiophore branching type, conidiophore wall, and phialid characteristics, then observed under a microscope (Gajera et al., 2015). The characteristics of the isolates obtained were then compared using the key to determining fungi.

RESULT AND DISCUSSION

99 fungal isolates were successfully isolated from soil contaminated with batik waste. This finding aligns with Kumar et al., (2012), which identified ten fungal isolates from soil contaminated with dyes from three home industries. The isolation success of fungi from soil samples is influenced by the availability of nutrients, oxygen, and biological, physical, and chemical characteristics of samples.

Qualitative screening for ligninolytic activity revealed positive results with forming brown zones around fungal colonies (Figure 1). These zones were subsequently scored (Table 2). The six isolates exhibited lignolytic ability, as evidenced by the presence of 7 SPG, 10 SPJ, 12 SPL, 19 SPS, 41 SRP, and 99 RQX. Illuri et al., (2021) reported that the brown zones surrounding the colonies indicate the presence of total polyphenol oxidase activity. Pandya et al., (2014) research on 12 *Trichoderma* sp. isolates demonstrated

positive reactions on tannic acid media, suggesting the secretion of ligninolytic enzymes. According to (Ingle & Mishra, 2016; Pérez-Cadena et al., 2020; Shanmugam et al., 2018), Trichoderma species such as *T. asperellum* strain BPLMBT1 and *T. asperelloides* LBKURCC2 produce extracellular laccase, which can be used in bioremediation processes.

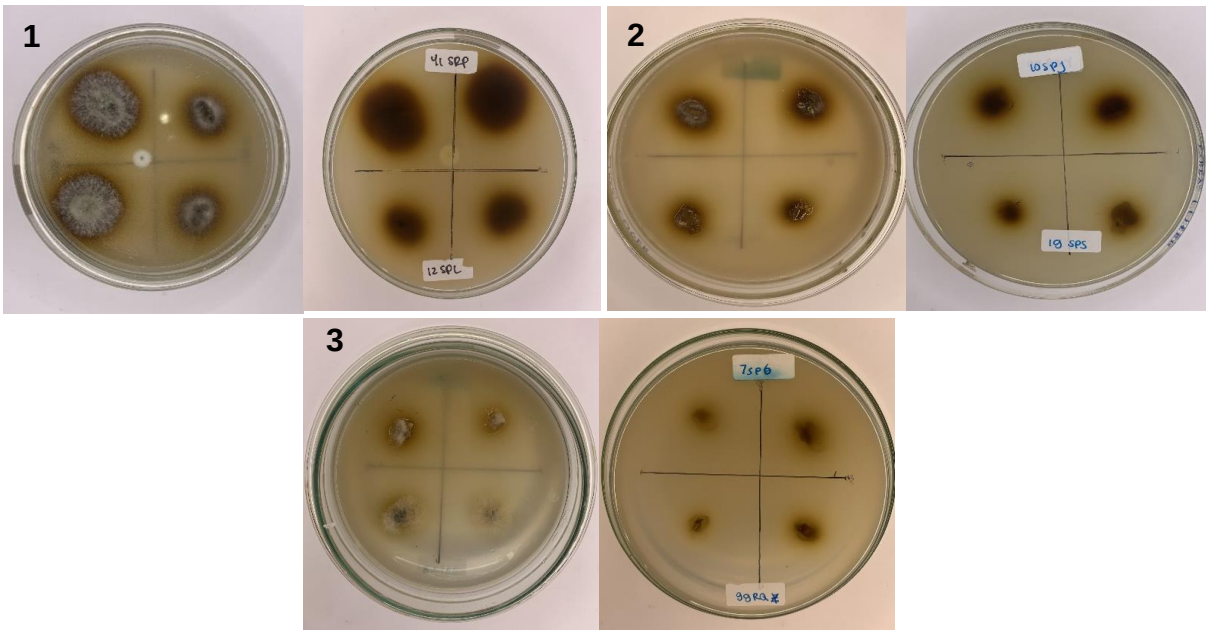

Figure 1: Screening results of ligninolytic activity using tannic acid after 7 days of incubation. (1: Forward and reverse colony of 41 SRP and 12 SPL, 2: Forward and reverse colony of 10 SPJ and 19 SPS, 3: Forward and reverse colony of 7 SPG and 99 RQX).

The decolorization of Remazol Red dye at 250 ppm for 120 hours by the potential fungi isolates 7 SPG, 10 SPJ, 12 SPL,19 SPS, 41 SRP, and 99 RQX (Table 3). All the potential fungal isolates were capable of decolorizing Remazol Red dye. However, the isolate with code 41 SRP exhibited the percentage decolorization ability of 87.72%. Using fungi as biological agents for dye waste remediation involves fungal mycelium's initial absorption of dye compounds. The electrostatic interaction between the negatively charged cell wall and the positively charged dye molecules facilitates absorption. This is followed by the enzymatic breakdown of complex chemical bonds, leading to a reduction in the color intensity of the dye (Munir et al., 2018). According to (Kaur et al., 2015), decolorization can occur through hyphae's passive absorption of dyes. This process involves the interaction of anionic dyes with positively charged components of the fungal cell wall, such as chitin, glucan, and cellulose. Subsequently, fungal enzymes degrade the absorbed dyes.

High dye concentrations in the media can be toxic to fungal growth. According to Parmar, (2014), the decolorization activity of acridine acid dye is influenced by the dye concentration in the media. The sensitivity of fungal isolates to increasing dye concentrations can inhibit fungal growth (Salem et al., 2019). (Kaur et al., 2015) reported that dye concentrations in the range of 200-1000 ppm in the media can reduce decolorization activity. Bergsten et al., (2009) identified *Penicillium simplicissimum* INCQS 40211 (CCT 6686) from various industrial waste-contaminated environments in São Paulo State, Brazil. Fungi have recently been identified as potential agents for biodegradation of complex industrial pollutants. Their unique ability to produce extracellular lignin-degrading enzymes, including manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase, enables them to effectively degrade complex organic and inorganic pollutants, yielding less toxic byproducts or even complete mineralization (Latif et al., 2023).

The differential decolorization rates among the fungi are influenced by their varying sensitivities to Remazol Red dye. According to Ranjusha et al., (2010), Remazol Reactive dyes, such as Remazol Red, have a high percentage of unfixed color in wastewater, leading to their accumulation in the aquatic environment, the factors including pH, inoculum concentration, and initial dye concentration influence biosorption of these dyes. While *Aspergillus flavus* can tolerate higher initial dye concentrations of 1000 mg/L, Bhatnagar et al., (2021) observed that the decolorization of malachite green dye was inhibited for two days, suggesting that the dye's antifungal properties may have hindered fungal growth. Additionally, fungal biomass decreased when inoculated in dye-containing media compared to control conditions, likely due to physiological stress induced by the dye.

The macroscopic and microscopic characteristics of fungi grown on PDA medium for seven days were observed (Figures 2 and 3). Morphological identification revealed that four isolates were *Trichoderma* sp. (12 SPL, 19 SPS, 41 SRP, dan 99 RQX). In comparison, the remaining two isolates were identified as *Penicillium* sp. (7 SPG) and *Fusarium* sp. (10SPJ) (Table 2).

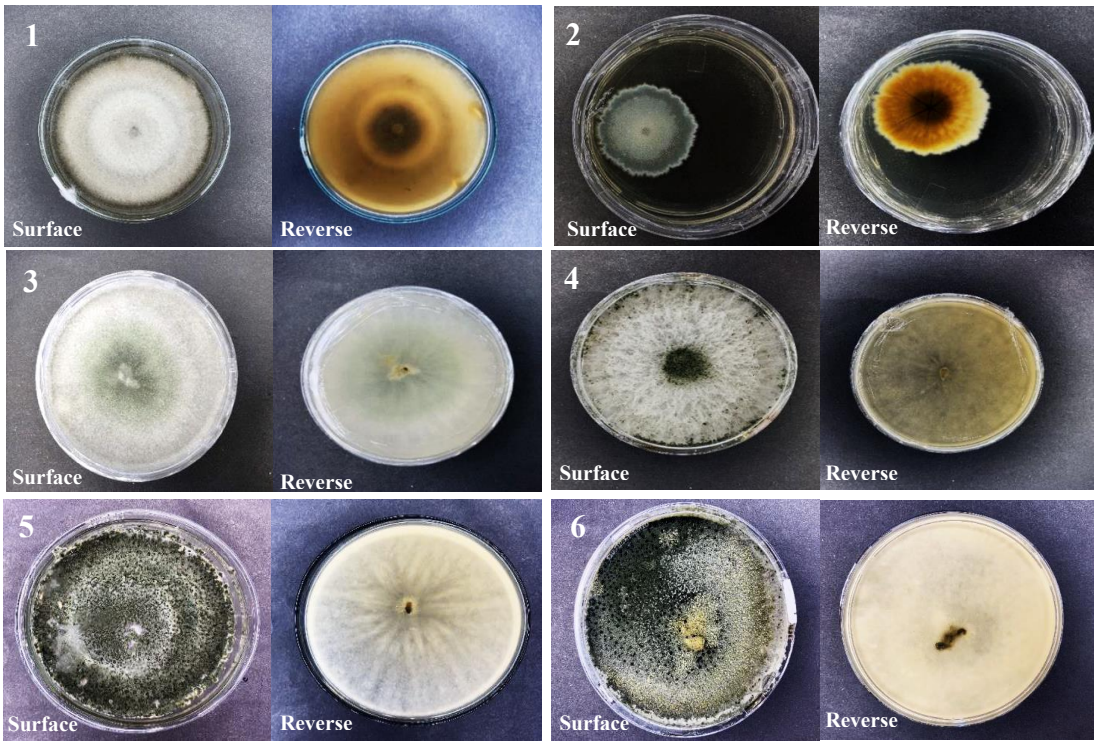

Figure 2. Morphology surface and reverse of potential fungi that have a ligninolytic positive reaction from soil-contaminated wastewater from Surakarta on PDA at seven days incubation (Isolate 1: 7 SPG, isolate 2: 10 SPJ, isolate 3: 12 SPL, isolate 4: 19 SPS, isolate 5: 41 SRP, and isolate 6: 99 RQX).

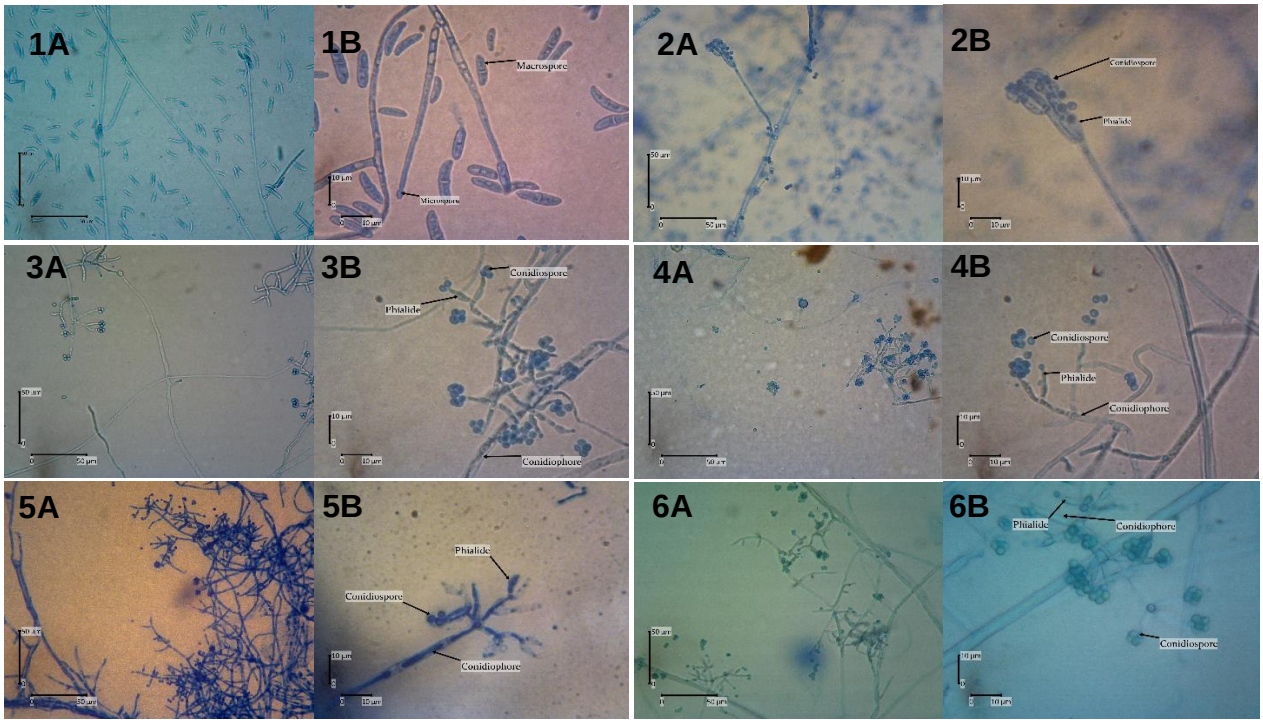

Figure 2. The microscopic character of potential fungi (Isolate 1A, B: 7 SPG, isolate 2A, B: 10 SPJ, isolate 3A, B: 12 SPL, isolate 4A, B: 19 SPS, isolate 5A, B: 41 SRP, and isolate 6A, B: 99 RXQ).

A single *Fusarium* sp. isolate (7 SPG) was identified in this study. Based on macroscopic characteristics such as a white to slightly reddish colony and flat surfaces with a cottony texture. Microscopic examination revealed macroconidia and microconidia. Ujat et al., (2021) reported that *Fusarium* sp. growing on PDA medium has a white colony with cottony and dense mycelia.

The isolate (10 SPJ) was identified as Penicillium sp. It exhibited macroscopic characteristics such as a bluish-green colony without white margins, no concentric circles, and a faint light yellow pigment. According to Saif et al., (2020), *Penicillium aurantiogriseum* has a green colony color, inconspicuous mycelium, and a light yellow soluble pigment. *P. aurantiogriseum* has verticillate conidiophores with strongly divergent branches. The conidia are grayish green, and the conidiospores are subglobose to ellipsoidal and smooth-walled.

Macroscopic characterization of isolates 12 SPL, 19 SPS, 41 SRP, and 99 RQX showed that isolates 41 SRP and 99 RQX had dark green colonies after seven days of incubation, while isolates 12 SPL and 19 SPS showed predominantly white hyphae with a green center. The reverse sides of the colonies were also white. The colony surfaces of all four isolates were smooth and fur-like. They had flat margins, concentric circles, and no exudates. Microscopic examination revealed flat, fibrous, and multi-branched hyphae and verticillate-branched conidiophores with phialid-shaped terminal cells. According to Hocking (2013), the morphology of Trichoderma in the isolates was light to dark green, had concentric circles, and was microscopically characterized by conidiophores, conidia, and phialids. Brito-Vega, (2020) states that conidiophores with common characteristics are long and branched and have two to three phialides, while (Romero-Arenas et al., 2009) state that Trichoderma has one to four phialides which are long and single-celled bottle-like with irregular small branches with conidia at the top which are globose, subglobose, or ellipsoidal.

CONCLUSION

This study successfully isolated and characterized indigenous fungi from batik waste-contaminated soil in Surakarta. Six fungal isolates were identified, including *Trichoderma* sp., *Penicillium* sp., and *Fusarium* sp. All isolates exhibited positive ligninolytic activity and decolorized Remazol Red dye at 250 ppm, with 41 SRP demonstrating the highest decolorization efficiency of 87.72%. These findings highlight Indigenous fungi's potential as bioremediation agents for treating batik wastewater. Future research could focus on optimizing the decolorization process using these fungal isolates and exploring their application in full-scale wastewater treatment systems.

TABLES

Table 1. Ligninolytic activity scoring of potential fungi isolates

Isolates	Scoring brown zone formed			
	+	++	+++	++++
7 SPG	+		+++	
10 SPJ				++++
12SPL				++++
19 SPS				++++
41 SRP				++++
99 RQX			+++	

Table 2. Description of macroscopic and microscopic characteristics of ligninolytic fungi isolated from soil-contaminated wastewater.

Isolate	Genus	Colony on PDA	Hyphae	Spore	Conidiophore	Phialide
7SPG	Fusarium sp	Color: white, reverse: reddish brown, texture: cottony	Setae	Macrospore and Microspore	Branched	-
10 SPJ	Penicillium sp.	Color: Green with grey in the middle, reverse: Yellow, texture: flat	Aseptate	Ellipsoidal joined into a chain	Biverticillate	Six phialide
12 SPL	Trichoderma sp.	Color: White with green in the middle, reverse: white with	Setae	Sub globose to globose	Upright, branching	Short and thick

		pale green in the middle, texture: Cottony					
19 SPS	Trichoderma sp.	Color: White with green in the middle, reverse: white, texture: Cottony with green granular	Setae	Sub globose to globose	Upright, branching	Short thick	and
41 SRP	Trichoderma sp.	Color: Green, reverse: white, texture: Cottony with green and yellow granular	Setae	Sub globose to globose	Upright, branching	Short thick	and
99 RQX	Trichoderma sp.	Color: Green, reverse: white, texture: Cottony with green granular	Setae	Sub globose to globose	Upright, branching	Short thick	and

Table 3. Decolorization assay of 250 ppm Remazol Red at 120 h by potential fungi

Isolate	Percentage of decolorization (%)
C	0.22 ±0.03
7 SPG	72.48 ± 0.13
10 SPJ	80.77 ± 0.15
12 SPL	76.18 ± 0.29
19 SPS	69.44 ± 0.06
41 SRP	87.77 ± 0.99
99 RXQ	75.56 ± 0.62

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