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Bioremediation, Biosorption, and Biodegradation of the Textile Dye Reactive Black 5 by Life Cultures of *Trichoderma asperellum* LBKURCC1

Bioremediasi, Biosorpsi dan Biodegradasi Pewarna Tekstil Reactive Black 5 oleh Kultur Hidup *Trichoderma asperellum* LBKURCC1

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ABSTRACT

Reactive black 5 (RB5) is an azo dye widely used in the textile industry for dyeing fabrics. It is categorized as a recalcitrant dye that is hard to degrade and an environmental pollutant. Therefore, textile waste effluents containing this dye must be treated to remove or degrade the dye, before being released into the environment. One method that can be used to degrade synthetic dyes such as RB5 is to use biological methods, by directly using live fungal cells or using laccase enzymes. *Trichoderma asperellum* LBKURCC1 is a filamentous fungus isolated from cacao plantation soil in Riau, Indonesia, and it is a laccase enzyme producer. To be able to determine the ability of *T.asperellum* LBKURCC1 life cultures to decolorize RB5 dye, several RB5 dye removal tests were carried out. Incubation of 50 ppm RB5 with life cultures of *T. asperellum* LBKURCC1 at room temperature (30°C, pH 6.5) for 24 hours resulted in 22% bioremediation, 3.2% biosorption and 19.1% biodegradation of the RB5 dye. The results of this study show that the live culture of *T.asperellum* LBKURCC1 is capable of biodegrading RB5. This is indicated by the degradation of RB5 by extracellular enzymes produced by these filamentous fungi.

Keywords: Azo dye, Laccase, Reactive black 5, *Trichoderma asperellum*

ABSTRAK

Reactive black 5 (RB5) adalah pewarna azo yang banyak digunakan dalam industri tekstil. RB5 dikategorikan sebagai suatu pewarna yang sulit didegradasi, dan dapat menjadi pencemar lingkungan sehingga tak boleh tersisa dalam limbah industri tekstil yang dilepas ke lingkungan bebas. Salah satu metode yang dapat digunakan untuk mendegradasi zat warna sintesis seperti RB5 adalah menggunakan metode biologis yaitu langsung menggunakan sel jamur hidup atau menggunakan enzim lakase. *Trichoderma asperellum* LBKURCC1 adalah jamur yang diisolasi dari tanah perkebunan coklat di Riau, Indonesia, yang dapat memproduksi lakase. Penelitian ini bertujuan untuk menentukan kemampuann degradasi zat warna RB5 oleh kultur hidup *T.asperellum* LBKURCC1. Beberapa uji degradasi atau penghilangan warna RB5 oleh kultur hidup *T.asperellum* LBKURCC1 dilakukan untuk mencapai tujuan tersebut. Uji penghilangan warna yang dilakukan adalah uji bioremediasi, biosorpsi dan biodegradasi. Inkubasi 50 ppm zat warna RB5 dengan kultur hidup *T. asperellum* LBKURCC1 pada suhu kamar (30°C, pH 6.5), selama 24 jam menghasilkan nilai bioremediasi RB5 sebesar 22%, biosorpsi RB5 sebesar 3,2% dan nilai biodegradasi RB5 sebesar 19,1%. Hasil penelitian ini memperlihatkan bahwa kultur hidup *T.asperellum* LBKURCC1 mampu melakukan biodegradasi terhadap RB5. Hal ini ditunjukkan oleh adanya degradasi RB5 oleh enzim ekstraselular produksi jamur filamen tersebut.

Kata Kunci: Pewarna Azo, Lakase, *Reactive black 5, Trichoderma asperellum*

INTRODUCTION

Remazol black 5 (RB5) is an azo-type dye. RB5 is one of the most widely used dyes in the textile industry, due to its ease of use to dye various textiles such as cotton, cellulosic fibers, nylon, and wool (El

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Bouraie and El Din, 2016). The azo-type dyes are used extensively due to their attractive colors. However, they are also among the most difficult to degrade, being also categorized as recalcitrant and toxic dyes (Slama et al., 2021). Azo dyes released into the environment may cause cancer and decline in aquatic life when ingested by aquatic organisms or mammals (Kishor et al., 2021). During the dyeing process of textiles, approximately 15% to 50% of azo dyes are not bound to the fabrics and therefore are subsequently present in textile wastewater (Al-Tohamy et al., 2022). Before release into the environment, these toxic dyes should be degraded.

Several chemical and physical methods for degrading textile dyes exist, among others are adsorption on various materials such as zeolite (Aljerf, 2018), $Ce_{1-x}Bi_xCrO_3$ (x = 0,0.5, 1) (Awin et al., 2018), coal fly ash (Eteba et al., 2023) and iron-oxide nanoparticles (Singh et al., 2020). Other physico-chemical methods include chemical photocatalysis (Vásquez et al., 2020) and Fenton oxidation (Liu and Li, 2018). However, these physical and chemical methods often are costly and can cause secondary problems in the disposal of sludge or dye-adsorbent waste (Donkadokula et al., 2020). Biological treatment of dye waste effluents provides an environmental and cost-effective alternative to physico-chemical methods (Singh et al., 2022). These biological treatments include the use of dye-degrading microbes (Anita et al., 2022; Varjani et al., 2020) or their isolated enzymes, such as laccase (Tavares et al., 2020).

Laccases are oxidoreductase multicopper-containing enzymes that can oxidize a wide variety of substrates, both phenolic and non-phenolic, including several synthetic textile dyes (Arregui et al., 2019). Biological oxidation of dyes by laccase leads to dye degradation, decolorization, and detoxification (Shanmugam et al., 2017). Among the producers of laccase are the filamentous fungi *Trichoderma* sp (Umar, 2021). *Trichoderma asperellum* LBKURCC1 is a strain isolated from the rhizosphere of cocoa plants in a plantation located in Riau Province, Indonesia. Although originally isolated based on its production of chitinase, it also is a laccase producer (Dahliaty et al., 2022). Since *T.asperellum* LBKURCC1 produces laccase, it has the potential to be used as a dye degrader, either directly as life cultures, or using their isolated enzymes. This study aims to determine the ability of *T.asperellum* LBKURCC1 to biodegrade RB5, by first determining the bioremediation of RB5 by this fungus.

MATERIALS AND METHOD

Fungal cultures

Trichoderma asperellum LBKURCC1 was obtained from the Laboratorium Biokimia Culture Collection (LBKURCC) facility of Universitas Riau, Pekanbaru, Indonesia. It is maintained on PDA slants, with periodic regeneration.

Chemicals and consumables

Reactive Black 5 (RB5) was from Merck Sigma-Aldrich Co. (Cat. Product No. 306452). Mineral media (MM) for dye degradation experiments at pH 4.5; 5.5; and 6.5 respectively was prepared with the following composition (1L media): 1 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 0.6 g KH_2PO_4, 22.3 g yeast extract, 1 g Tween-20, 0.046 g $CuSO_4.7H_2O$. Glass fiber circle filters GF/C for separation of mycelia from MM culture media was from Whatman™ Cat. No. 6780-2504 (pore size 1.2 μm).

Method to determine the bioremediation percentage of RB5 by fungal life cultures

Trichoderma asperellum LBKURCC1 spores were spread on PDA petri dishes and allowed to grow into lawns by incubation at room temperature (±30°C). Two plugs (1 cm in diameter) were taken from a mycelium lawn aseptically and inoculated into 30 mL MM in each 100 mL flask. These flasks were designated as fungal RB5 bioremediation test flasks. The flasks were incubated at room temperature with shaking at 120 rpm for 2 days until the fungal growth had reached the logarithmic phase. At this point, to the culture 750 μL of 0.002 g/mL RB5 were added to each test flask, together with 150 μL (5 g/L) chlortetracycline solution. The test flasks were then further incubated with shaking at 120 rpm at room temperature. At time 0 and 24 hours, aliquots were taken from each test flask for spectrophotometric analysis at 320 nm to 800 nm, using a UV-Vis Agilent Cary 60 spectrophotometer. Before spectrophotometric analysis, aliquots were centrifuged at 13,000 rpm for 10 minutes, to sediment any debris and fungal spores that may interfere with the supernatant spectrophotometric measurements. Photographs were also taken of these supernatant aliquots, for color documentation and visual comparison between samples. Control RB5 flasks were RB5 dye in MM media without the addition of fungal

plugs. Control RB5 flasks were also incubated at room temperature and treated the same as the fungal bioremediation test flasks. Acting as blanks for the spectrophotometric assay were MM media alone. All experiments were repeated twice. Bioremediation percentages (R) were calculated following equation (1), where A_c is the area under the absorption spectrum curve of control flasks starting at the wavelength where a difference between controls and fungal bioremediation tests was observed. A_t is the area under the absorption spectrum curve of fungal bioremediation test flasks at the same wavelength used to measure A_c.

$$R\ (\%)=\frac{Ac-At}{Ac} \times 100 \qquad \text{equation (1)}$$

Biosorption of RB5 assays by fungal mycelia

Mycelia from 24-hour incubated fungal test flasks were filtered using GF/C glass filter circles under vacuum suction. The fungal biomass obtained was washed with distilled water to remove any remaining MM. Washed biomass was added into 100 mL flasks containing 30 mL 1M NaOH pH 8 and shaken at room temperature at 180 rpm for 7 hours. After 7 hours of this shaking incubation process, 2 mL of the NaOH extract was then aliquoted and centrifuged at 13,000 rpm for 10 minutes, to sediment cell debris. The sediments were discarded, and the NaOH extract supernatant was transferred to a clean microtube. The spectrum of this supernatant was then measured at 320 nm to 800 nm with a UV-Vis Agilent Cary 60 spectrophotometer. Experiments were repeated twice. Biosorption percentages (S) were calculated using the following equation (2). A_c is the same as in equation (1). A_s is the area under the absorption spectrum curve of NaOH extract supernatant at the same wavelength used to measure A_c.

$$S\ (\%)=\frac{As}{Ac} \times 100 \qquad \text{equation (2)}$$

Enzymatic biodegradation of RB5 by life fungal cultures analysis.

Percentages of biodegradation by enzymes produced by life fungal cultures (D) were calculated using equation (3).

$$D\ (\%) = R-S \qquad \text{equation (3).}$$

RESULT AND DISCUSSION

Bioremediation of RB5 using life cultures of *T.asperellum* LBKURCC1 experiments were carried out at pH 4.5, 5.5, and 6.5 in the appropriate buffers. As shown by photographs of duplicated experiments (Figure 1), bioremediation/decolorization of RB5 by *T.asperellum* LBKURCC1 after 24 hours of incubation at room temperature was only observed at pH 6.5. Decolorization or bioremediation of RB5 at pH 6.5 by the fungal life cultures resulted in the reversion of the colored aliquot from a black-greenish color at time 0 hours, to the same color as MM blanks at time 24 hours. MM blanks and RB5 controls did not change color after 24 hours of incubation compared to 0 hours at all pH buffers tested (wells A and B for all pH and sampling times shown in Figure 1).

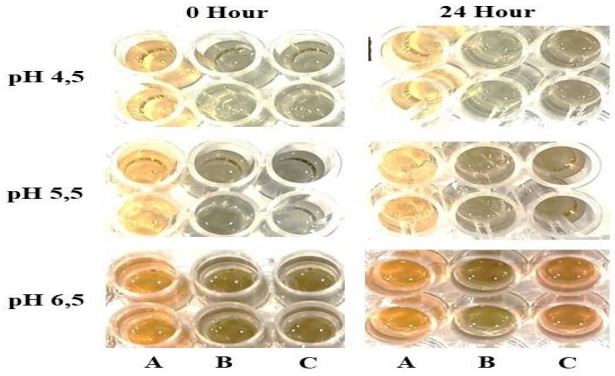

Figure 1. Visual color results of *T. asperellum* LBKURCC1 bioremediation test of RB5 test at times 0 and 24 hours of incubation at the indicated pH buffers in MM media. A = MM media blanks; B = Control RB5 in MM media; C = Fungal bioremediation RB5 test in MM media. Every pH is represented by two rows of duplicated experiments.

Spectrum analysis of RB5 24 hours after commencement of the fungal RB5 bioremediation test as shown in Figure 2 confirmed the visual color results, showing that bioremediation of RB5 by life *T.asperellum*

LBKURCC1 cells occurred only at pH 6.5. This was shown as a difference in the spectrum profiles of RB5 at pH 6.5 between RB5 controls and RB5 treated with life cultures of *T.asperellum* LBKURCC1 (Figure 2, panels E and F). Spectral maxima of RB5 controls in MM media at pH 6.5 were observed around 540 nm to 720 nm. This spectral maximum disappeared in the RB5 MM media that was treated with life cultures of *T.asperellum* LBKURCC1. No marked differences in the spectral maxima of RB5 controls and RB5 treated with *T asperellum* LBKURCC1 were seen at pH 4.5 and 5.5.

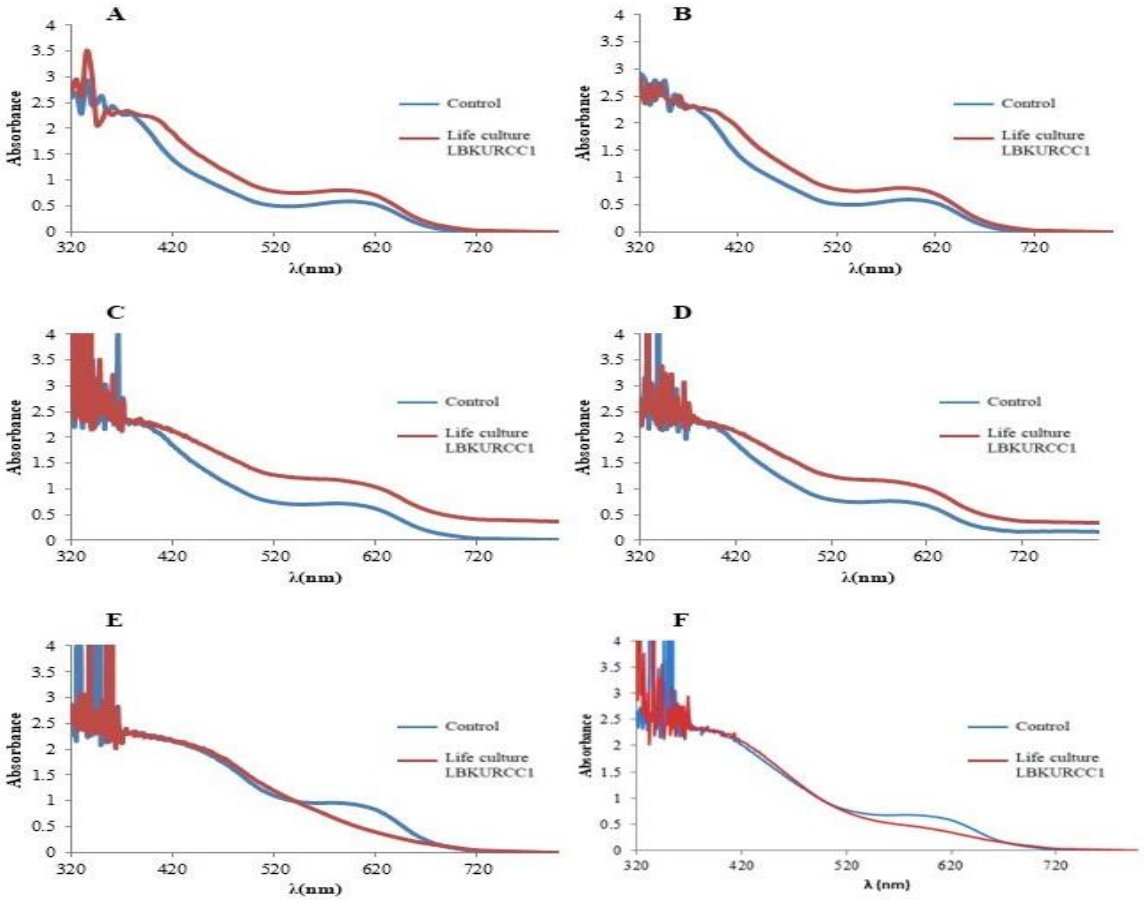

Figure 1. Visible light spectrum profiles of RB5 from 320 nm to 800 nm after 24 hours of incubation at room temperature. Blue solid lines = RB5 controls in MM media; Red solid lines = RB5 in MM media treated with life cultures of *T. asperellum* LBKURCC1. Panels A and B = duplicated experiments at pH 4.5; C and D = duplicated experiments at pH 5.5; E, and F = duplicated experiments at pH 6.5.

To determine the bioremediation, biosorption, and enzymatic biodegradation percentages at each pH, areas below the spectrum obtained from 540 nm to 720 nm were calculated. The choice of wavelength to measure areas below the spectrum was determined based on the bioremediation spectral profiles of pH 6.5, where a marked difference between RB5 controls and fungal-treated RB5 was observed. Compared to the other pH tests, bioremediation of RB5 at pH 6.5 had the highest percentage of 22.2% (Table 1). Bioremediation of RB5 by *T.asperellum* LBKURCC1 at pH 4.5 and 5.5 still could be detected, but it was very low compared to that at pH 6.5 and only contributed by fungal cell biosorption of the dye.

Since mycelia can also adsorb organic matter, it was important to determine the biosorption percentage of RB5 by *T.asperellum* LBKURCC1 fungal mycelia. Biosorption analysis showed that in all pH studies, biosorption percentages were almost the same in the range of 3 to 5%. At pH 6.5 the biosorption percentage was only 3.2%, while the biodegradation percentage was 19.1%. This shows that the contribution of biosorption to the bioremediation of RB5 by *T.asperellum* LBKURCC1 at pH 6.5 is only a small fraction compared to enzymatic biodegradation. The bulk of RB5 bioremediation at pH 6.5 in this study is attributed to biodegradation by enzymes produced and released by *T.asperellum* LBKURCC1 (Table 1).

Table 1. Bioremediation, biosorption, and biodegradation of RB5 by life cultures of *T. asperellum* LBKURCC1 at various media pH

Buffer pH of the media	Bioremediation (%)	Biosorption (%)*	Biodegradation (%)*
4,5	4,91 ± 0,39	4,91 ± 0,4	0
5,5	5,42 ± 0,40	5,4 ± 0,4	0
6,5	22,23 ± 11,39	3,16 ± 0,76	19,07 ± 0,76

Trichoderma asperellum LBKURCC1 is known as a laccase producer. It can produce laccase in a solid-state fermentation system using rice straw as its substrate (Rahayu et al., 2019). We have recently demonstrated that crude laccase extracts from *T. asperellum* LBKURCC1 can degrade the triphenyl methane textile dye methyl blue, which is also known as cotton blue (Dahlena et al., 2022). Therefore, the enzymatic biodegradation of RB5 observed in this study may be attributed to the production of laccase enzyme by *T. asperellum* LBKURCC1 in the MM media, in response to the presence of RB5.

Enzymatic dye degradation is possible by the release of extracellular enzymes into the media. There are several kinds of enzymes known to degrade dyes by oxidation, among others are the laccase enzymes, lignin peroxidases, and manganese peroxidases (Akhtar and Mannan, 2020). Studies show that in most cases the laccase enzymes play a more dominant role in the biodegradation of dyes compared to lignin peroxidases and manganese peroxidases (Adnan et al., 2017; Sing et al., 2017). Apart from that the peroxidases require the presence of hydrogen peroxide for their activities (Ilić Đurđić et al., 2020), plus the manganese peroxidases require the presence of Mn^{2+} for their activity (Kumar and Arora, 2022). Since the MM media used in this study did not contain peroxidase of Mn^{2+}, it is highly unlikely that lignin peroxidase and manganese peroxidase were responsible for the enzymatic biodegradation of RB5. Therefore, the most likely dominant mechanism in the bioremediation of RB5 by life cultures of *T. asperellum* LBKURCC1 at pH 6.5 observed in this study is due to the production and action of laccase.

This study shows that *T. asperellum* LBKURCC1 cultures can decolorize and degrade the widely used textile dye RB5. Different fungi degrade different dyes with different efficiency (Tang et al., 2022). The use of microbes for the degradation of textile dye effluents containing different kinds of dyes would require consortiums of several kinds of microbes. Therefore, it is important to build a library of known microbes that can degrade different kinds of dyes. This study contributes to this body of knowledge, for further utilization of filamentous fungi for bioremediation and biodegradation of textile dyes.

CONCLUSION

The filamentous fungal strain *T. asperellum* LBKURCC1 can decolorize reactive black 5 (RB5), through bioremediation using life cultures at pH 6.5. At pH 6.5, treatment of RB5 for 24 hours resulted in 22% bioremediation, 3.2% biosorption, and 19.1% enzymatic biodegradation of the RB5 dye. Bioremediation of RB5 at pH 4.5 and 5.5 is only 4.9% and 5.4% respectively and only contributed by biosorption of the dye by mycelia of the fungus.

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