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Decolorization of anthraquinone, azo and triphenylmethane dyes by laccase from newly isolated fungus, Cerrena sp. BMD.TA.1

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Abstract. The use of white-rot fungus for dyestuff degradation is alternative of eco-friendly strategy on removal of industrial effluents. This study was conducted to investigate the decolorization of dyes using laccase produced by Cerrena sp. BMD.TA.1 isolated from Gunung Rinjani National Park (GRNP), West Nusa Tenggara - Indonesia. This fungus had capability to decolorize three types of dyes, those: anthraquinone (Remazol Brilliant Blue R, RBBR), azo (Congo Red) and triphenylmethane (Fast Green FCF). The optimum laccase activity in this dyes treatment by fungal culture was achieved at 96 h pre-incubation, but the highest decolorization rate was reached at 144 h pre-incubation. After 72 h of dyes treatment, all dyes were removed at least 82%. The optimum decolorization for 100 mg L⁻¹ was similar for of all dyes types. The decolorization of RRBR was higher compared to Congo Red and Fast Green FCF at 1000 mg L⁻¹. Furthermore in purified laccase, the RBBR was decolorized without any addition of redox mediator. The decreasing of laccase activities and increasing of dye concentration resulted on the lowest decolorization. This study revealed that laccase produced by Cerrena sp. BMD.TA.1 contributed to decolorization process, and had potential industrial application on removal of dyes effluents.

1. Introduction

Synthetic dyes are widely applied in many industries; textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, and leather industries [1-3]. Based on chemical properties, dyes are classified into anthraquinone, azo and triphenylmethane. Anthraquinone dyes are important textile dyes released into sewage treatment system or the environment [4, 5]. Azo dyes are most important class used in textile processing and contributing about 70% of dyestuffs on the worldwide market [6]. Triphenylmethane dyes are widely used for various treatments of infectious diseases in food-producing animals and fish as well as for staining textiles, plastics and biomaterials [7]. The discharge of the dye effluents into environmental have led to carcinogenic, mutagenic and recalcitrant level, as well as they remain in the environment in long period of time [8-12]. Their existences in environment are necessary to be treated to reduce their levels of toxicity and to minimize their pollution impact.

In many cases, white-rot fungi have been used for decolorizing synthetic dyes because their extracellular laccase, lignin peroxidase (LiP) or manganese peroxidase (MnP) which is capable to

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degrade and decolorize synthetic dyes [6, 13-17]. Among of the three enzymes, laccase is now extensively used in the decolorization of dyes reaction for several reasons, those: not required H_2O_2 in the reaction, wide substrate specificity, and use molecular oxygen as a co-factor [18]. Previous study [19], we isolated a newly white-rot fungus *Cerrena* BMD.TA.1 from tropical forest at Gunung Rinjani National Park (GRNP), West Nusa Tenggara – Indonesia. The fungus showed MnP and Laccase activity and potential to degrade specific synthetic dye. In this study, fungal-culture of strain BMD.TA.1 was investigated for its ability to perform biological decolorization of anthraquinone, azo and triphenylmethane dyes. Moreover, there have been less studied concerning on the effect of the pre-incubation time on decolorization by fungi. The study was also undertaken to evaluate the effect of dye addition. The purified laccase of strain BMD. T.A.1 was also investigated for its laccase production and its potential for dye decolorization.

2. Materials and Methods

2.1. Chemicals

Remazol Brilliant Blue R (RBBR) and 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonate (ABTS) were purchased from Sigma (USA). Congo red was purchased from Merck (Germany). Fast Green FCF was purchased from BDH Chemicals (England). Agar, glucose and all other chemicals were provided from Himedia (India) at the highest purity.

2.2. Fungal and culture medium

Cerrena sp. BMD.TA.1 was isolated previously [19] and storage at -80°C in the Indonesian Tropical Culture collection (INTROF-CC), Laboratory of Forest Microbiology, Forest Research and Development Center, with identity number of INTROF-CC 06482. It was maintained as culture on 9-cm potato dextrose agar (PDA), containing glucose (20 g L⁻¹), potato (200 g L⁻¹), and agar (15 g L⁻¹), at 4°C prior to use. Decolorization by fungal culture was conducted using potato dextrose broth (PDB) containing glucose (20 g L⁻¹), and potato (200 g L⁻¹). The growth medium for production of laccase and for decolorization of dyes was prepared in solid-state fermentation medium (SFM) containing corncob and PDB (1:5, w/v).

2.3. Enzyme assay

Laccase activity was determined spectrophotometrically using 1 mM ABTS in 0.5 M sodium acetate buffer, pH 5 and measuring at 420 nm(ϵ_{420} = 36,000 mol⁻¹ cm⁻¹) [20]. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 nmol ABTS min⁻¹.

2.4. Purification of laccase

For the preparation of extracellular laccase purification, the fungus BMD.TA.1 was cultivated as described above. The purification of laccase was done in the time of maximum laccase activity. SFM medium was extracted using sodium acetate buffer (1:6, w/v) according to methods described by Falah et al (2018) [17] with slight modification. The extraction was conducted by grinding and followed by centrifugation at 10000 rpm for 20 min. Ammonium sulfate (60%, v/w) was added to the supernatant and incubated for 6 h, followed by centrifugation at 10000 rpm, 4°C for 30 min. The supernatants were decanted and the precipitant was dissolved in 0.5 M sodium acetic buffer (pH 5). This solution was stored at 4°C until further use.

2.5. Experimental of dyes decolorization by the fungus BMD.TA.1

The decolorization of anthraquinone, azo and triphenylmethane dyes was evaluated using PDB mention above. An inoculum of BMD.TA.1 for liquid culture was prepared, three agar plugs (5 mm in diam) punched out from fungal active growth on agar plate and inoculated in a 100 ml flask containing 20 mL of PDB. The content of RBBR, congo red and fast green FCF in the culture were 100 mg L^{-1} and 1000 mg L^{-1} added either after 48, 96, and 144 h of pre-incubation. After incubation for 24, 48 and 72 h, samples (5 mL) was collected and analyzed by measuring the decrease in absorbance at the

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absorbance maximal of each dye using a nano-spectrophotometer. Dye removal was calculated according to the formulation: decolorization (%) = $(C_0-C) \times 100/C_0$, where C_0 indicates the absorbance of the dye before decolorization and C is the absorption of the dye after decolorization at each sampling time. The laccase activity was also monitored before (48, 96, and 144 h of pre-incubation) and after (24, 48 and 72 h of incubation) addition of dyes.

2.6. Experimental of RBBR decolorization by the fungus BMD.TA.1 laccase

RBBR decolorization was measured in nano-spectrophotometer at 595 nm wavelength. The amount of RBBR (100, 250, 500, 750, 1000 mg L^{-1}) was stirred to final reaction of 160 µl containing purified laccase in sodium acetate buffer (pH 5). The reactions were incubated at 40°C in different time of the course of the experiment. The percentage of dyes decolorization was calculated using formula mentioned above. Experiments were performed in triplicate with controls (without enzyme addition).

3. Results and Discussion

3.1. Dyes decolorization by fungal liquid culture

The evaluation for ability of Cerrena sp. BMD.TA.1, to decolorize synthetic dyes solution were carried out by addition of several dyes type such as anthraquinone, azo and triphenylmethane to fungal culture. The mixing of each dyes solution was applied in different of time-point fungal culture preincubation (48, 96 and 144 h). The culture supernatant was analyzed for 24, 48, and 72 h to test dyes absorbance and the laccase activities. Fungus BMD.TA.1 was capable to decolorize all dyes tested (Figure 1) both in low (100 mg L⁻¹) and high (1000mg L⁻¹) concentration. In shorter pre-incubation (48 h), the lowest decolorization rate (only 57%) was obtained for triphenylmethane (Fast Green FCF) in 72 h, but other dyes were decolorized more than 80% at similar time (Figure 1 (I, A-C)). When the dyes solution was added to fungal culture after 96 and 144 h pre-incubation, the decolorization of all dyes increased more than 82% and achieved higher results at 144 h pre-incubation after 72 h incubation (Figure (I, A-C)). Interestingly when dyes solution was increased to be 1000 mg L^{-1} , the fungus BMD.TA.1 was also still able to decolorize all dyes with the same result with that of the lowest dyes solution (100 mg L⁻¹). At 144 h pre-incubation time, the decolorization of all dyes with initial concentration 100 mg L⁻¹ resulted in the same values (Figure 1 (I)), while the decolorization of triphenylmethane was lower than azo (congo red) and anthraquinone (RBBR) at 1000 mg L⁻¹ after 72 h incubation (Figure 1 (II)). According to dye class, as confirmed in previous studies [16, 21] decolorization will occurs when the chromophoric of dyes is cleaved [22]. The decolorization of dyes will be limited by its toxic properties, i.e structures and high dyes concentration [22,23].

In order way, biosorption by mycelium living or dead system of fungi might also play in the decolorization as the process that do not involve metabolic energy or transport. Some references reported that bioasorption have been occurred for only 5-10% [24] in few minutes and dyes will be removed by extending the incubation time [25]. In this study, the absorption was not evaluated because dyes were not present at the end of observation time. This indicated that decolorization was not influenced by absorption.

In Figure 1 showed laccase activity at time-point fungal culture pre-incubation and during decolorization of dyes. Laccase activity were vary depend on the time-point of dyes addition and the maximum activity was obtained at 96 h after pre-incubation. During decolorization, the laccase activity increased and the highest increasing was observed at 48 h after pre-incubation among all dyes tested. These results indicated that laccase in the fungal culture BMD.TA.1 played important role in the decolorization. This finding also suggest that the decolorization of given dyes was influenced by culture status of fungus, and that better decolorization for all dyes tested was obtained after 144 d pre-incubation, and the time-point of dyes addition was an important factor in obtaining most success for decolorization of gives by fungal.

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Figure 1. Percentage of anthraquinone (A), azo (B) and triphenylmethane (C) decolorization at initial concentration of 100 mg L⁻¹ (I) and 1000 mg L⁻¹ (II), and their laccase activity. Decolorization of dyes at 48 h (\blacktriangle), 96 h (\blacklozenge) and 144 h (\blacksquare) after pre-incubation to fungal culture. The laccase activity for 48, 96 and 144 d after pre-incubation was represented by bar clear to dark greycolor.

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3.2. Dyes decolorization by purified laccase

The optimum laccase activity produced by *Cerrena* sp. BMD.TA.1 was determined daily from 1 to 12 d after inoculation in SFM as described above. The laccase activity was obtained at 1 d after incubation (10.9 U mL⁻¹), reached maximum activity at 4 d (24.9 U mL⁻¹) after incubation, and decreased until 12 d (7.9 U mL⁻¹). The optimum period for laccase production in both SFM and liquid culture were reached at the same time (4 d), but the laccase activity itself was differed (Figure 1). The difference of laccase activity was probably influenced by critical nutritional factors, such as carbon and nitrogen sources, and how the supernatant of crude enzyme was prepared [26].



Figure 2. Effects of purified laccase activity (A) and dye concentration (B) on decolorization of RBBR. Incubation condition: A, RBBR (100 mg L⁻¹), pH 5, 40°C; B, laccase (2.3 U), pH 5, 40°C.

Proteins with laccase activity were purified using salting out process by addition of ammonium sulphate: 0-20%, 20-40%, 40-60%, and 60-80% saturation. Maximum laccase activity was observed at 60% saturation. Laccase activity after this step was 58.5 U mL⁻¹ with specific activity increased 30.5 fold purification (from 132.8 U mg⁻¹ to 4058.6 U mg⁻¹). This purified laccase was applied to evaluate its ability on decolorization of dyes. According on decolorization of dyes by fungal culture, the anthraquinone (RBBR) was selected as the substrate. The results showed that purified Cerrena sp. BMD.TA.1 laccase was able to decolorize of anthraquinone dyes (Figure 2) without addition of laccase mediator. Decolorization of RBBR increased with the increasing laccase activity (0.023 - 2.3)U), the decolorization achieved more than 50% with initial RBBR of 100 mg L^{-1} within 24 h and 2.3 U of laccase (Figure 1 B). The increasing dyes concentration by 250 mg L⁻¹, 500 mg L⁻¹, 750 mg L⁻¹ and 1000 mg L^{-1} would decrease decolorization rate to 50%, 48%, 46% and 45% (Figure 1 B). The reduction of decolorization in high dye concentration could be explained that chromophore structure of dye molecule may require numerous enzyme and the lowest enzyme activity results in less average enzyme attacks to dye molecule, and hence slower the rate of color removal [16, 27]. The optimum rate of decolorization was determined at pH 5 and temperature 40°C. The pH stability of *Cerrena* sp. BMD.TA.1 congruent with C. unicolor, that it was stable at pH 5 [28]. pH stability in decolorization plays important role on dyes removal by ligninolytic enzyme, including laccase [29-31]. The laccase was stable at 40-50°C, increasing temperature beyond 60°C resulted in faster enzyme maturation than the ezyme-catalyzed decolorization [32,33]. According to this study, laccase plays an important role in decolorization process applied by Cerrena sp. BMD.TA.1 culture.

4. Conclusion

Cerrena sp. BMD.TA.1, a fungus isolated from Gunung Rinjani National Park (GRNP), West Nusa Tenggara – Indonesia, was capable to decolorize various type of synthetic dyes, such as

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anthraquinone, azo and triphenylmethane. The decolorization of anthraquinone (RBBR) was higher compared to azo (azo) and triphenylmethane (Fast Green FCF) dyes. Optimum condition of RBBR decolorization by purified laccase occurred at pH 5 and the temperature 40°C. It is suggested that laccase produced by *Cerrena* sp. BMD.TA.1 could potentially used in biological approach to decolorize colored effluent of synthetic dyes.

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the data. AH led in writing, reviewed and edited the manuscript. All authors reviewed and approval the final manuscript.