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Decolorization of Remazol Brilliant Blue R by new isolated white rot fungus collected from tropical rain forest in East Kalimantan and its ligninolytic enzymes activity

Tito Sumandono[a], Henderson Saragih[a], Migirin[a], Takashi Watanabe[b], Rudianto Amirta[a]*

[a]*Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia*
[b]*Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan*

Abstract

In this research, decolorization of Remazol Brilliant Blue R (RBBR) was investigated using a new isolated white rot fungus, strain KRUS-G, collected from Mulwarman University Botanical Garden, Samarinda, East Kalimantan, Indonesia. The results showed that strain KRUS-G decolorized RBBR efectively compared to *Phanerochaete crysosporium* and *Ceriporiopsis subvermispora*. The highest decolorization was obtained at pH 4 with 89% loss of RBBR used. The effective decolorization was also observed in the high concentration of RBBR (1500 ppm).Concentration of RBBR at 500, 1000, and 1500 ppm caused slightly decreasing pattern on mycelia growth. Rapid decolorization was occurred within 4 days of incubation. We suggest that laccase played an important role on the decolorization even high activity of MnP was also detected.

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*Keywords:*white rot fungi; decolorization; MnP; Laccase; RBBR

* Corresponding author.
E-mail address: r_amirta@yahoo.com

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1. Introduction

A lot of synthetic dyes are used for many purposes such as textile, pulp and paper, food, and pharmacy. There are more than 100.000 dyes produced in the world. Approximately, 10-15 % of synthetic dyes are directly released to environment as effluent of industries, and 50 % are not confirmed exactly. Color removal of these dyes by chemical, physical, and electrochemical method demonstrated some good results, but these methods require high cost. Recently, many researchers utilize microorganisms like fungi which have ability to decolorize and degrade dyes[1].

White rot fungi are well known for their ability to degrade lignin of wood. This competence relates to their extracellular oxidative enzymes consisting of Manganese Peroxidase (MnP), Laccase, and Lignin Peroxidase (LiP). On the other hand, these enzymes are also involved in colour removal of synthetic dyes[2,3].

Utilizing some species of white rot fungi such as *Tram*etes sp, *Pleurotus ostrearus*, *Coriolus versicolor*, *Funaliatrogii*, *Dicomitus squalens*, *Ischnoderma resinosum*, *Pleurotus calyptratus*, *Bjerkandera adusta*, and also *Phanerochaete crysosporium* to degrade some synthetic dyes and textile-industries effluents have been reported[2,4, 9]. However, only a few reports have discussed utilization of white rot fungi which grow in tropical rain forest of East Kalimantan, Indonesia, related to their ability to decolorize synthetic dyes[10]. In this study, we investigated the capability of our new isolated white rot fungus namely KRUS-G from our tropical rain forest to decolorize Remazol Brilliant Blue R (RBBR) which is one of the most important dye used in textile industries and also toxic and recalcitrant [5].Ligninolytic enzymes and toxic effect of RBBR for mycelia growth were also discussed.

2. Materials and methods

2.1. Organisms

Phanerochaete chrysosporium ATCC 34541 and *Ceriporiopsis subvermispora* ATCC 90467 were obtained from Biomass Conversion Laboratory of Research Institute for Sustainable Humanosphere, Kyoto University, Japan. New isolated white rot fungus (KRUS-G) was selected among screened fungi growing on decayed woods in Mulawarman University Botanical Garden, Samarinda, East Kalimantan, Indonesia. All of the fungi were maintained on agar slant and petry dish containing potato dextrose agar (PDA) and refreshed periodically.

2.2. Reagent

Remazol Brillian Blue R (RBBR), Guaiacol, Potato Dextrose Agar (PDA), Polypepton, Glucose, Malt Extract, and Yeast werepurchased from Wako Chemical Co. Ltd, Japan. All of chemicals used in this research were of analytical grade.

2.3. Screening of new isolated white rot fungus

All of 24 fungi were inoculated into petry dishes containing 20 mL PDA and 500 ppm guaiacol each petry dish. This method was modified from Afrida *et al* (2009)[11]. Visual measurement was carried out to color change on PDA. The most effective fungus caused the highest color change from white to brown PDA. All of 24 fungi were also inoculated into petry dishes containing 20 mL PDA and 500 ppm RBBR each petry dish. Visual measurement of RBBR decolorization has been done to determine selected fungus.

2.4. Culture Media

Fungi effect on decoloration. Three mycelia plugs of KRUS-G were inoculated into 100 mL autoclaved erlenmeyer flask containing 20 mL liquid nutrient medium with the following compositions: 20 mg/L malt extract, 20 mg/L glucose, 1 mg/L polypeptone, 100 ppm RBBR, and distilled water[10]. Both *P. Crysosporium* and *C. Subvermispora* were also studied in the samewayof KRUS-G to evaluate their capability on decoloration of RBBR. In addition, erlenmeyer flasks containing nutrient, RBBR but no fungi werealso prepared as a control. Then, the flasks were incubated in shaking incubator for 6 days at 28^{o}C, 120 rpm. All of the treatments were carried out in triplicate at pH 4, 5 and 6.

RBBR concentration effect on decolorization. Three mycelia plugs of KRUS-G were inoculated into 100 mL autoclaved flask containing 20 mL GYP medium with comprised of 20 mg/L glucose, 5 g/L yeast extract, 5 g/L peptone, 1 g/L $MgSO_4.7H_2O$, distilled water[2], and RBBR. Sodium tartarate was used to adjust the medium at pH 4. The flasks were incubated in static incubator for 6 days and shaken periodically. All of the treatments were carried

out in triplicate at temperature of 28°C. Different concentrations of RBBR were 100, 500, 1000, 1500 ppm.

2.5. Toxic effect of RBBR on fungal mycelia growth

Toxic effect of RBBR was determined by the weighing of dry mycelia of the new isolated fungus. Dry mycelia weight (DMW) was obtained by filtering the contents of flask. Then a series of sample was also prepared in the absence of RBBR and used as the control. DMW from both conditions werecompared to evaluate the effect of RBBR on the growth of fungus.

2.6. Decolorization Measurement

All of free-mycelia liquid-media were centrifuged at 2000 g for 10 minutes (Hitachi CF15 RX II) to get supernatant. Decolorization was determined by measuring absorbance at 595 nm wavelength using UV-microplate reader spectrophotometer (Tecan Infinite M200, Wako).

2.7. Lininolytic enzymes assay

MnP activity was assayed using a reaction mixture containing 0.05 mL culture supernatant, 0.2 mM di-metoxy phenol (DMP), 25 mM Na-tartarate buffer (pH 4), 0.5 mM MnSO4, 0.1 H2O2, and 0.45 mL distilled water in total volume of 1 mL. **Laccase activity** was measured using a reaction mixture containing 0.05 mL culture supernatant, 0.2 mM DMP, 25 mM Na-tartarate buffer (pH 4), and 0.65 mL distilled water in total volume of 1 mL.

The reaction was monitored at 495 nm wavelength. One unit of enzyme activity was defined as amount of enzyme that increased the absorbance at 495 nm by 1.0 per minute. The methods were modified from Kamitsuji *et al.*[12]

3. Results and discussion

3.1. Screening of new white rot fungus for decolorization of RBBR

We observed activities of 24 fungi spesies collected from Mulawarman University Botanical Garden on agar-plate containing guaiacol. Fifteen species did not give brown color agar-plate but nine species showed positive effect (Table 1). This fact indicates that the nine ones have capability to oxidize guaiacol reflecting laccase activity[11]. It also means that the fungi are laccase positive strain.

Furthermore, among 9 fungi oxidizing guaiacol, none of them are able to decolorize RBBR but KRUS-G. This fungus was the only one fungus decolorizing PDA consisting of RBBR (Table 1). Decolorization has been observed since day 1 of inoculation till day 5 and whole RBBR on agar-plate was decolorized to dark-brown color by the fungus at day 5 (Fig 1). This result showed that KRUS-G was the most effective fungus among all collected fungi.

Table 1.Oxidation of guaiacol and decolorization of RBBR on agar plate by several fungi collected from Mulawarman University Botanical Garden

Fungi code	Oxidation of guaiacol (Bavendamm method)	Decolorization of RBBR	Fungi code	Oxidation of guaiacol (Bavendamm method)	Decolorization of RBBR
KRUS- A	✓	-	KRUS- M	-	-
KRUS- B	-	-	KRUS- N	-	-
KRUS- C	-	-	KRUS- O	✓	-
KRUS- D	-	-	KRUS- P	-	-
KRUS- E	-	-	KRUS- Q	✓	-
KRUS- F	✓	-	KRUS- R	-	-
KRUS- G	✓	✓	KRUS- S	-	-
KRUS- H	-	-	KRUS- T	✓	-
KRUS- I	-	-	KRUS- U	✓	-
KRUS- J	-	-	KRUS- V	-	-
KRUS- K	-	-	KRUS- W	✓	-
KRUS- L	-	-	KRUS- X	✓	-

Note : oxidation of guaiacol and decolorization RBBR were measured on day 5; concentration guaiacol and RBBR were 500 ppm

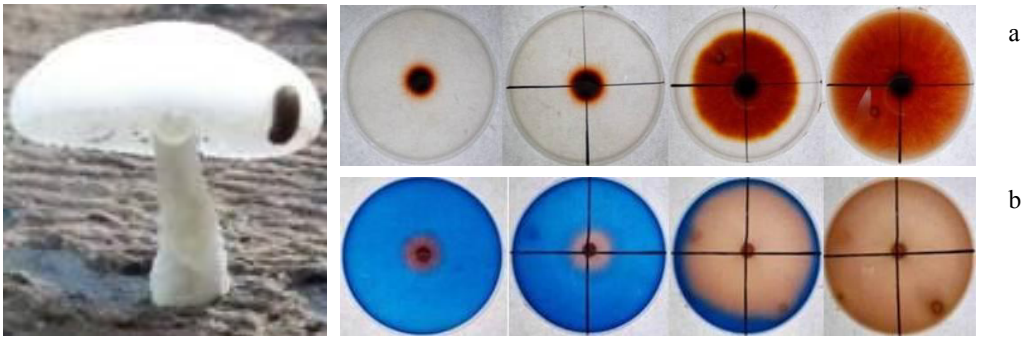

Fig 1. (a) Oxidation of guaiacol at concentration 500 ppm (b) Rapid decolorization of RBBR at concentration 500 ppm on PDA medium

3.2. Effective decolorization of RBBR by strain KRUS G

The experiment was carried out in liquid media containing 100 ppm RBBR. All of liquid media were incubated in shaking incubator for 6 days. Strain KRUS-G was compared to *P. crysosporium* and *C. subvermispora.* All of the fungi were cultured in liquid media consisting of RBBR at pH 4,5 and 6.

The decolorization pattern of KRUS-G was similar to both*C. subvermispora,* and *P.crysosporium.* All of three fungi increased decolorization of RBBR for 6 days at different pH(Fig 2). However, at the end of 6 days incubation period, KRUS-G showed better activity than *C. subvermispora* and *P.crysosporium*. More than 79 percent of RBBR was decolorized by KRUS-G while *C. subvermispora* and *P. crysosporium* decolorized 63-68 and 21-23 percent, respectively. Furthermore, this fungus gave the best performance at pH 4 among all of tested pH (Fig 2).

On the other hand, it was surprising that decolorization of RBBR by *P. crysosporium* was very low and limited although high decolorization capability of RBBR by *P. Crysosporium*was reported previously. Eiclerová *et al.* reported that whole of RBBR of 0.2 g L^{-1} on solid medium was decolorized by *P. crysosporium*[8].The fungus also reduced more than 90 % of azo dyes of 0.15 g L^{-1} in liquid medium[13]. However, our finding was not in agreement with these results. We obtained that decolorization rate of RBBR by *P. crysosporium* was very low even lower than both KRUS-G and *C.subvermispora.* Furthermore, related to dye decolorization by *C. subvermispora,* we found no paper discussing about it but one[14]. Accordingly, based on our knowledge, this is the second that reported dye-decolorization ability of *C. subvermispora.*

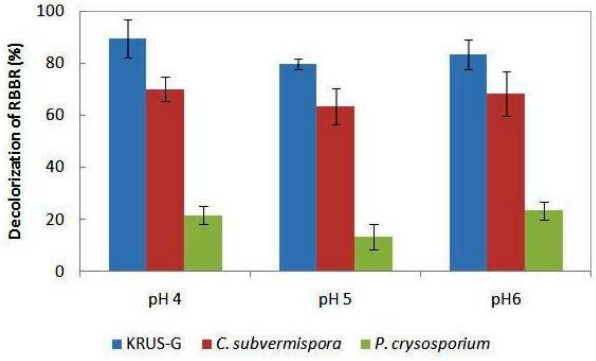

Fig. 2.Decolorization capacity of RBBR by KRUS-G, *C. subvermispora* and *P. crysosporium* in liquid media containing 100 ppm RBBR at 28ºC; Day 6

Effective decolorization of high concentration of RBBR by KRUS G was showed in Fig 3. KRUS-G successfully decolorized RBBR at various concentration in liquid media. After 6 days, 84% and 93.32%, of 100 and 500 ppm of

initial RBBR concentration were decolorized respectively. Decolorization was also occurred even at 1000 and 1500 ppm of initial RBBR (Fig 3). It was surprising that strain KRUS G could decolorize RBBR at high concentration while other fungi only at low concentration[4,5,7]. As shown at Fig 3, trends of decolorization at various concentration were similar. Rapid decolorizations occurred from day 1 until day 4, but decolorization rate became slow after day 4.

Laccase and MnP were produced by strain KRUS-G. Although MnP was detected in this experiment, we suggested that laccase played an important role on decolorization of RBBR by KRUS G. First, as evidence, we found at from Bavendamm test that this fungus positively produced laccase indicated by oxidation of guaiacol to brown-color appearing on solid media (Fig 1a). Second, in this research, decolorization of RBBR occurred in liquid media with the absence of Mn^{2+} whereas theoretically[15] the ion is essential for MnP cycle. Thus, it indicated that MnP was not involved in the decolorization mechanism.

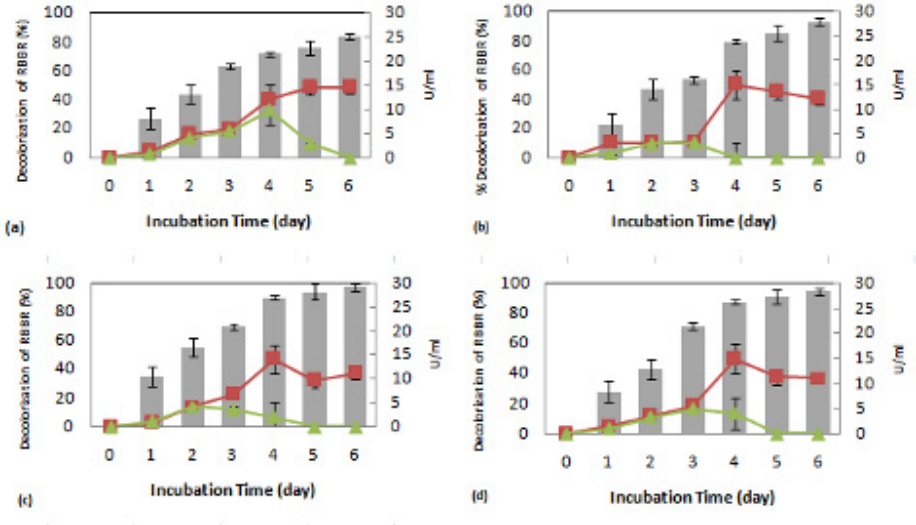

Fig 3. Decolorization of RBBR by strain KRUS G (a) 100 ppm; (b) 500 ppm; (c) 1000 ppm; (d) 1500 ppm. Symbols: MnP activity (■) laccase activity (▲)

Fig 4. Decolorization of RBBR (100 ppm) by KRUS G at pH 4; 28ºC

3.3. Fungal mycelia growth

Toxic effect of RBBR was determined by comparing DMW of liquid media containing RBBR to DMW of liquid media containing no RBBR (blank). As shown on Fig 5, DMW of blank or control sample achieved 0.267 g/20 mL while DMW of liquid media was not less than 220 g /20 mL. We found that the fungus could grow in liquid media containing 100 ppm RBBR as well as in liquid media containing no RBBR (control). The slight decreasing pattern of DMW onliquid media was appeared ranging from 500 to 1500 ppm. It means that concentration of RBBR at 500 ppm or more caused toxic effect to mycelia growth. This result is in line with Eiclerová *etal*[8]who explained that increasing concentration of dyes caused slight decreasing *Bjerkandera adusta* growth.

Related to dye toxicity to fungal growth, many authors have reported in several literatures. Erkurt *et al*[4] reported

that concentration of RBBR up to 100 ppm was not toxic to both *Coriolusversi color* and *Funaliatrogii*. However, at the same literature, unsimilar result occurred in *Pleurotus ostreatus*. Enhancement of RBBR caused reduction of DMW[4]. Associated with *P. ostreatus,* the fungus could only grow well in low concentration of RBBR[5].

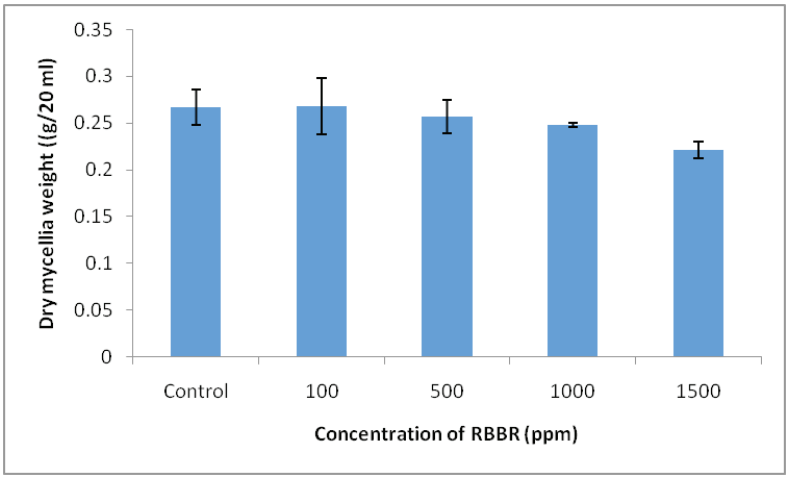

Fig5. Dry mycelia weight of KRUS G

4. Conclusion

Finally, we found that the new isolated white rot fungus strain KRUS G could effectively decolorize RBBR compared to *C. Subvermispora* ATCC 90467 and *P. Crysosporium* ATCC 34541 even at the high concentration of RBBR. Concentration of RBBR at 500, 1000, and 1500 ppm caused slight decreasing pattern on mycelia growth. We suggest that laccase played an important role on the decolorization even high activity of MnP was also detected.

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