Removal of Textile Dye, RBBR, via Decolorization by *Trametes hirsuta* AA-017

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Abstract. The use of synthetic dyes has an impact on the possibility of disposing such dyes into the environment. Fungal decolorization is one promising approach due to its capability to degrade dyes, thus, exploring fungi that can be applied in dye decolorization is essential. We investigated our potential strain of *Trametes hirsuta* to decolorize Remazol brilliant blue R (RBBR). The enzyme activity of laccase in various conditions was observed using Syringaldazine as a substrate, while fungal immobilization was conducted using calcium alginate as a solid support. The results indicated that $CuSO_4$ was the best inducer for the decolorization process. The fungus was able to perform 79.5% of RBBR decolorization for 48 hours in the presence of $CuSO_4$. Laccase was the prominent detected ligninolytic enzyme when decolorization was performed. The immobilized cells were able to decolorize 85% RBBR under 0.8 mM $CuSO_4$ and used for 3 cycles of decolorization. This study reveals the potential of fungal usage in the form of the immobilized and free cell to overcome the persistence of dye pollutants problem, as it is considered an effective, economic and eco-friendly approach for RBBR dye decolorization. These strategies can be suggested to encourage ecologically sustainable development for bioremediation.

Key words: *Trametes hirsuta*, decolorization, RBBR dye, laccase, immobilization

INTRODUCTION

Since its discovery in 1856, synthetic dyes have gained a more pivotal role in the recent era (Ziarani et al., 2018). They are organic substances having the ability to absorb visible light. Nowadays, more than 100,000 different dye structures have been synthesized (Tehrani-bagha & Holmberg, 2013). In correlation to its role in various industries, the consequences due to the disposal of the dyes to the environment are inevitable. Synthetic dyes, such as those used in the textile industry, will generate disastrous effects when released to the environment as effluents. The textile dyes alter the aesthetic quality of water, increase biochemical and chemical oxygen demand, impair photosynthesis, inhibit plant growth, and may promote toxicity, mutagenicity, and carcinogenicity (Lellis et al., 2019). Remazol brilliant blue R (RBBR) is one of the reactive textile dyes that is used extensively for dyeing in textile and other industrial applications. This dye belongs to the class of anthraquinone. Anthraquinone dyes, including RBBR, are often used as a starting material to produce polymeric dyes. Most of these compounds are highly toxic, carcinogenic, and very resistant to degradation (Eichlerova & Baldrian, 2020). For those reasons, many studies are conducted to explore and propose an approach for dye decolorization.

Approaches to decolorize dyes include the use of adsorbent (Ahmad et al., 2014; Pelosi et al., 2014), by employing microbial decolorization (Khan et al., 2013), enzyme decolorization (Šekuljica et al., 2015), through coagulation, via ozonation, photocatalysis (Gupta et al., 2015), etc. Physicochemical methods, as well as advanced approaches, are not feasible since they are often ineffective, time-consuming, expensive, and methodologically demanding (Eichlerova & Baldrian, 2020). In addition, there is a chance for the release of secondary pollutants from applying the physical-chemical method (Zin et al., 2020). For that reason, other alternatives are highly demanded.

Microbial decolorization, especially by fungi, has gained much interest since it is considered cost-effective and environmentally friendly. The decolorization by fungi may involve biosorption and biodegradation due to the secretion of extracellular enzymes (Sen et al., 2016). Fungi from various genera have been studied for their decolorization ability (Fu & Viraraghavan, 2001; Sen et al., 2016). Exploring fungi with the ability to decolorize dyes is required to provide promising alternatives amidst the extensive studies of physical-chemical methods.

Indonesia with its huge biodiversity is thus a great resource of potential fungus for dye decolorization.

White-rot fungus *Trametes hirsuta*, due to its ability to produce extracellular ligninolytic enzymes, is one of the fungi studied for dye decolorization. Several studies employing this fungus or its ligninolytic enzymes showed that this strain was a potential candidate (Anita et al., 2019; Dominguez at al., 2005). Previously, we also reported that local strain white-rot fungus, *Trametes hirsuta* AA-017, has the ability to decolorize Congo red, brilliant blue, and Coomassie brilliant blue (Andriani et al., 2019). These dyes are representatives of the diazo and triphenylmethane classes. Intensive research in our lab regarding the ligninolytic enzymes produced by this strain reveals its ability in degrading melanin. However, to be applied on an industrial scale, the decolorization process requires to be conducted in a continuous system. Immobilization enables the said purpose as well as improves the enzyme stability (Cipolatti et al., 2014).

In this study, we aimed to investigate the ability of *Trametes hirsuta* AA-017, a local strain isolated from Cibinong Botanical Garden Indonesia, to decolorize anthraquinone dye, Remazol brilliant blue R (RBBR), as well as optimizing the decolorization process using inducers. We also determined the ligninolytic activities of *Trametes hirsuta* AA-017 along the decolorization process to reveal which ligninolytic enzymes might have a major contribution in the decolorization process. We prepared an immobilized form of *Trametes hirsuta* AA-017 using calcium alginate, a cross-linking of calcium ion (Ca^{2+}) with natural polymer alginate. Alginate was chosen due to it allows for a mild immobilization process (Leenen et al., 1996), its biodegradability, hydrophilicity, and it offers mechanical stability (Arica et al., 2001). The immobilization process will enable the continuous process and will be useful for industrial application. To find the optimum condition for decolorization, we also varied the concentration of inducers and RBBR. The recent study presented here will highlight the potential of white rot fungus from our collection for dye decolorization as well as providing an alternative approach that is sustainable and environmentally benign.

METHODS

White-rot fungus *Trametes hirsuta* AA-017 (Figure 1) was selected as the most promising fungi to degrade RBBR dye among the screened fungi growing on decayed wood in germplasm garden (CSC) (Andriani et al., 2019). Malt extract agar (MEA) on the petri dish was used to maintain the fungus.

Figure 1. *Trametes hirsuta* AA-017 isolated from Cibinong Botanical Garden, Indonesia

Reagents

Remazol Brilliant Blue R (RBBR) and Syringal Dazin were purchased from Sigma-Aldrich. Malt Extract, Peptone, and Agar were purchased from BD DifcoTM. Sodium Azide, $MgSO_4.7H_2O$, $CuSO_4$, $MnSO_4.H_2O$ and H_2O_2 were purchased from Merck. Polypepton and Yeast Extract were purchased from Himedia. Veratryl Alcohol (VA) and 2,6-dimethoxyphenol (DMP) were purchased from Tokyo Chemical Industry (TCI). All chemicals used in this research were analytical grade.

Effect of various inducers on decolorization

Trametes hirsuta AA-017 was cultured for 7 days at 25°C, 150 rpm in 100 mL autoclaved flask containing 20 mL Glucose Yeast Peptone (GYP) medium with the following composition: 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, and 1 g/L $MgSO_4.7H_2O$. At incubation day 6, various inducers were added. The concentration of inducers added was 0.4 mM. The inducers used were $CuSO_4$, $MnSO_4$, and VA. After 7 days of incubation, 50 ppm RBBR was added as a synthetic pollutant. The decolorization rate was measured after the addition of RBBR at 0, 2, 4, 6, 24, and 48 h.

Ligninolytic enzymes assay

Enzyme activity assay for laccase and manganese peroxidase (MnP) were investigated to determine ligninolytic activity during degradation. Laccase activity and MnP activity was performed in accordance with Zavarzina and Zavarzin (2006) and Mariko et al. (2004), respectively, with modification as reported in the previous report (Andriani et al., 2016). Laccase activity assay was carried out in a total volume of 1750 μL with 0.5 mM syringaldazine in 0.1 M sodium acetate buffer containing 900 μL culture supernatant and the absorbance was measured at 525 nm. MnP activity was determined using a reaction mixture containing 1000 μL culture supernatant, 20 mM DMP, 20mM $MnSO_4$, 2 mM H_2O_2, and 50mM malonate buffer (pH 4.5) in a total volume of 3300 μL. The MnP activity was then measured at 470 nm.

Decolorization of RBBR by immobilized *Trametes hirsuta* AA-017 using submerged-immobilized beads (SIB)

Trametes hirsuta AA-017 was cultured in GYP medium for 7 days and induced by the addition of CuSO₄ at its optimum concentration at day 6. The fungus culture was immobilized in alginate beads. The fungus was immobilized by mixing it with sodium alginate (1.5% w/v) and stirring for approximately 2 h (Yanto et al., 2014). Into 1 mM CaCl₂ solution, the fungus culture-alginate solution was added drop by drop by pipette. The immobilized fungus appeared in the granules form at the surface of solution. A final concentration of 50 ppm RBBR was supplemented into flasks. To investigate the effect of mass-beads on the RBBR degradation ability, the addition of mass beads was varied from 3, 6, 9, and 12 g. Sampling was conducted at 0, 120, and 192 h. In addition, the decolorization on fungus beads was investigated by sampling at 0, 2, 4, 6, 24, and 48 h.

Effect of RBBR concentration on decolorization

Trametes hirsuta AA-017 was cultured with the aforementioned protocol and induced with 0.4 mM CuSO₄ after 6 days incubation. At incubation day 7, RBBR was added with a concentration in the 50-200 ppm range and the rate of decolorization was investigated at 0, 2, 4, 6, 24, 48, 72, 96, 120, 144, and 192 h.

Effect of inducer concentration on decolorization

Trametes hirsuta AA-017 was cultured with the aforementioned protocol and induced with varied concentration of CuSO4 ranging from 0.4-1 mM after 6 days incubation. At incubation day 7, RBBR was added with a concentration of 50 ppm and the rate of decolorization was investigated at 0, 2, 4, 6, 24, 48, 72, 96, 120, 144, and 192 h.

Determination of Decolorization

To get the supernatant, mycelia were removed by centrifugation at 4°C, 10000 rpm for 20 minutes (Tomy MX-307). Decolorization was determined by measuring absorbance at 592 nm wavelength using a UV-Vis spectrophotometer (UVmini-1240, Shimadzu).

RESULTS AND DISCUSSION

Decolorization activity under various inducers and ligninolytic enzyme activities

We observed the capability of *T. hirsuta* culture in producing laccase and decolorizing RBBR dye after being supplemented by various inducers. Aromatic compounds such as veratryl alcohol (VA) and metal ions including CuSO₄ and MnSO₄ were used as inducers and applied to fungal cultures. The result showed that CuSO₄ was the most effective and potential inducer to raise the decolorization of RBBR dye, followed by VA, and MnSO₄. As depicted in Figure 2, we found that the decolorization rate was increasing during incubation time and the highest level was attained at 48 h of incubation, with the decolorization of 79.5%, 69.7%, and 59.8%, by the respective inducers. In addition to the decolorization of RBBR, we determined the ligninolytic enzyme activities that might be involved in the decolorization process. We measured the activities of laccase and manganese peroxidase (MnP) for 48 h as can be observed in Table 1 and 2. We also tested the activity of lignin peroxidase. However, the value was the lowest and neglected (data not shown).

According to Table 1 and 2, along the 48 h of decolorization process, under the presence of various inducers, the activity of ligninolytic enzymes tested in this study, laccase and MnP, followed the same trend as the decolorization process. Based on the data, CuSO₄ seemed to be the most suitable inducer for both laccase and MnP activities. Laccase activity was dominant compared to MnP activity. The activity of laccase increased until it peaked at 48 h (178 U/L) of incubation. On the contrary, the level of MnP activity in the fungal culture remained low during the incubation. Based on the data presented, CuSO₄ could be a promising inducer to stimulate laccase instead of MnP in the *T. hirsuta* as well as indicating its potential to be applied in the decolorization process.

The preference of CuSO₄ over the remaining inducers might be understood since laccase was detected as the major contributing ligninolytic enzymes in this study. There have been some reports regarding the effect of copper on laccase production. Copper produces Cu^{2+} ions that are required in the laccase active site (Solé et al., 2012) and needed to enhance the level of laccase gene expression (Yang et al., 2016; Yang et al., 2013), whereas it can reduce the proteolytic activity (Piscitelli et al., 2011). The addition of Cu^{2+} results in better development of fungal mycelium (Shutova et al., 2008). Moreover, the synthesis of oxidase at the level of gene expression is also regulated by copper ions which cause the enhancement of laccase isoenzymes in copper-supplemented cultures (Palmieri et al., 2000). Therefore, the result obtained in this work corresponds with the finding of previous studies that the supplementation of Cu^{2+} ions contributed to improve the laccase activity.

Table 1. Profile of Laccase Activity during RBBR Decolorization under Various Inducers

Treatment	Enzyme activity (U/L)					
	0 h	2 h	4 h	6 h	24 h	48 h
CuSO$_4$	14.45±0.54	24.91±0.84	68.03±0.13	78.28±0.13	135.57±1.59	178.04±0.35
MnSO$_4$	n.d	n.d	n.d	n.d	n.d	n.d
VA	0.80±0.08	2.44±0.01	3.92±0.11	6.31±0.10	8.19±0.54	8.95±0.37
No Inducer	n.d	n.d	n.d	n.d	n.d	n.d

n.d = not detected; the data were obtained from duplicate experiments

Table 2. Profile of MnP Activity during Decolorization of RBBR under Various Inducers

Treatment	Enzyme activity (U/L)					
	0 h	2 h	4 h	6 h	24 h	48 h
CuSO$_4$	4.70±0.69	5.90±0.74	6.85±0.89	14.77±0.21	16.86±0.33	26.01±0.91
MnSO$_4$	0.66±0.04	1.08±0.01	2.08±0.17	3.27±0.20	4.61±0.01	6.12±0.38
VA	n.d	n.d	n.d	n.d	n.d	n.d
No Inducer	n.d	n.d	1.61±0.36	2.03±0.62	2.53±0.57	2.81±0.97

n.d = not detected; the data were obtained from duplicate experiments
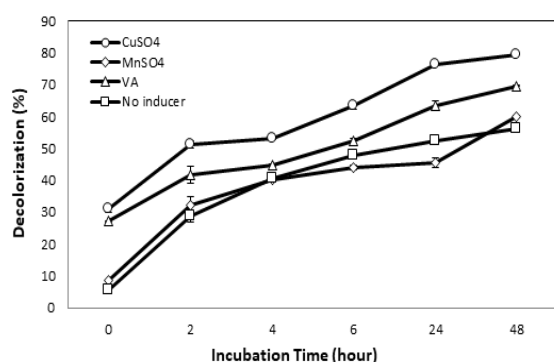

Figure 2. Decolorization rate of RBBR by *T. hirsuta* AA-017 culture under various inducers. The data was obtained from a duplicate experiment.

In addition to copper, aromatic compounds may also be applied as an inducer. Our earlier study has reported the application of aromatic compounds to increase the redox potential of some enzymes that impact dye decolorization such as laccase and manganese peroxidase (MnP) (Nuryana et al., 2019). Veratryl alcohol was one of the most effective inducers and thereby it was applied in this study. During the degradation process, induction by aromatic compounds was necessary to denote a protective response of fungi in the presence of potentially toxic compounds (De Souza et al., 2004; Elisashvili et al., 2010). With the occurrence of fungal protection, the rise of the decolorization rate could be obtained. Despite that, the decolorization level of MnSO$_4$ indicated no apparent difference in comparison to the control fungal culture with no inducer. It means that the supplementation of MnSO$_4$ in this study had no synergic effect on the activity of dye decolorization.

Immobilization of fungal cells and enzymes

Immobilization of biocatalyst beads has gained increasing attention because of its ability to develop efficient, stable, and recoverable systems for decolorization of textile wastewater (Andriani & Yanto, 2021; Bilal & Iqbal, 2019; Barapatre et al., 2017; Hayat et al., 2015). For the immobilization, Ca-alginate was used as the solid support for biomass. The immobilized white rot fungus achieved the highest decolorization at 192 h (77%) (Figure 5). Similar studies on RBBR decolorization using immobilized enzymes or immobilized fungal cells obtained 58-95% of decolorization (Table 3). Compared to previous studies on RBBR decolorization, the decolorization obtained in this study was relatively moderate and available for further improvements. Considering previous study which used *Trametes hirsuta* by Alam et al. (2021), the percentage of decolorization obtained by the study was higher than that achieved in this study (Table 3). However, when comparing the laccase activity, the value reported by earlier study (176 U L^{-1}) (Alam et al., 2021) was almost the same value as the one obtained in this study (178 U L^{-1}) (Table 1). Despite the similar laccase activity, the discrepancy on the RBBR decolorization is likely due to the difference on the matrix employed. In previous study by Alam et al. (2021), they applied solid support LECA (Light Expanded Clay Aggregate), which is mostly composed of silicate. The high porosity of the material might help in the biosorption mechanism, and thus improving the decolorization of dyes.

Compared to the *T. hirsuta* in free biomass, the decolorization of immobilized *T. hirsute* AA-017 was

lower. The highest decolorization by free *T. hirsuta* (79.5%) (Figure 2) was achieved at 48 h. At the same time point, the decolorization achieved by the immobilized cells was 2 times lower (42.8%) than that of free cells (Figure 3). The diffusional resistance may exist which lowers the reaction rate (Ahmedi et al., 2015). In addition, in a cell entrapment system, there is a possibility of mass transfer resistance due to the polymeric matrix (Arica et al., 2001). Despite the slower process, immobilization enables the continuation of a process, a significant quality

required for industrial application. For that reason, we reused the immobilized biomass for the decolorization process. The immobilized biomass was washed with deionized water prior to its use in the subsequent round of decolorization (Figure 4). According to Figure 4, the immobilized *T. hirsuta* was able to be used for 3 cycles. At the third cycle, the decolorization was decreased 1.6 times to be 46% compared to 77% at the first cycle. Even with a slower process, immobilization does enable the continuation of the decolorization process.

Table 3. Comparison of RBBR Decolorization Using Immobilized Ligninolytic Enzymes or Immobilized Whole-cell

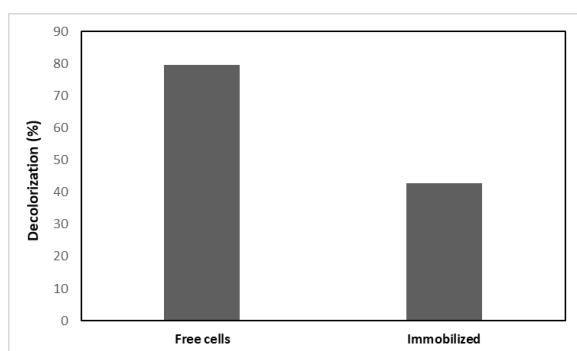| Origin	Enzyme	Method of immobilization	Immobilization matrix	Decolorization (%)	References
Trametes hirsuta AA-017	Laccase and MnP	Entrapment	Calcium alginate	77	This study
Trametes hirsuta D7	Laccase	Entrapment	Light Expanded Clay Aggregate (LECA)	95	Alam et al., 2021
Trametes versicolor	Laccase	Adsorption	PMMA/PANI electrospun fibers	87	Jankowska et al., 2020
Trametes versicolor	Laccase	Covalent binding	PMMA/PANI electrospun fibers	58	Jankowska et al., 2020
Trametes versicolor	Laccase	Entrapment	PILM	75	Hajkacem et al., 2020
Pleurotus ostreatus	Laccase, MnP, Lignin Peroxidase (LiP)	Adsorption	Calcium alginate	76	Dewi et al., 2019

Figure 3. Decolorization of RBBR (50 ppm) at 48 hours, under the influence of $CuSO_4$ as inducer in free and immobilized cells.
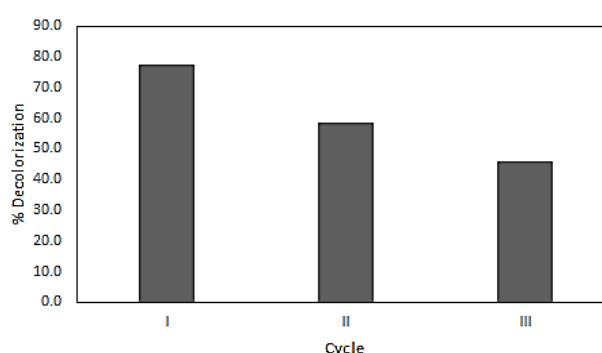

Figure 4. Decolorization process of immobilized *T. hirsuta* for 3 cycles of decolorization. Each cycle was conducted for 192 h, using 50 ppm of RBBR and 0.4 mM $CuSO_4$.

In the next step, we investigated the optimum concentration of RBBR and CuSO₄ as an inducer for the decolorization process. We varied the concentration of RBBR in the range of 50-200 ppm. The concentration of RBBR dye that could be degraded at the maximum level by *Trametes hirsuta* was 50 ppm (Figure 5). The decolorization was gradually decreased as the RBBR concentration increased. At the concentration of 200 ppm RBBR, the decolorization was achieved at 46%. Moreover, we also investigated the optimum concentration of CuSO₄ to be applied as an inducer (Figure 6). Copper is essential and functional at low concentrations. At high concentrations, it can be toxic and harmful for fungi (Baldrian & Gabriel, 2002; Susana et al., 2018). Furthermore, copper has been observed to inhibit fungal growth and laccase activity in the presence of the high concentration of copper (Revankar & Lele, 2006). Therefore, we investigated the effect of CuSO₄ at low concentration. According to the Figure 6, CuSO₄ at any concentration range (0.4-1 mM) could promote the decolorization of RBBR for 60% or more at 192 h. The highest level of decolorization, however, was achieved by employing CuSO₄ at the concentration of 0.8 mM, in which the decolorization achieved 85%, while the lowest RBBR decolorization (68.7%) was achieved when 1 mM CuSO₄ was applied. An earlier study has declared that the optimal concentration to produce the high laccase activity was 1.5-2.0 mM CuSO₄ (Palmieri et al., 2000). Meanwhile, another study has reported the activity of laccase increased at the concentration range of 0.6–1.2 mM CuSO₄, whereas at the higher concentration range of 1.5-1.8 mM, the activity decreased (Saparrat, 2004). Copper was added after 6 days of inoculation. The fungal cultures took time to grow and adapt in different conditions. After being added with copper, the decolorization rate was gradually increased due to the enhancement of laccase production in the cultures.
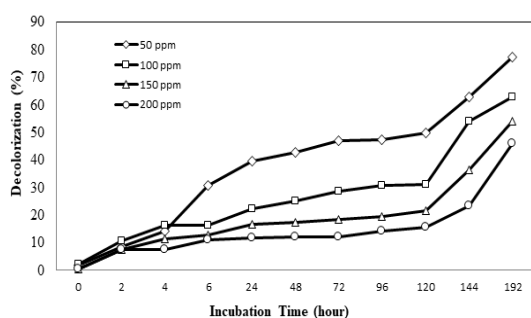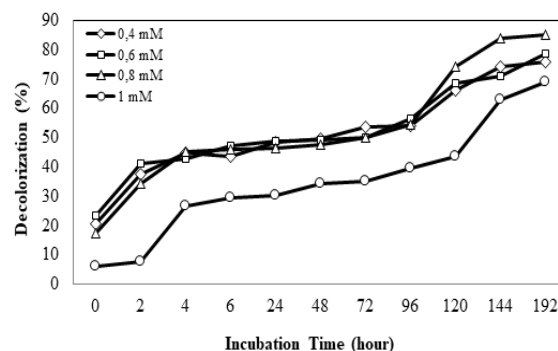

Figure 6. Decolorization of RBBR by *T. hirsuta* AA-017 culture under various copper concentrations.

To further optimize the degradation in its immobilized form, biomass of *T. hirsuta* was entrapped in various Ca-alginate beads-mass to investigate the influence of prepared bead mass on RBBR dye elimination (Figure 7a). It was noticed that RBBR dye in the experimental solution was diminished with a further increase in contact time. The dye removal increased with an increase in the incubation time due to higher diffusion of the dye inside the beads (Ramírez -Montoya et al., 2015). When 3 g of Ca-alginate bead mass was used, RBBR dye removal was started within 120 h and the dye was completely removed after 192 hr. Furthermore, the removal of RBBR dye was faster with an increasing amount of Ca-alginate beads. Our investigation showed that 6 g of beads was sufficient to completely degrade 50 ppm RBBR dye at 120 h, faster than when 3 g of beads-mass was used. It also indicates that increasing the amount of Ca-alginate beads to more than 6 g is not needed. It is likely due to more unoccupied pore on the surface of beads facilitated an enhancement on the adsorption dye with a higher dosage of beads-mass (Das et al., 2020). In addition, the decolorization of RBBR in fungal beads was investigated. The change of beads color from blue to pale brown was detected with an increase in contact time suggesting that the color of RBBR dye was degraded by fungal entrapped in beads and thereby changing the color of beads back to its original color after 48 h (pale brown) (Figure 7b).

This is the first report of this strain showing its ability to be applied in RBBR dye, commonly used in textile industry, either in a free or immobilized form. Using an immobilized *Trametes hirsuta* AA-017, we succeeded to establish a continuous decolorization process. Further optimization was achieved by varying inducer and RBBR concentration. The decolorization of RBBR was related to ligninolytic activity as laccase activity was detected during the process. The present study highlights the potential of local strain to be applied in dye wastewater treatment, allowing for a sustainable and environmentally benign process.
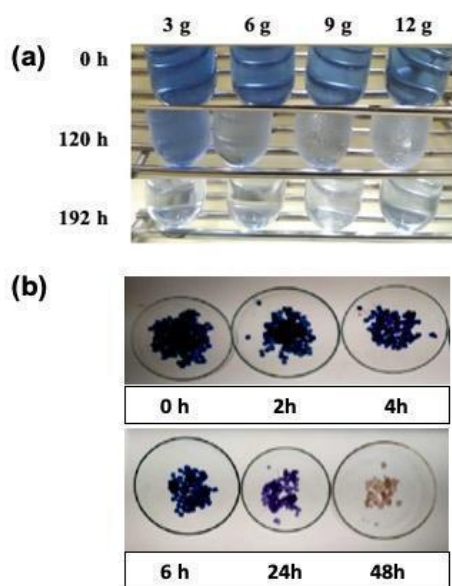

Figure 5. Decolorization under various concentrations of RBBR (50- 200 ppm).

Figure 7. Ca-alginate beads: (a) decolorization of RBBR in various Ca-alginate beads-mass; (b) changes of fungal beads in various incubation times.

CONCLUSION

This study shows that local strain, *Trametes hirsuta* AA-017, is a potential white rot fungus for RBBR decolorization. According to the data, the use of $CuSO_4$ as inducer provides the highest decolorization percentage among all inducers tested. During the decolorization process, laccase was the main ligninolytic enzyme involved in the process. In this study, we succeeded to establish a continuous process by immobilization. The immobilized white rot fungus was capable to decolorize 85% RBBR under 0.8 mM $CuSO_4$ and used for 3 cycles of decolorization.

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