



LIGNINOLYTIC ENZYME IMMOBILIZATION FROM *Pleurotus ostreatus* FOR DYE AND BATIK WASTEWATER DECOLORIZATION

R. S. Dewi*¹, M. Ilyas², A. A. Sari²

¹Faculty of Biology, Universitas Jenderal Soedirman,
Jalan Dr. Soeparno No. 63, Purwokerto 53122, Indonesia

²Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI)
Kawasan Cibinong Science Center, Cibinong, West Java 16911, Indonesia

³Research Center for Chemistry, Indonesian Institute of Sciences,
Kawasan PUSPITEK Serpong, Tangerang Selatan 15314, Indonesia.

DOI: 10.15294/jpii.v8i2.19372

Accepted: February 14th, 2019. Approved: June 28th, 2019. Published: June 30th, 2019

ABSTRACT

Batik wastewater is one of the environmental problems which has become a significant water pollution problem recently. It contains a large variety of synthetic dyes, chemicals, and has high chemical oxygen demand (COD). Synthetic dyes are difficult to degrade and have recalcitrant and toxic characteristics. Dyes can be degraded by ligninolytic enzymes, including laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). The immobilized ligninolytic enzyme is effectively used to enhance the degradation of the dye wastewater. *Pleurotus ostreatus*, the most abundant ligninolytic enzyme source, is the candidate to regenerate fungal biomass as the bioremediation agent. Immobilization of ligninolytic enzyme using alginate was observed in this study for its efficiency to decolorize and decrease COD concentration of RBBR dye and Naphtol batik wastewater in different time periods (0, 2, 4, 6, 24, 48, 72 h) and growth conditions (static and agitated). The results show that the Lac, MnP, LnP activities of *P. ostreatus* are 200.43, 9.714, 12938.60 U/l, respectively. Static conditions within 48 hours exhibit the highest percentage of decolorization of dye. Ligninolytic immobilized from its condition has decolorized RBBR dye up to 75.88%, while the percentage of decolorization of agitated culture is 68.09%. This ligninolytic immobilized has decolorized about 94.867% of batik wastewater within 24 hours under a static condition. It is also able to decrease the COD level of the batik wastewater containing Naphtol dye (504 to 233 mg/l) within 24 hours under a shaking condition. Immobilization of enzyme has been a promising alternative for decolorization of dye and batik wastewater.

© 2019 Science Education Study Program FMIPA UNNES Semarang

Keywords: COD, decolorization, immobilization, ligninolytic, *Pleurotus ostreatus*

INTRODUCTION

Synthetic dyes are used extensively in textile dyeing, such as in batik industry. Remazol Brilliant Blue R (RBBR) and Naphtol are widely used for batik dyes. Remazol Brilliant Blue R (RBBR) is a heterocyclic compound having for-

ming agent of quinone, comprising a benzene ring with a hydroxyl group called phenol. RBBR anthracene derivatives are a class of organopolutants that are toxic and difficult to disentangle (Vyas et al., 1995). Naphtol dyes are used to dye cellulose fibres and are not soluble in water. It is classified as a dye never found in a dipping solution but only formed after being in a solution

*Correspondence Address

E-mail: ratna.dewi0509@unsoed.ac.id

of fibre. In dyeing with naphthol dyes, the fabric is first worked with phenolic compounds that have an appeal to cotton and then worked with a stabilized diazonium salt solution, so that the dyes will form in the fabric (Sunarto, 2008). These dyes are often used for batik coloring process both in large scale industries and home industries. More than 2,000 different azo dyes have currently been used to dye various materials and contaminate the environment through wastewater (Sari et al., 2016). Synthetic dyes produce dye wastes that are difficult to decompose, which will disturb the environment.

Besides raising aesthetic problems, the disposal of textile wastewater directly into the environment can also threaten the preservation of aquatic ecosystems as it may inhibit the penetration of sunlight into the water thus disrupting the photosynthetic activity of the microalgae. Textile dyes are generally made of azo compounds (R-N = N-R) and their derivatives. When the azo compounds are too well in the environment, it will be the source of the disease because of its carcinogenic and mutagenic nature. Thus, it is necessary to find an effective alternative to decompose the waste (Husna et al., 2017).

Several studies of color removal and organic compounds present in industrial batik dye wastewater have been widely practiced, for example by using chemical means such as color degradation with oxidation reactions, anaerobic reactions, and photocatalysis reactions, and physically with coagulation, sedimentation, adsorption using activated carbon, silica, and biomaterials. Oxidative decolorization method is a useful technique in treating the wastewater soluble Ethyl orange dye (Manjunatha & Puttaswamy, 2016). The adsorption method using sawdust as biosorbent is successfully utilized for removing soluble congo red and azo dye from wastewater (Demirbas, 2009). The level of decolorization solutions of indigo carmine dye increases with increasing irradiation doses (Zaouak et al., 2018).

Several effective physical and chemical methods are used to reduce color wastewater contamination, but these methods require more expensive catalysts and reagents. Along with the development of biotechnology, a more effective way of reducing water pollution due to dye waste has been found, that is, by using white-rot fungi. White-rot fungi are organisms that can decolorize synthetic dyes, one of which is the *Pleurotus ostreatus* (Faraco et al., 2009; Willmot et al., 1998).

The mechanism of decolorization by fungi occurs through the process of adsorbing dye by the cell wall. The color change of the mushroom

mycelium from the primary color shows the decolorization process caused by the mechanism of dye adsorption by the fungus. Besides, to play a role in decolorization, white-rot fungi can also remodel lignin into cellulose (biodelignification) (Miyauchi et al., 2017; Rolz et al. 1986). Under certain conditions, white-rot fungi produce specific extracellular ligninolytic enzymes, lignin peroxidase, manganese peroxidase, laccase, and others in various combinations. The ligninolytic enzyme produced by wooden degrading fungi is ideally used for organopollutant biodegradation in the environment (Damián-Robles et al., 2017).

Dye decolorization with the immobilization of fungi and enzymes is an efficient, economical, and environmentally friendly method. Fungal immobilization has several advantages which increase enzymatic activities, such as improving thermal stability and operational stability, increasing selectivity, and sensitive to inhibitors. Immobilization is a limitation of cell mobility in a limited space. Other advantages of immobilization techniques are that they can obtain several high enzymatic activities, they have high operational stability, and they do not require enzyme purification. Further, the immobilization of biomass can be used for higher substrate concentrations and has longer cell lifespan. The immobilization system can increase bioreactor productivity and the resistance of environmental conditions, such as pH, temperature, organic solvents, and toxic substances (Homaei et al., 2013). Other advantages of fungal immobilization include having high cell concentrations, reusable cells, favorable microenvironment conditions such as nutrient gradient cell contact, pH gradient for cells, providing better biocatalysts (such as high rates), improving genetic stability, and being able to protect cells from damage (Shuler & Kargi, 1992). Immobilization of the enzyme is carried out to maintain the stability of the enzyme. Immobilization of enzymes is enzyme trapping in a polymer matrix or enzyme binding on a carrier material. However, it retains its catalytic properties (Tischer & Wedekind, 1999).

A wide variety of fungal organisms can decolorize dyes. Dye decolorization by using mushrooms is an effective method based on a reliable enzyme system and has been widely used by researchers for other waste degradation. Mushroom can degrade complex organic compounds in the presence of extracellular ligninolytic enzymes (Gomi et al., 2011). Mushrooms have a large surface area so they are easily separated from contaminants (Mishra & Malik, 2012). The mushrooms can also degrade organic and inorganic

contaminants (Awasthi et al., 2014).

The ability of the white-rot fungus to degrade dyes is related to the ability of the fungus to secrete ligninolytic extracellular enzymes such as Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase. Besides, the fungus can catalyze phenolic and non-phenolic compounds and has a large decolorization capacity for various synthetic dyes (Boubonnais & Paice, 1995).

Decolorization of dyes using enzymes currently becomes an interesting subject to investigate because it is easily applied on an industrial scale later. The use of enzymes is more efficient because they do not make new waste such as sediment (Vyas et al., 1995). Immobilization of enzyme can be used for decolorization since enzymes are not damaged as a biocatalyst due to environmental influences.

Immobilization of enzyme is a process whereby an enzyme is physically placed in a particular place such that its catalytic activity remains and can be used repeatedly. Other advantages of immobilization techniques are: 1) it enables for several stages of enzyme reactions, 2) it has high enzyme activity with immobilization techniques, 3) its general operational stability is high, 4) no enzyme extraction/purification step is required, and 5) immobilized biomass can be used for higher substrate concentrations and it has easy cell separation and extended cell life. Immobilization techniques are divided into two, namely active immobilization and passive immobilization. Active immobilization is entrapment or binding by physical or chemical forces. Physical trapping can use a variety of materials such as porous materials (agar, alginate, carrageenan, polyacrylamide, chitosan, gelatin, and collagen), a filter of porous metal, polyurethane, silica gel, polystyrene, and cellulose triacetate. On the other hand, passive immobilization can use an attachment, a form of a biofilm, which is a layer of cell growth on the surface of supporting media. These media can be either inert or biologically active (Liu, 2016).

An essential application of immobilization of fungi is dyestuff decolorization. Dye decolorization with the immobilization of this fungus is an efficient, economical, and environmentally friendly method. The result of dyestuff decolorization using enzyme immobilized in Ca-Alginate and glucose solids obtained by decolorization result is 90% within three days. Whereas in immobilization enzyme in immobilized laccase enzyme experiment inside calcium alginate can degrade antracinoïd dye, blue lancet, and red ponceau (Wu et al., 2005).

This study aims to develop immobilization material extracted from the ligninolytic enzyme of *Pleurotus ostreatus*. This immobilized enzyme is used to observe the ability of immobilized ligninolytic enzyme for decolorization of RBBR and naphthol dyes-contained batik wastewater. Further, reducing COD concentration in batik wastewater after decolorization process is also analyzed.

METHODS

Inoculum Preparation

Pleurotus ostreatus was grown on Potato Dextrose Agar sterile medium for 7-14 days at room temperature until the mycelium filled the Petri dish. The mycelium was inoculated on a medium of corn in a glass bottle (1 kg of milled corn, 100 g of CaCO₃ and a little bit of distilled water stirred evenly) which was previously sterilized by autoclaving 121 °C 2 atm, then incubated at room temperature for 21 days until the mycelium filled the bottle. Three plugs of inoculum in the corn media were added to the baglog substrate. The baglog substrate contained 3 kg wood sawdust, CaCO₃ 30 g, gypsum 30 g, corn starch 90 g, and TSP Triple Super Phosphate (TSP) 150 g, previously sterilized using an autoclave with a temperature of 121°C, for 45 minutes. The inoculum on the baglog substrate was incubated for 30-45 days at room temperature until the mycelium filled the baglog.

Extraction of Crude Enzyme

300 gr of mycelium of *Pleurotus ostreatus* in the baglog was then extracted with 1000 ml 50 mM malonate buffer (pH 4.5) using a homogenizer at 10,000 rpm for 10 min, then filtered and centrifuged at 8,000 rpm for 20 min at 4°C. The clear supernatant was added with 516 g/L ammonium sulfate, and the precipitate was discarded. This solution was centrifuged at 8,000 rpm for 20 min at 4°C. The precipitate (50 ml) was resuspended in 200 ml 50 mM malonate buffer. As much as 400 ml extract enzyme was kept in the freezer at -80°C until overnight, then freeze dried at -45°C, 0.0005 hPa until seven days (Sari et al., 2015).

Enzyme Assay

The measurement of Manganese Peroxidase (MnP) activity was done by monitoring the oxidation of 20 mM 2,6-dimethylphenol (2,6-

DMP) at a wavelength of 470 nm in 50 mM of malonate buffer solution (pH 4.5) containing 20 mM MnSO_4 and 2 mM H_2O_2 (Takano et al., 2004). Lignin Peroxidase (LiP) activity was monitored after the formation of 2 mM H_2O_2 and LiP buffer solution at 310 nm wavelength (Collins et al., 1997). The laccase activity was measured by monitoring the oxidation of syringaldazine to quinone form in a 0.1 M NaH_2PO_4 buffer solution, and then measured at a wavelength of 525 nm (Zavarzina et al., 2006). All reactions of the enzyme activity were carried out at a temperature of 20°C and during the measurement of absorbance value by UV-vis spectrophotometry 1 minute before the cuvet was inserted to a spectrophotometer. U/I units are the amount of enzyme needed to oxidize 1 μmol substrate for 1 min.

Enzyme Immobilization

A crude enzyme was measured as much as 0.6 grams, then mixed with 3.2 grams of sodium alginate and 40 ml of malonic acid buffer (pH 4.5), then homogenized with a magnetic stirrer. CaCl was prepared as much as 7.3 grams and then dissolved in 500 ml of distilled water, homogenized with the magnetic stirrer. The homogenized alginate was taken using a micropipette, then

dropped on CaCl solution being stirred, and then waited for 10 minutes. The alginate beads which had formed were filtered and rinsed with distilled water.

Assays for Decolorization

As much as 35 grams of beads were added on 100 ml of 100 ppm dye solution or batik wastewater in Erlenmeyer flasks 250 ml, and incubated on static and 100 rpm agitated condition at 30°C for 0, 2, 4, 6, 24, 48, and 72 hours. The pH was measured after assays by using pH meters. Beads were separated from dye and wastewater by centrifugation at 3000 rpm for 20 minutes. The absorbance value of supernatant was measured using spectrophotometry at 595 nm for RBBR and 425 nm for Naphtol wastewater, respectively. The percentage of decolorization was determined based on the equation as follows:

$$\text{Decolorization (\%)} = \frac{\text{last absorbance} - \text{first absorbance}}{\text{first absorbance}} \times 100\%$$

Effect of Treatment on COD Parameter

The measurement of the COD parameter was carried out before and after decolorization of Naphtol batik wastewater in 24 hours.



Figure 1. The Activity of Ligninolytic Enzyme Immobilized Alginate

RESULTS AND DISCUSSION

Enzyme Activity

The alginate immobilized enzyme activity was shown in Figure 1. Based on these data, it can be seen that the laccase enzyme activity immobilized alginate had a not-so-high activity (2000.4274 U/l). The highest activity was obtained at LiP with a value of 12938.6 U/l, while the lowest activity was found in MnP of 9.71371 U/l. From the data, it can be seen

that the most active enzyme is Lignin Peroxidase (LiP). It is because the enzyme works correctly against a particular substrate by the active side of the enzyme (Cichoke, 1999). LiP is an extracellular peroxidase enzyme whose activity depends on H_2O_2 . The primary function of extracellular enzymes (exoenzyme) is to carry out the necessary changes of the surrounding nutrients, enabling them to enter the cell (Chatter et al., 2009) and to be absorbed directly by the mould hyphae. After the crude enzyme was obtained, the activity of lignin peroxidase was analyzed. Veratril alcohol serves as a mediator

in redox reactions to stabilize the LiP-oxidation of lignocellulosic organic substrates (Nousiainen et al., 2014; Asgher et al., 2011). Indirectly, the difference in the value of the resulting LiP enzyme activity depends on the ability of the fungus to adapt to its constituent substrate and factors such as the biological, physical or chemical factors possessed by the enzyme. The suitability and effectiveness of the media substrate can maximize the enzyme's work to stimulate the high value of expected enzyme activity.

Enzyme immobilization is carried out to maintain the stability of the enzyme. Enzyme immobilization is the trapping of enzymes in the polymer matrix or binding of enzymes to a carrier material (Tischer & Wedekind, 1999). However, it still maintains its catalytic properties (Mohamad et al., 2015). Ligninolytic enzyme immobilization

uses adsorption techniques where interactions between material support and compounds can be Van Der Waals forces, hydrogen bonds, and hydrophobic interactions (Burn, 1986).

The RBBR Decolorization

Decolorization of RBBR was performed to find out how large the ligninolytic enzyme of *P. ostreatus* decomposed this dye. Three parameters were used: control parameters (decolorization using Ca-Alginate), agitation parameter (decolorization using Ca-Alginate + enzyme), and parameter without agitation (decolorization using Ca-Alginate + enzyme). The data on the percentage value of decolorization of RBBR are presented in Figure 2.

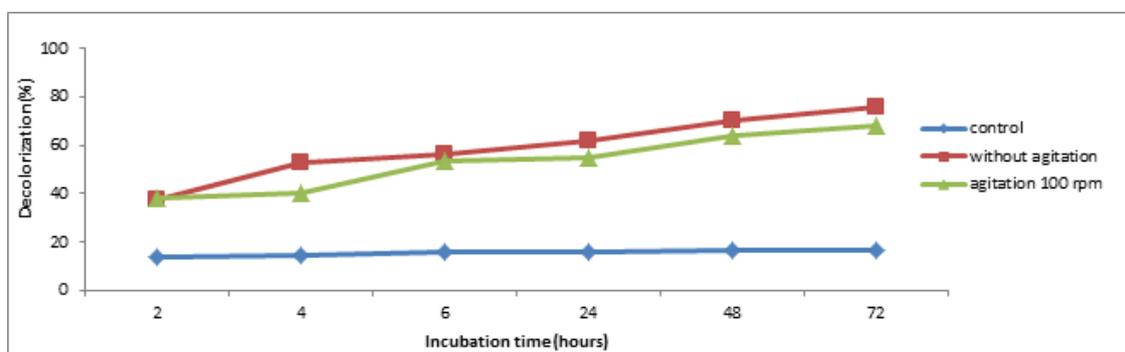


Figure 2. RBBR Decolorization Presentation within Various Incubation Times

The static parameter had a higher result than samples of agitation parameter. It shows that the RBBR decolorization was succeeded by enzyme immobilized compared to control using alginate. It proves that the immobilized enzyme had higher decolorization activity than the immobilization matrix without enzyme. This result is consistent with Hanifah (2018), who found that the crude enzyme extract weighing 80.24 g with laccase enzyme activity is 0.76 U/L and is successfully immobilized in the hydrotone matrix. The percentage of decolorization of reactive black 5 synthetic dyes with the hydrotone matrix is 10.89%, laccase enzyme is 75.56%, hydrotone matrix is immobilized by lacase enzyme by 97.54%.

Based on Figure 2, the incubation time resulting in the highest percentage of decolorization and the clearest appearance of the sample was 48 hours incubation time with 75.88% static condition. It means that 48-hour incubation time was the best time or the most optimum time in decolorizing batik wastewater. Following Alvarez et al. (2010) the incubation time affects the adsorption

in the process of decolorization of the wastewater. The longer the incubation time, the higher the absorbed dyestuff produced because the contact between the sorbent with the dyestuff will last too long. Based on Figure 2, the static treatment results in a higher percentage of decolorization compared with agitation treatment. It indicates that the static incubation system was able to decrease the percentage of decolorization compared to using the agitation incubation system.

The incubation system of agitation is an incubation system performed to speed up the dissolution process. Agitation on a laboratory scale is usually carried out by wiggling flasks containing sample solutions by using a shaker (shaker flask culture). The static incubation system is an incubation system performed without shaking the flask containing the sample of the solution until a predetermined time (Kartikasari et al., 2012).

Figure 2 also shows that all enzyme immobilized can decolorize the dye. This result is similar to the research of Wu et al. (2005) that found enzyme immobilized in Ca-Alginate can degrade antracinoind dye, blue lancet, and red ponceau

dyes with a decolorization result of 90% within 3 days.

The ligninolytic enzyme is capable of decolorizing RBBR dyes. Ligninolytic enzymes can be obtained from the *P. ostreatus*. Enzymes produced by *P. ostreatus* include laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) enzymes. Among these three enzymes, LiP is the most dominant. *P. ostreatus* degrades lignin to produce ligninolytic enzymes. Mushrooms are selected as one of the biodecolorization organizations capable of degrading the toxic color components because the fungus can transform, that is, to convert from harmful chemicals to waste into less or less harmful forms (Couto, 2009).

Ligninolytic enzymes, especially laccase, manganese peroxidase, and lignin peroxidase play an important role in degrading phenolic compounds, polyaromatic hydrocarbons, and textile dyes. The resulting laccase will reduce O_2 to H_2O in the phenolic substrate by the reaction of one electron to form a free radical that

can be likened to the cationic radical formed in the MnP reaction. LiP, a hemorrhoid-containing extracellular enzyme whose activity depends on H_2O_2 , has an enormous redox potential and a low optimum pH. MnP is an extracellular heme peroxidase which requires Mn^{2+} as its reducing substrate. MnP oxidizes Mn^{2+} to Mn^{3+} , which then oxidizes the phenolic structure into phenoxyl radicals. Mn^{3+} is formed very reactive and forms complexes by chelating organic acids such as oxalic acid or malate (Hatakka et al., 1994). *P. ostreatus* produces extracellular enzymes such as manganese peroxidase (Mn-P) and laccase (Lac) based on the resulting ligninolytic enzyme pattern. Mn-P and Lac are responsible for the biodegradation of organic pollutants because they have catalytic activity against different types of substrates. Lac on *P. ostreatus* can degrade the nonphenolic substrates by oxidizing them. Bioremediation and biodegradation of organic pollutants are conducted aerobically using the resulting extracellular enzyme (Chater et al., 2009).

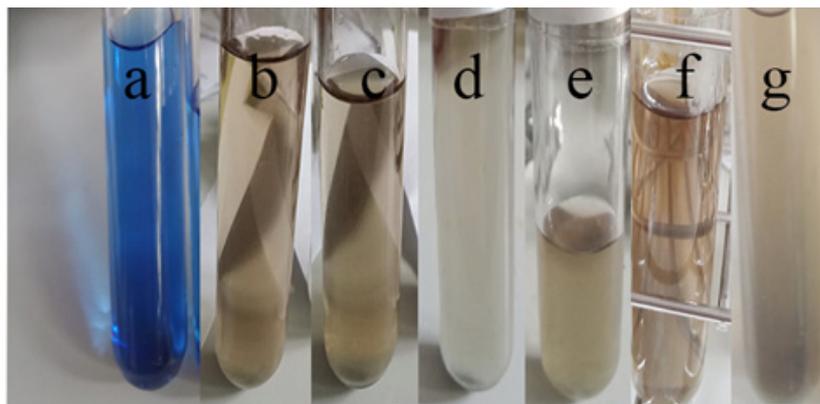


Figure 3. Decolorization Result of RBBR at Agitation Treatment. RBBR Samples with 0-hour (a), 2 hours (b), 4-hour (c), 6-hour (d), 24-hour (e), 48-hour (f), 72-hour (g) Incubation Time Treatments

Based on Figure 3, there is a decrease in the dyestuff content of each incubation time. The color of RBBR is decreased from blue into brown. The color change from blue to brown started at a 2-hour incubation time. It means that in this interval of time, the chromophore has already broken. The disappearing blue color implies the cleavage of the chromophore group (Osma et al., 2010). This result was also reported by Lonergan et al. (1993), that the ability of the mushroom is evaluated by looking at the color change (affirmative response), from blue to dark brown, lighter or colorless, due to the oxidation process and the breakdown of anthraquinone bonds. The blue color of RBBR comes from the chromophore, in this case, the anthraquinone that acts as a particular wavelength catcher antenna. The occurrence of

quinone bonding by ligninolytic enzymes makes the color of RBBR turn into dark brown, lighter, or unimportant depending on how many quinone bond changes occur. After 2 hours, decolorization of RBBR still appeared. It means that the degradation of the organic compound in RBBR dye process still occurred.

Osma et al. (2010) reported that two intermediates were identified after two hours of RBBR degradation using an immobilized enzyme ($m/z304.30$ and $m/z342.24$). However, no decolorization occurred during this interval of time. It indicated that the chromophore was not broken. After the degradation process for 95 h, the high peaks disappeared, and the other two peaks ($m/z207.16$) and ($m/z343.29$) appeared. Based on the appearance of the sample, in this interval

time, the disappearing blue color indicated that the chromophore was broken.

The decreased color is also caused by the adsorption process, in which the molecular substance leaves the waste solution and joins the surface of the mycelium. The adsorption process here serves to exclude aromatic compounds and dissolved organic compounds. The color concentration in the wastewater after receiving treatment from the isolation fungus has decreased the color concentration. In addition to the adsorption process, it is estimated that extracellular enzymatic systems play a role in this decolorization process. The occurrence of the decolorization process is suspected as a result of the adsorption process as a non-enzymatic system followed by the ability of degradation by isolates due to the occurrence of metabolic activity with the enzymatic system (Yang et al., 2009).

Based on Figures 2 and 3, the incubation time resulting in the highest percentage of decolorization and the clearest appearance of the sample was the 48-hour incubation time with 75.88% static condition. It means that the 48-

hour incubation time was the best time or the most optimum time in decolorizing batik wastewater. Following Satar & Husain (2009) the incubation time affects the adsorption in the process of decolorization. The longer the incubation time, the higher the absorbed dyestuff produced because the contact between the sorbent with the dyestuff will last too long. Based on Figure 2, the static treatment results in a higher percentage of decolorization compared with the agitation treatment. It indicates that the static incubation system was able to decrease the percentage of decolorization compared to using the agitation incubation system.

The agitation incubation system is an incubation system performed to speed up the dissolution process. Agitation in a laboratory scale is usually carried out by wiggling flasks containing sample solutions by using a shaker (shaker flask culture). The static incubation system is an incubation system performed without shaking the flask containing the sample of the solution until a predetermined time (Kartikasari et al., 2012).

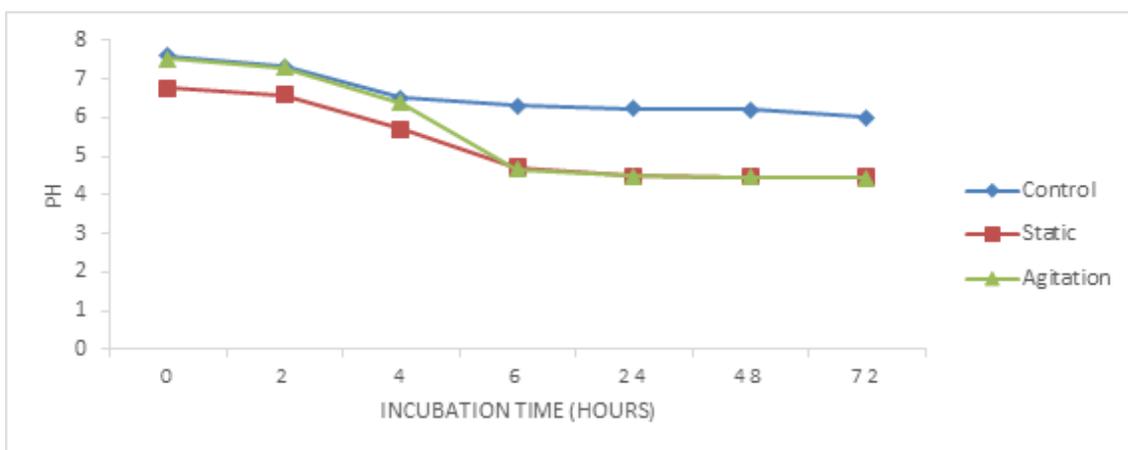


Figure 4. RBBR pH Analysis Results

Figure 4 shows the measuring of pH during the decolorization process. The results show that there is a decrease in the pH of batik wastewater after decolorization. The initial pH of batik wastewater at 0 hour with the parameter control was 7.6, and the final pH value of the research at 72 hours was 6. On the other hand, the initial pH value of batik waste at 0 hour with the parameter of 100 rpm agitation was 7.52, and the final pH value of the research at 72 hours was 4.44. Further, the initial pH value of batik wastewater at 0 hour with the parameter without agitation 100 rpm was 6.52, and the final pH value of the research at 72 hours was 4.45. Based on the results, the more acid of the pH value, the higher

percentage the decolorization is. This result is similar to Tian et al. (2013), in the increasingly acidic pH conditions, the absorbance value decreases so that the percentage of decolorization is more significant.

The presence of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase enzymes that are nonspecific to the substrate were allowed to decolorize batik wastewater. This enzyme is responsible for the breaking of aromatic bonds in complex color compounds. The enzyme in the process of decolorization causes the change of acidity (pH) degree of the dye. According to Ambarwati (2017), ligninolytic enzymes work well when their environmental

conditions are appropriate. The activity of RBBR decolorization by the ligninolytic enzyme is influenced by several factors, including nutrition and pH.

The Batik Wastewater Decolorization

Figure 5 demonstrates that the 24-hour incubation time of static conditions shows the

highest decolorization efficiency, but if it is continued up to 72 hours, the decolorization efficiency of the wastewater will decrease. The optimum condition for decolorization of batik Naphtol wastewater occurred at a 24-hour incubation time with an efficiency of color reduction of 94.867%. Meanwhile, the agitation conditions experienced efficiency at the second hour by 92.59% but it decreased until 72 hours.

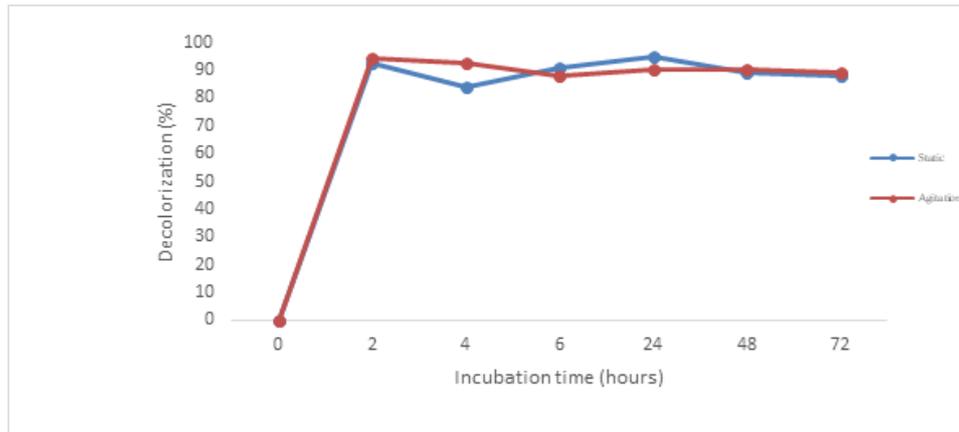


Figure 5. The Decolorization of Naphtol Batik Dye within Various Incubation Times

The ability of the immobilized alginate enzyme to decolorize wastewater is influenced by the incubation time. The results of the study show that the color reduction changed because it was influenced by the biosorption mechanism.

Biosorption is the process of passively absorbing a chemical using matrix. Adsorption is the process of passively absorbing a chemical independent of metabolism; this process occurs in a wall surface that involves ion bonding. The adsorption process using an adsorbent is called biosorption. The biosorption process is the binding of materials through adsorption (Abbas et al., 2014; Fomina & Gadd, 2014). Mixing adsorbent types is an alternative step to optimize

adsorption capacity in the biosorption process (Babu & Preetha, 2014). In this case, two types of biosorbent (ligninolytic enzyme and alginate) are mixed to increase their ability to decolorize wastes whose composition is more complicated than coloring.

Effect of Treatment on COD Parameter

COD is used to measure the concentration of organic compounds in water. The amount of COD determines the number of organic pollutants found in water. Therefore, a decrease in the COD value illustrates the reduced amount of pollutants in water.

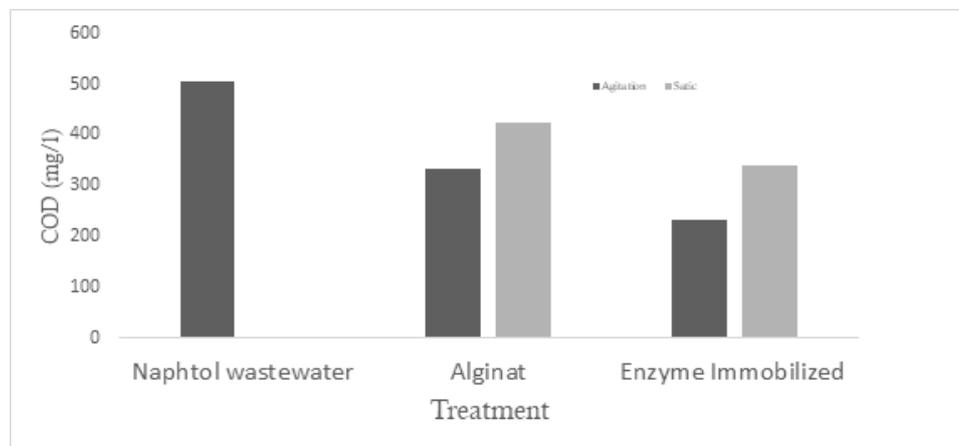


Figure 6. COD Values Before and After Decolorization

Figure 6 illustrates the COD parameters before decolorization (naphthol batik wastewater) and after decolorization (alginate/control and immobilized enzymes). The initial COD concentration in batik naphthol wastewater was 504 mg/l. After the decolorization process, a decrease in COD occurred in the alginate as a control (334.67 mg/l) and alginate immobilized enzymes (233 mg/l) at the agitated condition. The COD value decreased with the addition of immobilized and alginate enzymes. Although control of alginate was sufficient to reduce the value of COD, the immobilized enzyme treatment could reduce COD values much better. It proves that the mixture of 2 adsorbates enhanced the effectiveness of decreasing organic matter in batik waste pollutants.

The same thing occurred in the static condition treatment. The treatment of enzyme could reduce COD parameters (338.33 mg/l) as well as the treatment of alginate (424.67 mg/l). It shows that the immobilized enzyme treatment was better than the alginate treatment. These data also show that the enzyme treatment was better than the alginate treatment. However, for growth condition treatment, the COD value was not as good as the decolorization value. The agitation condition treatment was better than the static one on the COD parameter.

The continuous adsorption causes dissolved oxygen levels to decrease to the lowest level. The decrease in dissolved oxygen in the wastewater results in the decrease in the degradation process of organic matter as indicated by a decrease in COD. Processing using aeration is aimed to reduce organic carbon or organic nitrogen. COD is used as a measure or unit that describes the concentration of organic compounds. Organic matter in wastewater is broken down into carbon dioxide and ammonia (Angelidaki et al., 2011).

CONCLUSION

Based on the results obtained, it can be concluded that the Lac, MnP, LiP activities of *P. ostreatus* immobilized were 200.43, 9.714, 12938.60 U/l, respectively. The length of incubation time caused a higher decolorization of dyes when the optimum time was 48 hours. The immobilized ligninolytic enzyme in a static condition decolorized RBBR dye was up to 75.88%, while in an agitated condition decolorized RBBR it was up to 68.09%. In the application of batik wastewater treatment, this immobilized ligninolytic enzyme decolorized Naphthol dye was about 94.867% within 24 hours under a static condition. It was also able to decrease the COD concentration up to 53.7% (504 to 233 mg/l) within 24 hours under a shaking condition.

ACKNOWLEDGMENTS

This research was supported by Nagao Natural Environment Foundation Fiscal Year 2018.

REFERENCES

- Abbas, S. H., Ismail, I. M., Mostafa, T. M., & Sulaymon, A. H. (2014). Biosorption of Heavy Metals: A Review. *Journal of Chemical Science and Technology*, 3(4), 74-102.
- Alvarez, L. H., Perez-Cruz, M. A., Rangel-Mendez, J. R., & Cervantes, F. J. (2010). Immobilized Redox Mediator on Metal-Oxides Nanoparticles and Its Catalytic Effect in a Reductive Decolorization Process. *Journal of Hazardous Materials*, 184(1-3), 268-272.
- Angelidaki, I., Karakashev, D., Batstone, D. J., Plugge, C. M., & Stams, A. J. (2011). Biomethanation and Its Potential. In *Methods in Enzymology* (Vol. 494, pp. 327-351). Academic Press.
- Asgher, M., Ahmed, N., & Iqbal, H. M. N. (2011). Hyperproductivity of Extracellular Enzymes from Indigenous White Rot Fungi (*Phanerochaete chrysosporium*) by Utilizing Agro-Wastes. *BioResources*, 6 (4), 4454-4467.
- Awasthi, M. K., Pandey, A. K., Khan, J., Bundela, P. S., Wong, J. W., & Selvam, A. (2014). Evaluation of Thermophilic Fungal Consortium for Organic Municipal Solid Waste Composting. *Bioresource technology*, 168(2014), 214-221.
- Babu, E., & Preetha, B. (2014). Kinetics and Equilibrium Studies on Biosorption of Chromium (VI) by Mixed Biosorbents. *Int. J. Chem. Technol. Res*, 6(12), 4927-4933.
- Burn, R. G. (1986). Interaction of Enzymes with Soil Minerals and Organic Colloids, In Huang, P. M. and Schnitzer, M. *Interactions of Soil Materials with Natural Organics and Microbes*.
- Chater, K. F., Biro, S., Lee, K. J., Palmer, T., & Schrempf, H. (2010). The Complex Extracellular Biology of Streptomyces. *FEMS Microbiology Reviews*, 34(2), 171-198.
- Cichoke, A. J. (1999). *The Complete Book of Enzyme Therapy*. Penguin Inc.
- Collins, P. J., & Dobson, A. (1997). Regulation of Laccase Gene Transcription in *Trametes versicolor*. *Appl. Environ. Microbiol.*, 63(9), 3444-3450.
- Couto, S. R. (2009). Dye Removal by Immobilised Fungi. *Biotechnology Advances*, 27(3), 227-235.
- Damián-Robles, R. M., Castro-Montoya, A. J., Saucedo-Luna, J., Vázquez-Garciduenas, M. S., Arredondo-Santoyo, M., & Vázquez-Marrufo, G. (2017). Characterization of Ligninolytic Enzyme Production in White-Rot Wild Fungal Strains Suitable for Kraft Pulp Bleaching. *3 Biotech*, 7(5), 319.
- Demirbas, A. (2009). Agricultural Based Activated Carbons for the Removal of Dyes From Aqueous Solutions: A Review. *Journal of Hazardous Materials*, 167(1-3), 1-9.

- Faraco, V., Pezzella, C., Miele, A., Giardina, P., & Sanna, G. (2009). Bio-Remediation of Colored Industrial Wastewaters by the White-Rot Fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and Their Enzymes. *Biodegradation*, 20(2), 209-220.
- Fomina, M., & Gadd, G. M. (2014). Biosorption: Current Perspectives on Concept, Definition and Application. *Bioresource Technology*, 160, 3-14.
- Gomi, N., Yoshida, S., Matsumoto, K., Okudomi, M., Konno, H., Hisabori, T., & Sugano, Y. (2011). Degradation of the Synthetic Dye Amaranth by the Fungus *Bjerkandera adusta* Dec 1: Inference of the Degradation Pathway from an Analysis of Decolorized Products. *Biodegradation*, 22(6), 1239-1245.
- Hanifah, (2018). *Decolorization of Synthetic Dyes Reactive Black 5 by Laccase Immobilized in Hydroton*. (Thesis, Universitas Gadjah Mada/Faculty of Math and Science/ Chemical Department).
- Hatakka, A. (1994). Lignin-Modifying Enzymes from Selected White-Rot Fungi: Production and Role from in Lignin Degradation. *FEMS Microbiology Reviews*, 13(2-3), 125-135.
- Homaei, A. A., Sariri, R., Vianello, F., & Stevanato, R. (2013). Enzyme Immobilization: An Update. *Journal of Chemical Biology*, 6 (4), 185-205.
- Kartikasari, T. H., Lestari, S., & Dewi, R. S. (2012). Adsorpsi Zn dan Dekolorisasi Limbah Batik Menggunakan Limbah Baglog *Pleurotus ostreatus* dengan Sistem Inkubasi dan Volume Limbah Batik Berbeda. *Majalah Ilmiah Biologi BIOS-FERA: A Scientific Journal*, 29(3), 168-174.
- Liu, S. (2016). *Bioprocess Engineering: Kinetics, Sustainability, and Reactor Design*. Elsevier.
- Mishra, A., & Malik, A. (2012). Simultaneous Bioaccumulation of Multiple Metals from Electroplating Effluent Using *Aspergillus lentulus*. *Water Research*, 46(16), 4991-4998.
- Miyauchi, S., Navarro, D., Grisel, S., Chevret, D., Berin, J. G., & Rosso, M. N. (2017). The Integrative Omicsof White-Rot Fungus *Pycnoporus coccineus* Reveals Co-Regulated CAZymes for Orchestrated Lignocellulose Breakdown. *PLoS One*, 12(4), 1-17.
- Nousiainen, P., Kontro, J., Manner, H., Hatakka, A., & Sipilä, J. (2014). Phenolic Mediators Enhance the Manganese Peroxidase Catalyzed Oxidation of Recalcitrant Lignin Model Compounds and Synthetic Lignin. *Fungal Genetics and Biology*, 72(2014), 137-149.
- Osma, J. F., Toca-Herrera, J. L., & Rodríguez-Couto, S. (2010). Transformation Pathway of Remazol Brilliant Blue R by Immobilised Laccase. *Bioresource Technology*, 101(22), 8509-8514.
- Sari, A. A., Tachibana, S., Muryanto, & Hadibarata, T. (2016). Development of Bioreactor Systems for Decolorization of Reactive Green 19 Using White Rot Fungus. *Desalination and Water Treatment*, 57(15), 7029-7039.
- Satar, R., & Husain, Q. (2009). Applications of Celite-Adsorbed White Radish (*Raphanussativus*) Peroxidase in Batch Process and Continuous Reactor for the Degradation of Reactive Dyes. *Biochemical Engineering Journal*, 46(2), 96-104.
- Shuler, M. L., and Kargi, F. (1992). *Bioprocess Engineering: Basic Concepts*. Prentice-Hall International, Inc, New Jersey.
- Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An Overview of Technologies for Immobilization of Enzymes and Surface Analysis Techniques for Immobilized Enzymes. *Biotechnology & Biotechnological Equipment*, 29(2), 205-220.
- Sunarto, (2008). *Teknik Pencelupan dan Pencapan 3*. Departemen Pendidikan Nasional.
- Takano, M., Nakamura, M., Nishida, A., & Ishihara, M. (2004). Manganese peroxidase from *Phanerochaete crassa* WD 1694. *Bulletin of FFPRI*, 3(1), 7-13.
- Tian, C. E., Tian, R., Zhou, Y., Chen, Q., & Cheng, H. (2013). Decolorization of Indigo Dye and Indigo Dye-Containing Textile Effluent by *Ganoderma weberianum*. *African Journal of Microbiology Research*, 7(11), 941-947.
- Tischer, W., & Wedekind, F. (1999). Immobilized Enzymes: Methods and Applications. In *Biocatalysis from discovery to application*. Springer, Berlin, Heidelberg.
- Vyas, B. R., & Molitoris, H. P. (1995). Involvement of an Extracellular H₂O₂-Dependent Ligninolytic Activity of the White Rot Fungus *Pleurotus ostreatus* in the Decolorization of Remazol Brilliant blue R. *Appl. Environ. Microbiol.*, 61(11), 3919-3927.
- Wu, J., Xiao, Y. Z., & Yu, H. Q. (2005). Degradation of Lignin in Pulp Mill Wastewaters by White-Rot Fungi on Biofilm. *Bioresource Technology*, 96(12), 1357-1363.
- Yang, X. Q., Zhao, X. X., Liu, C. Y., Zheng, Y., & Qian, S. J. (2009). Decolorization of Azo, Triphenylmethane and Anthraquinone Dyes by a Newly Isolated *Trametes* sp. SQ01 and Its Laccase. *Process Biochemistry*, 44(10), 1185-1189.
- Zaouak, A., Noomen, A., & Jelassi, H. (2018). Gamma-Radiation Induced Decolorization and Degradation on Aqueous Solutions of Indigo Carmine Dye. *Journal of Radioanalytical and Nuclear Chemistry*, 317(1), 37-44.
- Zavarzina, A. G., & Zavarzin, A. A. (2006). Laccase and Tyrosinase Activities in Lichens. *Microbiology*, 75(5), 546-556.