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Covalent Immobilization of *Trametes polyzona* H18 Laccase Enzyme on Activated Carbon for Synthetic Dye Decolorization

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Laccase has a promising potential to be used as a bioremediation agent. In the present study, crude laccase produced by *Trametes polyzona* H18 isolated from Batam Botanical Garden, Batam Island was successfully immobilized on activated carbon and tested for Reactive Black 5 (RB5) dye decolorization. Activated carbon is recognized for its excellent adsorption capabilities, resulting from its extensive internal surface area and cavities, which enhance its adsorption capacity. Activated carbon was made from oil palm empty fruit bunches (OPEFB) through the pyrolysis process and modified by covalent binding using 3-aminopropyl-triethoxysilane (APTS) in variations of 5, 10, and 15% (v/v). Results showed that the highest immobilized laccase-activated carbon yield was 50.44% obtained by activated carbon modified by 15% (v/v) of APTS. The immobilized laccase-activated carbon improved the RB5 decolorization rate by 87.5 % compared to free laccase, which was only 35.5 % at 4 h of reaction time. These preliminary results suggested that immobilized laccase on activated carbon can be potentially developed for large-scale bioremediation of other synthetic dyes.

Keywords: Activated carbon, Decolorization, Immobilization, Laccase, *Trametes polyzona*

Nowadays, synthetic dyes are widely used in the coloring process of various industries, such as textiles, paper, plastics, leather, cosmetics, and other materials. Azo dyes are the largest and most versatile class of dyes that are used today. Approximately 70% or more than 2,000 different azo dyes are currently used in industry today. Azo dyes are stable in light and resistant to microbial degradation or fading away due to washing (Chung, 2016) (Benkhaya *et al.* 2020). However, it was estimated that about 5-10% of the dyestuff was released freely into the environment (Jin *et al.* 2007). Wastewater released by various synthetic dyes industries can pollute and harm the aquatic environment as well as human health. Decolorization of these dyes received much attention due to their high toxicity, carcinogenic agent, and mutagenic behavior (Atiq *et al.* 2010) (Sekuljica *et al.* 2015). The use of enzymes for the treatment or removal of environmental and industrial pollutants has attracted increasing attention because of their high efficiency, high selectivity, and environmentally friendly (Viswanath *et al.* 2014). Sekuljica *et al.* (2015) reported that the enzyme technology for dye degradation has several advantages such as greater specificity, the capability to operate over a broad concentration range of contaminants, better standardization, easy handling and storage, and independent microbial growth rates.

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Laccase has received attention from researchers for its ability to degrade various recalcitrant pollutants. Laccases have a wide substrate range, which can serve industrial purposes. Laccase can oxidize compounds that are structurally like lignin, including organic and inorganic substrates such as mono, di, polyphenols, amino phenols, and methoxy phenols as well as metal complexes which are the major reason for their attractiveness for dozens of biotechnological applications (Upadhyay *et al.* 2016). Over 60 fungal strains belonging to Ascomycetes, Basidiomycetes, and Deuteromycetes show laccase activity. Among the latter group, the lignin-degrading white-rot fungi are the highest producers of laccase. White rot fungi such as *Trametes versicolor* (Minussi *et al.* 2007) (Aydemir and Guler, 2015), *Trametes hirsuta* (Cuoto and Sanroman, 2007) (Anita *et al.* 2019) (Yanto *et al.* 2019), *Pleurotus ostreatus* (Pezzella *et al.* 2014), and *Leiotrametes flavida* (Falah *et al.* 2018) are reported can produce laccase enzyme.

However, laccases are not desirable for large-scale applications by using free enzymes because of their sensitivity to denature agents and non-reusability. Immobilization of free enzymes on various carriers is one way to overcome these limiting factors, which can protect them from inhibition, denaturation, improve their stability, maintain good catalytic activity, and reusability for reaction cycles. It is important to choose an appropriate carrier to optimize immobilization, including pore size, specific surface area, and biodegradability (Pezzella *et al.* 2014) (Anita *et al.* 2020). Other researchers have used materials such as magnetic chitosan–clay composite (Aydemir and Guler, 2015), perlite (Pezzella *et al.* 2014), hydrotalcite-like particles ($ZnAl_2$), light-expanded clay aggregate (LECA) (Anita *et al.* 2020), amorphous silica crystals, and glassy carbon surface (Castro *et al.* 2013), as a carrier for laccase immobilization.

In recent years, activated carbon has been used for the removal of dyes and other pollutants from wastewater due to its structural properties, high density, and high surface area. Activated carbon is a solid material consisting of pure carbon. Activated carbon is widely used in the process of purifying, discoloring, and removing odors at low cost and high efficiency. Activated carbons work on the principle of adsorption as a physical process in which the substances are adsorbed on the solid and do not induce any chemical reaction (Agrawal *et al.* 2017) (Khazravi *et al.* 2019). Some researchers have used activated carbon with or without enzyme immobilization for environmental treatments (Agrawal *et al.* 2017) (Khazravi *et al.* 2019) (Zhang *et al.* 2018).

However, the combination of an enzyme as a biological agent and a carbon compound is a great idea in terms of materials science and wastewater. The factor that affects the success of immobilization is not only the carrier characteristic but also the immobilization method. The immobilization yield values must be considered for the success of the immobilization method. Covalent binding was reported as the most immobilization strategy that is widely used for industrial applications (Castro *et al.* 2013) (Patel *et al.* 2016) (Zhang *et al.* 2018).

Trametes polyzona H18 was used in this experiment as a potential microbe due to its bioprospection to decolorization of various synthetic dyes. Previous studies show the ligninolytic enzyme system of this fungus is efficient in decolorizing several synthetic dyes. Perez-Cadena *et al.* (2020) reported *T. polyzona* ligninolytic enzymes were able to use Amaranth dye as the only carbon source starting at the beginning of the process which contributed to 95% decolorization process on average in an airlift reactor. Next, Uribe-Arizmendi *et al.* (2020) demonstrated the system of ligninolytic enzymes and cells of *T. polyzona* could decolorize amaranth, denim blue, and orange G textile dyes and a 200% increase in the decolorization rate was observed when the medium lacks glucose. Ramadhan *et al.* (2021) also showed impressive decolorization of anthraquinone (95.4% of RBBR, 89% of AB129) and azo dyes (94.8% of RB5 and 77.7% of AO7 dye) by *T. polyzona* H18.

In this study, the laccase enzyme produced by *Trametes polyzona* H18 was immobilized on activated carbon modified by covalent binding using 3-aminopropyl-triethoxysilane (APTS) in various concentrations. The immobilization process was

investigated in terms of immobilization yield and Reactive Black 5 (RB5) azo dye decolorization. Therefore, the objective of this study was to evaluate the ability of immobilized laccase enzyme on modified activated carbon in decolorizing azo dye.

MATERIAL AND METHODS

Organism, Raw Materials, and Chemicals. *Trametes polyzona* H18 a collection of the RC Applied Microbiology, BRIN, Cibinong, Indonesia was isolated from Batam Botanical Garden, Batam Island. Oil palm empty fruit bunch (OPEFB) fiber was obtained from an oil palm plantation in Cikasungka, West Java, and used as a substrate for laccase and activated carbon production. Malt extract [Himedia, India], glucose [Wako, Japan], and peptone [Merck, Germany] were used for fungal growth media. Glutaraldehyde was purchased from Wako Pure Chemical Industries, Ltd. (Japan). Reactive Black 5 (RB5) dye (table 1), (3-aminopropyl) triethoxysilane (APTS), 2,2-azino-bis-[3-ethyl benzothiazoline- 6-sulphonic acid] (ABTS) were purchased from Sigma Aldrich.

Fungal Culture Preparation. *Trametes polyzona* H18 was inoculated on malt extract agar (MEA) and incubated at room temperature. Four plugs of 7 days old fungal colony with 8 mm in diameter were inoculated into a 100 mL Erlenmeyer flask containing 20 mL of medium malt extract-glucose-peptone (MGP) broth. The flask was then incubated at room temperature under static conditions for 7-10 days. MGP broth medium consists of (g/L): malt extract 20, glucose 20, and peptone 1. After incubation finished, the culture was homogenized using a blender (Waring Commercial blender) at a high speed for 2 s. The homogenized mixture was used as a starter culture for laccase production.

Laccase Production. 100 g of 40-60 mesh OPEFB fiber was placed in a heat-resistant plastic bag, added with 200 mL of MGP broth medium, mixed, and covered tightly using a cotton cap. The mixture was sterilized at 121 °C for 15 min. After cooling at room temperature, the mixture was then inoculated with 10% (w/v) of starter culture and incubated at room temperature for 1 month.

Laccase Extraction. After incubation time finished, as much as 500 mL of acetate buffer 0.1 M (pH 4.5) was added to a solid substrate containing fungi. The mixtures were then homogenized by using a homogenizer (ACE AM-11 Nissei, Japan) at 10,000 rpm for 10 min under cold conditions and filtered. The filtrates were centrifuged at 10,000 rpm, 4 °C for 10 min, and the supernatant was added with ammonium sulfate 50% (w/v) and mixed for 2 h. Next, the mixtures were centrifuged at 10,000 rpm at 4 °C for 20 min. The pellet was then dissolved in 0.1 M acetate buffer, pH 4.5, and freeze-dried using a freeze-dryer (Labconco, USA) for 1–2 days. Then crude laccases powders were kept at -30 °C.

Laccase Activity Analysis. Laccase activity was observed spectrophotometrically according to Anita *et al.* (2020). One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute. Enzyme activity (U/mL) was calculated according to equation (1) (Baltierra-Tejo *et al.* 2015) with molar absorptivity (ε) of 36,000 M-1 cm-1 (Irshad *et al.* 2011).

$$\textbf{\textit{Enzyme activity}}\left(\frac{U}{mL}\right) = \frac{(Abs.final - Abs.initial)\,x\,Vtotal\ mixture\ (mL)\,x\,10^3}{(\varepsilon\ x\ Venzym\ (mL)\,x\ t)}$$

(Chung, 2016)

Where: 10^3 :correction factor (µmol mol^{-1})

Preparation and Modification of Activated Carbon. OPEFB were mashed and sieved into sizes 40-60 mesh. OPEFB was then activated through a one-stage pyrolysis reaction using water vapor flow in an electric furnace at 700 °C for 4 h. This process produced activated carbon. Next, activated carbon was modified by (3-aminopropyl) triethoxysilane (APTS) to get an amino-terminated surface. About 100 mg of activated carbon were immersed with 5, 10, and 15% (v/v) APTS at 80 °C for 2 h in a water bath. Then modified activated carbon was neutralized with distilled water. APTS-modified activated carbon was soaked into a 10% (v/v) glutaraldehyde solution for 2 h at room temperature and neutralized with distilled water. Modified activated carbon is then used as a carrier for the laccase in the immobilization process.

Immobilization of Laccase. The laccase immobilization process was carried out on modified

activated carbon. As much as 75 mg of modified activated carbon was soaked into 100 mL of laccase enzyme solution. The initial laccase activity used in this study was 7.6 x 10^{-3} U/mL. The mixtures then were incubated at 4 °C overnight. After 24 h incubation time, the mixtures were filtered, and the immobilized laccase-activated carbon was kept at 4 °C. The filtered solution was analyzed as free laccase activity. The immobilized laccase-activated carbon was calculated by subtracting the initial laccase activity from the free laccase activity. While immobilization laccase yield was estimated by the ratio of immobilized laccase activity to the initial laccase activity and multiplied by 100.

Characterization of Immobilized Laccase on Activated Carbon. FTIR was used to characterize the functional groups of the activated carbon before and after the immobilization process. An activated carbon sample of as much as 0.1 mg was placed on a diamond plate and then IR spectra were recorded in absorption mode with a scan count of 4 per sample and a resolution of 4.0 cm^{-1} in the wave number range of 4000 to 400 cm^{-1} at room temperature using spectrum two Perkin Elmer software.

Dye Decolorization. About 75 mg of laccase-activated carbon was added into 20 mL of 100 mg/L RB5 dye at 100 mL Erlenmeyer flask and covered by a plastic cap. Flask then was incubated at an incubator shaker at 100 rpm, 30 °C for 4 h. Samples were taken at specified time intervals and all samples were centrifuged at 10,000 rpm for 10 min. Finally, the dye decolorization was measured using a UV-Vis spectrophotometer at a wavelength of 599 nm. Activated carbon without laccase and free laccase were used as control. Dye decolorization was calculated using equation (Benkhaya *et al.* 2020).

$$\textit{Decolorization}\ (\%) = \frac{(\textit{Initial absorbance} - \textit{Final absorbance})}{\textit{Initial absorbance}} x\ 100$$

(Benkhaya *et al.* 2020)

RESULT

Laccase Immobilization on Activated Carbon. The changes in functional groups of modified activated carbon and its enzyme immobilization spectrum are shown in Figure 1. The FT-IR spectrum of modified activated carbon (Figure 1a) shows a peak in the 3100-3500 cm^{-1} corresponding to OH vibration, a peak in 1739 cm^{-1} attributed to the carbonyl groups (C=O), and 1219-1369 cm^{-1} associated with phenol groups. After laccase immobilization on activated carbon (Figure 1b), there is an increase in the intensity of peak 3210-3589 cm^{-1} indicating vibration of OH groups and N-H groups from laccase. Additionally, the peak at 1639 cm^{-1} indicated CHO and $CONH_2$ vibrations associated with the activated carbon reaction between glutaraldehyde and the laccase enzyme. The peak at 1031 cm^{-1} also indicated the presence of Si-O covalent binding from activated carbon and APTS. The proposed immobilization process of laccase on activated carbon is shown in Figure 2.

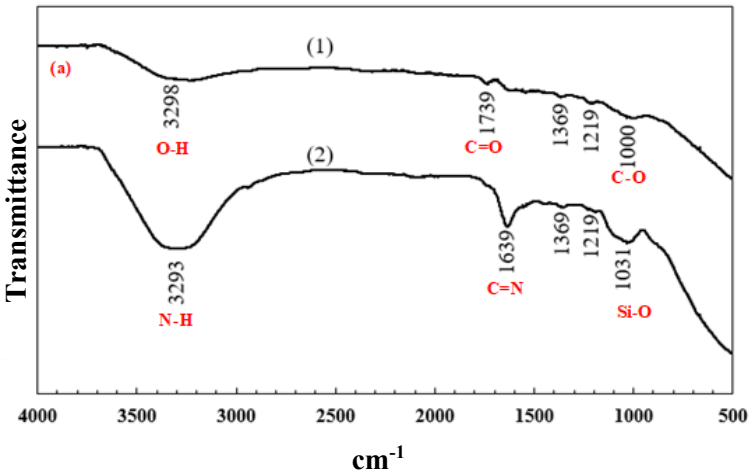

Fig 1 FT-IR spectra of activated carbon (a) and its immobilized enzyme spectrum (b).

To study the effect of silanization agents in the immobilization process, APTS was used at various concentrations: 5, 10, ad 15% (v/v). As shown in Table 1, the immobilization yield increased with the increase in APTS concentration. The highest immobilized laccase yield, at 50.4%, was achieved with an APTS concentration of 15%, corresponding to laccase activity of 3.8 x 10^{-3} U/mL.

Synthetic Dye Decolorization Assay. Laccase-activated carbon modified by various concentrations of APTS was used to decolorize Reactive Black 5 (RB5) dye for 4 h. The solution of RB5 dye before and after immobilized enzyme decolorization is presented in

Figure 3.

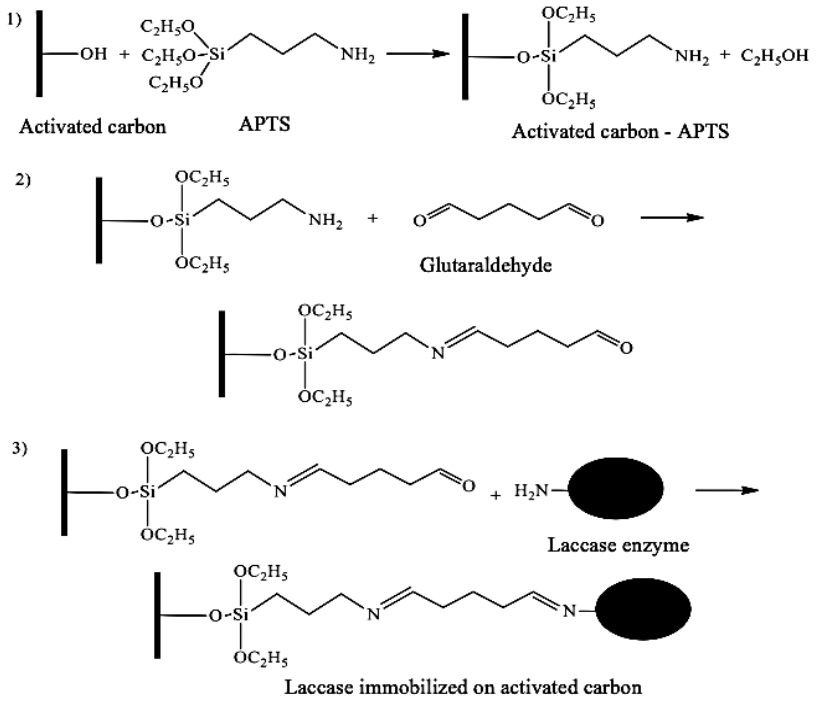

Fig 2 Proposed mechanism of laccase immobilization of activated carbon.

Based on the results, it was confirmed that 4 h is the most suitable time because its prolongation does not increase the biodegradation efficiency (unpublished data). It should be noted that the decolorization efficiency reached 87.5% using immobilized laccase on activated carbon modified by APTS 15% (Figure 4).

The decolorization of RB5 dye was compared using immobilized laccase on activated carbon, free laccase and activated carbon as control. The solution of RB5 dye after a 4 h reaction time is shown in Figure 5. The results showed that activated carbon could adsorb RB5 dye by 73.9% while free laccase decolorized 35.5% of RB5 dye. Enhanced decolorization was observed when laccase was immobilized on activated carbon, resulting in 87.5% decolorization of RB5 (Figure 6).

Table 1 Immobilization yield of laccase on activated carbon at various silanization agent concentration.

APTS Concentration (%)	Initial laccase activity (x 10^{-3} U mL^{-1})	Free laccase activity (x 10^{-3} U mL^{-1})	Immobilized laccase activity (x 10^{-3} U mL^{-1})	Immobilized yield (%)
5	7.6	5.6	2.0	26.8
10	7.6	5.3	2.3	30.7
15	7.6	3.8	3.8	50.4

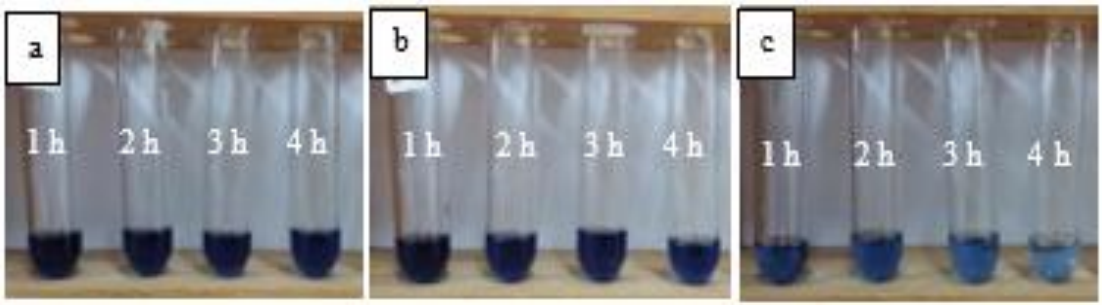

Fig 3 The solution of Reactive Black 5 dye after 4 h reaction using immobilized laccase on activated carbon modified by APTS 5% (a), 10% (b), and 15% (c).

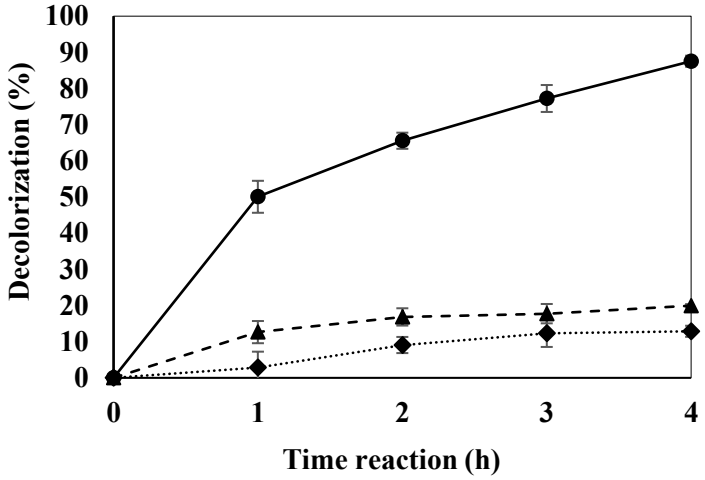

Fig 4 Decolorization percentage of dye after 4 h reaction using immobilized laccase on activated carbon modified by APTS 5% (◆), 10% (▲), and 15% (●).

DISCUSSION

Immobilization through covalent binding between enzyme and carrier is the most stable method that applied in the industrial sector. Modification of the carrier surface is necessary to optimize the covalent binding system. The surface modification of activated carbon is carried out by the silanization process using APTS. Silanization with APTS, a trifunctional organosilane, provides reactive amino groups that are sensitive to the further activation process. Glutaraldehyde is a good cross-linking agent that can react with amine groups of enzyme and carrier surfaces through the formation of imine bases (Pezzella *et al.* 2014). Modification of activated carbon with a functionalizing agent, APTS, provides the link between the carrier surface and the enzyme. Higher APTS concentration provides more enzyme binding sites, thus increasing the yield of immobilized enzymes. Zhang *et al.* (2013) reported that immobilization methods, immobilization carrier materials, and immobilization enzyme loading are important factors for enzyme immobilization. Parameters such as APTS concentration, pH, temperature, and glutaraldehyde concentration also affect the surface properties of the carrier and the yield of immobilization (Pezzella *et al.* 2014).

FTIR analyses were conducted to verify the

laccase the bonding and immobilization of laccase on activated carbon. The important peaks presented in this analysis were at 3100-3500, 1639-1739, and 1000-1031 cm^{-1}. A peak in the 3100-3500 cm^{-1} corresponds to OH vibration, a peak of 1639-1739 cm-1 attributed to the carbonyl groups (C=O), and 1219-1369 cm^{-1} associated with phenol groups (Pallares *et al.* 2008). After laccase immobilization on activated carbon, the intensity of peak 3210-3589 cm^{-1} indicates vibration of OH groups and N-H groups from laccase (El-Batal *et al.* 2014). These results demonstrated the successful immobilization of the laccase enzyme on the activated carbon surface. The immobilization will enhance the decolorization of dyes by simultaneous adsorption by activated carbon and biodegradation by laccase.

The present study shows that the differences between decolorization efficiency using immobilized laccase on activated carbon modified by APTS 5% and 10% is due to the reduced binding site on the carrier surface thus resulting in less immobilized laccase. In another publication, Pezzella *et al.* (2014) showed that perlite modified by APTS at 4% yielded 45% immobilization with 71% of decolorization efficiency. This value was higher than using perlite modified by APTS 0.4%, which resulted in immobilized yield and decolorization efficiency of only 29% and 28%, respectively.

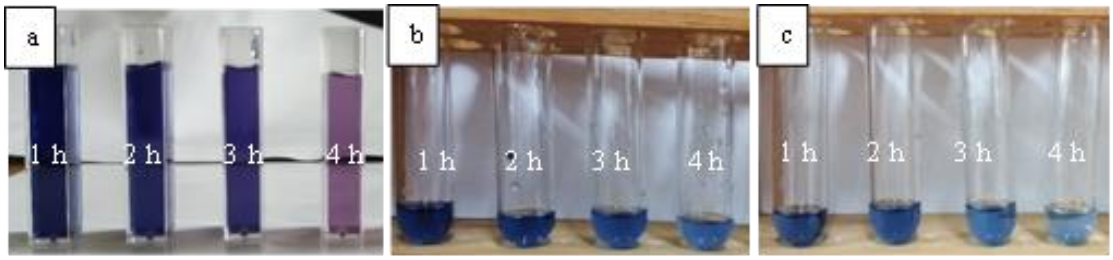

Fig 5 The solution of Reactive Black 5 dye after 4 h reaction using free laccase enzyme (a), activated carbon modified by APTS 15% (b), and immobilized laccase on activated carbon modified by APTS 15%(c).

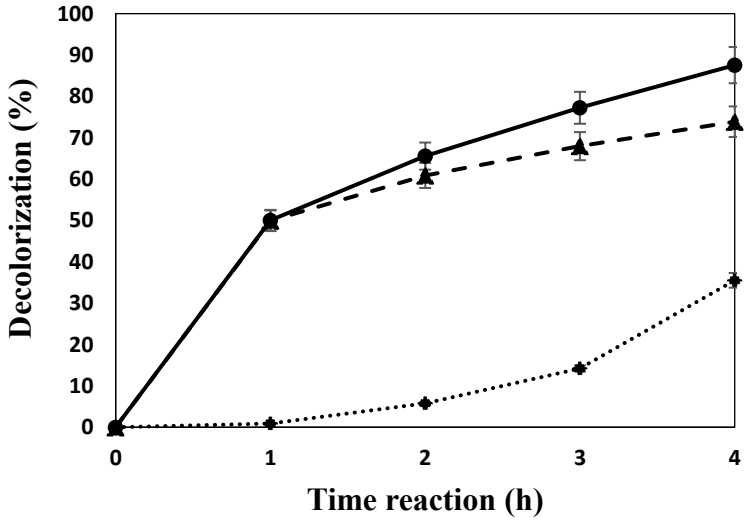

Fig 6 Decolorization percentage of dye after 4 h reaction using free laccase enzyme (◆), activated carbon modified by APTS 15% (▲), and immobilized laccase on activated carbon modified by APTS 15% (●).

Moreover, the decolorization using immobilized laccase in activated carbon was 1.2 and 2.5 times higher compared to the activated carbon without immobilized enzyme and free laccase enzyme, respectively. These results explained that the decolorization process occurs through both adsorption by activated carbon and degradation by laccase. Other research also reported that decolorization or Reactive Blue 19 dye by immobilized laccase on fiber-activated carbon is due to both dye adsorption and degradation of the dye by the laccase enzyme (Khazravi *et al.* 2019).

Decolorization of RB5 dye by using immobilized laccase on activated carbon not only exhibited higher efficiency but also reduced reaction time compared to the free enzyme. In this research, immobilized laccase on activated carbon achieved 87.5% decolorization of RB5 dye within 4 h, whereas free laccase only achieved 35.5% decolorization at the same time reaction. Anita *et al.* (2019) reported that decolorization of RB5 dye by free laccase from *Trametes hirsuta* D7, at the enzyme activity of 0.25 U/mL, achieved more than 80% decolorization 7 h of incubation time at room temperature.

CONCLUSION

Laccase enzyme has been immobilized on activated carbon for Reactive Black 5 dye removal. The highest immobilized laccase-activated carbon yield was 50.4% obtained by activated carbon modified by 15% (v/v) of APTS. The immobilized laccase-activated carbon improved the RB5 decolorization rate by 87.5% compared to free laccase, which was only 35.5% at 4 h of reaction time. The results indicated effective adsorption and degradation of azo dye using the laccase immobilized on activated carbon used in this study.

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