Immobilization of *Trametes hirsuta* D7 in Light Expanded Clay Aggregate for Decolorization of Synthetic Dye

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**Abstract.** Light Expanded Clay Aggregate (LECA) in a granular form was used for the immobilization of fungus *Trametes hirsuta* D7 to decolorize Remazol Brilliant Blue R (RBBR) dye. The efficacy of LECA as a media for immobilization was assessed using steam activation, contact time, immobilization method and dosage of sorbent. Batch experiments were carried out for decolorization and the results showed that activated LECA has the capability to remove 35% RBBR for 24 h of contact time. Non-freeze dry immobilization process demonstrated a higher decolorization than that of freeze dry process with maximum 88% and 22% removal, respectively. Different dosage of activated and non-freeze dry-LECA was tested to achieve optimum decolorization and it was found that 0.2 g/ml dye could remove 76% dye for 3 hours and reached the maximum removal for 94% at 24 hours with high laccase activities (460 U L-1). This study found that fungus *T. hirsuta* D7 and LECA could be used to decolorize RBBR and LECA had the potential to be an alternative matrix for fungi immobilization.

**1. Introduction**

Textile industry, particularly in Indonesia, is a fast-growing industry and has positive impacts to national economy and poverty. However, it also becomes a big threat as significant wastewater produced from its processes. It is estimated that 1000-3000 m3 of wastewater is reproduced after processing 12-20 ton textiles per day while Indonesia produced about 6 million ton of textile products annually [1, 2].

Most of textile wastewater consist of dyes which remain from dying and printing processes. About 10-15% of total dyes used is found in textile wastewater due to the low affinity for textile substrates of dyes hydrolysed form [3, 4]. If the textile wastewater is directly discharged to environment, the unfixed dyes can remain for an extended period of time [5]. The accumulation amount of dyes leads to reduction of light absorption that results in the depletion of dissolved oxygen, causing anoxic condition that are lethal to aquatic organisms. Moreover, the dyes released has toxic and carcinogenic characteristics which are harmful to human health, especially to skin and oral cavity [6, 7].

Physical and chemical decolorization have restrictions such as higher cost, sludge production, other constituent effects and operational limitations [8, 9]. Thus, biological treatment has been attracted to decolourize textile wastewater in particular by using white-rot fungi (WRF). WRF produce Lignin Modifying Enzymes (LMEs) which consist of lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac). LMEs have a low substrate specificity hence they are able to degrade various xenobiotic compounds, such as dyes [4][10, 11].

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Most of decolorization studies using WRF still used liquid or solid culture which still require the development of efficient dyes removal process [4][11][12]. Thus, immobilized culture has received increasing attention as it is simple to reuse, easier solid-liquid separation, reduce clogging and it can perform in continuous-flow system [13]. Moreover, it has been found that immobilized culture could minimize the risk of contamination, resistance to toxic compounds and show a higher activity level [10, 14].

Several experiments have been studied to decolorize using WRF immobilization such as stainless steel sponge, polyurethane foam, nylon sponge [15], alginate [15–18], chitosan [19], carbon supports from lignocellulosic wastes [20], sorghum [21], controlled porosity carrier-silica beads [22] and activated carbon [23]. However, relatively few or none studies have been conducted with WRF immobilized in Light Expanded Clay Aggregate (LECA). LECA is produced from the expansion of a certain type of clay that is shaped as a pellet and heated in a high temperature around 1200°C. LECA’s characteristics are light, porous, non-toxic, chemically inert, has a neutral pH, resilient to frost and chemicals as well as non-biodegradable [24, 25]. LECA is used in gardening as it can retain moisture in the soil, as a media for filtration and other wastewater and water treatment facilities such as heavy metal removal, phosphor, and Polycyclic Aromatic Hydrocarbons (PAHs) [25–27]. However, LECA has not reported as an alternative matrix for fungi immobilization.

This study is aim to investigate the ability of immobilized T. hirsuta D7 in LECA to decolorize synthetic dye, Remazol Brilliant Blue R (RBBR). The efficacy of LECA was assessed using steam activation, contact time, immobilization method (freeze dry and non-freeze dry process) and dosage of sorbent.

2. Materials and methods

2.1 Chemicals and fungal culture preparation
The experiments were conducted in Laboratory of Biomass Process Technology and Bioremediation, Research Center for Biomaterials, Indonesian Institute of Sciences. T. hirsuta D7 was newly isolated from peat swap forest Bengkalis, Riau, Indonesia [28]. LECA were purchased from local company while chemicals such as RBBR was purchased from SIGMA and malt extract, glucose, peptone, tartaric acid, ABTS and acetate buffer were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

2.2. LECA preparation
LECA is used in granular form with the size around 7.8 – 16 mm. Prior to the studies, samples were pre-treated by steam activation at 800-900°C for 1 hour [29]. Next, samples were washed Lawith distilled water to remove dust and were placed in the oven for 24 hours in 60°C to remove extra water and humidity. LECA were placed in container before application.

2.3. Pre-culturing of the fungus in Erlenmeyer flasks
T. hirsuta D7 was grown on Malt Extract Agar (MEA) then incubated at room temperature (25-30°C) for 7 days. Erlenmeyer flasks (100 mL) containing 20 mL MGP medium broth (20 g L⁻¹ malt extract, 20 g L⁻¹ glucoses and 1 g L⁻¹ peptone and was adjusted to pH 4.5 and sterilized in autoclave for 15 minutes at 121°C) was inoculated with three agar plugs from an actively growing fungus on MEA. The Erlenmeyer flasks were capped with cotton to allow passive aeration and incubated statically at room temperature for 7 days. After that, the fungal culture was homogenized by using homogenizer

2.4. Fungi immobilization in LECA
This study used entrapment within porous matrices as the immobilization technique. The prepared LECA samples were added to MGP medium broth in Erlenmeyer flask 250 mL then were sterilized in autoclave for 15 minutes in 121°C. The sterilized LECA were placed in 300 mL-Erlenmeyer flask and homogenate was transferred into the flask until the LECA samples were submerged and they were incubated in reciprocal shaker at 100 rpm and 28°C for 3 days. After that, it was filtered to separate the
liquid and immobilized LECA. Next, the immobilized LECA were processed by two different methods: firstly, dried aseptically at room temperature by ambient air (non-freeze dry) and secondly, dried using freeze dry.

2.5. Synthetic dye decolorization test

Decolorization tests by immobilized *T. hirsuta* D7 in LECA were evaluated by several steps (Figure 1). Decolorization was conducted in reciprocal shaker at room temperature and 100 rpm in 100 mL Erlenmeyer flasks containing an initial volume of 20 mL RBBR solution and 1% (w/v) glucose (for the testes with fungi only). The dye removal was measured at 0, 1, 2, 3, 4, 5 and 24 hours using UV-Vis spectrophotometer (Shimadzu UV-1800) at $\lambda$ max of RBBR 592.5 nm. Prior to the measurement, the samples were centrifuged (WiseSpin CF-10) at 1000 rpm for 10 minutes. The experiments were done triplicate and the reported results are shown as averages with standard deviations. The percentage of decolorization was calculated as follows:

$$
\% \text{ decolorization} = \frac{C_0 - C_i}{C_0} \times 100\%
$$

\(C_0\) = Initial absorbance of dye (Abs)

\(C_i\) = Absorbance of dye at i-hour (Abs)

![Figure 1. Experiment steps for decolorization using immobilized *T. hirsuta* D7 in LECA.](image)

2.6. Enzyme assay

Laccase activity in the dye solution was determined using ABTS (2,2'-azino-di-[3-ethylbenzothiazoline-(6)-sulfonic acid] as substrate. The reaction mixture contained 0.1 M acetate buffer 400µL, 2 mM ABTS 500µL and 100µL dye solution. It was monitored at 420 nm using UV-Vis spectrophotometer and calculated from the molar extinction coefficient ($\epsilon$) of 6.500 M$^{-1}$cm$^{-1}$.

3. Results and discussion

The first experiment was conducted to study the effect of steam activation on the absorption of RBBR by LECA granules. In general, the results showed that activated LECA has a higher dye removal than that of the non-activated LECA (Figure 2). The maximum decolorization was 35% and 29% for activated LECA and non-activated LECA, respectively, which was achieved at 24 hour of contact time. Thus, it indicated that the LECA granules could adsorb the synthetic dye and its removal efficiency increased with steam activation process. This result is in line with Rodriguez-Reinoso et al (1995) which found that steam activation process widens the microporosity and gives a large development of meso- and macroporosity that may contribute for increasing the dye adsorption [29].
Different method of immobilization was studied in order to evaluate the optimum removal dye efficiency. It was tested under the certain condition (dye concentration = 100 mg/dm$^3$, contact time = 4 hours, adsorbent dosage = 0.25 g/mL, and incubation time = 3 days). According to the result, non-freeze dry process demonstrated a higher decolorization than freeze dry process with maximum dye removal was 88% and 22% respectively (Figure 3). Better decolorization was still showed by activated LECA although it was slightly decreased in the non-freeze dry process. Therefore, the activated LECA granules and non-freeze dry process were selected as optimum parameters to be studied in further experiments. Moreover, the higher dye removal also showed that the immobilized fungi involved in the decolorization process.

The effect of adsorbent dosage was studied by using various adsorbent weight in 20 mL of RBBR solution with 1% glucose as a nutrient addition from 1 g to 5 g of activated LECA granules. The results in Figure 4 show that at 24 hours of contact time, all variations of dosage had the maximum decolorization between 91.5 to 95%. However, the fastest dye removal could be achieved at 5 g and 4 g dosage which decolorize 74.5% and 76% RBBR at 3 hours, respectively.
Laccase activities were examined for different adsorbent dosage between 1 to 5 g/20 mL of dye (Table 1). At 1 hour of contact time, the enzyme activity increased with the increasing adsorbent dosage. It is expected that this occurred due to the secretion of laccase enzymes within medium which produced by the immobilized cells [30]. This laccase enzyme is predicted to be involved in the decolorization process since it was reported that laccase can directly oxidize anthraquinone dyes, such as RBBR, which acts as enzyme substrate [12]. Moreover, laccase can also oxidize other various organic and inorganic compounds, including diphenols, polyphenols, diamines and aromatic amines by forming a free radical through losing a single electron from the substrate which may then being oxidized by laccase [31]. Although the value of enzyme activities varies at each contact time, the 4 g of activated LECA with non-freeze dry process showed the optimum parameters for secretion of laccase. It is in accordance with the decolorization process that demonstrated optimum at 4 g/20 mL of dye.

**Table 1. Laccase activity (U/L) immobilized in activated LECA.**

| Immobilized LECA (g) | 1 h  | 2 h  | 3 h  | 4 h  | 5 h  | 24 h |
|----------------------|------|------|------|------|------|------|
| 5                    | 39 ± 2 | 53 ± 4 | 38 ± 2 | 74 ± 4 | 79 ± 4 | 39 ± 4 |
| 4                    | 31 ± 2 | 374 ± 27 | 460 ± 44 | 81 ± 2 | 158 ± 8 | 68 ± 4 |
| 3                    | 17 ± 2 | 419 ± 20 | 49 ± 5 | 302 ± 30 | 95 ± 9 | 31 ± 2 |
| 2                    | 4 ± 2 | 7 ± 1 | 8 ± 1 | 10 ± 1 | 11 ± 1 | 18 ± 2 |
| 1                    | 3 ± 1 | 5 ± 1 | 5 ± 2 | 8 ± 1 | 7 ± 1 | 5 ± 1 |

**Figure 4.** Decolorization of RBBR by *Trametes hirsuta* D7 immobilized into LECA with different adsorbent dosage.

4. **Conclusions**

This study was found that LECA has a potential to be an alternative matrix for fungi immobilization and can be used to decolourize textile dye. Activated LECA is able to decolourize 35% RBBR in 24 hours of contact time and activated immobilized LECA with non-freeze dry process showed better dye removal by 88% maximum decolorization for 4 hours. The optimum decolorization process was given by 0.2 g/mL dye dosage which can remove 76% dye for 3 hours. Further studies are required to optimize process parameters for larger scales as well as it is needed to be tested using textile wastewater sample.
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References
[1] Ghaly A E, Ananthashankar R, Alhattab M, Ramakrishnan VV 2014 Production, characterization and treatment of textile effluents: a critical review J Chem Eng Process Technol 5(1) 1–19
[2] Indonesia C 2018 Industri Tekstil RI: “Hidup Segan, Mati Tak Mau.” https://www.cnbcindonesia.com/news/20180914151304-4-33156/industri-tekstil-ri-hidup-segan-mati-tak-mau. Accessed 26 Aug 201
[3] Hao O J, Kim H, Chiang P C 2000 Decolorization of wastewater Critical reviews in environmental science and technology 30(4) 449–505
[4] Rodriguez E, Pickard M A, Vazquez-Duhalt R 1999 Industrial dye decolorization by laccases from ligninolytic fungi Curr Microbiol 38(1) 27–32
[5] Weber E J and Stickney V C 1993 Hydrolysis kinetics of reactive blue 19-vinyl sulfone Water Research 27(1) 63–67
[6] de Campos Ventura-Camargo B and Marin-Morales M A 2013 Azo dyes: characterization and toxicity – a review Textiles and Light Industrial Science and Technology 2(2) 87–103
[7] Hayat H, Mahmood Q, Pervez A, Bhatti Z A, Baig S A 2015 Comparative decolorization of dyes in textile wastewater using biological and chemical treatment Sep Purif Technol 154 149–153
[8] Van der Zee F P and Villaverde S 2005 Combined anaerobic–aerobic treatment of azo dyes—a short review of bioreactor studies Water Research 39(8) 1425–1440
[9] Robinson T, McMullan G, Marchant R, Nigam P 2001 Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative Bioreour Technol 77(3) 247–255
[10] Couto S R 2009 Dye removal by immobilised fungi Biotechnol Adv 27(3) 227–235
[11] Glenn J K and Gold M H 1983 Decolorization of several polymeric dyes by the lignin-degrading basidiomycete Phanerochaete chrysosporium Appl Environ Microbiol 45(6) 1741–1747
[12] Mechichi T, Mhiri N, Sayadi S 2006 Remazol Brilliant Blue R decolourization by the laccase from Trametes trogii Chemosphere 64(6) 998–1005
[13] Ting Y P and Sun G 2000 Use of polyvinyl alcohol as a cell immobilization matrix for copper biosorption by yeast cells J Chem Technol Biotechnol 75(7) 541–546
[14] Shin M, Nguyen T, Ramsay J 2002 Evaluation of support materials for the surface immobilization and decoloration of amaranth by Trametes versicolor Appl Microbiol Biotechnol 60 218 – 223
[15] Couto S R, Sanromán M A, Hofer D, Gübitz G M 2004 Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus Trametes hirsuta for decolourization of textile dyes Bioreour Technol 95(1) 67–72
[16] Park C, Lee B, Han E J, Lee J, Kim S 2006 Decolorization of acid black 52 by fungal immobilization Enzyme Microb Technol 39(3) 371–374
[17] Yanto D H Y, Zahara S, Laksana R P B, Anita S H, Oktaviann M, Sari F P 2017 Proc. Int. Symp. on Applied Chemistry (Tangerang) vol 1803, ed Silvester Tursilodadi (USA: AIP Publishing) p 020062
[18] Ramsay J A, Mok W H W, Luu Y S, Savage M 2005 Decoloration of textile dyes by alginate-immobilized Trametes versicolor Chemosphere 61(7) 956–964
[19] Asgher M, Noreen S, Bilal M 2017 Enhancing catalytic functionality of Trametes versicolor IBL-04 laccase by immobilization on chitosan microspheres Chem Eng Res Des 119 1–11
[20] Ramírez-Montoya L A, Hernández-Montoya V, Montes-Morán M A, Cervantes F J 2015 Correlation between mesopore volume of carbon supports and the immobilization of laccase from Trametes versicolor for the decolorization of Acid Orange 7 J Environ Manage 162 206–214

[21] Zahmatkesh M, Spanjers H, van Lier J B 2018 A novel approach for application of white rot fungi in wastewater treatment under non-sterile conditions: immobilization of fungi on sorghum Environ Technol 39(16) 2030–2040

[22] Champagne P P and Ramsay J A 2010 Dye decolorization and detoxification by laccase immobilized on porous glass beads Bioresour Technol 101(7) 2230–2235

[23] Sari A A, Hanifah U, Parmawati Y, Permadi R 2018 Development of immobilized activated carbon-enzyme for decolorization of black liquor Key Eng Mater 775 402–407

[24] Shokoohi R, Samadi M T, Samarghandi M R, Ahmadian M, Karimaian K, Poormohammadi A 2017 Comparing the performance of granular coral limestone and Leca in adsorbing Acid Cyanine 5R from aqueous solution Saudi J Biol Sci 24(4) 749–759

[25] Nkansah M A, Christy A A, Barth T, Francis G W 2012 The use of lightweight expanded clay aggregate (LECA) as sorbent for PAHs removal from water J Hazard Mater 217 360–365

[26] Malakootian M, Nouri J, Hussain H 2009 Removal of heavy metals from paint industry’s wastewater using Leca as an available adsorbent Int J Environ Sci Technol 6(2) 183–190

[27] Johansson L 1997 The use of leca (light expanded clay aggregates) for the removal of phosphorus from wastewater Wat Sci Tech 35 87–93

[28] Hidayat A, Yanto D H Y 2018 Biodegradation and metabolic pathway of phenanthrene by a new tropical fungus, Trametes hirsuta D7 J Environ Chem Eng 6 2454–2460

[29] Rodríguez-Reinoso F, Molina-Sabio M, González M T 1995 The use of steam and CO2 as activating agents in the preparation of activated carbons Carbon 33 15–23

[30] Wesenberg D, Kyriakides I, Agathos S N 2003 White-rot fungi and their enzymes for the treatment of industrial dye effluents Biotechnol Adv 22 161–187

[31] Kiiskinen, L L, Viikari, L, Kruus K 2002 Purification and characterisation of a novel laccase from the ascomycete Melanocarpus albomyces Appl Microbiol Biotechnol 59 198–204