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Judul Artikel : Effects of Mediators for Ligninolytic Enzyme Production and Kinetic Studies on Degradation of Pentachlorobenzene by *Trametes versicolor* U80

Nama Jurnal : Water, Air, Soil Pollution

Reputasi : Terindeks Scopus Q3 (SJR=0,59)

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papernya

ajeng arum sari <ajeng_as@yahoo.co.id> Mon, Apr 11, 2016 at 4:09 PM
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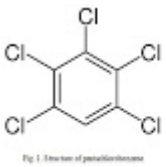
ini papernya. untuk bagian2 tertentu belum aku revisi yaa.. makasih.

6 attachments



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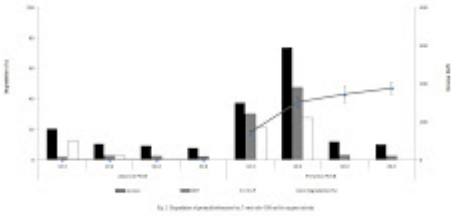

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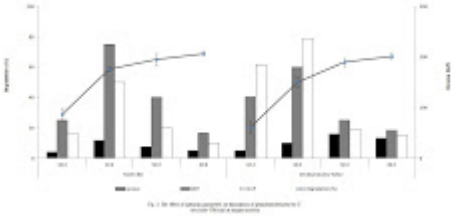

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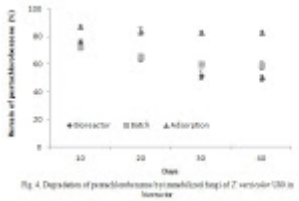

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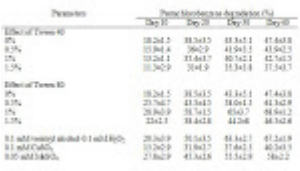

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WATE <em@editorialmanager.com> Fri, Apr 15, 2016 at 9:24 AM
Reply-To: WATE <mariecres.briones@springer.com>
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Submission ID: WATE-D-16-00716
Re: "Effects of Mediators for Ligninolytic Enzyme Production and Kinetic Studies on Degradation of Pentachlorobenzene by Trametes versicolor U80"
Full author list: Ajeng Arum Sari; Hasbi Yasin; Sanro Tachibana; Tony Hadibarata

Dear Mr Hasbi Yasin,

We have received the submission entitled: "Effects of Mediators for Ligninolytic Enzyme Production and Kinetic Studies on Degradation of Pentachlorobenzene by Trametes versicolor U80" for possible publication in Water, Air, & Soil Pollution, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr. Ajeng Arum Sari who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

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To Prof. Sanro Tachibana,

Hello, sensei. How are you doing? Last week I got email from editor about asking revision of manuscript. I revised it and please find our revised manuscript. By the way, thank you for asking Sumitomo Foundation. I will try another research grant later. Thank you so much.

Sincerely yours,

Ajeng Arum Sari

From: ajeng arum sari
Sent: Friday, June 24, 2016 2:30 PM
To: 橘 燦郎
Subject: published paper

To: Prof. Sanro Tachibana,

Dear Sensei, how are you doing? I would like to inform you that our manuscript has been published in AIP Conference Proceedings. http://scitation.aip.org/content/aip/proceeding/aipcp/10.1063/1.4953477

Another manuscript about pentachlorobenzene degradation is still under review. I do not know why it takes so long time (more than 2 months). Now, I am preparing our manuscript about dioxane degradation. I will send the draft later.

By the way, several days ago I read about grant for environmental research from Sumitomo Foundation. Unfortunately, all documents should be applied in Japanese. I could not understand Japanese so I can not apply it.

Thank you for your understanding, Sensei.

Sincerely yours,

Ajeng Arum Sari

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2 attachments

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Effects of Mediators for Ligninolytic Enzyme Production and Kinetic Studies on Degradation of Pentachlorobenzene by *Trametes versicolor* U80

Ajeng Arum Sari, Hasbi Yasin, Sanro Tachibana, Tony Hadibarata

Fig. 2.

The % of degradation is the highest after some time (not at the beginning and not at the end). After maximum od degradation, microbes loose their food so % of degradation decrease. Figure 2 is correct by my opinion.

Thank you for your opinion and I also added one sentence (line 173)

Fig. 4:

Figure 4 is not so clear for me, because in title is written that they used bioreactor, but on the figure are also presented results from batch and adsorption system. Otherwise results are OK, because in bioreactor bioremediation is the best due to good oxigen distribution, so during the time the most of pentachlorobenzene is destroyed.

Thank you for your suggestion. I made mistake in the title for Fig. 4. I edited it. “Fig. 4. Degradation of pentachlorobenzene by immobilized fungi of *T. versicolor* U97”

Tables:

For Tables I have no comment, because I think that they are good. Tween that they used are surfactants and there are parameters of different types of Tween and different concentrations.

Thank you for your opinion and I agree with you. Tween is good mediators to enhance the degradation of pollutant by fungi.

Title page

Manuscript type

Original Paper

Effects of Mediators for Ligninolytic Enzyme Production and Kinetic Studies on Degradation of Pentachlorobenzene by *Trametes versicolor* U80

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Abstract

Pentachlorobenzene is one new POPs that has been recently added to the Stockholm Convention on Persistent Organic Pollutants (POPs). Based on this reason, one treatment having ability to degrade this compound is needed. The microbiological process by using white-rot fungus was used in this experiment. Free cell of *Trametes versicolor* U80 degraded pentachlorobenzene 43% in liquid medium at 40 d incubation. The rapid initial uptake of pentachlorobenzene was obtained in the first 20 d. The results based on ionization potential and the partial least square function indicated that both enzymatic systems of lignin peroxidase and P-450 monooxygenase involved in the degradation of pentachlorobenzene. By using addition of Tween 80, $MnSO_4$, and veratryl alcohol, degradation of pentachlorobenzene could be improved. Based on kinetic study, the use of 1% of Tween 80 showed the highest degradation rate (2.0619/day) and the degradation of pentachlorobenzene by 50% can be shortened up to 24 days. Application of *T. versicolor* U80 in soil and bioreactor degraded pentachlorobenzene 43% and 50% at 40 d, respectively. *T. versicolor* U80 shows good capability degrading pentachlorobenzene in soil and bioreactor although it is lower than in liquid due to the difference of pollutant accessibility and transfer oxygen. Finally, strain *T. versicolor* U80 can be proposed as a excellent candidate for remediation application in pentachlorobenzene pollution.

Keywords: *Trametes versicolor*; pentachlorobenzene; lignin peroxidase; Tween 80; kinetic study

1. Introduction

In addition to the original Stockholm Convention on Persistent Organic Pollutants (POPs), nine new POPs have been recently added, including pentachlorobenzene. This

compound has also been proposed for inclusion in the POPs protocol of the Longrange Transport of Atmospheric Pollutants Convention of the UNECE. In the past, pentachlorobenzene was used to reduce the viscosity of polychlorinated biphenyls (PCBs) products during heat transfer and it was also used in electrical equipment mixed with PCBs (Bailey et al. 2009). This compound can also be produced as a byproduct by industrial processes using chlorine and carbon. Pentachlorobenzene 0.4 ng/L was found in water and sediment in the Yangtse River near Nanjing, China (Jiang et al. 2000). Because of its persistent, long-range transportable nature and toxic biological effects, the presence of pentachlorobenzene in the environment should get attention. Bailey et al. (2009) stated that based on its characteristics, the naturally degradation of pentachlorobenzene in the water and soil is estimated to be months to years. Based on this reason, one treatment having ability to degrade this compound is needed.

The microbiological process for degradation of toxic organic pollutant is now considered as a promising method for the problem of environmental pollution. Bacteria has ability to degrade di- and trichlorobenzene even it has low activity to degrade highly chlorinated benzenes (Takagi et al. 2009). White-rot fungus *Tramates versicolor* has ability to degrade 1,2,3-trichlorobenzene and 1,2,4-trichlorobenzene by using P-450 monooxygenase (Marco-Urrea et al. 2009). However, white-rot fungi have been extensively studied for pollutant removal because they mainly produce ligninolytic enzymes i.e. laccase, lignin peroxidase, and manganese peroxidase. Unfortunately, few researches have studied pentachlorobenzene degradation.

Laccase is *N*-glycosylated extracellular multicopper oxidases that play a key role in the depolymerization of lignin (Wells et al. 2006; Hadibarata and Nor 2014). Manganese peroxidase is a low molecular weight diffusible ligninolytic oxidant, which oxidize Mn^{2+} to Mn^{3+}. It was secreted in carbon and nitrogen limited media and enhanced with

supplementation of Mn^{2+} and veratryl alcohol (Asgher et al. 2008). Lignin peroxidase is capable of mineralizing a variety of aromatic compounds (Shrivastava et al. 2005). This enzyme is effective for degradation of pollutant in the presence of hydrogen peroxide and mediators. The role of ligninolytic enzymes in the degradation of pentachlorobenzene mainly depends on the composition of the culture medium and its mediators. The redox mediators have the potential to mediate oxidation reaction between a pollutant and an enzyme and enhance the enzymatic activity (Yamanaka et al. 2008; Jamal et al. 2011).

Oil palm empty fruit bunches (OPEFB) contains lignin that used for pre-grown source of white-rot fungi during degradation of pollutant (Sari et al. 2014). A study for degradation of pentachlorobenzene is soil is necessary to be conducted because the possibility of this pollutant is settled in soil. However, the extremely low solubility of pentachlorobenzene should get the attention. On the other hand, for technical application, immobilized fungi to degrade organopollutant compounds has been also developed. Immobilized fungi offers advantages such as easy recovery, easy packaging, short retention time, and protection of cells from pollutants (Sari et al. 2015).

Although biodegradation of organochlorine compounds has been widely observed, the effect of mediators on degradation of pentachlorobenzene has not been extensively investigated. Kinetic studies were performed by determination of the residual pentachlorobenzene concentration in samples collected during degradation. This is the first study to evaluate the ability of *Trametes versicolor* to degrade pentachlorobenzene based on its degradation kinetics. In this study, the potential of free cells and immobilized fungus of *Trametes versicolor* U80 to degrade pentachlorobenzene in liquid medium (batch and bioreactor) and soil was evaluated. This study also investigated the enzymatic activities involving the degradation of pentachlorobenzene after addition of several mediators. In order

100 to understand the fate of pentachlorobenzene, the biodegradation mechanisms for its
101 decomposition was clarified.
102
103 **2. Materials and methods**
104 2.1 Chemicals and preparation of fungus

105 Pentachlorobenzene and all solvents were purchased from Wako Pure Chemical
106 Industry, Osaka, Japan. The structure of pentachlorobenzene is shown in Fig. 1. Oil palm
107 empty fruit bunches (OPEFB) was obtained from PTPN V, Sumatera, Indonesia. *Trametes*
108 *versicolor* U80 was collected from decaying wood which native to Matsuyama, Japan and
109 preserved in Faculty of Agriculture, Ehime University, Japan. This strain cultured on a malt
110 extract agar medium was stored at 25° C for several days and kept at low temperature 4° C.

111 2.2 Degradation test with pentachlorobenzene
112 A malt extract liquid medium containing (in g/L) malt extract (20), glucose (15), and
113 polypeptone (1) was used for degradation experiments (Sari et al. 2015). Three 5 mm plugs
114 were added into the Erlenmeyer flask containing 20 mL of medium. Each inoculated flasks
115 was pre-incubated for 7 d. At the incubation stage, 0.1 mM of pentachlorobenzene diluted in
116 dimethylformamyde was added to the Erlenmeyer flasks. As the control, fungal culture after
117 pre-incubation was autoclaved and pentachlorobenzene was added. The mediators
118 ligninolytic activity enhancer were used to know the change of enzyme activity affecting
119 degradation of pentachlorobenzene. Several concentrations of Tween 40 and Tween 80 (0.5%,
120 1%, and 1.5%) were added to the cultures of *T. versicolor* U80 in liquid medium,
121 respectively. Furthermore, 0.1 mM of $CuSO_4$, 0.05 mM $MnSO_4$, or 0.1 mM veratryl alcohol-
122 0.1 mM H_2O_2were added to the cultures in liquid medium.

Soil from paddy field in the Faculty of Agriculture, Ehime University was used for this experiment. It was air-dried and sieved through a fraction passing Ø 3 mm mesh and stored at room temperature before use. Thirty grams sterilized soil was mixed with 3 g of *T. versicolor* U80 pre-grown in OPEFB, 10 ppm pentachlorobenzene diluted in DMF, 5% (w/w) glucose, and 10% (w/w) nutrient source called shiitake no sato. Control treatment was performed with only soil medium and 10 ppm of pentachlorobenzene without inoculated fungus.

A 45 mL glass column was used for the bioreactor. Growing fungus in malt extract medium was homogenized 10,000 rpm for 10 min and then used for the inoculum (Sari et al. 2015). 1.5% sodium alginate was mixed with crude fungal and then droply added into 0.1 M $CaCl_2$ diluted in water. The beads were added into the column and the bioreactor was filled up with 100 mL pentachlorobenzene solution (final concentration 0.1 mM). The flowrate used was 1 mL min^{-1}. All the samples were incubated in the dark place at 25 °C for 10-40 d.

2.3 Pentachlorobenzene degradation analysis:

After harvesting, the culture from liquid medium and bioreactor were extracted using ethyl acetate three times. On the other hand, the sample in soil was extracted by using soxhlet apparatus with dichloromethane for 16 h. All samples were purified by column chromatography with C200 silica gel (hexane:dichloromethane, 9:1). GC-MS Shimadzu QP-2010 was dissolved used to analyze samples. It was equipped with a TC-1 column (30 m, id: 0.25 mm), helium as a carrier gas, a flow rate 1.5 mL min^{-1} with column pressure 100 kPa, and interface temperature at 120°C. The temperature program was started at 120°C hold for 2 minutes, raised to 180°C with a rate 20°C min^{-1}, then 2°C min^{-1} to 210°C, then 5°C min^{-1} to 310°C, and finally maintained for 3 min to allow eluting peak to exit the column. Aliquot of 1 µL of sample was injected into the chromatographic system.

148

149 2.4 Enzyme assays

150 Lignin peroxide (LiP), manganese peroxide (MnP), and laccase were assayed by
151 determination of absorbance of sample using a Shimadzu UV/Vis-1600 spectrophotometer.
152 In the harvest time, the culture from liquid medium were filtered on a filter paper. On the
153 other hand, 5 g of the culture from soil was diluted with 30 ml distilled water, and then
154 homogenized at 10,000 rpm for 10 min. After mixing, the mixture was filtrated (Sari et al.
155 2013). MnP activity due to oxidation of 2,6-dimethoxyphenol malonate buffer in $MnSO_4$
156 solution was assayed at 470 nm (Takano et al. 2004). LiP activity was determined by
157 monitoring the formation of 2 mM H_2O_2 and LiP buffer at 310 nm (Collins et al. 1997).
158 Laccase activity was determined by syringaldazine oxidation at 525 nm (Zavarzina and
159 Zavarzin 2006). The enzyme activities were expressed in Unit/liter.

160

161 2.7. Statistical analyses

162 All data were expressed as mean ± SD (standard deviation) from triplicate experiments.
163 Partial least square and linear regression to obtain kinetic study were calculated by using
164 MINITAB 17.

165

166 **3. Results and discussion**

167 3.1 Degradation of pentachlorobenzene in liquid medium and its mechanisms

168 In control experiment, pentachlorobenzene was degraded only 5%, so that the
169 adsorption mechanism by using the dead fungal was not occurred. The result explains that
170 biosorption was not playing a role in bioremoval mechanism. Fig. 2 shows that
171 pentachlorobenzene was degraded by approximately 43% during the 40 d incubation period.
172 The rapid degradation of pentachlorobenzene was obtained in the first 20 d, and then

followed by a slower rate up to 40 d. The slower degradation rate was caused by the decreasing of nutrient source. A few informations providing degradation of pentachlorobenze by white-rot fungi were obtained. Nineteen strains of basidiomycetes were found to be capable of growing in soil contaminated hexachlorobenzene (HCB) with concentration range of 5000-50,000 mg of HCB kg^{-1} soil. *Psilocybe castanella* CCB444 and *Lentinus zeyheri* CCB274 were the most capable of degrading HCB in soil during a 65-day study period (Matheus et al. 2000). *T. versicolor* (ATCC42530) has able to degrade 1,2,3- and 1,2,4-trichlorobenzene in initial concentration 6 mg L^{-1}. After 7 d of incubation, the percent degradation of 1,2,3- and 1,2,4- trichlorobenzene were 91.1% and 79.6%, respectively (Marco-Urrea et al. 2009).

Trametes versicolor U80 secreted all enzymes during the absence and presence of pentachlorobenzene on days 10 and 20. The enzyme activity increased 5-10 fold in the presence of pentachlorobenzene at 20 d and this means that catalysis of the decomposition of the recalcitrant aromatic compounds by *T. versicolor* U80 is affected by ligninolytic enzymes. This result was also in line with the rapid degradation at that days.

The pollutant oxidation by white-rot fungi are not rapid but efficient, but they are very nonspesific (Hammel 1995). Organopollutants are usually chemically resistant because of delocalization of their energy and, moreover, the dense clouds of Π-electrons on both sides of the ring structures make them highly resistant to nucleophilic attack (Cajthaml and Svobodová 2012). Therefore, ligninolytic enzymes are needed to breakdown this compound through reaction with electron in ring structure of aromatic compounds. However, these enzymes require the presence of H_2O_2 as the electron acceptor to oxidize organopollutants (Ruiz-Duenas and Martinez 2009). *Phanerochaete chrysosporium* degraded several polycyclic aromatic hydrocarbons (PAHs) by using LiP and MnP (Hammel 1995). LiP oxidize certain PAHs directly based on their ionization potential, whereas MnP co-oxidize

198 them indirectly during enzyme-mediated lipid peroxidation. The mechanism involves
199 applying required energy to remove one-electron, so-called ionization potential and
200 transforms substrates into radical, which used to degrade pollutant compounds.
201 Pentachlorobenzene has ionization potential 8.8-9.21 eV. LiP-H_2O_2 catalyze the oxidation of
202 pentachlorobenzene based on ionization potential of pollutant. Since ionization potential LiP
203 7.35 eV, it seems that pentachlorobenzene was difficult to be degraded by LiP itself. It
204 indicates that degradation of pentachlorobenze is not only affected by LiP. The another
205 possibility is role of P450-monooxygenase. Ligninolytic fungi has able to decompose
206 organopollutant using ligninolytic enzymes and cytochrome P-450 monooxygenase
207 (Cajthaml, 2015). To confirm this hypotesis, partial least square analysis was used later.
208
209 3.2 Effects of mediators on degradation in liquid medium
210 In order to demosntrate enhancement the ligninolytic activity of *T. versicolor* U80,
211 some mediators were used in this study. The surfactans as mediators, Tween 40 and Tween
212 80 in several concentrations, were employed on degradation of pentachlorobenzene in liquid
213 medium (Table 1). The surfactant is easily catabolized without any effect on the production
214 ligninolytic enzymes for degradation of pollutant compounds (Leonardi et al. 2007). The
215 result showed that 1% Tween 80 improved the degradation 1.5 fold. However, addition of
216 Tween 40 did not improve degradation of pentachlorobenzene.
217 The bioavailability of a pollutant is one of the factors which determines the success of
218 bioremediation approaches (Leonardi et al. 2007). The objectives in using mediators to
219 enhance bioremediation processes through improvement solubilization of pollutant in the
220 liquid phase (Fava et al. 2004). The structure of a Tween consist of a nonionic
221 polyoxythylene-sorbitan head group and a hydrophobic linear hydrocarbon chain where the
222 difference between Tween 40 (monopalmitate) and Tween 80 (monooleate) is in their

hydrophobic. The growth of *T. versicolor* U80 was not affected by the presence of Tween 80. By contrast, Tween 40 suppressed the growth of this strain. Degradation of phenanthrene by *Pleurotus ostreatus* did not improve with addition of Tween 40 at 14 days was also obtained by Marquez-Rocha et al. (2000).

Presence of Tween 80 caused the bioavaibility of pentachlorobenzene more increased which has high stability in the media. Tween 80 as surfactants could reduce surface and interfacial tensions and also increase the solubility and mobility of hydrophobic organic compounds (Singh et al. 2007). Addition of 1% Tween 80 affected on the highest degradation of pentachlorobenzene. After addition of this surfactant, enzyme activity of *T. versicolor* U80 was increased (Fig. 3). The presence of a monounsaturated acryl chain in Tween 80 also could be the possible occurrence of peroxidation reactions in process (Zheng and Obbard 2001). Venkatadri and Irvine (1990) stated the possible effects of Tween 80 are protecting ligninase from being mechanically inactivated, increasing ligninase activity by hydrolysis mechanism, increasing an extracellular energy source in its hydrolysis products during secondary metabolism.

Addition of other mediators to enhance the ligninolytic enzymes resulted the change of degradation of pentachlorobenzene (Table 1). These mediators act as natural diffusible redox mediators of ligninolytic enzymes. The addition of 0.1 mM $CuSO_4$ to the reaction mixtures had no effect on improvement pentachlorobenzene degradation even the growth of fungus was not suppressed. It indicated that laccase has no role in this degradation. Laccase is more difficult to degrade some xenobiotic compounds than LiP because laccase ionization potential is lower than that of Fe^{3+} from LiP (Li et al. 1999). Addition of $MnSO_4$ slightly improved the degradation of pentachlorobenzene. Addition of $MnSO_4$ to induce MnP improved degradation of pollutant (Zhao et al. 2010; Marco-Urrea and Reddy 2012). MnP acts by oxidizing Mn^{2+} to Mn^{3+} which then diffuses into the pollutant. Reaction between

248 veratryl alcohol and hydrogen peroxide enhanced LiP production in pentachlorobenzene
249 degradation. This result affected in the increasing degradation of pentachlorobenzene (Fig. 3).
250 Veratryl alcohol naturally presence in white-rot fungi as a secondary metabolite. It is useful
251 as a mediator for the degradation of lignin. Futhermore, it protects LiP against inactivation
252 (Christian et al. 2005). However, due to the short life span of veratryl alcohol cation radical,
253 the addition of veratryl alcohol is needed to improve degradation of pollutants. The
254 formation of a veratryl alcohol-LiP complex and the production of veratryl alcohol radicals
255 may play a role in the enhanced activity of LiP (Vazquez-Duhalt et al. 1994). Furthermore,
256 partial least squares regression showed the most important enzyme for the degradation of
257 pentachlorobenzene by *T. versicolor* U80 was LiP. The regression equation is given below.

258 $$\text{Degradation (\%)} = 29.4541 - 0.0118\ \text{Laccase} + 0.0138\ \text{MnP} + 0.1517\ \text{LiP} \quad (1)$$

259

260 LiP catalyses the oxidation of non-phenolic aromatic compounds ring opening and
261 side chain cleavage reactions, C-C cleavage in side chain of lignin, cleavage of aromatic ring,
262 oxidation of benzyl alcohols to aldehydes, oxidative dechlorination reactions, and
263 methoxylations. The constant 29.45 is a value that can not be represented by the ligninolytic
264 enzymes. The study assumed that this enzyme was P-450 monoxygenase. Recent studies
265 shows ligninolytic fungi have P-450 type genes that are useful to attack many pollutant
266 compounds in the early step of degradation (Cajthaml 2015). It catalyses hydroxylation and
267 reduction. The efficient functioning of an intracellular catabolic system was related with
268 adsorbed ability of pollutant compound. The proteins associated with fungal membranes
269 probably play a major role in the steps of degradation (Shary et al. 2008). *Phlebia brevispora*
270 degraded polychlorinated biphenyls (PCB) by using P-450 monooxygenase and formed the
271 methoxy metabolites via hydroxylated PCBs (Kamei et al. 2006). The role of P-450
272 monooxygenase in degrading pollutant, chlorobenzoates, were also reported by Stella et al.

(2013). Purfied P-450 monooxygenase has able to degrade chlorobenzoates upon 1 h incubation through hydroxylated derivatives.

3.3Degradation kinetics of pentachlorobenzene

Aplication of kinetic study is conducted to estimate kinetic parameters for growth of fungi on a pollutant and subtrates (i.e. mediators) (Helbling 2015). Degradation rate constants and half live periods of pentachlorobenzene were calculated from the data in Table 1, using first-order reaction model (Zhang et al. 2016). Table 2 shows the calculated results of degradation kinetics. The degradation of pentachlorobenzen in liquid medium during 40 days incubation fitted well to first-order reaction model. It was reflected by the regression coefficient R^2 (0.9487-0.9797). The use of 1% of Tween 80 showed the highest degradation rate (2.0619/day). On the other hand, the use of 1.5% of Tween 40 showed the lowest degradation rate (1.0960/day). Our result showed that the different mediators led to the different degradation kinetics. Furthermore, the time needed to degrade pentachlorobenzene by 50% was 37 days, while after addition of 1% Tween 80, degradation of pentachlorobenzene can be shortened up to 24 days. These results were consistent with the previous result in section 3.2. Tween 80 enhanced an extracellular energy to break down pentachlorobenzene during secondary metabolism.

3.4 Degradation of pentachlorobenzene in soil

In 30 days, *T. versicolor* U80 pre-grown in oil palm empty fruit bunches (OPEFB) had ability to degrade pentachlorobenzene in soil by 40% (Fig. 4). Previously, OPEFB was used as pre-grown source for *T. versicolor* U97 to degrade DDT (Sari et al. 2013). It was concluded that OPEFB mechanisms during the degradation of DDT were adsorption, carbon source utilization, and stimulation of ligninolytic systems used for secondary metabolism

(Sari et al. 2014). Degradation in soil was still lower than in liquid medium even the concentration of pentachlorobenzene in liquid medium was higher than in soil. The high electrophonic chlorine atoms on the benzene ring make aerobic oxidative degradation of pentachlorobenzene became difficult (Kengara et al. 2013). The low accessibility of pentachlorobenzene in soil because of limitation of mass transfer and low diffusion into organic matter that affected pentachlorobenzene is retained in the soil pores caused desorption hysteresis (Mougin et al. 1997; Fujian et al. 2001; Gao and Jiang et al. 2010). Pollutants may have different concentration-dependent relationships with soil conditions, which influences the fate of organic chemicals in soil (Zhang et al. 2006).

Furthermore, the same result with the enzyme activity in liquid medium, *T. versicolor* U80 in soil secreted the high enzyme activities at 10 and 20 d. However the degradation was not only affected by the enzyme activity but also depended on the medium and accessibility attack enzyme to pollutant.

3.5 Degradation of pentachlorobenzene in bioreactor:

Continuous flow column system was designed to determine the ability of immobilized of microbial strain at the water interface to degrade pentachlorobenzene. Fig. 5 shows that degradation by immobilized fungi in bioreactor was higher than in the batch condition after 10 d meaning that transfer oxygen in bioreactor process was higher than in batch condition, and it will affect on the improvement of pentachlorobenzene degradation.

4. Conclusion

Free cell of *Trametes versicolor* U80 degraded pentachlorobenzene by 43% in liquid medium during 40 days incubation. Based on the ionization potential and the partial least square function, the results indicated that both lignin peroxidase and P-450 monooxygenase

enzymatic systems involved in the degradation of pentachlorobenzene. By using the addition of Tween 80, $MnSO_4$, and veratryl alcohol, the degradation of pentachlorobenzene could be improved. Based on kinetic study, the use of 1% of Tween 80 showed the highest degradation rate (2.0619/day) and the degradation of pentachlorobenzene by 50% can be shortened up to 24 days. Application of *T. versicolor* U80 in soil and bioreactor degraded pentachlorobenzene 43% and 50% at 40 d, respectively. Furthermore, the metabolite products of pentachlorobenzene after the degradation is still needs further investigation.

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REFERENCES

Asgher, M., Bhatti, H.N., Ashraf, M., Legge, R.L. (2008). Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. *Biodegradation, 19*, 771–783.

Bailey, R.E., van Wijk, D., Thomas, P.C. (2009). Sources and prevalence of pentachlorobenzene in the environment. *Chemosphere, 75* (5), 555-564.

Cajthaml, T., Svobodová, K. (2012). Biodegradation of aromatic pollutants by ligninolytic fungal strains. In *Microbial Degradation of Xeobiotics*. Singh, S.N. (ed). Berlin, Germany: Springer-Verlag, 291-316.

Cajthaml, T. (2015). Biodegradation of endocrine-disrupting compounds by ligninolytic fungi: mevhanisms involved in the degradation. *Environmental Microbiology, 17* (12), 4822-4834.

Christian, V., Shrivastava, R., Shukla, D., Modi, H., Vyas, B.R.M. (2005). Mediator role of veratryl alcohol in the lignin peroxidase-catalyzed oxidative decolorization of Remazol Brilliant Blue R. *Enzyme and Microbial Technology, 36* (2), 327-332.

Collins, P.J., Field, J.A., Teunissen, P., Dobson, A.D.W. (1997). Stabilization of lignin peroxidase in white rot fungi by tryptophan. *Applied and Environmental Microbiology, 63* (7), 2543-2548.

Fava, F., Berselli, S., Conte, P., Piccolo, A., Marchetti, L. (2004). Effects of humic substances and soya lecithin on the aerobic bioremediation of a soil historically

355 contaminated by polycyclic aromatic hydrocarbons (PAHs). *Biotechnology and*
356 *Bioengineering, 88*, 214–223.

357 Fujian, X., Hongzhang, C., Zuohu, L. (2001). Solid-state production of lignin peroxidase
358 (LiP) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium* using steam-
359 exploded straw as substrate. *Bioresource Technology, 80* (2), 149-151.

360 Gao, H.J., Jiang, X. (2010). Effect of initial concentration on adsorption-desorption
361 characteristics and desorption hysteresis of hexachlorobenzene in soils. *Pedosphere, 20* (1),
362 104-110.

363 Hadibarata, T., Nor, N.M. (2014). Decolorization and degradation mechanism of Amaranth
364 by *Polyporus* sp. S133. *Bioprocess and Biosystems Engineering, 37* (9), 1879-1885.

365 Hammel. K.E. (1994). Mechanisms for Polycyclic Aromatic Hydrocarbon degradation by
366 ligninolytic fungi. *Environmental Health Perspectives, 103*, 41-43.

367 Helbling, D.E. (2015). Bioremediation of pesticide-contaminated water resources: the
368 challenge of low concentrations. *Current Opinion in Biotechnology, 33*, 142-148.

369 Jamal, F., Qidwai, T., Pandey, P.K., Singh, R., Singh, S. (2011). Azo and anthraquinone dye
370 decolorization in relation to its molecular structure using soluble *Trichosanthes dioica*
371 peroxidase supplemented with redox mediator. *Catalysis Communication, 12*, 1218-1223.

372 Jiang, X., Martens, D., Schramm, K.W., Kettrup, A., Xu, S.F., Wang, L.S. (2000).
373 Polychlorinated organic compounds (PCOCs) in waters, suspended solids and sediments
374 of the Yangtse River. *Chemosphere, 41*, 901-905.

375 Kamei, I., Sonoki, S., Haraguchi, K., Kondo, R. (2006). Fungal bioconversion of toxic
376 polychlorinated biphenyls by white-rot fungus, *Phlebia brevispora*. *Appl Microbiol*
377 *Biotechnol, 73*, 932-940.

378 Kengara, F.O., Doerfler, U., Welzl, G., Ruth, B., Munch, J.C., Schroll, R. (2013). Enhanced
379 degradation of ^{14}C-HCB in two tropical clay soils using multiple anaerobic-aerobic cycles.
380 *Environmental Pollution, 173*, 168-175.

381 Leonardi, V., Sasek, V., Petruccioli, M., Annibale, A.D., Erbanova, P., Cajthaml, T. (2007).
382 Bioavailability modification and fungal biodegradation of PAHs in aged industrial soils.
383 *International Biodeterioration & Biodegradation, 60*, 165–170.

384 Li, K., Xu, F., Eriksson, K.E.L. (1999). Comparison of fungal laccases and redox mediators
385 in oxidation of a nonphenolic lignin model compound. *Applied and Environmental*
386 *Microbiology, 65* (6), 2654–2660.

387 Marco-Urrea, E., Perez-Trujillo, M., Caminal, G., Vicent, T. (2009). Dechlorination of 1,2,3-
388 and 1,2,4-trichlorobenzene by the white-rot fungus *Trametes versicolor*. *Journal of*
389 *Hazardous Materials, 166*, 1141-1147.

390 Marco-Urrea, E., Reddy, C.A. (2012). Degradation of chloro-organic pollutants by white rot
391 fungi. In S.N. Singh (Ed). Microbial Degradation of Xenobiotics, 31-66. Heidelberg:
392 Springer.

Marquez-Rocha, F.J., Hernandez-Rodriguez, V.Z., Vazquez-Duhalt, R. (2000) Biodegradation of soil-adsorbed polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. *Biotechnology Letter, 22*, 469–472.

Matheus, D.R., Bononi, V.L.R., Machado, K.M.G. (2000). Biodegradation of hexachlorobenzene by basidiomycetes in soil contaminated with industrial residues. *World Journal of Microbiology and Biotechnology, 16* (5), 415-421.

Mougin, C., Pericaud, C., Dubroca, J., Asther, M. (1997). Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete *Phanerochaete chrysosporium*. *Soil Biology and Biochemistry, 29* (9), 1321-1324.

Ruiz-Dueñas, F.J., Martínez, A.T. (2009). Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. *Microb Biotechnol, 2*, 164-177.

Sari, A.A., Kristiani, A., Tachibana, S., Sudiyani, Y., Abimanyu, H. (2014). Mechanisms and optimization of oil palm empty fruit bunch as a pre-grown source for white-rot fungus to degrade DDT. *Journal of Environmental Chemical Engineering, 2* (3), 1410-1415.

Sari, A.A., Tachibana, S., Limin, S.G. (2013). Enhancement of ligninolytic activity of *Trametes versicolor* U97 pre-grown in agricultural residues to degrade DDT in soil. *Water, Air, & Soil Pollution, 224* (7), 1-9.

Sari, A.A., Tachibana, S., Muryanto, Hadibarata, T. (2015). Development of bioreactor systems for decolorization of Reactive Green 19 using white rot fungus. *Desalination and Water Treatment, 57* (15), 7029-2038.

Shary, S., Kapich, A.N., Panisko, E.A., Magnuson, J.K., Cullen, D., Hammel, K.E. (2008). Differential expression in *Phanerochaete chrysosporium* of membrane associated proteins relevant to lignin degradation. *Appl Environ Microbiol, 74*, 7252-7257.

Shrivastava, R., Christian, V., Vyas, B.R.M. (2005). Enzymatic decolorization of sulfonphthalein dyes. *Enzyme Microbial Technology, 36*, 333–337.

Singh, A., Van Hamme, J.D., Ward, O.P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology Advances, 25* (1), 99-121.

Stella, T., Covino, S., Kresinova, Z., D'Annaibale, A., Petruccioli, M. Cvancarova, M., Cajthaml, T. (2013). Chlorobenzoic acid degradation by *Lentinus (Panus) tigrinus*: in vivo and in vitro mechanistic study-evidence for P-450 involvement in the transformation. *J Hazard Mater, 260*, 975-983.

Takagi, K., Iwasaki, A., Kamei, I., Satsuma, K., Yoshioka, Y., Harada, N. (2009). Aerobic mineralization of hexachlorobenzene by newly isolated pentachloronitrobenzene-degrading *Nocardioides sp.* strain PD653. *Applied and Environmental Microbiology, 75* (13), 4452-4458.

Takano, M., Nakamura, M., Nishida, A., Ishihara, M. (2004). Manganese peroxidase from *Phanerochaete crassa* WD 1694. *Bulletin of FFPRI, 3* (1), 7-13.

Vazquez-Duhalt, R., Westlake, D.W.S., Fedorak, P.M. (1994). Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. *Applied and Environmental Microbiology, 60* (2), 459-466.

Venkatadri, R., Irvine, R.L. (1990). Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology, 56* (9), 2684-2691.

Wells, A., Teria, M., Eve, T. (2006). Green oxidations with laccase mediator systems. *Biochem Soci Trans, 34*, 304-308.

Yamanaka, R., Soares, C.F., Matheus, D.R., Machado, K.M. (2008). Lignolytic enzymes produced by *Trametes villosa* ccb176 under different culture conditions. *Brazilian Journal of Microbiology, 39* (1), 78-84.

Zavarzina, A.G., Zavarzin, A.A. (2006). Laccase and tyrosinase activities in lichens. *Microbiology, 75* (5), 546-556.

Zhang, X.X., Cheng, S.P., Zhu, C.J., Sun, S.L. (2006). Microbial PAH degradation in soil: degradation pathways and contributing factors. *Pedosphere, 16*, 555–565

Zhang, Y.H., Xu, D., Zhao, X.H., Song, Y., Liu, Y.L., Lin, H.N. (2016). Biodegradation of two organophosporus pesticides in whole corn silage as affected by the cultured *Lactobacillus plantarum*. *3 Biotech*, DOI 10.1007/s13205-016-0364-3

Zhao, Y.C., Zhang, M., Liu, L., Ma, W.J., Yi, X.Y. (2010). Fundamental study of degradation of dichlorodiphenyltrichloroethane in soil by laccase from white rot fungi. International *Journal of Environmental Science and Technology, 7*(2), 359-366.

Zheng, Z.M., Obbard, J.P. (2001). Effect of non-ionic surfactants on elimination of polycyclic aromatic hydrocarbons (PAHs) in soil-slurry by *Phanerochaete chrysosporium*. *J.Chem. Technol. Biotechnol. 76*, 423–429.

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