Feasibility of Aspergillus keratitidis InaCC1016 for synthetic dyes removal in dyes wastewater treatment

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Abstract. Several industries produce waste that can not be degraded naturally or toxic to a living organism, i.e., dyes waste. Fungi were considered as the best candidates for dyes waste treatment among other microorganisms because of fungi more resistance in the lack of nutrient conditions. Besides, their biomass can also function as an adsorbent that was able to absorb dyes so that it is more effectively applied. This study aims to evaluate the feasibility Aspergillus keratitidis to degrade Congo Red (CR) and Methylene Blue (MB) in the solid and liquid state. Dyes decolorization in the solid-state was observed based on clear zone produced, and in the liquid state, decolorization was determined spectrophotometrically. A. keratitidis was able to decolorize synthetic dyes in both media, solid and liquid state. CR was more effective dyes to be removed by A. keratitidis than MB. This fungus able to decolorize about 96% of 200 ppm CR within seven days and 63% of 100 ppm MB within ten days. Moreover, MB was more toxic dyes than CR, which inhibited A. keratitidis growth. A. keratitis was suggested involved lignolytic enzyme on dyes decolorization due to it can degrade lignin compound, but it needs a further study to prove it. Based on our knowledge, this is the first report about a potential study of A. keratitidis in dyes decolorization and lignin degradation activity.

1. Introduction
Nowadays, the environmental pollution issue is being a concern of people in over the world. The increase in population over the past few decades has boosted industrial growth to grow faster to meet the demand. Industrial activities have a detrimental effect on environmental sustainability. Several industries produce waste that can not be degraded naturally or toxic to a living organism. For example, textile industries produce wastewater containing synthetic dyes that mostly were hardly degraded naturally, toxic, and carcinogenic [1][2]. The textile industry is the largest supplier of wastewater because this industry consumes large amounts of water and around 17-20% of the total water consumed will be discharged in the form of waste [3][4]. When these waste released into the environment, it can hamper not only the aquatic environment but also human health [5][6][7].

Many dye wastewater treatment technologies have been developed which involve oxidation methods (photocatalytic oxidation, ozone, H₂O₂, Fenton process) and physical methods (adsorption and filtration) [3]. However, the existing methods have not been effectively utilized because of their high costs, need complicated equipment, and produce new pollutant [8]. Biological method (fungi, algae and,
bacteria) is one of the best alternatives at this time because this method is considered quite effective, cheaper and environmentally friendly [3][9]. Fungi is the best candidates among other microorganisms because of fungi more resistance in the lack of nutrient conditions. Besides, their biomass can also function as an adsorbent that can absorb dyes so that it is more effectively applied. Unfortunately, the degradation of using microorganisms, including fungi, still faces challenges, where the derivative products produced, are toxic even more toxic than the parent compounds [2]. Therefore, the study related to finding the candidates that can degrade dyes compound still needs to be done thoroughly.

This study aims to evaluate the feasibility the fungus isolate KT2 62, which was later identified as *Aspergillus keratitidis*, to degrade dyes compound. Based on our knowledge, there are no studies related to the potential of *A. keratitidis* in decolorization of dyes. The dyes used in this study were Congo Red and Methylene Blue, and the experiment was carried out in two types of media, namely solid and liquid state. The ability of fungi to degrade dyes is often associated with their ability to degrade lignin, so this study also confirmed the ability of *A. keratitidis* to degrade lignin.

2. Material and Methods

2.1. Microorganism source and growth
Fungus isolate was isolated from wood decay in tropical rain forest located in East Kalimantnan, Indonesia. Fungus isolate was identified and deposited in the Indonesian Culture Collection (InaCC) with accessing number InaCCF1016. The fungus was grown in the Potatoes Dextrose Agar (PDA) and incubated at 30°C for seven days before decolorization assay.

2.2. Molecular identification of fungus isolate
Fungal mycelia collected from 72 h Potatoes Dextrose Broth (PDB) were used to DNA extraction. The extraction of fungal DNA was performed using the nucleon PHYTOPure (Amersham LIFE SCIENCE) nucleon reagent. Strain identification was carried out by PCR amplification in ITS using ITS 4: (5′-TCCTCCGCTTATTGATATGC-3′) and ITS 5: (5′-GGAAGTAAAAGTCGTAACAAGG-3′) [10][11]. DNA sequencing results were analyzed using the ChromasPro version 1.7.5 program (Technelysium Pty Ltd, Australia). Sequence alignment was conducted between the new sequences and the closest sequences to the search results for Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) using MUSCLE (Edgar 2004 ) in the MEGA 7 program [12]. Phylogenetic trees were constructed by Neighbor-Joining (NJ) analysis [13] using the MEGA 7 software program and Tamura 3-G+C parameter model as the best evolutionary model [14]. Bootstrap (BS) analysis was performed based on 1000 replications [15]. About 22 ITS rDNA sequences obtained from BLAST search results were used as ingroup, and *Trichoderma viride* (AM498467) was chosen as outgroup.

2.3. Synthetic dyes decolorization assay
Dyes decolorization by *A. keratitidis* was firstly examined in the solid-state. Congo red (CR) and Methylene Blue (MB) was chosen as a model of synthetic dye. The media was used containing 80% (w/v) PDB, 2% (w/v) agar, and 200 ppm of dyes. One disc (8 mm) of mycelia carried out from 7 days incubation culture was placed on the center of solid media, and then, the culture was incubated at 30°C for seven days. The diameter of mycelia and clear zone produced was measured, and the decolorization index was calculated following equation [16]:

$$\text{Decolorization index (\%)} = \frac{DD}{MD} \times 100\%$$

(1)
Where DD was decolorization zone diameter and MD was mycelia diameter of fungus on media containing dyes.

The decolorization of synthetic dyes also performed on the 50 ml liquid media that has the same formula as the solid media without agar addition. Two discs of mycelia taken from 7 days incubation fungus culture were added into the 300 ml Erlenmeyer flask, and the culture was incubated in a shaker incubator at 110 rpm, 30 °C for ten days. The uninoculated media was used as a control, and the experiment was set with three replicates. Culture media was withdrawn from the flask at 3, 7, and 10 days time interval, and the CR decolorization was monitored spectrophotometrically at wavelength 480 nm, and the MB was at 660 nm. The effect of dyes initial concentration on dyes decolorization rate also was performed on 100 and 200 ppm for CR and 50 and 100 ppm for MB. Biomass of fungus was also collected in the final time of incubation through a paper filter, and biomass was dried at 60 °C for 24 h. Biomass production was expressed as g dry weight of mycelia was produced in one L of media (g L⁻¹).

2.4. Lignin degradation assay

The ability of fungus isolate to degrade lignin was observed using two methods of confirmation. First, fungus isolate was grown on solid media consisted of minimal salts media MSM supplemented with lignin extract as a sole carbon source (MSM-L) and 2% (w/v) agar. MSM consisted of 4.5 g / 1 K₂HPO₄; 0.53 g / 1 CaCl₂ 2H₂O; 0.5 g / 1 MgSO₄.7H₂O; 5 g / 1 NH₄NO₃; 0.001 g / 1 CuSO₄.5H₂O; 0.001 g / 1 FeSO₄.7H₂O; 0.001 g / 1 MnSO₄.7H₂O; ZnSO₄.7H₂O. Final concentration of lignin extract in MSM was 0.25 % (v/w). Lignin extract was prepared from the empty fruit oil palm waste. The extraction process followed a method that was used by Barapatre et al. [17]. One disc of fungus mycelia carried out from 7 days culture incubation was inoculated on the center of solid-state media. The culture was incubated at 30 °C for seven days, and the diameter of mycelia was measured on the final day incubation. The fungal grow index was used as an indicator of fungal ability to degrade lignin. As a control, the fungal isolate was grown on rich media, PDA. Fungal growth index was calculated using the following equation:

\[
\text{Growth Index (\%)} = \frac{\Phi_s}{\Phi_c} \times 100\%
\]

Where \(\Phi_s\) was a diameter of fungus mycelia on minimal media (MSM-L) and \(\Phi_c\) was a diameter of fungus mycelia on control media (PDA)

The second observation was performing in a liquid state containing MSM with the addition of black liquor as a sole carbon source. Lignin was a major compound in black liquor. Two discs of mycelia taken from 7 days incubation fungus culture were added into the 250 ml Erlenmeyer flask containing 50 ml sterile MSM-BL. The culture was incubated in a static condition at room temperature (28-30 °C) for 21 days. The degradation of lignin was observed on the change of the absorption of supernatant media at 280 nm. This wavelength was specific for lignin compound absorption. The liquid media without inoculant was used as a control. The culture media was observed within time interval 3, 7, 10, 14, and 21 days. All of the experiment conducted in this study was set up in 3 replication.
3. Results and Discussion

3.1. Fungal identification (molecular phylogenetic analysis)

The phylogenetic tree originating from the NJ analysis consists of 1 InaCC F1016 sequence and 22 closest sequences derived from BLAST results in GenBank (Figure 1). The phylogeny tree shows that the InaCC F1016 sequence is in a clade with *Aspergillus keratitidis* (KY980627, KY980626, and KY980647) with a 99% bootstrap value. The position of the InaCC F1016 sequence on the phylogenetic tree is one clade with three sequences of *Aspergillus keratitidis* BLAST search results with a bootstrap value of 99%. This results showed that InaCC F1016 is *Aspergillus keratitidis*.

![Phylogenetic tree of ITS4, and ITS5 rDNA regions sequences of Aspergillus keratitidis InaCC F1016 and related taxa with Trichoderma viride as an outgroup. The phylogeny tree constructed by the Neighbor-Joining method with more than 50% bootstrap values by 1000 replications.](image)

*Aspergillus keratitidis* belong to subgenus *Polypaecilium* that was first described as *Sagmonella keratitidis* isolated from corneal scraping by Hsieh *et al.* [18] and then it was also found as the most frequently isolated species from house dust as reported by Tanney *et al.* [19]. This fungi species was considered as xerophiles microorganism than can growth on low water activity environment [19]. Based on our observation, there is a limited report regarding the functional characterization of this fungus. This fungus culture has been deposited in the Indonesian Culture Collection, LIPI with the catalog number InaCCF1106.
3.2. Synthetic dyes decolorization assay

Feasibility of *Aspergillus keratitidis* to decolorize Congo Red, and Methylene Blue was investigated in two conditions, solid-state and liquid state. To our knowledge, this is the first time report for *A. keratitidis* decolorization analysis. Our finding showed that *A. keratitidis* was able to decolorize 200 ppm CR and MB on solid-state, which was presented as a decolorization index number. The decolorization index (DI) was used as a tool to reflect the ability of fungus on dyes decolorization. More highest the ID number, better the ability in decolorize dyes. Decolorization can be observed as a clear zone surrounding the fungus mycelia. This finding showed that *A. keratitidis* has a better ability to decolorize CR than MB, 4.04, and 1.78, respectively (Table 1). Based on our observation, CR and MB decolorization by *A. keratitidis* was high compared to other reports. *Fomitopsis rosea* was able to decolorize CR and MB with DI number about 1.27 and 1.40, respectively [16]. *Fusarium solani* decolorized MB in solid media with DI about 0.8 [20].

**Table 1.** Decolorization of synthetic dyes on the solid-state by *A. keratitidis* after seven days of incubation

| Synthetic Dyes    | MM (mm)       | DD (mm)       | DI  |
|-------------------|---------------|---------------|-----|
| Congo Red         | 10.1 ± 0.06   | 44.5 ± 0.13   | 4.04|
| Methylene Blue    | 8.1 ± 0.06    | 14.5 ± 0.34   | 1.78|

MD: Mycelia diameter, DD: Decolorization diameter, DI: Decolorization index, n = 3

![Graph](image.png)

**Figure 2.** Synthetic dyes decolorization in the liquid state by *A. keratitidis*. CR = Congo Red; MB = Methylene Blue
Decolorization of CR and MB in liquid medium was monitored within ten days, and the results showed that \textit{A. keratitidis} more rapidly decolorized CR than MB (Figure 2). \textit{Aspergillus keratitidis} able to decolorize 100 ppm CR about 98% within seven days. However, the decolorization decreased to 89% at ten days because the medium turn into yellowish that disturbed the measurement. The change in color medium might be due to the fungus produces a pigment or enzymatic reaction that produced color [16]. Besides, our finding showed that \textit{A. keratitidis} hardly decolorized MB. It can be seen in Figure 2 that only about 63% of MB can be decolorized within ten days. CR and MB belong to different group of dyes. CR is a dye that was classified as an azo dye group that contains an azo group, \(-\text{N=N-}\), as part of the structure, while MB was a heterocyclic group. The difference in the chemical structure of these two compounds was thought to affect the decolorization process of dyes by fungi [4]. Hsueh and Chen [21] reported that azo dyes with different properties of a substituent on the aromatic ring could affect the efficiency of biodecolorization.

Many studies were reported regarding MB and CR decolorization. The decolorization of dyes by fungi is strain-dependent. Lyra \textit{et al}. [22] studied the degradation of MB dan CR by white-rot fungi strains. The result showed that \textit{Pycnoporus sanguineus} was more effective to decolorize CR than MB, that is 72.6% and 54.5% respectively. In other hands, \textit{Datronia caperata} has the best ability to decolorize MB than CR, that is 74.3% and 20.3% respectively. Other fungus strain has a good ability in both dyes such as \textit{Pycnoporus cinnabarinus} was able to decolorize about 90% of 100 ppm CR and about 80% of 100 ppm MB within 20 days [16].

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Effect of dye initial concentration on decolorization of CR by \textit{A. keratitidis}}
\end{figure*}

In this study, the effect of the dyes initial concentration on decolorization of CR and MB by \textit{A. keratitidis} was also examined. The decolorization of CR become slower in the first three days when the initial concentration of CR was increased to 200 ppm (Figure 3). However, high concentration of CR did not inhibit the decolorization of CR in the future incubation. The decolorization of 200 ppm CR reached to 80% in 7 days of incubation, and there was no significant difference of decolorization with
100 ppm CR in the final day of incubation. This finding can be explained that fungus biomass and dyes concentration ratio affects decolorization efficiency. The fungus biomass was remained constant while the dyes concentration increased, so it needs more time to decolorize all of the dye compounds compare to the lower one [23]. In another side, the effect of dye concentration on MB degradation was performed in lower concentration, that was 50 ppm. We tend to examine the degradation of MB in low concentration due to MB was hardly decolorized than CR. Figure 4 showed that the decolorization of MB become faster while the concentration of MB was low. In the seven days incubation, 50 ppm MB was successfully decolorized until 50%, two-fold higher than 100 ppm MB in the same day. However, in the final day incubation, there was no significant difference in decolorization percentage between two concentration of MB.

![Figure 4. Effect of dye initial concentration on decolorization of MB by A. keratitidis](image)

The toxicity effect of dyes on A. keratitidis growth was investigated through measuring the biomass production in the liquid medium. Our investigation result can be seen in Table 2. Fungal biomass production was lower in medium containing MB compare to CR at the same dye concentration. Increased on dye concentration from 100 to 200 ppm did not affect fungal growth in CR. The opposite result was found on MB, which increased dye concentration gave a negative effect on fungal growth. Biomass production was decreased when the dye concentration was increased from 50 ppm to 100 ppm. High concentrations of dyes cause inhibition of the metabolic processes of microorganisms and affect fungal growth. This finding also showed that MB was more toxic than CR because a toxic compound can inhibit fungal growth [23]
Table 2. Biomass production by A. keratitidis in CR and MB medium

| Dyes Concentration (ppm)  | Congo Red (CR)  | Methylene Blue (MB) |
|---------------------------|-----------------|---------------------|
|                           | 100             | 200                 | 50      | 100     |
| Biomass Production (g L⁻¹) | 3.20 ± 1.05     | 3.20 ± 0.60         | 1.80 ± 0.69 | 1.20 ± 0.20 |

3.3. Lignin degradation assay

The ability of fungi to decolorize dyes is often associated with their ability to degrade lignin, so this study also confirmed the ability of A. keratitidis to degrade lignin. Moreover, there was no study have been reported related to A. keratitidis ligninolytic activity. A. keratitidis was cultured on minimal salt medium containing lignin as sole carbon (MSM-L) to evaluate its ability to degrade lignin. Our finding showed that A. keratitidis able to degrade lignin proven by its ability to grow in media that only provided lignin as the only carbon source. Fungus growth ability can be quantified as a growth index (Table 3). Growth index resulted from the comparison of fungal growth between lignin medium (MSM-L) and control medium (PDA). Growth index as 100% implies that fungal in the minimal medium can grow as well as in the control medium. Because A. keratitidis has 90.3% of growth index, it implied that this fungus could grow in the minimal medium containing lignin but not as well as in the control medium that contain rich nutrients. Nevertheless, these results inform that A. keratitidis can degrade lignin and utilize it as a carbon source to grow. Carbon is one of the main components needed by microorganisms to grow, namely as an energy source. Before it can be used as an energy source, lignin must be degraded first into simple compounds involved some lignolytic enzymes, such as laccase, lignin peroxidase, and manganese peroxidase [24]. This evidence proves that A. keratitidis can degrade lignin.

Table 3. Fungal growth observation on lignin degradation assay

| Mycelia Diameter (mm) | Mycelia Thickness | Growth Index (%) |
|-----------------------|-------------------|------------------|
|                       | PDA               | MSM-L            |                   |
|                       | 15.5 ± 0.05       | 14.0 ± 0.10      | ++                |
|                       |                   |                  | +                 |

Note: +++ = thick; ++ = medium; + = thin; - = not appear

In addition to using solid media, lignin degradation ability was confirmed in liquid media. Moreover, in liquid media, the number of lignin degraded can be quantified but not in solid media. The presence of lignin degradation can be seen by observing changes in absorbance at wavelengths of 280 nm. This wavelength is specific for lignin compounds [25][26]. The results showed that there was a significant decrease in absorbance at wavelength 280 nm (Figure 5). This results implied that there had been a decrease in lignin compounds due to the degradation by A. keratitidis. A. keratitidis can degrade lignin as 36% and 44% relative to control along 7 and 14 days of incubation, respectively. This second proof reinforces that A. keratitidis can degrade lignin.

The ability of lignolytic fungi to degrade dyes depend on lignolytic enzymes produced [9]. Lignolytic enzymes produced by lignin-degrading fungi such as laccase, LiP, and MnP are non-specific enzymes [27]. This enzyme can degrade other compounds that have a similar structure to lignin. Lignin is an aromatic polymer compound, while most synthetic dyes compound also has aromatic rings. The similarity of structure with dye stuffs is the basis for why lignolytic fungi can degrade synthetic color compounds [28]. Further study was needed to understanding the decolorization mechanism by A.
keratitidis weather involved biodegradation through ligninolytic enzyme or biosorption or both of mechanism.

![Figure 5. Day-wise absorption spectra of lignin degradation by A. keratitidis within 21 days of incubation](image)

4. Conclusion
A. keratitidis was able to decolorize synthetic dyes in both media, solid and liquid state. CR was more effective dyes to be removed by A. keratitidis than MB. Moreover, MB was more toxic dyes than CR, which inhibited A. keratitidis growth. A. keratitis was suggested involved lignolytic enzyme on dyes decolorization due to it can degrade lignin compound, but it needs a further study to prove it.

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