

## Optimization of Laccase Production by *Megasporoporia* sp. for Bioremoval of Methyl Orange and Cr(VI)

Titan Dwikama Putra<sup>1,2</sup>, Wichanee Bankeeree<sup>2</sup> and Sehanat Prasongsuk<sup>2,\*</sup>

<sup>1</sup>Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

<sup>2</sup>Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

(\*Corresponding author's e-mail: [sehanat.p@chula.ac.th](mailto:sehanat.p@chula.ac.th))

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### Abstract

Industrial expansion has accelerated the discharge of hazardous pollutants, particularly azo dyes such as methyl orange (MO) and heavy metals such as hexavalent chromium [Cr(VI)], which pose serious environmental and health risks, especially when both contaminants coexist in wastewater. Enzymatic bioremediation offers a sustainable alternative to conventional physicochemical treatments, and this study aimed to optimize laccase production by the white-rot fungus *Megasporoporia* sp. and evaluate its ability to simultaneously remove MO and Cr(VI). Laccase biosynthesis was optimized using a One-factor-at-a-time (OFAT) approach by systematically varying nutritional and physicochemical factors, including carbon sources, nitrogen sources, copper concentration, pH, temperature, agitation speed, and incubation time. The highest yield was achieved under optimal conditions: glucose or sucrose as the carbon source, a combination of yeast extract and ammonium sulfate as the nitrogen source, 1.5 - 2.0 mM CuSO<sub>4</sub>, pH 5.0, incubation at 28 °C for 5 days, and static cultivation. The crude enzyme exhibited strong pollutant removal in the single-pollutant systems, achieving 74.17% MO removal and 98.67% Cr(VI) removal, whereas its performance declined in the dual-pollutant system due to inhibitory interactions between the two contaminants (42.46% MO removal and 87.57% Cr(VI) removal). Phytotoxicity assays using *Vigna radiata* demonstrated that laccase substantially mitigated toxicity in the single-pollutant treatments, improving germination from 22% to 64% for MO and from 16% to 82% for Cr(VI), whereas only partial recovery was observed in the dual-pollutant system. These findings indicate that *Megasporoporia* sp. can efficiently produce high laccase yields under mild, low-cost conditions, and that the crude enzyme is highly effective for individual MO and Cr(VI) remediation, although simultaneous removal remains constrained by pollutant interactions. The study highlights the potential of laccase-based bioremediation for wastewater treatment while underscoring the need for further strategies, such as enzyme immobilization, to enhance performance in complex contaminant systems.

**Keywords:** Optimization, One-factor-at-a-time (OFAT), Laccase, *Megasporoporia* sp., Bioremoval, MO, Cr(VI), Simultaneous removal

### Introduction

As industrialization has advanced, the global industrial sector has undergone rapid expansion to meet market demands. Examples of such industries include textiles, dye manufacturing, leather, and paper pulp production. Although this development contributes significantly to national economic growth, these industries also generate a massive amount of wastewater

[1,2]. This issue becomes critical when waste management practices are inadequate, leading to the direct discharge of effluents into the environment. The resulting impact can lead to severe environmental problems, as industrial effluents generally contain hazardous substances that are persistent and harmful to living organisms. Among these pollutants, synthetic

dyes and heavy metals are xenobiotic compounds frequently found in such wastewater [3-5].

Azo dyes are among the most widely used types of synthetic dyes in various industrial processes. At least 70% of dyes used in these industries are azo dyes [6]. One example of a commonly used azo dye is methyl orange (MO; 4-dimethylaminoazobenzene 4'-sulfonic acid sodium salt). Studies have shown that MO and its intermediates, such as dimethyl benzyl-1,4-diamine, sulfonamide, and 1,4-diaminobenzene, are carcinogenic, mutagenic, and teratogenic compounds [7,8]. Meanwhile, heavy metals such as hexavalent chromium [Cr(VI)] can also be found in leather industry. This is because chromium sulfate (Cr(OH)SO<sub>4</sub>) is usually used as a tanning agent. Although it is predominantly present as trivalent chromium [Cr(III)], a fraction of it can be oxidized to Cr(VI) under certain conditions [9]. Cr(VI) represents the most toxic oxidation state of chromium and is reported to be up to 100 times more toxic than Cr(III). Due to its structural similarity to sulfate ions, Cr(VI) can easily enter cells through sulfate channels, leading to cellular damage [10,11]. Moreover, MO and Cr(VI) have been reported to coexist in various types of wastewater, thereby enhancing its toxicity [12].

Several studies have developed various physicochemical methods for wastewater treatment containing MO and Cr(VI). These methods include adsorption [13], electrocoagulation [14,15], ion exchange [16,17], and catalytic reduction [18,19]. Although these approaches show promising results, they generally have several drawbacks, such as high operational costs, secondary sludge generation, incomplete pollutant removal, and limited applicability to large-scale systems [20]. In contrast, biological methods utilizing microorganisms have been considered as alternative approaches since they can overcome some of the limitations of physicochemical techniques. However, under actual wastewater conditions, nutrient limitations and extreme environmental factors can inhibit microbial growth, thereby reducing their remediation efficiency [21]. Considering these challenges, enzyme-based bioremediation has recently attracted increasing attention. Instead of employing whole microbial cells, this approach utilizes the enzymes produced by microorganisms to transform hazardous compounds into less toxic or non-toxic forms.

Laccase (EC 1.10.3.2) is an oxidoreductase class enzyme that mediates the oxidation of aromatic and non-aromatic substrates by reducing molecular oxygen to water [22]. Laccase has been reported to be produced by various microorganisms, although it is predominantly known to be synthesized by white-rot fungi (WRF) [23]. Among microbial enzymes, laccase holds significant economic value and has demonstrated great potential in wastewater treatment. The major advantage of this enzyme lies in its broad catalytic ability to degrade a wide range of pollutants [24,25]. Silveira *et al.* [26], reported that laccase derived from *Marasmiellus palmivorus* efficiently decolorizes MO. Another study also highlighted the potential of laccase produced by *Ganoderma multipileum* in reducing Cr(VI) [27]. Nevertheless, to the best of our knowledge simultaneous enzymatic remediation of MO and Cr(VI) has not been previously reported. Therefore, this study aims to optimize laccase production from the WRF *Megasporoporia* sp. and to evaluate its potential to simultaneously remediate MO and Cr(VI).

## Materials and methods

### Fungal culture

The WRF used in this study, *Megasporoporia* sp., was obtained from the Plant Biomass Utilization Research Unit (PBURU), Department of Botany, Faculty of Science, Chulalongkorn University. The culture was maintained on Potato Dextrose Agar (PDA) containing (g/L): 200 potato infusion, 20 dextrose, and 15 agar, and stored at 4 °C. Prior to further experiments, the WRF was routinely subcultured on fresh PDA plates and incubated at room temperature for 1 week.

### Laccase production and optimization

*Megasporoporia* sp. was previously reported by Lestari *et al.* [28] to produce laccase in a liquid medium containing (g/L): 10 glucose, 1 KH<sub>2</sub>PO<sub>4</sub>, 0.5 MgSO<sub>4</sub>, 0.14 CaCl<sub>2</sub>, 0.0025 thiamine, and 0.4 mM veratryl alcohol, adjusted to pH 5.0. However, the resulting laccase yield was relatively low. Therefore, in this study, laccase production by *Megasporoporia* sp. was optimized using a one-factor-at-a-time (OFAT) approach with a modified medium composition according to Othman *et al.* [29] (g/L): 10.5 glucose, 5 yeast extract, 2 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 K<sub>2</sub>HPO<sub>4</sub>, 0.5 MgSO<sub>4</sub>, 0.02 FeSO<sub>4</sub>, 0.3 CaHPO<sub>4</sub>, 0.2 ZnSO<sub>4</sub>, 0.2 MnSO<sub>4</sub>, and

0.25 CuSO<sub>4</sub>, adjusted to pH 5.0. Five mycelial plugs (Ø ~1 cm) from a 1-week-old culture were inoculated into the liquid medium. Subsequently, the crude enzyme was obtained by separating the culture supernatant from the fungal biomass using Whatman No. 1 filter paper.

Several factors were evaluated to determine the optimal conditions for laccase production by *Megasporoporia* sp. These factors included the carbon sources (rice straw, sugarcane leaf, rice husk, glucose, sucrose, starch), organic nitrogen sources (beef extract (BE), meat extract (ME), peptone (PE), yeast extract (YE)) paired with inorganic nitrogen sources (ammonium chloride (AC), sodium nitrate (SN), potassium nitrate (PN), ammonium sulfate (AS)), copper concentration (0.5 - 2.0 mM), pH (3.0 - 8.0), temperature (25 - 30 °C), agitation speed (0 - 200 rpm), and incubation time (5 - 21 days). Each factor was evaluated independently to identify conditions that maximized laccase production by *Megasporoporia* sp.

#### Laccase activity and protein content assay

Laccase activity in the crude enzyme was determined spectrophotometrically based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm. The reaction mixture consisted of 50 µL of crude enzyme and 500 µL of 2 mM ABTS, with the final volume adjusted to 1000 µL using 0.1 M citrate buffer (pH 3.0). One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute, calculated according to the formula described by Anita *et al.* [30] (Eq. (1)).

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta Abs \times V_{\text{total}} (L) \times 10^3}{\varepsilon \times V_{\text{enzyme}} (L) \times t} \quad (1)$$

where  $\Delta Abs$  is the difference between the final and initial absorbance,  $10^3$  is the correction factor (µmol/mol),  $\varepsilon$  is the molar absorptivity ( $3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ), and  $t$  is the reaction time (min).

Meanwhile, the total protein content in the crude enzyme was determined using the method of Lowry *et al.* [31], with bovine serum albumin (BSA) used as the standard.

#### Bioremoval of MO and Cr(VI)

The laccase produced by *Megasporoporia* sp. was evaluated for its potential to remove MO and Cr(VI) from synthetic wastewater. In this study, crude enzyme with an initial activity of 1 U/mL was added to 5 mL of solution containing 50 mg/L of MO or Cr(VI) for a single-pollutant system, and 50 mg/L each of MO and Cr(VI) for a dual-pollutant system. The mixtures were then incubated at 28 °C and shaken at 150 rpm for 48 h in the dark to prevent photodegradation. Samples were collected periodically to determine the removal of MO and Cr(VI). The decolorization of MO was analyzed spectrophotometrically at 462 nm [32], while the reduction of Cr(VI) was measured using the 1,5-diphenylcarbazide (DPC) method, in which the resulting purple complex was quantified at 540 nm [33]. The bioremoval efficiencies of MO and Cr(VI) were calculated using the following formula (Eq. (2)):

$$\text{Bioremoval efficiency (\%)} = \left(\frac{OD_i - OD_f}{OD_i}\right) \times 100 \quad (2)$$

where  $OD_i$  is the optical density before removal, and  $OD_f$  is the optical density after removal.

#### Phytotoxicity evaluation

The phytotoxicity evaluation was conducted to determine whether the bioremoval products of MO and Cr(VI) by the laccase produced by *Megasporoporia* sp. still exhibited phytotoxic effects on plants. In this assay, mung bean (*Vigna radiata*) seeds were used as a plant model due to their sensitivity to azo dyes and heavy metals [34]. Prior to use, the seeds were surface-sterilized by soaking in 70% ethanol for 10 min, followed by several rinses with sterile deionized water (DI). Ten seeds were placed in a Petri dish containing pre-sterilized Whatman No. 1 filter paper, onto which 5 mL of the treated synthetic wastewater was added. DI water served as the negative control, while untreated synthetic wastewater served as the positive control. The plates were then incubated in the dark for 1 week [35]. The percent germination (Eq. (3)), germination index (Eq. (4)), and percent germination index (Eq. (5)) were calculated using the following formulas [36]:

$$\% \text{ Germination} = \frac{\text{germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (3)$$

$$GI = l \times n \quad (4)$$

$$\% \text{ Germination Index} = \frac{GI \text{ of each test group}}{GI \text{ of control}} \times 100 \quad (5)$$

where  $l$  is the total sum of root lengths of the germinated seeds in each replicate, and  $n$  is the number of germinated seeds.

### Statistical analysis

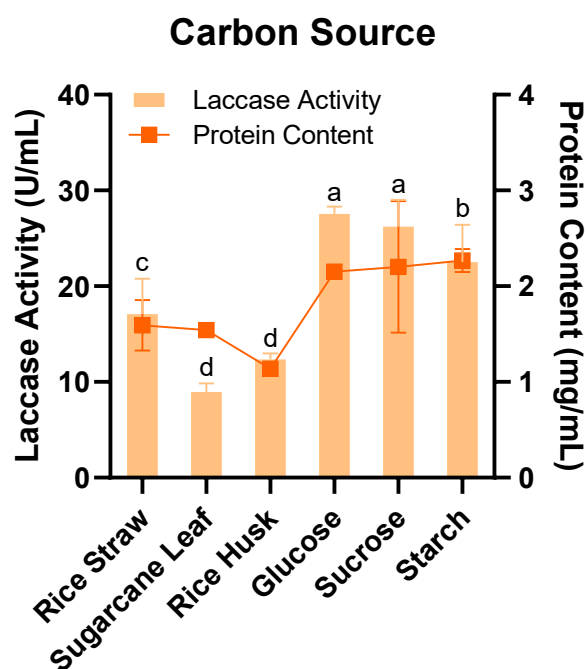
All experiments were conducted in triplicate ( $n = 3$ ), while the phytotoxicity evaluation was performed in 5 replicates ( $n = 5$ ). Statistical differences among treatments were assessed using 1-way ANOVA with a significance level of  $p < 0.05$ . When significant differences were detected, Tukey's HSD post-hoc test was applied for pairwise comparisons. Data visualization and statistical analyses were carried out using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA).

## Results and discussion

### Laccase production and optimization

#### Effect of carbon source

The type of carbon source plays a significant role in laccase production by WRF. In this study, when glucose was used, *Megasporoporia* sp. exhibited the highest laccase activity. However, this value was not significantly different from that obtained with sucrose (26.22 U/mL), as shown in **Figure 1**. A similar observation was reported by Umar and Ahmed [37], who demonstrated that both the monosaccharide (glucose) and the disaccharide (sucrose) enhanced laccase activity in *Ganoderma leucocontextum*. These findings suggest that, in certain fungal species, glucose and sucrose may induce the expression of laccase-encoding genes more effectively than other carbon sources [38]. Based on the results of this study, sucrose may therefore serve as an alternative carbon source for laccase production by *Megasporoporia* sp., considering that it is more cost-effective than glucose [39].



**Figure 1** Effect of different carbon source on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD ( $n = 3$ ). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

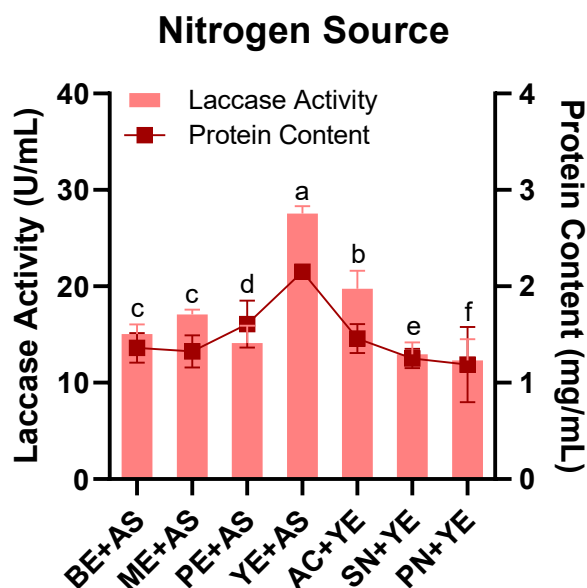
#### Effect of nitrogen source

Ardila-Leal *et al.* [40] explained that the simultaneous use of organic and inorganic nitrogen

sources can enhance laccase activity due to nitrogen assimilation. In **Figure 2**, the combination of yeast extract and ammonium sulfate is shown to significantly

increase laccase activity compared to other nitrogen combinations. Similar findings have also been reported in several other WRF species, such as *Pleurotus ostreatus* [41], *Pleurotus sajor-caju* [42], and *Pycnoporus sanguineus* [43], which demonstrated a positive effect of yeast extract supplementation on laccase productivity. This preference for yeast extract in

fungal laccase production is likely due not only to its role as a nitrogen source but also to the presence of protein hydrolysates and vitamins that enhance laccase biosynthesis [44]. Meanwhile, Umar and Ahmed [37] also reported that ammonium sulfate can effectively stimulate laccase production in *Ganoderma leucocontextum*.

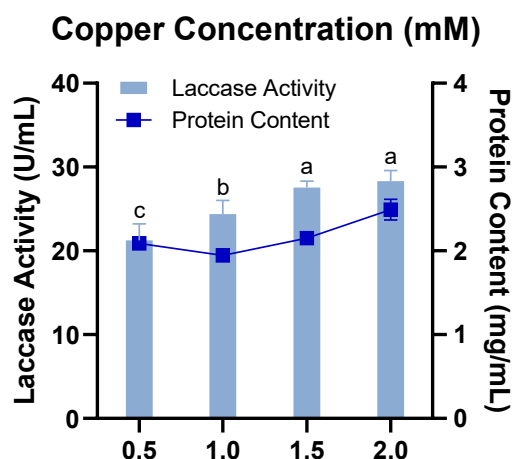


**Figure 2** Effect of different nitrogen source on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

#### *Effect of copper concentration*

Since the catalytic active site of laccase contains copper atoms, the availability of copper in the culture medium plays a crucial role in regulating laccase biosynthesis. Copper acts as an inducer by activating the transcription of laccase-encoding genes. However, at higher concentrations, copper can exert toxic effects on fungal cells, impairing growth and consequently reducing enzyme productivity [45]. In this study, laccase activity increased proportionally with copper concentration and reached its maximum at 2.0 mM

(Figure 3). A previous report also demonstrated that supplementation with 0.5 - 2.0 mM copper enhanced *pel3* gene transcription and extracellular laccase activity in *Pleurotus eryngii* KS004 [46]. Despite this, the enzyme activity observed at 1.5 mM was not significantly different from that at 2.0 mM. Therefore, 1.5 mM can be considered the optimal concentration for inducing laccase production, as it provides high activity while being more cost-effective and reducing potential cytotoxic stress.

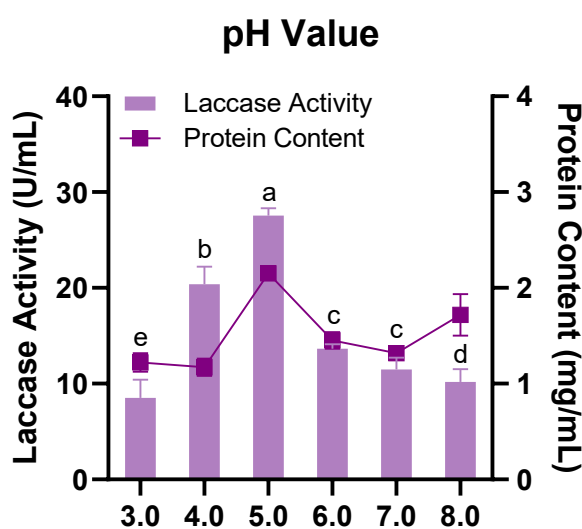


**Figure 3** Effect of copper concentration on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

#### Effect of pH value

At the lowest growth medium pH (pH 3.0), the laccase activity was the lowest. However, as the pH slightly increased, laccase activity also increased, reaching its highest peak at pH 5.0 (Figure 4). The optimal pH for laccase production varies among fungi, although many species generally produce laccase most effectively within the pH range of 3.0 - 6.0 [47]. The results also show that further increases in pH led to a gradual decline in laccase activity. A similar pattern was

reported for *Pestalotiopsis* sp. CDBT-F-G1, where laccase activity decreased when the growth medium pH exceeded 5.0 [48]. According to Salem et al. [49], the increased presence of  $\text{OH}^-$  ions at higher medium pH can interact with the type 2 and type 3 copper centers in laccase. These copper centers form the catalytic sites of the enzyme; thus, higher pH conditions can reduce the redox potential of the substrate and consequently decrease laccase activity.

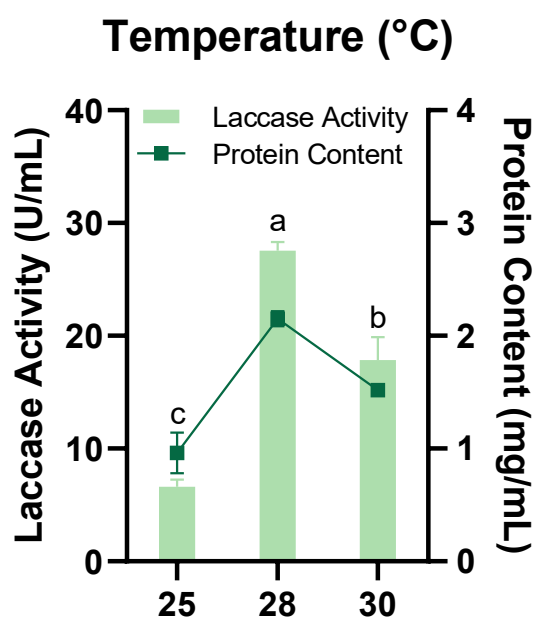


**Figure 4** Effect of medium pH on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

### Effect of temperature

Laccase production by *Megasporoporia* sp. was highest at 28 °C (Figure 5). The lowest enzyme activity occurred at 25 °C, which is likely associated with reduced metabolic and enzymatic reaction rates at lower temperatures. Temperatures that are too low or below the optimal range can slow fungal growth and consequently reduce its enzymatic activity [50]. Conversely, temperatures exceeding the optimum may lead to partial enzyme denaturation, thereby diminishing catalytic efficiency [51]. These results are consistent with findings by Edae and Alemu [52], who reported 28

°C as the optimal temperature for laccase production in *Pleurotus* sp. Similarly, Sura *et al.* [53] observed comparable temperature-dependent behavior in *Trichoderma harzianum*. In general, each fungal species has a specific temperature range that supports maximal enzyme biosynthesis, and most studies indicate that optimal laccase production commonly occurs between 20 - 30 °C [42]. Based on these results, it can be concluded that *Megasporoporia* sp. can produce high levels of laccase at room temperature, indicating that no additional temperature control is required to achieve optimal enzyme production.

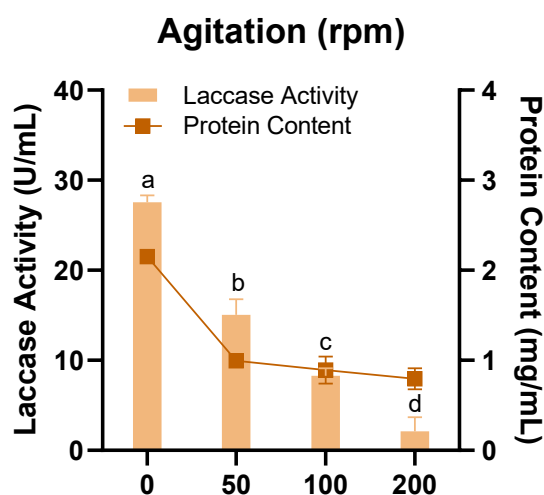


**Figure 5** Effect of temperature on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

### Effect of agitation speed

Laccase production under different agitation speeds was evaluated. Although previous reports generally indicate that shaking conditions enhance laccase production in filamentous fungi due to improved oxygen transfer and homogenized nutrient distribution [54,55], the present study demonstrated that *Megasporoporia* sp. exhibited higher laccase productivity under static cultivation (Figure 6). In contrast, cultures subjected to increased agitation speeds showed a pronounced decrease in laccase activity, with the most significant reduction observed at 200 rpm. A study by Krumova *et al.* [56] reported similar results in

*Trametes trogii*, which produced higher laccase activity under static conditions compared to shaking conditions. It is likely that the cell walls of *Megasporoporia* sp. possess relatively low shear resistance. Under agitated conditions, hydrodynamic shear stress could negatively affect mycelial integrity and growth, and this suppression of biomass development would directly reduce laccase production [57,58]. Moreover, high agitation rates generate strong shear forces that can disrupt fungal physiological homeostasis, ultimately inhibiting enzyme biosynthesis [59].

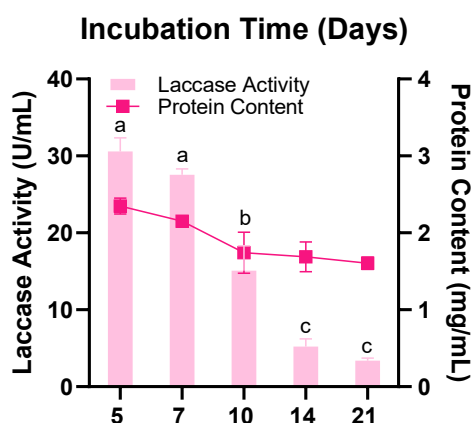


**Figure 6** Effect of agitation speed on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

#### Effect of incubation time

Determining the optimal incubation time is necessary to identify the appropriate harvesting period for laccase. After 5 days of incubation, laccase activity reached its highest level at 30.57 U/mL (Figure 7). In the subsequent incubation intervals, the activity showed a slight decline, and by days 14 and 21, the laccase activity dropped sharply to 5.22 and 3.37 U/mL,

respectively. Similar findings were reported for *Aspergillus niger* IBP2013 and *Curvularia lunata* MY3, which also exhibited an optimal incubation time of 5 days for laccase production [60,61]. Therefore, it can be concluded that *Megasporoporia* sp. is able to produce high laccase activity within a relatively short incubation period, which is advantageous for production efficiency.



**Figure 7** Effect of incubation time on laccase activity and protein content produced by *Megasporoporia* sp. Data are presented as mean  $\pm$  SD (n = 3). Bars sharing different letters differ significantly in enzyme activity ( $p < 0.05$ ; 1-way ANOVA followed by Tukey's HSD test).

Collectively, this study demonstrates that optimized conditions resulted in more than a 30-fold increase in laccase production compared to previous unpublished data obtained by Lestari *et al.* [28].

Although optimization using the OFAT approach successfully enhanced laccase production, this method is unable to reveal interactions among factors. Therefore, in future studies, the most influential factors

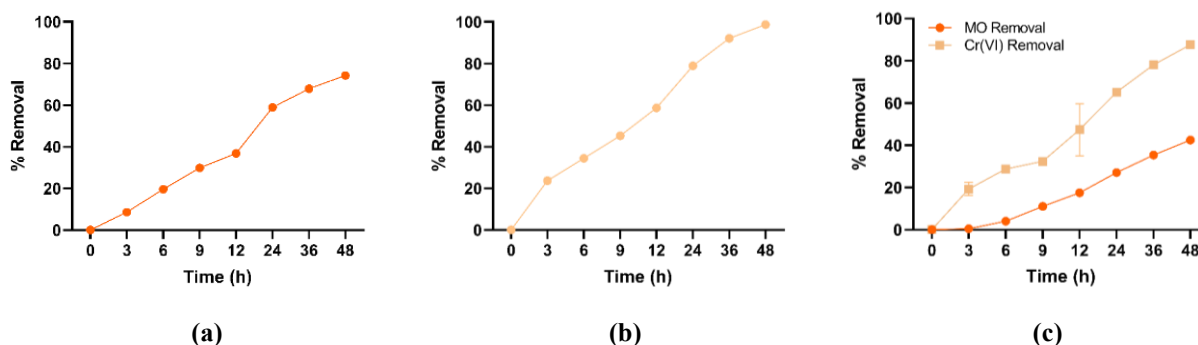
(e.g., carbon source, nitrogen source, and copper concentration) should be further investigated to determine their interactive effects on laccase production by *Megasporoporia* sp., for example by applying response surface methodology (RSM) [62].

### Bioremoval of MO and Cr(VI)

In the single-pollutant system, laccase showed remarkable efficiency in removing both MO and Cr(VI), achieving approximately 74.17% and 98.67% removal, respectively (**Figures 8(a)** and **8(b)**). This strong performance highlights the enzyme's versatility in acting on both metal and organic pollutants. The higher Cr(VI) removal can be explained by the enzyme's ability to reduce toxic Cr(VI) to its less toxic Cr(III), a process that has been reported in previous studies. For example, the laccase produced by *Ganoderma multipileum* was found to eliminate over 94% of Cr(VI) [27]. Furthermore, the MO decolorization in this study showed a comparable result to that reported by Girelli *et al.* [63], where the application of laccase for MO decolorization demonstrated notably high catalytic efficiency, achieving around 71% decolorization under optimized conditions. MO decolorization involves

oxidative cleavage of the azo ( $-N=N-$ ) bond and breakdown of aromatic ring reactions that are often slower and less complete [64,65].

When both MO and Cr(VI) were present together (dual-pollutant system), the removal efficiency of each pollutant decreased slightly compared to the single-pollutant setup (**Figure 8(c)**). Cr(VI) removal remained relatively high (87.57%), whereas MO removal dropped more noticeably (42.46%). This pattern suggests that interactions between the 2 contaminants influence enzymatic activity and reaction kinetics. However, when pollutant concentration is high, combined toxicity may inhibit enzyme activity, leading to an overall reduction in removal performance [66]. Therefore, the observed differences between the single- and dual-pollutant systems likely reflect a balance between synergistic stimulation of enzyme activity and the inhibitory effects caused by the coexisting pollutants. To address this limitation, enzyme immobilization for further applications is highly recommended, as it can enhance both the stability and catalytic performance of the enzyme, thereby potentially improving its bioremoval efficiency toward pollutants [67].



**Figure 8** (a) MO removal in the single-pollutant system; (b) Cr(VI) removal in the single-pollutant system; and (c) Simultaneous removal in the dual-pollutant system (orange line: MO; yellow line: Cr(VI)).

### Phytotoxicity evaluation

The phytotoxicity evaluation using *V. radiata* showed that both MO and Cr(VI) exhibited strong inhibitory effects on seed germination and growth, indicating their toxicity to plants (**Table 1**). The addition of laccase reduced this toxicity in both MO and Cr(VI) treatments, as reflected by higher germination (from 22% to 64% for MO and from 16% to 82% for Cr(VI)) and higher germination indices (from 2.24% to 37.57% for MO and from 0.83% to 68.50% for Cr(VI)), along

with lower growth inhibition compared to the pollutants alone. This suggests that laccase effectively detoxifies these contaminants, likely by degrading MO's azo bonds and reducing Cr(VI) to the less toxic Cr(III) form. Moreover, in the dual-pollutant system, phytotoxicity remained pronounced (8% germination and 0.17% GI), and although laccase treatment slightly improved germination (60%) and GI (31.60%), the enzyme's protective effect was less substantial than in the single-pollutant systems. The higher toxicity of MO and

Cr(VI), particularly in combination, is consistent with previous findings that both pollutants induce oxidative stress, inhibit nutrient uptake, and disrupt photosynthetic activity in plants [68, 69]. The coexistence of MO and Cr(VI) likely causes synergistic

stress, increasing reactive oxygen species accumulation and cellular damage. Meanwhile, laccase's partial detoxification effect aligns with its known ability to oxidize aromatic dyes and reduce metal toxicity [70].

**Table 1** Germination, root elongation, and GI of *V. radiata* under MO, Cr(VI), and mixed-pollutant treatments.

| Treatment                     | % Germination           | Root elongations (cm)     | % GI                       |
|-------------------------------|-------------------------|---------------------------|----------------------------|
| DI Water                      | 100 ± 0.00 <sup>a</sup> | 65.50 ± 0.35 <sup>a</sup> | 100 ± 5.47 <sup>a</sup>    |
| MO                            | 22 ± 8.36 <sup>d</sup>  | 5.94 ± 0.24 <sup>d</sup>  | 2.24 ± 1.51 <sup>e</sup>   |
| Laccase-treated MO            | 64 ± 8.94 <sup>c</sup>  | 38.02 ± 0.39 <sup>c</sup> | 37.57 ± 8.62 <sup>c</sup>  |
| Cr(VI)                        | 16 ± 11.4 <sup>de</sup> | 2.56 ± 0.17 <sup>d</sup>  | 0.83 ± 0.76 <sup>f</sup>   |
| Laccase-treated Cr(VI)        | 82 ± 13.03 <sup>b</sup> | 53.66 ± 0.93 <sup>b</sup> | 68.50 ± 22.3 <sup>b</sup>  |
| MO and Cr(VI)                 | 8 ± 4.47 <sup>e</sup>   | 1.20 ± 0.06 <sup>e</sup>  | 0.17 ± 0.11 <sup>f</sup>   |
| Laccase-treated MO and Cr(VI) | 60 ± 10.0 <sup>c</sup>  | 33.80 ± 0.53 <sup>c</sup> | 31.60 ± 10.06 <sup>d</sup> |

Description: Different superscript letters within the same column indicate significant differences ( $p < 0.05$ ;  $n = 5$ ; 1-way ANOVA followed by Tukey's HSD test).

## Conclusions

The OFAT optimization of laccase production by *Megasporoporia* sp. resulted in the following optimal conditions: glucose or sucrose as the carbon source, a combination of yeast extract and ammonium sulfate as the nitrogen source, 1.5 - 2.0 mM CuSO<sub>4</sub>, pH 5.0, incubation at 28 °C for 5 days, and static cultivation. The crude enzyme produced by this fungus demonstrated considerable potential for MO and Cr(VI) removal in the single-pollutant system, achieving 74.17% MO removal and 98.67% Cr(VI) removal. Meanwhile, in the dual-pollutant system, the crude enzyme achieved 42.46% MO removal and 87.57% Cr(VI) removal. In addition, the phytotoxicity evaluation using *V. radiata* revealed that laccase treatment improved germination from 22% to 64% for MO and from 16% to 82% for Cr(VI), while the dual-pollutant system showed a more limited improvement (from 8% to 60% germination). These results indicate that laccase from *Megasporoporia* sp. holds promising potential as an eco-friendly and safe approach for treating wastewater containing MO and Cr(VI) in the future.

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## Declaration of generative AI in scientific writing

The authors admit the use of generative AI tool (ChatGPT by OpenAI) for language and grammatical editing during the arrangement of this manuscript. AI tool did not contribute to content or data interpretation. All authors remain the responsibility for the manuscript's content and conclusions.

## CRedit author statement

**Titan Dwikama Putra:** Methodology; Investigation; Data curation; Formal analysis; Writing - Original Draft. **Wichanee Bankeeree:** Project administration; Supervision; Writing - Review & Editing; Resources. **Sehanat Prasongsuk:** Conceptualization; Validation; Supervision; Funding acquisition; Project administration.

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