Removal of malachite green toxicity using water hyacinth (Eichhornia crassipes) biomass

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Abstract. Malachite green (MG), a common synthetic dye that is used extensively in many industries including aquaculture, is highly toxic to cells. One of its metabolites, leucomalachite green, poses more toxic effects to cells compared to MG itself. Adsorption of this synthetic dye seems to be a promising method in removing dye without the formation of other harmful products. This study evaluated the potency of water hyacinth (Eichhornia crassipes) biomass of both root and leaf part of the plant in removing MG dye toxicity. Biomass from both root and leaf part of the plant showed high decolorization efficiency in less than 1 hour of incubation. Results from thin layer chromatography (TLC) analysis showed no formation of other degradation metabolites, implying the decolorization process that occurred was solely adsorption. Toxicity test towards Saccharomyces cerevisiae showed that water hyacinth biomass could reduce MG toxicity. Therefore, the root and leaf biomass collected from E. crassipes does have the potential to detoxify aqueous solution contaminated by MG.

1. Introduction

Synthetic dyes have been commonly used in many industries, such as textiles, food, pharmaceutical, cosmetic, aquaculture, pulp and paper industries to color products [1]. Once disposed of, the wastewater ends up in the environment, contaminating the soil and water bodies. Dyes are very detectable contaminants, because of their intense color in water bodies. Dyes are more stable and resistant to microbial degradation due to their complex aromatic molecular structures [2]. Moreover, colored dye effluents are known to be very toxic and are potential mutagenic and carcinogenic agents [3].

One of the most extensively used toxic synthetic dye is Basic Green 4, commonly known as malachite green (MG). MG, belonging to the triphenylmethane dye group, is highly soluble in water and is commonly used in textile industry (for dyeing silk, wool, jute, leather, ceramics, and cotton), in the aquaculture industry (as a fungicide, parasiticide, and disinfectant), as well as a cytochemical staining agent [4,5]. MG has been extensively used in aquaculture as a topical treatment by bath or flush methods to control skin and gill flukes [4]. However, the use of MG brings controversy due to the toxic side effects it produces. Though the use of MG as a biocide or coloring agent has been banned in several countries, many parts of the world still use this dye due to its low cost, ready availability and efficacy [6].

MG is highly toxic to mammalian cells, even in concentrations of less than 0.1 ppm. It is reported to cause carcinogenesis, mutagenesis, chromosomal aberration, and respiratory toxicity [7]. It is also...
known to increases liver tumor formation in rats and generates reproductive abnormalities in rabbits and fish [8]. One of MG degradation product, leucomalachite green (LMG) is known to be more toxic than MG itself and remains in cells for a longer time period [3,9]. Many methods have been tested for the removal of MG from aquatic environments. Current methods of detoxifying and decolorizing MG involve the use of boron-doped diamond (BDD) anodes and electro-Fenton systems, peroxicoagulation, all of which are neither readily available nor effective when dealing with wastewater that has entered the environment [2]. Degradation of MG by various methods might not fully detoxify the products and might be even more toxic. There is therefore a need for an affordable and efficient method to remove these effluents from wastewaters.

One efficient method in removing dyes is adsorption. Adsorption is a promising approach for the removal of various pollutants from water and wastewater. Agricultural solid wastes and biosorbenst, such as biopolymers and biomass adsorbents, have shown interesting adsorption abilities to remove MG [10]. One promising biosorbent is water hyacinth (Eichhornia crassipes) biomass. Water hyacinth is a wild aquatic plant that often grows in lakes, rivers, swamps, or ponds. It can easily grow in extensive amount that causes environmental problems and can be considered a weed by some, therefore, it has little to no economic value in public. Nevertheless, water hyacinth has the potential to remove pollutants, when used as a biological filtration system. It has also been reported that the water hyacinth has excellent tolerance towards toxic pollutants. The water hyacinth’s biomass has large potential in decolorizing textile dyes and able to adsorb heavy metals [11,12]. Therefore, this study was aimed to evaluate the potential of water hyacinth biomass in removing MG dye toxicity through a yeast assay system.

2. Materials and methods

2.1. Materials
Malachite green (927.01 g/mol, dye content ≥ 90 %) and CuSO₄·5H₂O were purchased from Merck. Potato dextrose agar (PDA) and broth (PDB) were obtained from Pronadisa. The solvents (acetic acid, ethyl acetate, n-propanol) were purchased from CV Anugrah. Saccharomyces cerevisiae was isolated from a commercial baker’s yeast (Fermipan). Samples of Eichhornia crassipes were collected from Danau Kelapa Dua, Tangerang, Indonesia.

2.2. Biomass preparation
Water hyacinth were cleaned completely with tap water for several times to remove dirt and all the soil particles followed by separation of the leaf and root from the stem. Root and leaf were separated, and both samples were washed with distilled water. Samples were dried in the oven of 70 °C for 24 hours, then were put in desiccator for another 24 hours. The dried samples were grounded, and the powder was used in this study.

2.3. MG decolorization assay
MG decolorization assay was carried out by putting 0.1 g of both root and leaf biomass into a 100 ml solution of 50 ppm MG. The samples were incubated at 25 °C in shaker incubator at 120 rpm speed. Samples were withdrawn at time intervals (0, 1, 3, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 180, 240, and 300 minutes) and the residual MG concentration in solution was measured immediately using UV/VIS spectrophotometer (BioDrop) in wavelength 617 nm. Absorbance spectra was measured at 300-800 nm wavelength to see the overall change. This experiment was repeated for four times [3,9,13]. The absorbance value was converted into concentration (ppm) using linear equation obtained from standard curve.

Based on the absorbance value using UV/VIS spectrophotometer, percentage of decolorization was measured [3]. The decolorization efficiency of the adsorption was calculated using the following equation:

\[
\text{Decolorization (\%) } = \frac{(A_0 - A)}{A_0} \times 100
\]
In which $A_0$ is the initial absorbance and $A$ is the absorbance of medium after dye decolorization at 617 nm. All the values shown were mean ± standard deviation of four replicates. Percentage of decolorization for each time interval between root and leaf sample were compared by two sample t-test in Microsoft Excel.

2.4. Thin layer chromatography (TLC) assay for metabolites

After complete adsorption, metabolites produced in decolorized dye solution were extracted with equal volume of ethyl acetate and a small amount of CuSO$_4$·5H$_2$O. The metabolites formation was examined by TLC using silica gel. The solvent mixture used was n-propanol: acetic acid: ethyl acetate: distilled water (6:1:1:2 v/v). The separated products were examined directly [14].

2.5. Toxicity test

Toxicity test of the decolorized product was done using *Saccharomyces cerevisiae*. *S. cerevisiae* was isolated from a commercial baker’s yeast by adding a certain amount of the yeast into PDB media as a starter culture. The culture was incubated for 24 hours in room temperature. The culture was then streaked into PDA and incubated for 24 hours in 37 °C until pure culture was obtained.

The toxicity test was done using a well-diffusion assay. PDA was poured in petri dish; depth of agar was 3–4 mm. With sterile cotton swab, *S. cerevisiae* was spread evenly over the plate successively in two directions to obtain an even inoculum. The plate was allowed to dry for 5 min. Wells of 7 mm diameter were cut on the surface of the agar. Twenty microliters of each treatment (MG solution treated for 0, 5, 15, 30, 60, 180 and 300 minutes) were added in one well. This was done in four replicates for both root and leaf samples. In addition, a standard was made by adding untreated MG solution of different concentrations (0, 5, 7.5, 10, 25 and 50 ppm) to each well. All test plates were incubated aerobically at 37 °C for 24 hours and observed for the inhibition zones. The zone of inhibition was measured by a scale to the nearest mm excluding well diameter [15,16]. Means between groups were compared by One Way ANOVA followed by Tukey Honest Significant Difference (HSD) post-hoc analysis in Minitab 18.

3. Result and discussion

3.1 MG decolorization assay

MG decolorization assay was done to evaluate the ability of *E. crassipes* biomass in removing MG. Both root and leaf biomass of *E. crassipes* were dried, grounded and added to the MG solution. The initial concentration of the dye was 50 ppm. The test was carried out by examining the absorbance spectra between 300 - 800 nm. Figure 1 shows the graph of absorbance spectra.

The change in absorbance at 617 nm indicates reduction of MG concentration in solution. As time progressed, the peak of A617 decreased correspondingly with MG concentration in the solution (Table 1). Barapatre *et al.* [17] reported that untreated MG has three main absorbance peaks at 315 nm, 420 nm and 617 nm, while degraded MG by ligninolytic fungus *Aspergillus flavus* has increased absorption peaks at 220, 254 and 370 nm. Mukherjee & Das [18] also reported that the absorption peak of MG at 617 nm was completely lost while three new absorption peaks appeared at 612, 335, 321 nm due to biodegradation by enzymes of *Enterobacter asburiae* XJUHX-4TM. Meanwhile, if the decolorization is due to the adsorption, the original absorption peak would decrease proportionally. Therefore, the process of decolorization in this study indicates adsorption of the dye by *E. crassipes* biomass.

Table 1 showed that both root and leaf could decolorize MG up to 90% in less than 1 hour. In comparison between root and leaf part of the plant, the root section showed a significantly (p<0.05) higher adsorption ability up to 180 minutes of incubation. The root part also showed faster decolorization ability where it reached equilibrium at 20 minutes compared to the leaf which needed 45 minutes. In comparison with other biomass, Burca *et al.* [19] reported that *Camellia sinensis* leaves could reach equilibrium in 80 minutes for removal of MG with concentration of 75-140 ppm.
Meanwhile for other dyes, Tan et al. [20] reported that whole part of *E. crassipes* plant could remove methylene blue and methyl orange up to 98% and 67% respectively in 20 days in open air and exposed to sufficient sunlight. This indicates that the use of *E. crassipes* biomass in this study has a promising decolorization ability towards MG in a short period of time.

Overall, the root biomass of *E. crassipes* performed better than the leaf biomass in this study. This is likely due to the cellulose content of the plant parts. Cellulose fibres are hydrophilic due to the hydroxyl groups found on the surface of the structure and could therefore contribute to the effectiveness in which the root biomass absorbs the dye. Lara-Serrano *et al.* [21] showed that the roots of *E. crassipes* contained significantly higher amounts of cellulose, holocellulose and hemicellulose when compared to the plant’s leaves. It is therefore likely that the root biomass has better absorption properties the leaf biomass.

A. Root
Figure 1. Absorbance spectra of MG solution after treated with *E. crassipes* biomass addition, taken every five minutes. The initial MG concentration was 50 ppm. The samples were incubated in a shaker incubator at 25 °C and 120 rpm speed. Data shown were mean of four replicates.

Table 1. Decolorization percentage of *E. crassipes* biomass towards MG and its concentration.

| Incubation time (min) | Dye concentration (ppm) | Decolorization percentage (%) |
|-----------------------|--------------------------|-------------------------------|
|                       | Root                     | Leaf                          | Root                        | Leaf                        |
| 0                     | 40 ± 2.4                  | 37 ± 2.5                      | 0 ± 0.0                     | 0 ± 0.0                     |
| 5                     | 12 ± 1.1                  | 12 ± 0.7                      | 71 ± 1.2*                  | 70 ± 0.5                    |
| 10                    | 5 ± 0.2                   | 9 ± 0.4                       | 89 ± 0.4*                  | 79 ± 0.6                    |
| 15                    | 4 ± 0.2                   | 6 ± 0.2                       | 93 ± 0.4*                  | 86 ± 0.3                    |
| 20                    | 3 ± 0.2                   | 5 ± 0.3                       | 94 ± 0.6*                  | 88 ± 0.6                    |
| 25                    | 3 ± 0.2                   | 5 ± 0.4                       | 95 ± 0.6*                  | 88 ± 0.5                    |
| 30                    | 3 ± 0.2                   | 5 ± 0.3                       | 95 ± 0.6*                  | 89 ± 0.4                    |
| 45                    | 3 ± 0.2                   | 4 ± 0.3                       | 95 ± 0.7*                  | 91 ± 0.6                    |
| 60                    | 3 ± 0.2                   | 4 ± 0.4                       | 95 ± 0.7*                  | 91 ± 0.7                    |
| 90                    | 3 ± 0.2                   | 4 ± 0.4                       | 95 ± 0.7*                  | 92 ± 0.7                    |
| 120                   | 3 ± 0.2                   | 4 ± 0.3                       | 95 ± 0.7*                  | 92 ± 0.5                    |
| 180                   | 3 ± 0.2                   | 4 ± 0.4                       | 94 ± 0.7*                  | 92 ± 0.7                    |
| 240                   | 4 ± 0.2                   | 4 ± 0.4                       | 93 ± 0.8                   | 92 ± 0.6                    |
| 300                   | 4 ± 0.2                   | 4 ± 0.3                       | 92 ± 0.9                   | 92 ± 0.8                    |

* Indicates significant difference of decolorization percentage (p < 0.05) between root and leaf during the same time.

Note: The values shown were mean ± standard deviation of four replicates. Means were compared using two sample t-test. Dye concentration was determined using standard curve of MG.
3.2 TLC analysis of metabolites

When metabolized, MG can form a colorless yet more toxic product called LMG, which is also known to retain in the muscles and cells of fish for longer periods of time [22]. A TLC analysis using the product of the adsorption process was therefore used to determine if the adsorption conducted by the biomass produces any other metabolites. The samples collected from different time points of the adsorption process (Figure 2Aa-g and 2Ba-g) were run through the TLC with Figure 2A1-5 and 2B1-5 as controls. The controls, which contain MG on its own, display a single spot at the same retardation factor (Rf) value of around 0.7 with a decrease in intensity and size in proportion to the concentration of MG present in the sample. Similar to the controls, a large, single, vivid spot at the same Rf value as the control samples can be seen at time 0-300 minutes that decreased in intensity and size over time (Figure 2Aa-g and 2Ba-g). This is especially true for the samples incubated with the root biomass as the spot formed by the sample at 300 minutes is much more faded and smaller than the spot formed by the lowest concentration of the control samples, which therefore implies a concentration of MG of less than 5 ppm in the sample, measured as 4 ppm (Table 1). The decrease in intensity of the spots formed indicates that a process of decolorization and adsorption occurs when the leaf and root biomass of *E. crassipes* are incubated with the MG.

TLC analysis also showed that no other metabolites were present in the final product and that the biomass was able to adsorb the product without degrading MG. Wanyonyi *et al.* [14] reported that TLC of degraded MG by crude protease enzymes showed the appearance of three additional spots with Rf values of 0.96, 0.92 and 0.90 as compared to control Rf value of 0.87. This indicates that the process of decolorization demonstrated by the biomass in this study was indeed adsorption and not degradation.

![Figure 2](image.png)

**Figure 2.** TLC analysis of metabolites produced. The arrows show separated dye compounds being extracted with Rf value of ± 0.7. 1: MG 50 ppm; 2: MG 25 ppm; 3: MG 10 ppm; 4: MG 7.5 ppm; 5: MG 5 ppm; a: 0 min; b: 5 mins; c: 15 mins; d: 30 mins; e: 60 mins; f: 180 mins; g: 300 mins.
3.3 Toxicity analysis of metabolites in yeast assay system

Despite evidence that adsorption has occurred, a decrease in the intensity of the color does not necessarily mean that the samples are detoxified and therefore a toxicity assay was conducted using baker’s yeast (S. cerevisiae) to determine the toxicity of the samples incubated with the root and leaf biomass of E. crassipes.

### Table 2. Toxicity test of root and leaf metabolites

| Time (minutes) | Root            | Leaf            |
|----------------|-----------------|-----------------|
| 0              | 10.3 ± 0.80<sup>a</sup> | 10.0 ± 0.70<sup>a</sup> |
| 5              | 2.0 ± 0.70<sup>c</sup>  | 4.5 ± 1.50<sup>b</sup>  |
| 15             | 0<sup>c</sup>    | 1.3 ± 0.40<sup>c</sup> |
| 30             | 0<sup>c</sup>    | 0<sup>c</sup>     |
| 60             | 0<sup>c</sup>    | 0<sup>c</sup>     |
| 180            | 0<sup>c</sup>    | 0<sup>c</sup>     |
| 300            | 0<sup>c</sup>    | 0<sup>c</sup>     |

Note: Clear zone area was calculated by excluding diameter of wells. Means that do not share a letter are significantly different (p < 0.05).

The samples incubated with the root and leaf biomass were plated into the wells at different time points and the results can be seen in Table 2. As seen in Table 2, the diameter of the clear zone at 0 minute was similar to the diameter of that MG concentration 50 ppm (data not shown) and gradually decreases as time passes. After 5 minutes, the clear zone diameter was reduced significantly for both root and leaf sample. However, the diameter for the leaf samples was still significantly higher (p<0.05) than the root samples. This might be due to the slower adsorption by the leaf samples compared to the root samples at 5 minutes. After 15 minutes, both samples toxicity were comparable to MG 0 ppm (data not shown). This indicates a lower growth inhibition effect of the yeast colonies surrounding the wells and thus demonstrating a gradual decrease of the toxicity of the samples up until 300 minutes. The adsorption that occurs when the root and leaf biomass are incubated with MG can therefore decolor and detoxify MG contaminated areas. Based on the results, it can be concluded that the root and leaf biomass collected from E. crassipes does have the potential to detoxify aqueous solution contaminated by MG.

4. Conclusion

E. crassipes root and leaf biomass were proved to have potential in decolorizing MG solution by adsorption process. Both the root and leaf biomass could decolorize up to 90% MG in less than 1 hour. Moreover, TLC analysis showed that decolorization process occurred did not produced other metabolites. It was supported by yeast toxicity test which proved decreased toxicity of treated MG compared to untreated MG. In conclusion, E. crassipes biomass has potential to detoxify MG, therefore can be used in bioremediation of industrial waste water as an affordable source of biosorbent.

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