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Biotransformation of Methylene Blue by Mixed Fungal Cultures of *Gloeophyllum trabeum* and *Aspergillus oryzae*

Adi Setyo Purnomo[1*], Adelia Sabilah Prameswari[1], Hamdan Dwi Rizqi[1], Taufiq Rinda Alkas[1], Ratna Ediati[1], Yuly Kusumawati[1]

[1]*Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember (ITS), Kampus ITS Sukolilo, Surabaya, 60111, Indonesia*

Abstract. This study investigated the biotransformation of methylene blue (MB) by mixed fungal cultures of *Gloeophyllum trabeum* and *Aspergillus oryzae*. Equal volumes of *A. oryzae* and *G. trabeum* cultures were applied to Erlenmeyer containing MB and incubated at 30°C for 7 days. The change in absorbance of the MB control solution and the solution after application, measured with a UV-Visible spectrophotometer, was used to calculate the decolorization. The addition of *A. oryzae* to *G. trabeum* cultures showed MB biodecolorization reaching 69.34%, greater than single cultures of *G. trabeum* and *A. oryzae*, which were 31.50% and 36.82%, respectively. Metabolite identification from MB biodecolorization by mixed culture using LC-QTOF-MS found the following metabolites: $C_{16}H_{20}N_3S$, $C_{19}H_{22}N_3SO_4$, $C_{31}H_{48}N_3S^+$. The results of this study showed that the addition of *A. oryzae* enhanced the percentage of MB decolorization from *G. trabeum* culture.

Keywords: *Aspergillus oryzae*; Biodecolorization; *Gloeophyllum trabeum*; Methylene blue; Mixed culture; Pollutants

1. Introduction

Indonesia is one of the main textiles producing countries in Asia, which one of the world's top 10 exporters of textiles and textile products, along with countries such as China, India, Thailand, Brazil, and the United States. Indonesia's main attractions are its cheap labor force and large domestic market (AHK Indonesien, 2022). With the increasing population growth rate, textile production as a need for clothing increases rapidly.

This industrial process always produces waste, especially liquid waste. Many methods have been tried to manage textile wastewater, such as ozonation, photochemistry, adsorption, ion exchange, floatation, and electrokinetic coagulation. Removal efficiencies are in the range of 70-95%, but these processes still have drawbacks. The disadvantages of these methods include the need for large areas and the generation of a large amount of sludge, which causes problems with waste disposal (Istirokhatun et al., 2021). However, sludge from industrial by-products can also be processed and activated into biosorbents (Extracellular polymeric substances/EPS) (Kistriyani et al., 2020).

Approximately 10.000 types of dyes are used in the textile industry, and during the dyeing process, 10-15% of the textile dyes used will be removed with wastewater. One of

*Corresponding author's email: adi_setyo@chem.its.ac.id, adi.spurnomo@yahoo.com, Tel.: +62-31-5943353, Fax: +62-31-5928314
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the dye often used in the textile industry is methylene blue (MB) because it is economical, easy to obtain, and has a very strong adsorption power (Pratiwi et al., 2021). Meanwhile, although MB has some advantages, it can be toxic to humans and the environment. It can also cause human health problems such as respiratory disorders, stomach disorders, blindness, and digestion and mental disorders. Furthermore, MB also triggers nausea, diarrhea, vomiting, cyanosis, shock, gastritis, jaundice, methemoglobinemia, tissue necrosis, and increased heart rate, causing premature cell death in tissues and skin/eye irritation (Khan et al., 2022). The toxicity of MB dye was studied and reported had the no observed adverse effect level (NOAEL) value of 25 mg kg^{-1} for MB in rats (Bharti et al., 2019). Besides, MB had the LD_{50} 1180 mg kg^{-1} (oral acute toxicity rat), LC_{50} 18 mg L^{-1} (96 h, *Mystus vittatus*), and EC50 2.26 mg L^{-1} (48 h, *Daphnia magna*) (LabChem, 2019). The MB molecular structure is figured out in Figure 1.

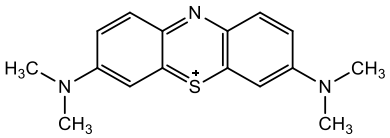


Figure 1 MB molecular structure

Many methods have been applied for MB degradation including advanced oxidation processes (AOPs), photodegradation, ozonation, oxidation with UV/H_2O_2, photocatalytic degradation etc. Several studies using nanocomposites have been carried out with excellent photodegradation results (88-100% removal), such as CuO/Bi_2O_3 nanocomposites, SnO_2-bentonite, TiO_2/Seashell, and ZnO-nanorods/activated carbon fibers (Khan et al., 2022). However, most of them need high costs and require elevated energy costs.

One of the effective methods for reducing dye wastewater is using microorganisms as a biological activity through biodegradation. Biodegradation, also called bioremediation, is a very broad field and the most reliable mechanism for removing organic and inorganic pollutants from the environment is by using microorganisms (Zahari et al., 2022). One of the microorganisms used for biodegradation is brown rot fungi (BRF). BRF produce hydroxyl radicals generated from the Fenton reaction to degrade cellulose, hemicellulose, and some dyes (Purnomo et al., 2022). According to Riadi et al. (2021), the initial organic compound degradation reaction is faster and more economical with Fenton's reagent than other chemical treatments, and the degradation yield can reach 70–99% (Riadi et al., 2021). In addition to producing hydroxyl radicals, these fungi produce cellulase enzymes used to degrade cellulose as a source of carbon and energy. It is an advantage of brown rot fungi compared to white-rot fungi which only use ligninolytic enzymes as degrading agents (Kim et al., 2014).

In a previous study by Purnomo et al. (2021), MB biodegradation was carried out using brown rot fungus *Gloeophylum trabeum* in liquid PDB media resulting in a decolorization percentage of 71.61% for 14 days of incubation. This result indicates that the biodegradation of MB using brown rot fungus *G. trabeum* takes a long time. Hence, it is necessary to modify the culture through mixed cultures. *Aspergillus oryzae* can decolorize several types of azo dyes from aqueous solutions such as Direct Red 23 and Direct Violet 51 because *A. oryzae* is used as a biosorption substrate by azo dyes (Corso et al., 2012). *A. oryzae* was reported that it can be used for the remediation of hydrocarbon polluted soils (Asemoloye et al., 2020) and degrade a mycotoxin compound (Ochratoxin A) that can contaminate agricultural products (Xiong et al., 2021). The combination of brown rot fungus *G. trabeum* and filamentous fungi *A. oryzae* is a new combination that has never been studied for MB degradation. This study aims to determine the degradation ability of this

combination against methylene blue dye, predict the metabolite products, and propose the degradation pathways.

2. Methods

2.1. Fungi and Chemicals

The fungi used in this study include *G. trabeum* and *A. oryzae* taken from the Microorganism Chemistry laboratory collection. The chemicals were used such as methylene blue (SAP Chemicals), potato dextrose agar (PDA, Merck), potato dextrose broth (PDB, Difco), distilled water (Brataco), alcohol (70%, Brataco), and filter paper (Whatman).

2.2. Microorganisms Culture conditions

Stock cultures of *G. trabeum* and *A. oryzae* from the collection were taken ± 1 cm^2 of mycelium and then inoculated on PDA sterile petri dish that had been incubated statically at 30°C for 7 days. *G. trabeum* and *A. oryzae* mycelia (diameter 1 cm) were inoculated into 9 mL of PDB medium separately and then pre-incubated statically for 7 days at 30°C. The regenerated *G. trabeum* and *A. oryzae* fungi were put into a sterile cup blender (Waring, J-SPEC LB10BUJ) containing 25 mL of sterile distilled water and then homogenized until evenly crushed. The homogenate (1 mL) was inoculated into Erlenmeyer containing 9 mL of PDB liquid medium using a micropipette and then pre-incubated statically for 7 days at 30°C (Pratiwi et al., 2021).

2.3. Biotransformation of MB by Single Culture

The fungal culture of *G. trabeum* or *A. oryzae* 10 mL after pre-incubation (section 2.2) was added to 9 mL of PDB media and then added with 1 mL of MB 2000 mg/L (final concentration of MB 95.24 mg/L, total volume 20 mL). The culture was incubated in an incubator (Lovibond, ET 651-8) at 30°C for 7 days. After incubation, the cultures were separated by centrifuge (Thermo, IEC CL40R) at 3000 rpm for 5 minutes. The absorbance of the supernatant was measured using a UV-Vis spectrophotometer (Thermo, Genesys 10S UV-Vis). Abiotic control was made from 20 mL PDB liquid medium which added with 1 mL MB dye to reach a final concentration of 95.24 mg/L. In contrast, the biotic control was made from the fungal culture of *G. trabeum* or *A. oryzae,* which was killed by using autoclave before adding MB dye. The percentage of MB dye decolorization was calculated using Equation 1, where Ak and At are control absorbance and treatment absorbance, respectively (Pratiwi et al., 2021).

$$\text{Decolorization (\%)} = \frac{Ak-At}{Ak} \text{ x } 100\% \quad (1)$$

2.4. Biotransformation of MB by Mixed Cultures

A. oryzae liquid culture (10 mL) was added to the pre-incubated *G. trabeum* culture (10 mL), and then added 1 mL of 2000 mg/L MB (final culture concentration 95.24 mg/L). The mixed cultures were incubated at 30°C for 7 days. The cultures were separated by using a centrifuge at 3000 rpm for 5 minutes after 7 days, the supernatant was analyzed by using a UV-Vis spectrophotometer. Abiotic control was made from 20 mL PDB liquid medium added with 1 mL MB dye to reach a final concentration of 95.24 mg/L. In contrast, the biotic control was made from mixed cultures of *G. trabeum* and *A. oryzae* fungi which were turned off by heating with an autoclave before adding MB. The percentage of MB dye decolorization was calculated using Equation 1.

2.5. Analysis of Biotransformation of MB and Its Metabolite Products

Analysis of biotransformation of MB and its metabolites was carried out by analyzing the supernatant using the LC-QTOF MS (Bruker impact II™). The ionization source was

ionization electro spheres with a mass range of 50-350. The elution method used the gradient method with a flow rate of 0.2 mL min-1 after 7 days in the first 3 minutes and the next 7 minutes using a flow rate of 0.4 mL min-1. The mobile phase used methanol with a ratio of 99:1 for the initial three minutes and 61:39 for the 7 minutes remaining. The column used was Acclaim TM RSLC 120 C18 with a size of 2.1x100 mm and the column temperature is 33°C (Nabilah et al., 2022).

3. Results and Discussion

3.1. Biotransformation of MB by Single and Mixed Cultures

After the 7th day of adding MB, an analysis was carried out with a UV-Vis spectrophotometer to determine the ability of *G. trabeum* and *A. oryzae* cultures to decolorize MB. The use of a UV-Vis spectrophotometer in this study was due to the ease of measuring the amount of dye absorbed. The ability of fungal cultures to remove color was assessed from the difference between the initial and final absorbance compared to the initial absorbance. The analysis was carried out by separating the fungal biomass from the culture medium solution containing MB by centrifugation at 3000 rpm for 5 minutes. The supernatant was taken with a syringe so the mycelium did not mix and affect the absorbance value. From the results of the analysis, the absorbance profile of degradation by *G. trabeum* and *A. oryzae* is shown in Figure 2.

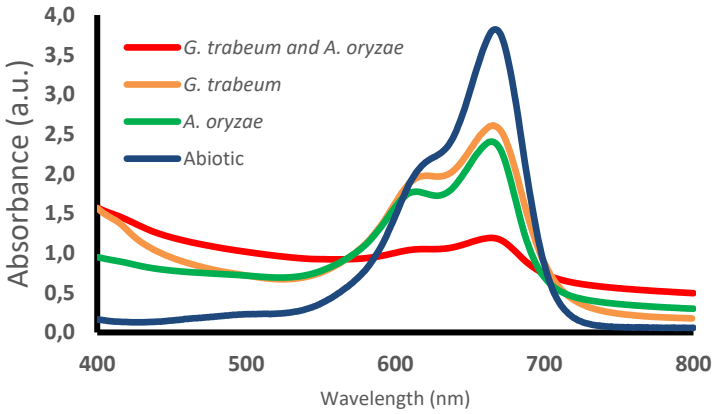


Figure 2 The absorbance profile graph of the MB decolorization result on the 7th day

Mixed cultures of *G. trabeum* and *A. oryzae* fungi on decolorization of MB dye were also analyzed using a UV-Vis spectrophotometer. The results of UV-Vis analysis showed that at the MB wavelength of 665 nm the obtained absorbance was 1.191, so the percentage of color removal using mixed culture was 69.34 %. Based on these results, it can be concluded that *A. oryzae* addition to *G. trabeum* culture can improve its decolorization ability.

Visualization of the MB solution at the mixed culture color after the incubation period was from deep blue to green. The color of the abiotic control was solid blue as a reference. This indicated that the process of transformation of MB by cultures occurred. The mixed cultures have a greener color than the single cultures of *G. trabeum* and *A. oryzae*, indicating an effect after adding *A. oryzae* on the biotransformation of MB by *G. trabeum*. The absorbance profile of MB decolorization was shown in Figure 2. Figure 2 showed the same graphic pattern at a wavelength of 665 nm and the intensity decreased from the abiotic control, especially in the mixed culture, there was the lowest decrease in absorbance compared to single cultures of *G. trabeum* and *A. oryzae*.

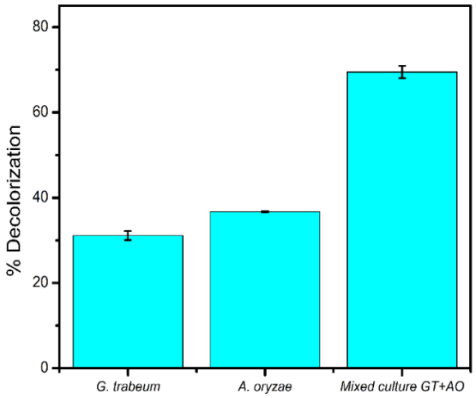


Figure 3 The decolorization of MB by all treatments

Figure 3 shows that the decolorized MB can be determined by the decolorization percentage of all culture treatments after 7 days. The addition of *A. oryzae* mold into the brown-rot fungus (BRF) *G. trabeum* culture could increase the decolorization of MB in a PDB liquid medium. Decolorization of mixed cultures was greater than single culture, which decolorized MB by 69.34 % after incubation for 7 days at 30°C, 37.84 % greater than single culture *G. trabeum* only 31.50 %, while *A. oryzae* only decolorized the MB of 36.82 %. This fact had the same conclusion as our previous study which mixed *G. trabeum* with *Trichoderma viride* fungus. These mixed cultures also demonstrated a higher decolorization than the single cultures (Pratiwi et al. 2021).

The increase of decolorization percentage in mixed cultures can occur by two mechanisms. First, the presence of a mixture of enzymes belonging to both fungi, xylanase (Kim et al., 2014) and laccase (Arimoto et al., 2015) in *G. trabeum* and lignolytic enzymes belonging to *A. oryzae* (Asemoloye et al., 2020) or hydrolytic enzymes such as cellulase, xylanase, azoreductase, pectinase, etc. (Corso et al., 2012). The second was the Fenton reaction mechanism that belongs to *G. trabeum* which produces hydroxyl radicals (Purnomo et al., 2020). Thus, adding *A. oryzae* s could increase the decolorization ability of MB in liquid media.

3.2. Identification of Metabolites of MB Biotransformation by Single Culture of G. trabeum and A. oryzae

Based on the chromatogram, there was the same peak between the control and treatment at a retention time of 5.57 min (Figure 4). Based on the QTOF-MS analysis, the two peaks have m/z of 284 which was the m/z of the MB. This assumption was based on the research by Rauf and colleagues in 2010 where MB had a peak m/z of 284 (Rauf et al., 2010) and Rizqi and Purnomo (2017) also found this peak in MB decolorization by *Daedalea dickinsii* fungus. The MB peak intensity in treatment culture was lower than the MB peak in the abiotic control. This phenomenon showed that MB had been transformed (degraded) and the MB concentration was reduced. In the chromatogram treatment, new peaks appeared at the retention times of 1.63, 2.64, 3.04, 4.66, and 5.40 mins. The identification of metabolite was performed based on references of previous studies and databases (Table 1).

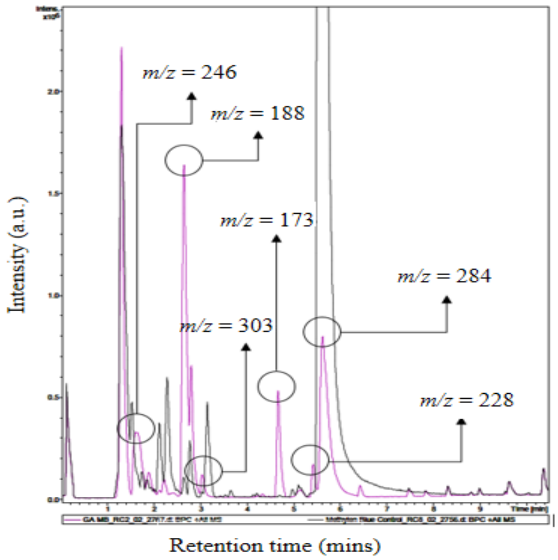
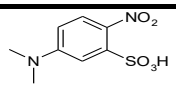
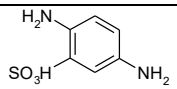
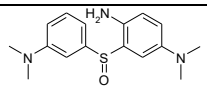
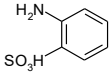
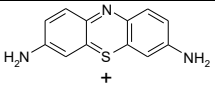


Figure 4 Profile Chromatogram of MB Biotransformation by *G. trabeum*. Black chromatogram: abiotic control (MB + PDB), while Red: treatment chromatogram (*G. trabeum*)

Table 1 Metabolites of MB decolorization by *G. trabeum*

Retention time (mins)	*m/z*	Molecular Formula	Molecular
1.63	246	$C_8H_{10}N_2SO_5$	 5-(dimethylamino)-2-nitrobenenesulfonic acid
2.64	188	$C_6H_8N_2SO_3$	 2,5-diaminobenzenesulfonic acid
3.04	303	$C_{16}H_{20}N_3SO$	 3-((3-dimethylamino) phenyl) sulfinyl-N-N-dimethylbenzen-1,4-diamine
4.66	173	$C_6H_7NSO_3$	 2-aminobenzenesulfonic acid
5.40	228	$C_{12}H_{10}N_3S$	 3,7-diaminophenothiazin-5-ium

The chromatogram profile of MB biotransformation by *A. oryzae* showed the same peak between control and treatment at a retention time of 6 min, with m/z of 284.122 identified as MB (Figure 5). The chromatogram treatment showed a new peak at the 5.17 min retention time, which was identified as $C_{31}H_{48}N_3S^+$ (Table 2).

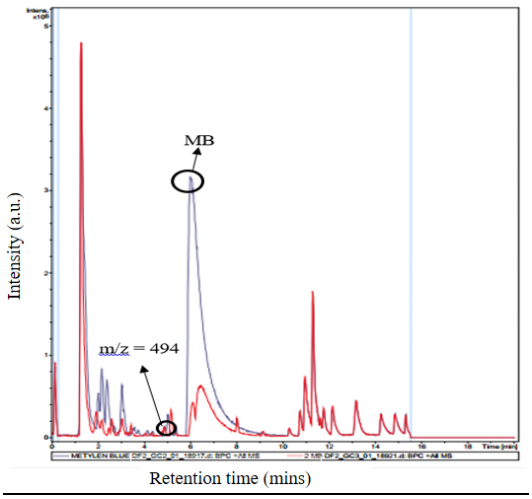
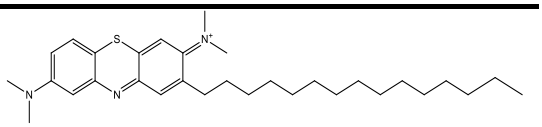


Figure 5 Profile Chromatogram of MB Biotransformation by *A. oryzae*, Black chromatogram: abiotic control (MB + PDB), while Red: treatment chromatogram (*A. oryzae*)

Table 2 Metabolites of MB decolorization by *A. oryzae*

Retention time (min)	*m/z*	Molecular Formula	Molecular
5.17	494	$C_{31}H_{48}N_3S^+$	 N-(8-(dimethylamino)-2-pentadecyl-3H-phenothiazin-3-ylidene)-N-methylmethanaminium

3.3. Identification of Metabolites of MB Biotransformation by Mixed Cultures

The LC chromatogram showed the same peak between control and treatment at a retention time of 5.78 mins, which identified MB (*m/z* 284). Based on the chromatogram in Figure 6, the MB peak on treatment showed a lower intensity than the MB peak in control. This indicates a decolorization process in the treatment so that the MB concentration was reduced. The treatment chromatogram showed that new peaks appeared at the retention times of 2.79, 4.81, and 7.44 mins (Table 3).

The LC-QTOF MS profile showed a decrease in the intensity of MB in treatment compared to control, which indicated a decrease in MB concentration. The appearance of several new peaks in the treatment indicated the metabolites of MB degradation. MB biodegradation pathway was proposed, as shown in Fig. 7. *G. trabeum* transformed MB via 3 initial pathways were oxidation of the sulfide group be 3-((3-dimethylamino) phenyl) sulfinyl-N-N-dimethylbenzen1,4-diamine; oxidation be 5-(dimethylamino)-2-nitrobenzenesulfonic acid; and demethylation to 3,7-diaminophenothiazin-5-ium, then oxidation cleavage to 2,5- diamino benzenesulfonic acid, and to 2-aminobenzenesulfonic acid.

Besides the transformation process of MB by *A. oryzae,* the product metabolites were N-(8-(dimethylamino)-2-pentadecyl-3H-phenothiazin-3-ylidene)-N-methylmethanaminium. From the transformation process of MB by mixed cultures, the product metabolites were 3,7-bis (dimethylamino)-9a,10 dihydrophenothiazin-5-ium; 3-2-amino-3-methyl-5-(N-methylformamido) phenyl) sulfinyl) 2,6-dimethylphenyl) (methyl) carbamic acid; and N- (7-(dimethylamino)-2-pentadecyl-3H-phenothiazin-3-ylidine)-N-methylmethanaminium. The estimated pathway for MB degradation using mixed culture *G. trabeum* and *A.*

oryzae is shown in Figure 7. This study indicated that the mixed culture could be used to transform MB.

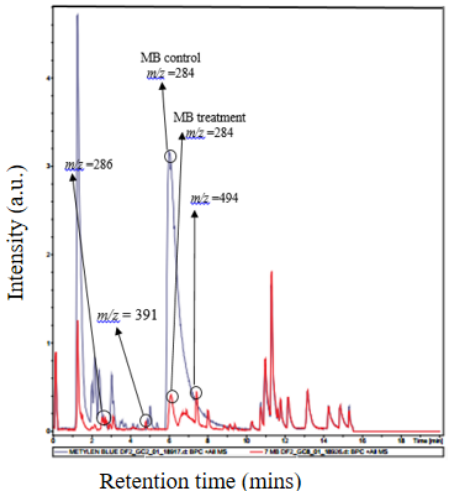
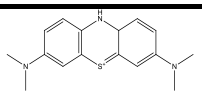
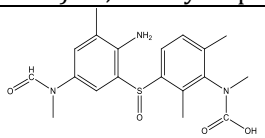
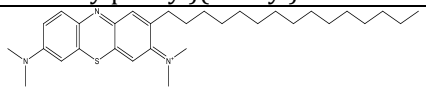


Figure 6 Profile Chromatogram of MB Biotransformation by mixed cultures, Black chromatogram: abiotic control (MB + PDB), while Red: treatment chromatogram (mixed cultures)

Table 3 Metabolites of MB decolorization by mixed cultures

Retention time (mins)	*m/z*	Molecular Formula	Molecular
2.79	286	$C_{16}H_{20}N_3S$	 3,7-bis(dimethylamino)-9a,10 dihydrophenothiazin-5-ium
4.81	391	$C_{19}H_{22}N_3SO_4$	 3-2-amino-3-methyl-5-(N-methylformamido) phenyl) sulfinyl)2,6dimethylphenyl)(methyl) carbamic acid
7.44	494	$C_{31}H_{48}N_3S$	 N-(7-(dimethylamino)-2-pentadecyl-3H-phenothiazin-3-ylidine)-N-methylmethanaminium

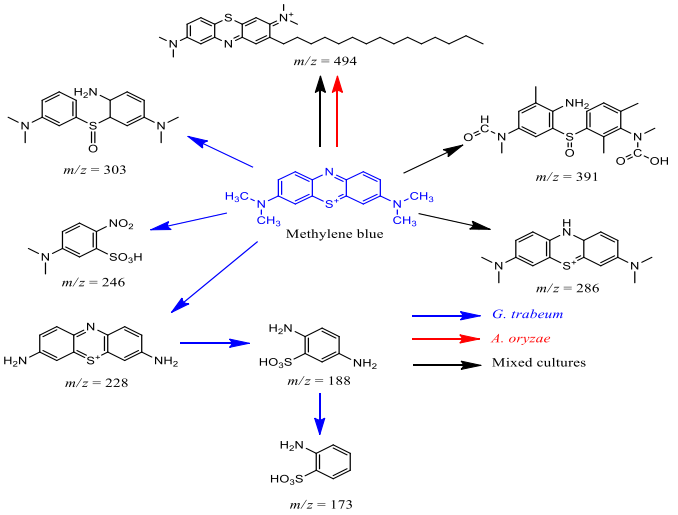


Figure 7 MB degradation proposed pathway by single cultures and mixed cultures

4. Conclusions

The single culture of *G. trabeum* and *A. oryzae* decolorized methylene blue (MB) by 31.50% and 36.82%, respectively. In comparison, the mixed cultures of *G. trabeum* and *A. oryzae* decolorized MB by 69.34% after incubation for 7 days. Based on the LC-QTOF MS analysis, the MB metabolite from biodecolorization by *A. oryzae* was $C_{31}H_{48}N_3S^+$, while that by *G. trabeum* were $C_6H_8N_2SO_3$, $C_6H_7NSO_3$, $C_8H_{10}N_2SO_5$, $C_{16}H_{20}N_3SO$, and $C_{12}H_{10}N_3S$. On the other hand, the MB metabolites by mixed cultures were $C_{16}H_{20}N_3S$, $C_{19}H_{22}N_3SO_4$, and $C_{31}H_{48}N_3S^+$. This study indicated that mixed cultures of BRF of *G. trabeum* and filamentous fungus *A. oryzae* were effective in decolorizing MB dye.

Acknowledgments

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