Involvement of Fenton Reaction on Biodecolorization and Biodegradation of Methylene Blue Dye by Brown Rot Fungi *Daedalea dickinsii*

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**Abstract:** The disposal of dye wastewater has become a major global concern. Meanwhile, microorganisms have shown high potential in the treatment of wastewater pollutants. In this study, the involvement of the Fenton reaction in the biodecolorization and biodegradation of methylene blue (MB) by the brown rot fungus *Daedalea dickinsii* was investigated. Subsequently, *D. dickinsii* is a fungus capable of producing hydroxyl radicals (•OH). This experiment was conducted with an initial MB concentration of 75 mg/L, and different incubation times of 0, 7, 14, 21, and 28 d respectively. The result showed that the Fenton reaction played an important role, and this was demonstrated by the addition of FeSO₄ as a Fe²⁺ source. The removal of MB by *D. dickinsii* with the addition of Fe²⁺ reached 91.454% at 28 d in a mineral salt medium. It was higher compared to *D. dickinsii* culture treatment without Fe²⁺ addition, 86.427%. Furthermore, the metabolic degradation product was analyzed using LC-TOF/MS and identified as 2-amino-3-hydroxy-5-(methylamino) benzenesulfonic acid and N-(3,4-dihydroxy phenyl)-N-methyl formamide.

**Keywords:** decolorization; degradation; *Daedalea dickinsii*; methylene blue; Fenton reaction

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**INTRODUCTION**

Synthetic dyes are widely used in various industrial sectors, such as textiles, paper, cosmetics, and leather, due to their long-term stability, low cost, and ability to produce derivative colors [1]. However, it is undeniable that these industries also generate dye wastewater, which can cause severe environmental problems. This dye wastewater is toxic and harmful to aquatic life when it is not properly treated, as it reduces light penetration and hinders the photosynthesis process in water bodies [2]. Moreover, the accumulation of toxic synthetic dyes in animals and human bodies can pose health risks [3], particularly as many dyes contain heavy metals and are carcinogenic [4].

Methylene blue (MB) is a synthetic dye with a stable molecular structure commonly used for coloring textiles such as wool and silk, as well as microorganisms [4]. To prevent its discharge into the environment, it is important to carry out pre-treatment for removing MB. Various physical and chemical methods can be used to eliminate dye effluents from the environment through adsorption, flocculation, filtration, and irradiation [5]. However, they have some drawbacks, such as high cost, limited efficiency, and potentially produced hazardous by-products [5]. Meanwhile, the biological method using microorganisms or enzymes is categorized as an eco-friendly and costs less method [6].

Brown rot fungi (BRF) are functional microorganisms that contribute to biomass recycling and soil fertility by breaking down wooden structures such as hemicellulose and cellulose. However, BRF cannot degrade lignin as they do not produce ligninolytic enzymes [7]. BRF produces hydrogen peroxide (H₂O₂) and utilizes Fe²⁺ from the media and substrate to carry out the Fenton reaction, leading to hydroxyl radical (•OH) production. These fungi were
reported using hydroxyl radicals produced through the Fenton reaction (Fe$^{2+}$ + H$_2$O$_2$) to degrade xenobiotic compounds [8]. Previous studies have shown that BRF species such as Gloeophyllum trabeum, Fomitopsis pinicola, and Daedalea dickinsii can degrade 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) by the involvement of Fenton reaction [9]. Moreover, G. trabeum also could improve lignocellulose degradation efficiency [10]. BRF isolated from sludge of textile industry effluent can also eliminate azo dyes acid red and orange II [11].

BRF D. dickinsii was also successfully decolorized and degraded MB [12]. However, the involvement of the Fenton reaction was not analyzed yet. Therefore, in this study, a further experiment involving MB removal by the Fenton reaction produced by D. dickinsii was observed. This was induced by adding Fe$^{2+}$ to the treatment media (MSM), and there may be confirmation that the Fenton reaction plays a role in MB degradation. It was also supported by LC-TOF/MS for knowledge of its degradation product. Moreover, the degradation pathway was also proposed in this study.

**EXPERIMENTAL SECTION**

**Materials**

In this experiment, MB and Potato dextrose agar (PDA) media were purchased from Merck. Potato dextrose broth (PDB) media was purchased from Himedia. Furthermore, all of the Merck chemicals including magnesium sulfate (MgSO$_4$), calcium chloride dihydrate (CaCl$_2$·2H$_2$O), boric acid (H$_3$BO$_3$), cobalt sulfate (CoSO$_4$·7H$_2$O), copper sulfate pentahydrate (CuSO$_4$·5H$_2$O), ammonium molybdate tetrahydrate ((NH$_4$)$_6$Mo$_7$O$_24$·4H$_2$O), manganese sulfate monohydrate (MnSO$_4$·H$_2$O), zinc sulfate (ZnSO$_4$), and ferrous sulfate heptahydrate (FeSO$_4$·7H$_2$O) were in analytical grade.

**Instrumentation**

Petri dish, inoculation loop, Erlenmeyer 100 mL, measuring glass, beaker glass, micropipette, UV-vis spectrophotometer (GENESYS 10S, Thermo scientific), time-of-flight liquid chromatography-mass spectrometry (LC-TOF/MS) spectroscopy (Model, manufacturer) were used in this work.

**Procedure**

**Microorganism and culture condition**

D. dickinsii NBRC 31163 (NITE Biological Resource Center, Japan) was obtained from the Microorganism Chemistry Laboratory, Department of Chemistry, ITS, Indonesia. D. dickinsii was cultivated on PDA media for 7 d and incubated at 30 °C [12]. Then, the mycelium was homogenized in 50 mL of sterile demineralized water by using the sterilized blender. Furthermore, 1 mL fungal culture (9.2 × 10$^4$ CFU/mL) was inoculated into 9 mL PDB for 7 d within static incubation at 30 °C. Then the culture media was removed from D. dickinsii after its pre-incubation, and the mycelium was washed three times with sterile demineralized water [12].

**Decolorization and degradation of MB using D. dickinsii**

Biodecolorization and biodegradation of MB were performed on MSM, prepared according to modified Kirk’s medium [8], by dissolving 0.8 mM MgSO$_4$, 0.2 mM CaCl$_2$·2H$_2$O, 12 μM H$_3$BO$_3$, 0.4 μM CoSO$_4$·7H$_2$O, 0.2 μM CuSO$_4$·5H$_2$O, 0.04 μM (NH$_4$)$_6$Mo$_7$O$_24$·4H$_2$O, 2 μM MnSO$_4$·H$_2$O, and 0.4 μM ZnSO$_4$ in 1 L of demineralized water. Then it was sterilized at 121 °C for 20 min. For Fe$^{2+}$ source involvement, MSM was added to 100 μM FeSO$_4$·7H$_2$O [8].

The mycelium culture of D. dickinsii was inoculated to MSM and supplemented with MB at its final concentration of 75 mg/L. It was incubated under the static condition at 30 °C for various times 7, 14, 21, and 28 d respectively. The involvement of the Fenton reaction on MB degradation was evaluated by comparing treatment within Fe$^{2+}$ and without Fe$^{2+}$ addition. The supernatant was obtained by centrifugation at 3000 rpm for 10 min. The decolorization percentage was calculated by using Eq. (1):

$$\text{Decolorization(%) = } \frac{A_0 - A_1}{A_0} \times 100\%$$

where $A_0$ and $A_1$ are the initial absorbances and the absorbance of the treatment culture, respectively.
Statistical analysis was conducted by the average measurement from triple analysis results. Significant differences between the discoloration and degradation process groups were determined using a t-test within a confidence level of 5% (P < 0.05).

**Identification of metabolite products**

Degradation metabolites of MB were detected by analysis of the supernatants by LC-TOF/MS. At a mass range of 50–350, the ionization source used the electrospray ionization (ESI) technique. A flow rate of 0.2 mL/min was used for the first 3 min of the gradient elution procedure, followed by 0.4 mL/min for the next 7 min. The mobile phase used was methanol and water in a volume ratio of 99:1 for the first 3 min and 61:39 for the next 7 min. Furthermore, an Acclaim TM RSC 120 C18 type column (2.1 × 100 mm, 33 °C) was used [12].

**RESULTS AND DISCUSSION**

**Decolorization and Degradation of D. dickinsii**

Biodecolorization and biodegradation of MB by *D. dickinsii* were carried out throughout the incubation phase. The various incubation times for degrading MB were 0, 7, 14, and 28 d, respectively. Furthermore, the supernatant was examined using a UV–vis spectrophotometer at 200–800 nm after centrifugation at 3000 rpm for 10 min. Fig. 1(a) shows MB decolorization by *D. dickinsii* without Fe²⁺ addition, while Fig. 1(b) shows MB decolorization with Fe²⁺ addition. Both absorbance profiles decreased since the increment of incubation time. The lowest absorbance at MB maximum wavelength 665 nm was obtained after 28 d of culture incubation [13]. Fe²⁺ was added to induce the involvement of the Fenton reaction in MB removal. Therefore, this can be a validation that MB decolorization was also supported by the Fenton reaction.

Fig. 2 shows the percentage results of MB decolorization by *D. dickinsii* without Fe²⁺ addition and with Fe²⁺ addition. The ability of *D. dickinsii* to decolorize MB was increased throughout its incubation time. The highest results were 86.420% and 90.084% for 28 d of incubation, respectively. The culture of *D. dickinsii* with Fe²⁺ addition showed the highest percentage of decolorization compared to treatment without Fe²⁺ addition, and the gap was approximately 3.657%. The result indicates that Fe²⁺ addition supported *D. dickinsii* to enhance MB decolorization and degradation. On the first day of treatment (0 d), according to Tables 1 and 2, *D. dickinsii* decolorized MB by 5.041% and 20.062% for without and within the addition of Fe²⁺, respectively. Subsequently, *D. dickinsii* within Fe²⁺ addition showed the highest result from the beginning. Fungi have a special ability that can adsorb pollutants, including dye, through their cell interaction [14]. Therefore, biosorption was processed by the interaction between active and inactive cell surface microorganisms within the functional group of pollutants (e.g. electrostatic interaction and ion exchange) [15].
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Table 1. Percentage of MB bio decolorization by *D. dickinsii* without Fe$^{2+}$ addition

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Control concentration (ppm)</th>
<th>Treatment concentration (ppm)</th>
<th>Decolorization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.782</td>
<td>52.970</td>
<td>5.041</td>
</tr>
<tr>
<td>7</td>
<td>55.782</td>
<td>36.801</td>
<td>33.962</td>
</tr>
<tr>
<td>14</td>
<td>55.782</td>
<td>25.554</td>
<td>54.146</td>
</tr>
<tr>
<td>21</td>
<td>55.782</td>
<td>15.596</td>
<td>72.014</td>
</tr>
<tr>
<td>28</td>
<td>55.782</td>
<td>7.564</td>
<td>86.427</td>
</tr>
</tbody>
</table>

Table 2. Percentage of MB bio decolorization by *D. dickinsii* with Fe$^{2+}$ addition

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Control concentration (ppm)</th>
<th>Treatment concentration (ppm)</th>
<th>Decolorization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62.636</td>
<td>50.070</td>
<td>20.062</td>
</tr>
<tr>
<td>7</td>
<td>62.636</td>
<td>31.441</td>
<td>49.803</td>
</tr>
<tr>
<td>14</td>
<td>62.636</td>
<td>12.907</td>
<td>79.394</td>
</tr>
<tr>
<td>21</td>
<td>62.636</td>
<td>9.866</td>
<td>84.249</td>
</tr>
<tr>
<td>28</td>
<td>62.636</td>
<td>6.211</td>
<td>90.084</td>
</tr>
</tbody>
</table>

A study found that *D. dickinsii* can produce extracellular hydroxyl radicals (•OH) caused by the Fenton reaction during the incubation process [16]. The Fenton reaction also influenced the biodegradation of DDT by the BRF *D. dickinsii* and *Fomitopsis pinicola*, which only produced very low levels of •OH (5.1 and 1.2 M, respectively) [9]. In addition to the Fenton reaction, Singh discovered that some fungi could generate degradative enzymes such as laccase and peroxidase [17].

Based on the experiment carried out by Rizqi and Purnomo [12], it was reported that *D. dickinsii* decolorized MB on PDB media by approximately 53.55% after 14 d of incubation. However, this study was significantly different, which obtained about 79.394% by Fe$^{2+}$ addition in MSM. These experiments proved that the Fenton reaction was involved in this MB decolorization. In addition, it can also happen because •OH was highly produced. Purnomo et al. [9] found that the addition of Fe$^{2+}$ to the medium boosted •OH production.

**Identification of Metabolite Products**

LC-TOF/MS identified the MB biodegradation metabolite products. MB was found in a relative mass of 284 g/mol with a retention time of 16.09 min (Fig. 3(a)), this is shown in the chromatogram Fig. 4(a). The intensity of the MB treatment peak was lower than the
control peak, indicating that it was degraded. Furthermore, at retention times of 7.80 and 8.67 min, two metabolites were found in the chromatogram treatment (Fig. 4(b) and 4(c)), respectively. Table 3 shows the proposed metabolites products of MB degradation. At a retention time of 7.80 min, the peak had \( m/z = 218 \) (Fig. 3(b)), which was identified as 2-amino-3-hydroxy-5-(methylamino)benzenesulfonic acid. Moreover, the peak with \( m/z = 167 \) (Fig. 3(c)) at a retention time of 8.67 min was identified as \( N-(3,4\text{-dihydroxy phenyl})-N\text{-methylformamide} \). These metabolite results were reported previously by Houas et al. [18], who revealed the photocatalytic degradation pathway on MB in the water.

The MB degradation product was also analyzed for its functional group by using FTIR spectroscopy analysis (Fig. 5). Compared with MB dye analysis, the result shows that broad peak spectra at around 3000 nm\(^{-1}\) were observed as –OH stretching vibration overlapping within the amine group (–NH). At the same time, peaks at 2935 nm\(^{-1}\) were analyzed from the C–H symmetrical stretching band. However, there was a new peak on degraded MB result at 1725 nm\(^{-1}\) that was different within MB spectra, referring to the C=O group [19]. This result was consistent with the purpose of MB metabolite degradation above. Meanwhile, C=C from aromatic rings were identified at a wavenumber of 1600 nm\(^{-1}\) [20].

Based on the identification of metabolite products, the MB biodegradation pathway was proposed in Scheme 1. MB was transformed into two pathways, both

![Fig 4. Chromatogram profile of MB degradation by D. dickinsii with Fe\(^{2+}\) addition](image)

![Fig 3. Images of mass spectra of (a) control MB, (b) peak at 7.80 min, and (c) peak at 8.67 min](image)
Table 3. Metabolites of MB degradation by D. dickinsii with Fe²⁺ addition

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Relative mass</th>
<th>Chemical formula</th>
<th>Molecular structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.80</td>
<td>218</td>
<td>C₁₇H₁₉N₂O₄S</td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
<tr>
<td>8.67</td>
<td>167</td>
<td>C₆H₄NO₃</td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
</tbody>
</table>

Fig 5. FTIR analysis result of MB degradation

paths have only one benzenic ring affected by •OH attack. In the first pathway, MB was oxidized by the formation of a sulfide group, followed by cleavage of the chromophore bond in NH₂ because of •OH generated by the Fenton reaction. The amine group was demethylated, and one site on the benzenic ring was hydroxylated by continuous •OH attack from the Fenton reaction. The second pathway, amine (chromophore) and sulfide bonds MB were hydroxylated, dissociating another ring. Then the •OH attacked the methyl group, and the amine group became the amide (aldehyde) group.

Wood rot fungus can be classified into three types based on the type of decay and their breakdown. There are BRF, white rot fungi (WRF), and soft rot fungi (SRF) [21]. Subsequently, WRF can degrade lignin and other compounds in particular when it ingests food [22]. The reason is WRF has a special ligninolytic enzyme system mechanism, including laccase, lignin peroxidase (LiP), mangan peroxidase (MnP), and other extracellular enzymes, which are capable of synergistically degrading lignin even on mild media conditions [23]. According to a previous study reported by Zeng et al. [24], semi-solid-state fermentation of agricultural waste rice straw mixed with Phanerochaete chrysosporium fungus can be degraded and decolorize MB by 84.8% at an initial concentration of 0.4 g/L. Furthermore, white rot fungi Pleurotus ostreatus also can degrade MB, proved by the increasing decolorization percentage at 25–700 mg/L concentration [25]. SRF typically attack higher moisture and degrade lower lignin cell wood. Although, there are still no studies that mention the enzymatic degradation system [26].

BRF are capable of breaking down wood substrate both enzymatically and non-enzymatically [7], resulting in a brownish discoloration of the cell wall. Naturally, BRF preferentially targets polysaccharides such as

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cellulose and hemicellulose for nutritional purposes rather than lignin and possesses a cellulolytic enzyme system that can partially oxidize lignin without the need for ligninolytic enzymes [7]. However, the enzymes produced by BRF are unable to penetrate the cell wall’s pores, which are larger than the enzymes themselves. Hence, BRF needs a low-molecular-weight-biochemical agent, which can penetrate cell-wall pores and degrade/depolymerize cellulose [27]. This mechanism is caused by extracellular oxidants (free radicals), which are small, diffusible, and could react on distance from hyphae [16].

BRF possess a unique non-enzymatic system that allows them to produce •OH through the Fenton reaction (Scheme 2), enabling them to decay wooden cells [27]. BRF can produce more H₂O₂ from glucose than WRF does. The Fenton reaction has been proven to be an effective technology, which uses a catalytic oxidation process by mixing H₂O₂ and Fe²⁺ to produce •OH. These •OH radicals could be used as the strongest oxidant currently available in biological systems to degrade a wide range of xenobiotic and organic contaminants [28].

According to Su et al. [29], Fenton-like catalysis using iron-containing minerals could be an alternative optimum method for eliminating refractory organic pollutants. The •OH radicals are non-selective robust species in water which could oxidize organic substrates, with an estimated oxidation potential of 2.8 and 1.9 V at pH 0 and 14, respectively [29]. The removal of one hydrogen atom from hydrocarbons and other organic substances can be affected by •OH. This is possible because the hydrogen bond has a lower energy than the O–H bond, which has an energy of 109 kcal/mol [29].

Studies have suggested that •OH is particularly effective at breaking down wood components compared to other related oxygen species [30]. Due to its strong oxidizing agent capabilities, it can degrade raw material feedstock (complex organic chemical component). The •OH can react with complex organic compounds containing an aromatic ring in two ways: 1) by introducing an electrophile to the aromatic ring or 2) by abstracting a proton (H⁺) from aliphatic [21]. Under low pH conditions, •OH not only abstracts the H⁺ but also can eliminate H₂O and produce an aryl cation. This is followed by carbon-carbon linkages cleavage and other degradation [21].

The Fenton chemical reaction requires Fe²⁺ and H₂O₂ as the main components to produce •OH. Naturally, BRF reduces Fe³⁺ to Fe²⁺ and produces H₂O₂ through the extracellular hydroquinone-quinone redox cycle, as shown below [31] where Q is quinone, H₂Q is hydroquinone, HQ• is semiquinone, and •OOH is perhydroxyl radical (Scheme 3). It further suggested that cellulbiose dehydrogenase or iron-binding catechols reducing Fe³⁺ outside the BRF mycelium can initiate and generate Fenton chemistry [32]. The hypothesis that the Fenton reaction plays a role in the BRF mechanism can be supported by the addition of FeSO₄, which can ionize Fe³⁺ as additional Fe²⁺ to aid the Fenton reaction in the decolorization of MB by D. dickinsii. Fe²⁺ oxidizes and then becomes Fe³⁺ [8]. Moreover, the concentration of H₂O₂ produced by D. dickinsii could be analyzed by using the ferrous ion oxidation-xylene orange (FOX) method. Purnomo et al. [9] showed that by the FOX method, D. dickinsii could produce 21 mM H₂O₂ during 14 d of incubation time.

Another previous study reported by Purnomo et al. [33] showed that three species of BRF Gloephyllum trabeum, Fomitopsis pinicola, and D. dickinsii could degrade synthetic dye methyl orange. The proposed metabolite degradation pathway consists of three-step processes: (1) demethylation, (2) desulfonylation, and (3) hydroxylation. Furthermore, Alkas et al. [34] reported that D. dickinsii immobilized into sodium alginate-polyvinyl alcohol supported by UiO-66 could adsorb and degrade MB within its maximum capacity of 0.329 mg/g. The metabolite products C₁₆H₂₀N₃S (m/z = 286), C₂₂H₃₂N₃O₅S (m/z = 450), and C₁₂H₁₃N₃O₇ (m/z = 311)
were also identified. Furthermore, another BRF species that can degrade Violet S3RL, Yellow Brown S2RFL, Red W4BS, Yellow SRLP and Red S3B was successfully analyzed [35].

To identify metabolite products in this recent study, LC-TOF/MS was used for the analysis. The MB peak is found at 16.09 min retention time with m/z 284 g/mol, as shown in the chromatogram (Fig. 4). The MB treatment was degraded because the peak intensity was generally lower than the MB's control peak. The metabolites products were detected on two new peaks at retention times of 7.80 (m/z = 218) and 8.67 min (m/z = 167), which are proposed as 2-amino-3-hydroxy-5-(methylamino) benzenesulfonic acid and N-(3,4-dihydroxyphenyl)-N'-methyl formamide, respectively. These metabolites were discovered during a research project on the photocatalytic degradation of MB in water [18].

Rizqi and Purnomo [12] reported that D. dickinsii can degrade MB into three metabolites, namely 3-(dimethyl amino)-7-(methylamino)phenothiazine (C15H16N3S+, m/z = 280), 3,7-bis(dimethylamino)-4H-phenothiazine-5-one (C16H19N3OS, m/z = 300), and 4-(dimethylamino)-2-[m-(dimethylamino)phenylsulfinyl]benzene-amine (C16H21N3OS, m/z = 303). Meanwhile, Houas et al. [18] experiment also showed that MB can be degraded into C16H19N3OS with m/z = 303, by the attack of •OH. It also explained that MB was attacked by •OH on C-S+=C functional group, and to open the center ring, double bond conjugation conservation was required. The sulfoxide group of C16H21N3OS was attacked again by •OH and dissociated the two aromatic rings (not detected/reaction Scheme 4) [18].

Furthermore, the sulfone can be attacked by a third •OH, resulting in the formation of a sulfonic acid found in metabolites with m/z of 218 and 158. According to Houas et al. [18], sulfur has attained its maximum oxidation degree (+6) in reaction 8. Furthermore, SO42− ions could be attacked again by •OH until they become phenolic.

\[
\begin{align*}
\text{NH}_2\text{C}=\text{C}\text{H}_2\text{H}_2\text{S}\text{O}_4 + \cdot\text{OH} & \rightarrow \text{NH}_3\text{C}=\text{C}\text{H}_2\text{H}_2\text{SO}_4 + \text{C}_6\text{H}_5\text{R} \\
\text{NH}_2\text{C}=\text{C}\text{H}_2\text{H}_2\text{S}\text{O}_4 + \cdot\text{OH} & \rightarrow \text{NH}_3\text{C}=\text{C}\text{H}_2\text{H}_2\text{SO}_4 + \text{C}_6\text{H}_5\text{R}
\end{align*}
\]

**Scheme 4.** Two kinds of aromatic ring were dissociated by hydroxyl radical

**CONCLUSION**

This study showed that BRF D. dickinsii with the addition of Fe2+ has a greater capability to decolorize and degrade MB in MSM compared to D. dickinsii culture without Fe2+ addition. The highest results of decolorization were achieved at 90.084 and 86.427%, respectively, at 30 °C for 28 d of incubation, and a decolorization gap of 3.657%. These results suggest that the Fenton reaction is involved in MB removal by •OH production. The MB degradation metabolites by D. dickinsii were analyzed by LC-TOF/MS and FTIR spectroscopy and identified as 2-amino-3-hydroxy-5-(methylamino) benzenesulfonic acid and N-(3,4-dihydroxyphenyl)-N'-dimethylformamide.

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