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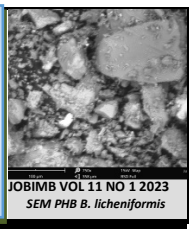


# Biodegradation of Synthetic Reactive Orange 16 Dye in Anaerobic Reactor Conditions

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**ABSTRACT**

Industrial use of azo dyes has been linked to severe environmental damage in the textile and apparel sectors. The breakdown products of many azo dyes are poisonous and mutagenic, posing a threat to human health and the environment. Hence, azo dye wastewater bioremediation is gaining interest. The objective of this study was focused on the biodegradation of an azo dye, Reactive Orange 16 (RO16), in an anaerobic continuous tank reactor operated with digested sludge sample. The digested sludge was used as the organic biodegradation agent for azo dye. The aim was to investigate the fate of the anaerobic RR16 breakdown products during the degradation process. Gas Chromatography-Mass Spectrophotometry (GC-MS) was used to identify RO16 biodegradation metabolites and their corresponding molecular weight. As a result, RO 16 showed about 85.91% decolorization capacity, and the degradation metabolites were detected as pentanoic acid and phenol after seven days of incubation. The degradation mechanism was completed as the azo bond of RO16 was successfully cleaved based on the result of GC-MS, and the proposed pathway of the degradation mechanism is described. The digested palm oil sludge with dye degradation enzymes may provide new avenues for biological waste treatment, especially wastewater bioremediation.

## INTRODUCTION

The dye pollutants that are generated by the textile sector pose a severe threat to the environment's wastewater discharge. High quantities of dye are produced by the textile industry, which releases 5,000 tons of dying ingredients into the environment annually. As a result, it is bad for both human health and aquatic life. According to Zhu et al. [1], industrial wastewater contains various harmful chemicals that are very difficult to break down, such as metals, cyanides, alkaline cleaning solutions, degreasing solvents, oil, and fat. Additionally, some dyes have the potential to break down into aromatic amines or dyes that contain pyridine, cyanide, and heavy metals. The content of the product was carcinogenic, mutagenic, and cytotoxic effects [2,3]. Thus, it is essential to create efficient techniques for removing colors and other organic pollutants from wastewater. The common wastewater treatment methods include physical, chemical, and biological water treatment. Other well-known methods to treat textile effluents include the destruction of colored particles and physicochemical methods such as sand filtration, membrane separation, and adsorption using carbon activated and coagulation-flocculation [4]. However, all those methods are expensive and involve the formation of concentrated sludge, creating a secondary disposal problem. Therefore, bioremediation offers a convenient solution in terms of low cost and environmentally friendly. Microorganisms such as bacteria, fungi, and algae have been known to decolorize and metabolize azo dyes under anaerobic and aerobic conditions, including *Staphylococcus equorum* RAP2 [5]; basidiomycetes (*Phanerochaete chrysosporium* ATCC 24725) [6] and *Fucus vesiculosus* [7].

At the same time, bacteria such as *Enterococcus* sp. and *Pantoea* sp. showed highly effective at removing toxic and cationic Malachite Green and azo Methyl Red dyes [8]. Meanwhile, *Bacillus stratosphericus* is known to have a significant potential to effectively remove color from reactive orange 16 dye (RO16), a widely utilized dye in the textile sector

[9]. Due to their practical and compatible reactive orange 16 decolorizing isolates from tannery and textile effluents, a novel Bacillus consortium was developed [10].

In a recent review, anaerobic was the best condition where the reduction mechanisms of azo compounds are well understood [11]. A broad range of facultative and strictly anaerobic species of microbes, such as facultative anaerobic (*Streptococcus faecalis, Proteus vulgaris*) and strictly anaerobic (*Eubacterium* sp.; *Bacteroides* sp.; *Clostridium* sp.) had an excellent ability to decolorizing azo dye. Symmetric cleavage of the azo dye group (-N=N-) is always correlated with the microbial degradation of azo dye in either anaerobic or aerobic conditions. Symmetric cleft could be arbitrated by several mechanisms such as enzymes, sulphide biogenic reductants, low molecular weight redox mediators, or a mix of those mechanism reductions [12]. An enzymatic mechanism for the degradation of the azo compound has been studied widely, resulting in many types of potential biodegradation way.

Common enzymes such as azoreductase, laccase, tyrosinase, peroxidase, NADH-DCIP reductases, and MG reductase were reported to involve azo compound degradation. This study aimed to elucidate the degradation of azo dyes from textile wastewater effluent using digested sludge in anaerobic thermotolerant conditions. Besides that, further investigation is proposed by analyzing metabolites produced after the decolorization process and a possible azo dye degradation pathway.

## MATERIALS AND METHODS

### Digested Sludge Sampling

Digested sludge was collected from a biogas reactor at Palm Oil Mill facilities in Negeri Sembilan, Malaysia (2.9916° N, 102.4812° E). Felda Global Ventures Bhd. (FGV) was the owner of this palm oil mill facility.

### Azo dye preparation

The reactive Orange 16 (RO16) with λ max at 493 nm was prepared in 100 ppm, 200 ppm, and 300 ppm [13]. The dye was prepared in 1 L for each concentration and stored at room temperature. The dye concentrations were used to determine the effect of increasing dye concentration on the percentage of dye degradation.

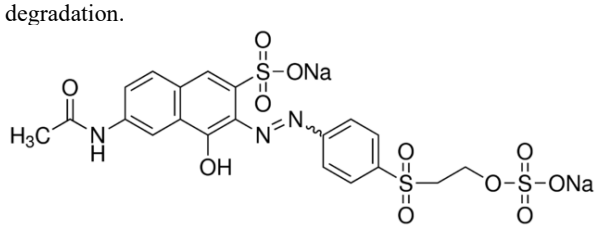


**Fig. 1.** Chemical structure of Reactive Orange 16.

### Biodegradation of Azo dyes using the digested sludge

The biodegradation process was carried out in small-scale reactors using serum bottles with a volume of 125 mL. The fully covered batch reactor design was suitable for the anaerobic condition. In each serum bottle, 70% of the space was used for the degradation process, while 30% was used as headspace for gas space during the degradation.

Reactive Orange 16 (RO16) at 100 ppm, 200 ppm, and 300 ppm were mixed with digested sludge in the batch reactors. In each reactor, the digested sludge and substrate were prepared in a 2:1 ratio to a volume of 87.5 mL (70% of the space in the serum bottle).

The digested sludge and substrate were combined in the serum container through manual mixing using a micropipette. Subsequently, the headspace of the reactor was purged with pure nitrogen gas for approximately 1.5 min to eliminate any dissolved oxygen present. Subsequently, the serum bottle was tightly sealed using a rubber stopper and an aluminum crimp. Each type of sample was prepared in triplicate and one control sample. The control sample contains no digested sludge, only MSM and azo dyes.

The batch reactors were then incubated in an incubator shaker for seven days at 35 °C/55 °C and 200 rpm.
The biodegradation process was conducted for 168 hours of incubation. The medium was then centrifuged for 10 min at 5000 rpm. The absorbance of the supernatant was measured with a UV-Vis Spectrophotometer (UV1800) (Shimadzu, Japan). The measurement was conducted using the maximum wavelength of the dye (493 nm). The capability for decolorization (percent) was calculated using the following formula:

$$Decolorization\,\% = (Initial\ Absorbance - Final\ Absorbance)/(Initial\ Absorbance)\ X100$$

### Extraction of biodegradation products

Reactive Orange 16 dye metabolites were analyzed in the batch reactor culture medium on Day 0 and Day 7 of incubation. The samples were centrifuged at 5000 rpm for 10 min. The supernatants obtained were filtered using filter paper to remove large particles inside the flask. The filtered supernatants were then acidified to pH 2 with 2 M hydrochloric acid. Then, liquid-liquid extraction was done by mixing the supernatants with ethyl acetate in a 1:1 ratio using a separating funnel. Next, the organic extract comprising azo dye metabolites was dried with anhydrous Na2SO4 and kept at room temperature. The thoroughly dried organic extracts were then dissolved in 1 mL of HPLC-grade methanol for further analysis by Gas Chromatography-Mass Spectrometry (GCMS-QP2010 Ultra, Shimadzu) [14].

### Metabolite analysis using Gas Chromatography-Mass Spectrometry

The extraction sample was analyzed using Gas Chromatography (GCMS-QP2010 Ultra, Shimadzu) equipped with a DB-5 MS capillary column. One ppm of samples was filtered using a 0.2 µm membrane filter and filled into 2 mL autosampler vials. The GC-MS was set in split mode, with an injector temperature of 300 °C. The initial temperature of the injection port was 80 °C for 2 mins, then increased linearly at 10 °C $min^{-1}$ to 290 °C. The helium gas flow rate as a carrier was set to 1.0 mL $min^{-1}$. NIST spectral library stored in the computer software was used to identify the metabolites by comparing the retention time and fragmentation pattern [14].

### Data Analysis

Analysis of variance (ANOVA) was used to study the significance of different temperature conditions and concentrations of azo dyes toward the degradation process. The ANOVA was performed using the SPSS version 16.0 software. ANOVA, Tukey's HSD test, and effects were significant when the P-value was < 0.05.

## RESULTS AND DISCUSSION

### Degradation of azo dye using digested sludge

**Fig. 2** depicts the dye removal rate for various initial dye concentrations and temperatures under anaerobic conditions. As demonstrated, increasing the incubation duration to 7 days increased the removal efficiency at all initial concentrations of Reactive Orange 16 (RO16) dye, such that the removal efficiency at 100 and 300 mg/L at 35ºC of RO16 was 46.08 and 74.95%, respectively. At an incubation temperature of 55 ºC, the removal efficiencies were 78.04 and 85.91%, respectively. The decolorization activity was significantly higher in the reactor incubated under the thermophilic condition (55 °C) at 300 ppm. There was a significant difference in concentration between 100 ppm and 200 ppm but no significant difference between 200 ppm and 300 ppm at 55 °C.

The 300 ppm samples resulted in higher decolorization capacity than two other lower concentrations. This is supported by[15], who showed that the decolorization of azo dye increases upon increasing the dye concentration from 200 to 800 ppm. A previous study by [16] showed the same trends as the decolorization level increased when the dye concentration and temperature were increased. Even though the results look similar, the concentration range was lower than 100 ppm. Furthermore, the report by [17] showed that 200 ppm of mixed azo dyes was the optimum concentration that could be degraded in 5 days of incubation. A previous study [18], showed 78.6% decolorization of RO3R which is similar in molecular weight to RO16, but the incubation condition was mesophilic and the time frame was 24 hours only.

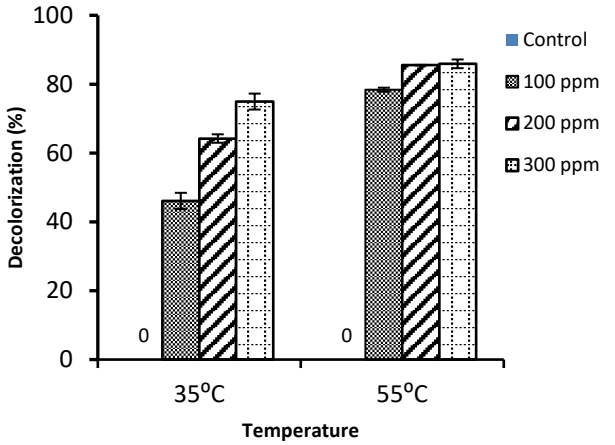


**Fig. 2**. Reactive Orange 16 (RO16) decolorization at 35°C and 55°C after 7 days. The data displays the mean ± standard deviation of triplicates. Significant differences within each group are shown by different letters in mean data (Tukey's HSD test, $p < 0.05$).

### Metabolite production in azo dye degradation

Reactive Orange 16 (RO16) degradation metabolites have been analysed using GC-MS. The mass spectroscopy result revealed the presence of several simple compounds and metabolites from the dye degradation process. The molecular structures of RO16 were quite similar to other dyes in the sulphonate group. Dissociation of the sulphonate group would be initiated in aqueous solution to become anionic dye ions [19]. The differences might be in the molecular size between these two dyes. Consequently, the degradation products detected by mass spectroscopy were also similar. The GC-MS analysis showed that the metabolites had high similarity with pentanoic acid and phenol as the significant by-products of both RO16 dye degradations. Phenol is an easy substrate for laccase enzyme to form simpler products such as hexanoic acid and pentanoic acid. The proposed mechanism pathway was constructed and shown in **Fig. 3** based on the results obtained.

**Fig. 3** shows that the asymmetric cleavage of the -N=N bond as the first suggested step of RO16 degradation results in the formation of unknown intermediates and (4-sulfophenyl) diazenyl. After that, oxidative desulfonation is applied to the other intermediate, 4-sulfophenyl diazenyl, resulting in phenyldiazenyl. Phenyldiazene loses nitrogen ($N_2$) as a gas molecule in its radical form, and phenol is created when the aromatic ring reacts with a hydroxyl radical (OH). Laccase, which is the enzyme that uses phenols as a substrate, cleaves the ring to form fatty acids such as pentanoic acid and alcohols such as hexanol. A similar pathway was observed for the degradation of Acid Orange 7 using a Microbial Fuel Cell (MFC) system aided with the laccase enzyme [20]. However, the experiment's condition was maintained at 30 °C and in an aerobic environment. In addition, [21] also showed asymmetric cleavage of RO16 and also with laccase present, but there was no formation of phenol as a by-product after degradation.

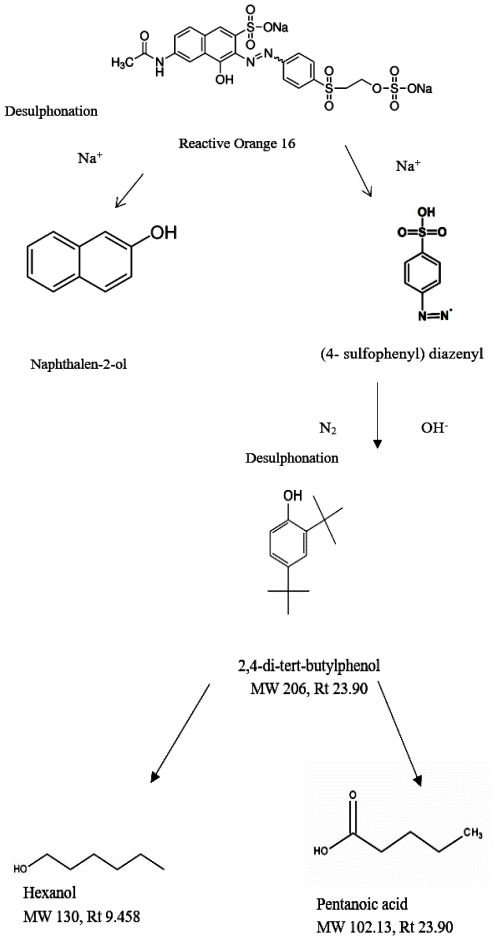


**Fig. 3**. Proposed pathway for Reactive Orange 16 (RO16) degradation.

Based on the RO16 dye used in this study, it has a sulphate group and is very likely to produce a phenolic compound as the degradation by-product [22]. Consequently, the enzymatic mechanism focusing on azo bond cleavage and phenolic degradation will be highlighted. In addition to azoreductase and NADH-DCIP reductase, laccase is one of the enzymes that can be employed to break down azo dyes [23]. According to research by Guo et al. [24], laccase's action during the breakdown of the azo bond is really through a non-specific oxidation mechanism.

The laccase enzyme was reported to be expressed by bacteria and fungi. [25] studied the potential of a white-rot fungi, *Tinea versicolor,* that could express laccase to degrade azo dye and phenol compounds in wastewater. Moreover, bacterial laccase also significantly impacted the degradation of textile wastewater with a broad range of working temperatures, easy to be cloned and cost-effective [26]. Meanwhile, the anaerobic condition is also favourable for the laccase enzyme to assist in the biodegradation process. Anaerobic digestion processes that produce biogas as the end-product could also be enhanced by the presence of laccase. In another study, the fungus *Trametes versicolor* could secrete laccase enzyme to increase methane yield in an anaerobic digester [27]. Meanwhile, a study by [28] showed the synergistic effect of laccase enzyme with peroxidases that resulted in an effective phenolic toxicity removal and lowered the inhibition of the biogas formation process under anaerobic conditions.

## CONCLUSIONS

Synthetic reactive Orange 16 dye could be degraded and discolored by thermotolerant digested sludge containing unknown bacteria. Due to the efficiency of anaerobic digestion, the thermophilic environment (55°C) was found to significantly speed up the degradation process compared to the mesophilic condition (35 °C). The decolorization capability was 87%, demonstrating the digested sludge's potential for use in dye biodegradation. After seven days of incubation, the degradation metabolites were detected as pentanoic acid and phenol. This study showed that organic digested sludge is a good source of energy and degradation agents. Strategic operation using an anaerobic digester can be a viable option for effective bioremediation of textile wastewater.

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