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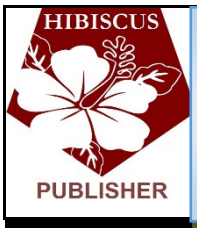

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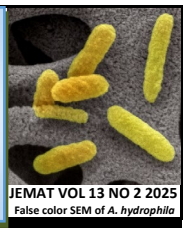

Biodecolorization of Metanil Yellow by *Serratia marcescens* MM06 Under Variable Environmental Conditions for Soil and Water Remediation

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Abstract

Metanil Yellow is a common pollutant coming from the dyeing industry. Its removal using decolorizing bacteria offers a sustainable approach to remediate polluted soils and water bodies. This research examines the impact of several parameters, such as initial dye concentration, temperature, pH, and NaCl concentration, on the decolorization efficacy of Serratia marcescens strain MM06 for the dye Metanil Yellow. The percentage of decolorization studied across different dye concentrations from 100 to 700 mg/L shows the highest decolorization at dye concentrations of between 100 and 300 mg/L. The decolorization was highest at 25°C. The effect of pH on the decolorization showed that the best decolorization occurred between pH 7 and 8. The effect of salinity on the decolorization for future coastal areas remediation showed that the best decolorization (90–100%) happened at NaCl levels up to 15 g/L. The decolorization effectiveness decreased as the NaCl concentration increased, reaching 10% at 30 g/L. Decolorization was severely inhibited by mercury, silver, and copper at 1 mg/L, which shows that chelating or sequestering agents may need to be added to the contaminated soil or water bodies before decolorization can take place. Gaining a thorough understanding of the decolorization characteristics is an important preliminary study before field studies are carried out to understand the limitations of the decolorization bacterium.

INTRODUCTION

Bioremediation of textile waste can be accomplished through the utilization of both biocatalysts and microorganisms [1–3]. Bioremediation is a promising treatment option for effective dye degradation under various conditions, often better than physicochemical methods, and offers numerous advantages such as reduced costs, minimized potential hazardous chemical exposure, a sustainable approach, and the absence of sludge production. Bioremediations clean up soil and water bodies back to their original pristine state, which allows farming and agricultural activities [4,5]. The target bacteria or native indigenous bacteria naturally mineralize this pollutant and leave little to no residue behind. Conventional physicochemical methods, which include electrochemical precipitation, utilize high voltage, solvents such as methanol, acids including sulfuric acid, N-dimethylformamide, sodium thiosulfate, and potassium iodide introduce more toxic pollutants to both the environment and health, for the treatment of textile effluents [6]. Various microbial strains have been effectively isolated and employed to eliminate or neutralize hazardous pollutants from identified contaminated locations [7]. Bioremediation can be done in situ

or ex-situ with mixed consortia or pure bacterial strains due to the flexibility in terms of design and operating conditions [8]. Engineered technologies such as the use of bioreactors and enzymatic catalytic breakdown of the dye molecules under controlled conditions, make it even easier to get rid of the dye pollutants.

Metanil Yellow, also known as Acid Yellow 36, is a synthetic azo dye that is mostly used in the textile dyeing industry, including the Batik industry. Due to the dye posing serious health and environmental risks, its uses need mitigation as far as pollution is concerned. In the textile industry the dye is used to dye cotton, wool, and silk, giving them a bright yellow color. The paper industry also uses it to make colored papers and stationery while the leather industry also uses it to make leather products bright yellow in appearance. Metanil Yellow is used to color synthetic plastics and polymers, in the plastics and polymers industry [9–12]. In the past, the dye has been used in the food and drink industry to color food such as candies, drinks, and processed foods. However, many countries soon banned or limited the its use in food products as its metabolite produced under anaerobic environments often found in the intestine yielded compounds that can cause cancer [4,13–15]. Metanil Yellow exposure can also lead to allergic reactions and other health problems. The improper or intentional disposal of the dye can result in water pollution, significantly impacting aquatic life due to its moderate persistence in the ecosystem [13,16,17].

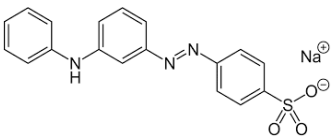

Fig. 1. Structure of Metanil Yellow.

Microbial bioremediation of this dye is a more economic option that uses microorganisms to break down azo dyes into safer substances. Microorganisms like bacteria, fungi, and algae break down dyes through enzymatic processes like reductive cleavage by azoreductases. Before microbial bioremediation can take place, preliminary works on the parameters that can enhance or impede remediation need to be studied. This is the main aim of this study, where a previously isolated dye-degrader was studied for its decolorization ability of this dye.

MATERIALS AND METHODS

Growth of Metanil Yellow-degrading *Serratia marcescens* strain MM06

Serratia marcescens strain MM06 was previously isolated as a Reactive red 120 dye degrader [18]. The decolorization medium (pH 7.0) containing (g/L) was as follows: $(NH_4)_2.SO_4$ (0.3%), $NaNO_3$ (0.2%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.05%), NaCl (0.5%), Na_2HPO_4 (0.705% or 50 mM). This medium was supplemented with Metanil Yellow (200 mg/L) [5]. Decolorization was monitored at 434 nm [19].

Assay of dye-decolorizing enzyme azoreductase

Assay for azoreductase was performed according to a previous method [20]. Bacterial growth cultures starting from 12 to 48 h grown in MSM medium supplemented with 200 mg/L of Metanil Yellow at 150 rpm on an orbital shaker were harvested by centrifugation at 10,000 × g for 10 minutes at 4ºC. The cell pellets were washed thrice with 50 mM potassium phosphate buffer (pH 7.0) and broken up through a sonication program of a sonicating time of 30 seconds at 70% output (Model 505 Sonic Dismembrator) and a 4 min cooling time on ice for a total sonicating time of 30 min. The sonicated cells were centrifuged to remove debris at 15,000 × g for 10 minutes at 4ºC. The assay mixture contained 100 µL of crude enzyme in 50 mM phosphate buffer (pH 7.0) with 300 mg/L Metanil Yellow. The enzyme was incubated for 3 min in the assay mixture to allow equilibration to room temperature before 2 mM NADH was added to start the reaction. Azoreductase activity was measured at 340 nm in a quartz cuvette using a Shimadzu UV-Vis 1201 spectrophotometer. One unit of enzyme activity equals the oxidation of 1 µmol of NADH as a substrate per min.

RESULTS AND DISCUSSION

Azoreductase activity

Microorganism uses metabolic pathways to degrade synthetic azo dyes. The degradation of azo dyes started with the reductive cleavage of the azo bond (–N=N–), which is facilitated by the azoreductase enzymes [20]. The result (**Fig. 2)** demonstrates that Metanil Yellow decolorization by *Serratia marcescens* strain MM06 commenced within the first 12 hours, facilitated by the high starting inoculum concentration. Concurrently, the azoreductase activity was found to steadily increase, which maximizes around 24 hours, which shows a similar profile to the profile seen in the decolorization of metanil yellow by a previously-isolated bacterium [21].

The decolorization of the dye by Bacillus sp. strain AK1 and *Lysinibacillus* sp. strain AK2 occurred at a similar pace, which is within the first 24 h, while the azoreductase activity was found to peak at this time [20]. Guo et al. [5] reported on the decolorization of the dye by a bacterial consortia isolated from extreme environments with high salt, alkaline, and hot environments. The decolorization occurred near 200 mg/L, similar to this study, with the azoreductase activity peaking at this concentration. In another study, *Bacillus* -3330 decolorization of metanil yellow exhibits near 90% decolorization after 24-hour of incubation and the azoreductase activity also peaked within this time period [22] that. Other Metanil yellow decolorization experiments that report on azoreductase activity are by Mansur et al. [23] and Muliadi et al. [24]. The central theme in all of these reports, including this study, is a consistent pattern of quick early removal of dye concurrent with an increase in azoreductase activity. Azoreductase activity in other azo dyes is also reported such as during the decolorization of sulfonated azo dyes by *Kerstersia* sp. strain VKY1 [25] and *Galactomyces geotrichum* MTCC 1360 [26].

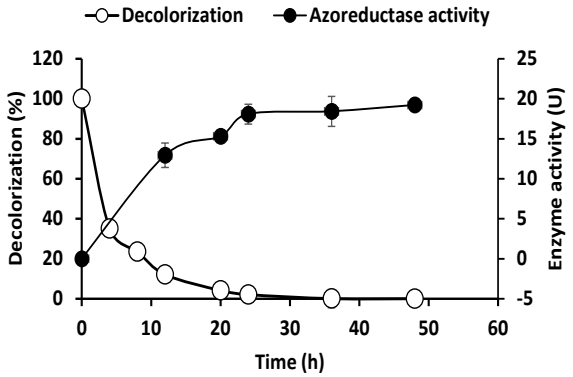

Fig. 2. Azoreductase activity of the Metanil Yellow-decoloring *Serratia marcescens* strain MM06. The experiment was replicated thrice, and error bars represent standard deviation.

Effect of initial dye concentration

The effectiveness of the Metanil Yellow-decolorizing bacterium was tested at different concentrations of the dye, ranging from 0 to 700 mg/L. The decolorization efficiency was maximal at low concentrations from 100 to 300 mg/L, achieving nearly 95% of decolorization. ANOVA analysis indicated no significant difference ($p<0.05$) between these values. The efficiency started to decline at higher concentrations, reaching only 10% decolorization at the highest concentration tested at 700 mg/L (**Fig. 2**). This phenomenon was also observed by [21], who reported that the best concentration was 200 mg/L. The decrease in decolorization efficiency, especially at higher dye concentrations, can be caused by the dye or its metabolites impeding the metabolic activity and enzyme performance required for the decolorization process [6,27–29].

Previous studies demonstrated that different bacterial strains degrade Metanil Yellow differently, depending on the dye concentration and cell reuse. For instance, Anjaneya et al. [20] showed that *Bacillus* sp. strain AK1 and *Lysinibacillus* AK2 could decolorize Metanil Yellow with a longer period needed at larger dye concentrations. The azoreductase activity was high, reaching 1615 and 1630 nmol/mg protein/min, respectively. This high enzyme activity stayed moderately high after repeated use, with a lower efficiency observed. Another halophilic alkalithermophilic bacterial consortia was able to decolorize Metanil Yellow even at high dye concentrations by over 90% in 48 hours [5]. This capability to decolorize dye with repeated use allows the consortium to be acceptable for high-salinity wastewater treatment.

In another study, a newly obtained *Bacillus* strain decolorized Metanil Yellow within 24 hours and completely within 72 hours, according to Lal and Chand [22]. The bacterium decolorized efficiently after repeated reuse and exhibited high azoreductase activity. The bacterium *Bacillus* sp. strain Neni-10 decolorized the dye after 24 hours under ideal conditions [23]. In a biostimulation experiment, Muliadi et al. [24] were able to stimulate a Malaysian agricultural soil microbial population to decolorize Metanil Yellow by over 80% within 48 hours. [30] shows that *Oenococcus oeni* ML34, was able to decolorize Fast Red in 36 hours and Metanil Yellow in a similar period. It appears that reusing of the dye-decolorizing microorganisms is a trend, which makes the process sustainable.

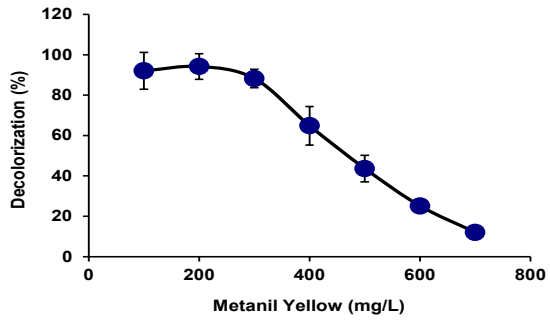

Fig. 3. Metanil Yellow decolorization by *Serratia marcescens* strain MM06 at various dye concentrations. The experiment was replicated thrice, and error bars represent standard deviation.

Effect of temperature on decolorization of Metanil Yellow

The stimulatory or inhibitory effects of temperature on the decolorization of Metanil Yellow between 20 °C and 50 °C were studied. The process of decolorization exhibited a moderately broad peak maximum between 25 and 30 °C, which showed that there was a clear temperature range that worked best for removing dye.There was a gradual decrease in decolorization after 30°C. At 40°C, the removal rate was reduced to 40%, and at 40°C, the decolorization dropped even further at 45°C, it was very hard to get rid of the dye, and less than 25% of it was removed. The lower performance at higher temperatures is due to thermal stress damaging cells and making redox enzymes less stable. On the other hand, low temperatures slow down the decolorization process because they slow down the turnover of enzymes and the transfer of electrons [31–34].

Temperature affects the decolorization of synthetic azo dyes like Metanil Yellow, which optimizes wastewater treatment biodegradation. Much research has studied how different bacterial strains react to temperature changes during Metanil Yellow decolorization. [20] observed that *Bacillus* sp. strain AK1 and *Lysinibacillus* strain AK2 decolorized best from 30°C to 40°C. A halophilic alkalithermophilic bacterial consortia decolorized Metanil Yellow G efficiently at higher temperatures, particularly at 50°C, according to [5]. The study found that consortium enzymatic activity and metabolic rates rose with temperature, improving decolorization.

Lal and Chand [22] found that a newly obtained *Bacillus* strain decolorized Metanil Yellow at 30–35°C. *Bacillus* sp. strain Neni-10 decolorized best at 37°C, according to Mansur et al. [23]. The study found that temperatures above 40°C hindered the strain's decolorization, emphasizing the need for appropriate temperature. Muliadi et al. (2021) used biostimulation to improve Malaysian agricultural soil microbial populations, decolorizing Metanil Yellow at 30°C. *Oenococcus oeni* ML34 decolorized Fast Red at 30°C, according to [30]. [21] observed the best temperature for the decolorization of 200 mg/L Metanil Yellow by the bacterium *Pseudomonas* sp. strain UPM291 at 35 °C. Malaysian tropical climates mean that most organic pollutants would be degraded faster all year round.

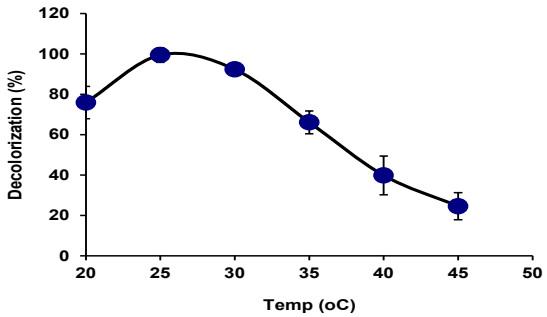

Fig. 4. Metanil Yellow decolorization by *Serratia marcescens* strain MM06 at various temperatures. The experiment was replicated thrice, and error bars represent standard deviation.

Effect of pH on decolorization of Metanil Yellow

The ability of the bacterial isolate to decolorize Metanil Yellow was assessed across a pH range of 5.5 to 8.0. ANOVA analaysis indicates that the best pH was between 7 and 8.0 (**Fig. 5**). decolorization was suboptimal under acidic environments, resulting in reduced efficacy in removing color as observed in several other studies [31–34]. [21] observed the best pH for the decolorization of 200 mg/L Metanil Yellow by the bacterium *Pseudomonas* sp. strain UPM291 was at pH 6.5. Decolorization of synthetic azo dyes like Metanil Yellow is affected by pH, a key element in wastewater treatment biodegradation optimization. Various studies have studied how different bacterial strains react to pH changes during Metanil Yellow decolorization [20]. It was observed that *Bacillus* sp. strain AK1

and *Lysinibacillus* sp. strain AK2 decolorized best at pH ranges of 7–9. [5] found that the halophilic alkalithermophilic bacterial consortia could decolorize Metanil Yellow G in alkaline circumstances, notably at pH 10. Lal and Chand [22] found that a newly isolated *Bacillus* strain decolorized Metanil Yellow at pH 7–8. [23] reported that *Bacillus* sp. strain Neni-10 decolorized best at pH 8. The strain's decolorization was negatively influenced by pH levels below 6 or over 9, emphasizing the necessity of an appropriate pH. Muliadi et al. [24] used biostimulation to improve Malaysian agricultural soil microbial populations and decolorize Metanil Yellow best at pH 7.5. *Oenococcus oeni* ML34 decolorized Fast Red at pH levels around 7 according to [30].

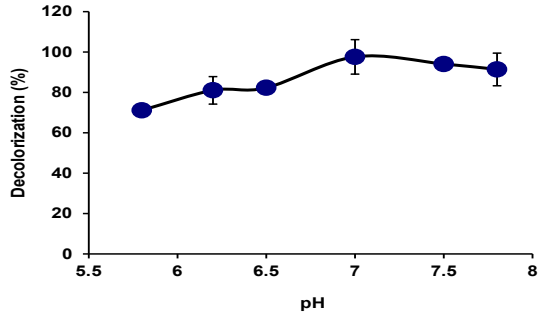

Fig. 5. Metanil Yellow decolorization by *Serratia marcescens* strain MM06 at various pHs. The experiment was replicated thrice, and error bars represent standard deviation.

Effect of NaCl concentration on decolorization of Metanil Yellow

An investigation was conducted to examine the impact of NaCl content on the efficacy of decolorizing Metanil Yellow by the bacterium. The findings suggest that the effectiveness of decolorization is affected by the concentration of salt, with the best results reported at moderate levels of NaCl. The effectiveness of the bacterium in removing color from Metanil Yellow was evaluated at various NaCl concentrations ranging from 0 to 30 g/L. The decolorization efficiency increased at low NaCl concentrations (0 to 5 g/L), reaching nearly 85% at 5 g/L. The efficiency consistently remained at a high level, ranging from 90% to 95%, at NaCl concentrations of up to 15 g/L. ANOVA analysis showed no difference in decolorizing ability between 10 and 15 g/L.

The plateau indicates that the bacterial isolate exhibits a high efficacy level in decolorizing Metanil Yellow under moderately salty circumstances. Nevertheless, when the Concentration of NaCl exceeded 15 g/L, a decrease in decolorization efficiency was noted. The efficiency reduced to around 75% at a concentration of 20 g/L and dropped to around 10% at a concentration of 30 g/L (**Fig. 6**). The decrease in decolorization efficacy at elevated NaCl concentrations can be ascribed to the osmotic stress caused by high salt levels, which can have a detrimental impact on bacterial metabolism and enzyme function essential for the decolorization process. High salt levels can cause cellular dehydration and hinder metabolic processes, decreasing bacterial effectiveness [35–37]. Synthetic azo dyes like Metanil Yellow decolorize depending on NaCl concentration, which optimizes biodegradation in saline wastewater.

Various studies have studied how different bacterial strains react to NaCl levels during Metanil Yellow decolorization. Anjaneya et al. [20] observed that *Bacillus* sp. strain AK1 and *Lysinibacillus* strain AK2 decolorized best at NaCl concentrations up to 5%. A halophilic alkalithermophilic bacterial consortia decolorized Metanil Yellow G efficiently at increased NaCl concentrations, particularly 10%, according to [5]. Lal and Chand [22] found that a newly obtained *Bacillus* strain decolorized Metanil Yellow at 3% NaCl. Mansur et al. [23] reported that *Bacillus* sp. strain Neni-10 decolorized within 24 hours at NaCl concentrations up to 4%. Muliadi et al. (2021) reported decolorization at NaCl concentrations up to 5%. *Oenococcus oeni* ML34 decolorized Fast Red at 3% NaCl concentrations, according to El Ahwany [30]. [21] observed that the bacterium *Pseudomonas* sp. strain UPM291 can tolerate up to 15 g/L of NaCl for the decolorization of 200 mg/L Metanil Yellow. A summary of selected Metanil yellow-degrading bacterium is shown in **Table 1**.

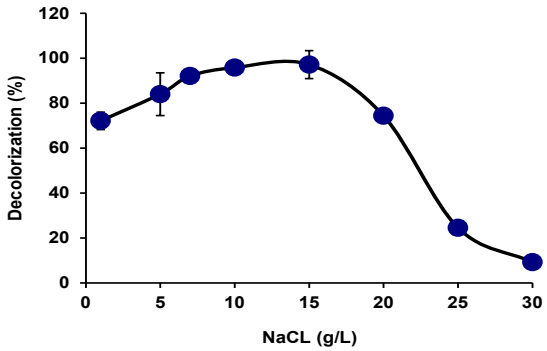

Fig. 6. Metanil Yellow decolorization by *Serratia marcescens* strain MM06 at various salt concentrations. The experiment was replicated thrice, and error bars represent standard deviation.

The effect of heavy metals on the Metanil Yellow decolorization

Almost all studies done on the decolorization of Metanil Yellow do not study the effect of heavy metals, and this should be studied since many polluted sites contain not only organic but also inorganic pollutants, including heavy metals [39]. The results indicate that the heavy metals mercury, silver and copper, all at 1 p.p.m, strongly inhibit decolorization of the dye (**Fig. 6**). This inhibition is likely through the binding to the sulfhydryl group of enzymes. This indicates that some form of heavy metals detoxification additives or treatments need to be added to ensure remediation of the dye is not affected.

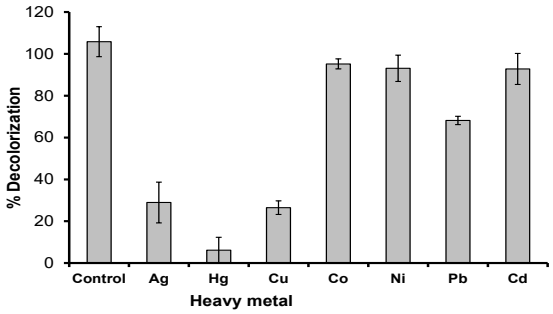

Fig. 6. Metanil Yellow decolorization by *Serratia marcescens* strain MM06 in the presence of several heavy metals. Data represent mean ± standard deviation, n=3.

Table 1. Metanil Yellow-decolorizing bacteria.

Microorganism	Best pH	Best Temperature	Maximum concentration Degraded (mg/L)	Time (hours)	Extra Carbon Source/Yeast Extract	Ref
Bacillus sp. strain Neni-10	7.0	37°C	50	48	Yeast Extract	[23]
Bacillus -3330	8.0	30°C	100	24	Glucose	[22]
Halophilic alkalithermophilic consortium	9.0	50°C	200	12	Starch	[5]
Mixed culture FN3	7.5	35°C	75	36	Yeast extract	[24]
Oenococcus oeni ML34	4.5	30°C	60	48	None	[30]
Bacillus sp. AK1	7.2	40	200	27	Yeast extract	[20]
Lysinibacillus sp. AK2	7.2	40	200	12	Yeast extract	[20]
Isolates NHG and NH2	7.0	25	50	48	Glucose	[38]
Pseudomonas sp. strain UPM291	6.5	35	200		Yeast extract	[21]
Serratia marcescens strain MM06	7.0 to 8.0	25	300	24	Yeast extract	this study

CONCLUSION

The findings of this study indicate that the capacity of the bacterial strain to decolorize Metanil Yellow is significantly influenced by the initial dye concentration, temperature, pH, and NaCl concentration. The best conditions for the removal of the dye were when the concentrations were between 100 and 300 mg/L, the temperatures were around 25°C, the pH was between 7 and 8, and the NaCl concentrations were less than 20 g/L. Mercury, silver, and copper, which are cationic heavy metals, strongly inhibited declorization. This suggests that chelating or sequestration agents may need to be added to neutralize the toxic effect of these metal ions on the decolorization process to the polluted soil or water bodies. These results are a testament to the importance of keeping the right environmental conditions or being aware of the limitations to the dye removal process in field remediation works.

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