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Decolorization and Degradation of Methylene Blue by *Arthrobacter globiformis*

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Methylene blue (MB) has been commercially used in textile industries for dyeing of nylon, silk, and leather. The removal of the dye from the industrial wastewater has received considerable attention from an environmental standpoint (Mckay *et al*. 1999; Khattri and Singh 2000; Al-Ghouti *et al*. 2003). The ozonation (Javier *et al.* 1993), photocatalytic degradation (Lakshimi *et al*. 1995), and chemical oxidation (Dutta *et al*. 2001) of the dye in aqueous solutions have been reported.

The microbial decolorization and *N*-demethylation reactions of MB have been also investigated (Kling and AraújoNeto 1991; Ollikka *et al.* 1993; Ferreira *et al*. 2000). However, no other detailed investigation is reported on the degradation of the dye by microorganisms. In the screening of microorganisms with the ability to degrade MB, I have found the degradation of the dye by *Arthrobacter globiformis* (*A. globiformis*). This study reports the decolorization and degradation of MB by *A. globiformis.*

MATERIALS AND METHODS

Chemicals: Methylene blue (MB; C.I. 52015), Azure B (C.I. 52010), Azure C (C.I. 52002), and Thionine (C.I. 52000) were purchased from Aldrich Chemical Co. (USA). MB and Thionine were purified by preparative thin layer chromatography (TLC) eluting with methanol-chloroform-acetic acid (80:20:0.1, v/v). The other dyes were used without any purification steps. Thionolin and leuco MB were synthesized according to the literature, respectively (Granick *et al*. 1940, Obata 1961).

Microorganism and growth determination: *Arthrobacter globiformis* (*A. globiformis*) IFO 12137 was found as a MB-degrading bacterium. The strain was kindly supplied by the Institute for Fermentation, Osaka (IFO), Japan. It was maintained on 702 medium (10g of polypeptone, 2g of yeast extract, and 1g of $MgSO_4 \cdot 7H_2O$ per L of distilled water, pH 7.0) agar slants at 6℃. The measurement of growth (dry cell weight) of *A. globiformis* was performed by filtering 50mL of culture medium through a 0.3 μm cellulose nitrate membrane filter, followed by drying to constant weight at 80℃.

Assay of decolorization: Decolorization of MB and Thionine by *A. globiformis* was measured spectrophotometrically (Jasco model V-570 UV/VIS spectrophotometer). One loop of the stock culture was inoculated into 20 mL of 702 medium in a 50 mL Erlenmeyer flask and preincubated for 16 hr at 37℃. The 1 mL of culture medium was inoculated into 50 mL of 702 medium in a 100 mL Erlenmeyer flask containing the dye. Then the flask was incubated at 37℃ with rotary shaking at 120 rpm. Aliquots (1 mL) of the incubated culture were removed at selected intervals, salted out with NaCl, and extracted with 10 mL of 1-butanol. The alcohol layer was dried over anhydrous Na_2SO_4. The absorbance of the alcohol extracts was measured at λ max of the dye. Decolorization was calculated as follows: Decolorization (%) = [(initial absorbance - observed absorbance) / initial absorbance] $\times$ 100.

Identification of the metabolites: The metabolites formed from MB and Thionine were identified by comparison with *Rf* value on TLC and λ max of authentic standards. The cultures (50 mL $\times$ 4; total volume of 200 mL) of *A. globiformis* containing the dye (conc.: 0.1 mM) incubated as described above. The incubated cultures were adjusted to pH 10 with 1N NaOH, salted out with NaCl, and extracted twice with an equal volume of chloroform. The extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure at 40℃. The residue was redissolved in a small amount of methanol and subjected to TLC on Merck Silicagel $60F_{254}$ with ethanol-chloroform-acetic acid (85:10:5, v/v). The bands of the metabolites were scraped and extracted with methanol for recording of their UV-visible spectra.

RESULTS AND DISCUSSION

The decolorization of MB by *A. globiformis* depended upon the initial concentration of MB in the cultures. The effect of initial concentration of MB on the decolorization by *A. globiformis* is shown in Figure 1. At concentration of 0.1 and 0.2 mM, the decolorization (%) of MB increased with increasing the incubation time and reached > 95% and about 80% in 72 hr, respectively. At concentration of 0.3 and 0.4 mM, the decolorization was only about 15% in 72 hr.

Figure 2 shows growth curves of *A. globiformis* in the cultures containing MB. At concentration of 0.1 mM, *A. globiformis* exhibited good growth. However, the growth of *A. globiformis* in the cultures was inhibited with increasing concentration of MB during 72hr incubation. At concentration more than 0.3 mM, growth was not apparent with the exception of slight growth after 8 hr. These results indicate that the decolorization of MB by *A. globiformis* is effectively observed only at low concentration, below 0.2 mM, which does not cause significant inhibition of growth of *A. globiformis*.

TLC analysis showed that three metabolites were in the chloroform extract of the culture containing MB after 24 hr of incubation (Table 1). These metabolites 1-3 were determined as Azure B, Azure C, and leuco MB by comparison with *Rf* value

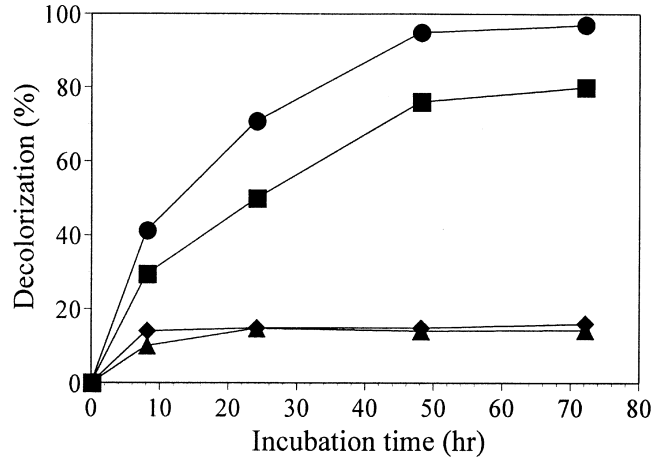

Figure 1. Decolorization of MB by *A. globiformis*. Initial weight of *A. globiformis:* 1.2 mg/50 mL. Initial MB concentration (mM): -●- 0.1, -■- 0.2, -◆- 0.3, -▲- 0.4.

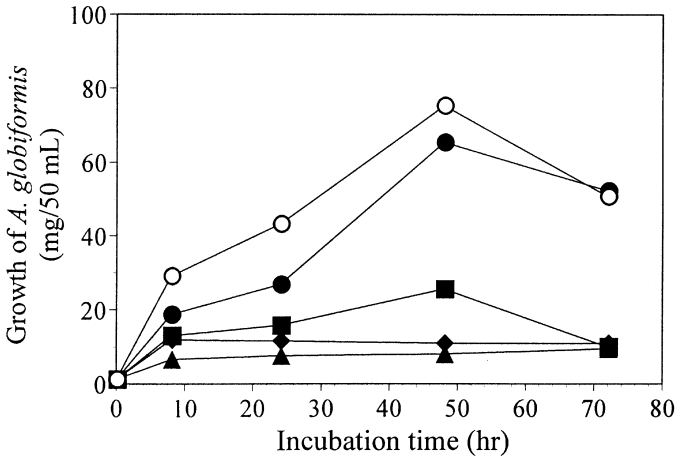

Figure 2. Growth of *A. globiformis* in the cultures containing MB. MB concentration (mM): -○- 0 (control), -●- 0.1, -■- 0.2, -◆- 0.3, -▲- 0.4.

Table 1. *Rf* value on TLC and λ max of the metabolites formed from MB and those of the authentic standards.

Metabolite	*Rf* value	λ max (nm)	Standard	*Rf* value	λ max (nm)
1	0.06	638	Azure B	0.06	638
2	0.21	614	Azure C	0.21	613
3	0.59	257	Leuco MB	0.59	257

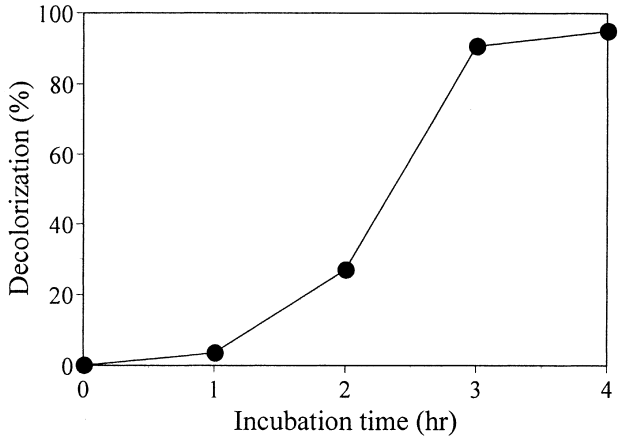

Figure 3. Decolorization of Thionine by *A. globiformis*. Initial weight of *A. globiformis*: 1.2 mg/50 mL.

Table 2. *Rf* value on TLC and λ max of the metabolites formed from Thionine and those of the authentic standard.

Metabolite	*Rf* value	λ max (nm)	Standard	*Rf* value	λ max (nm)
4	0.74	589	Thionolin	0.74	589
5	0.86	544			

on TLC and λ max of authentic standards. A putative *N*-demethylated metabolite from Azure C, Thionine, was not detected in the extract of the cultures.

Figure 3 shows the decolorization of Thionine by *A. globiformis*. After 3 hr of incubation, the decolorization (%) of Thionine was more than 90%. Thionine was quickly decolorized in the initial incubation period by *A. globiformis*. This result explains why Thionine was not detected as a metabolite during degradation of MB by *A. globiformis*. Similarly, trace amounts of the metabolites were found in the chloroform extract of the culture containing Thionine after 4 hr of incubation (Table 2). The metabolite 4 was determined as Thionolin. The metabolite 5 was not identified. These metabolites were not found in the chloroform extract of the culture after 72 hr of incubation. On the basis of these results, a proposed pathway for the initial degradation of MB by *A. globiformis* is shown in Figure 4.

Kling and AraújoNeto (1991) reported that lignin peroxidase from *Phanerochaete chrysosporium* degraded MB by sequential *N*-demethylation, and the final metabolite of degradation of MB was azure C. However, Azure C still exhibits absorption at the visible region. The present study demonstrated that *A. globiformis* decolorized and degraded MB by the reduction, the *N*-demethylation, and the deamination. *A. globiformis* will be a promising bacterium for biological treatment of wastewater containing MB. Further studies necessary to reach the stage for practical use of this bacterium.

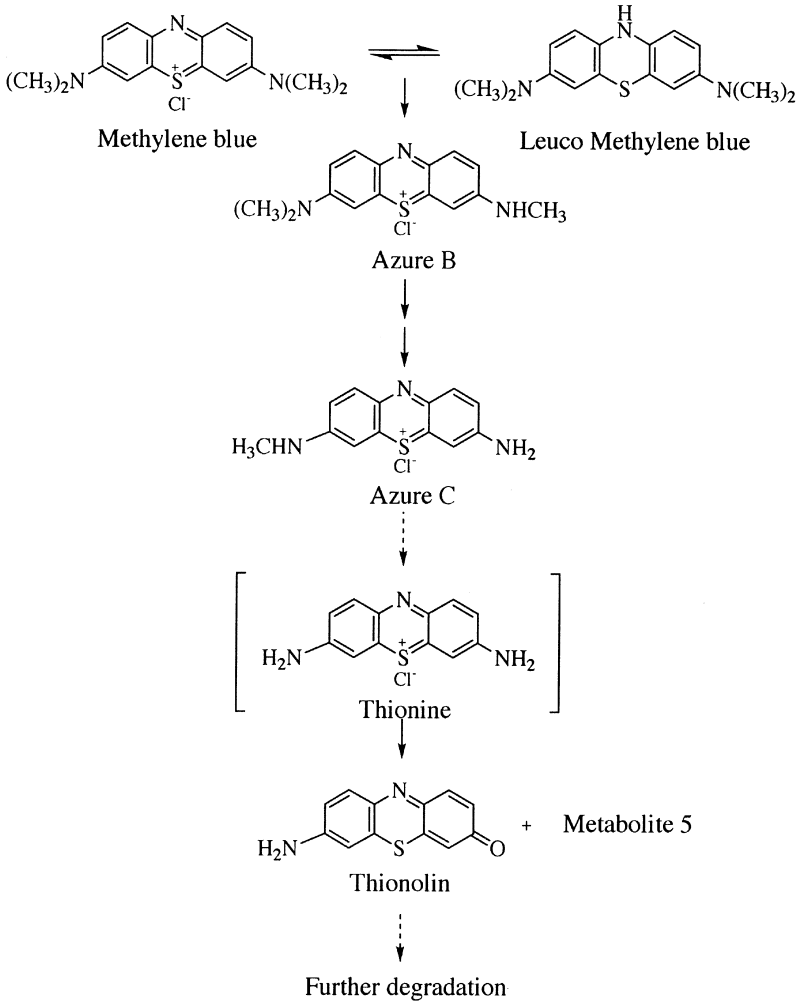

Figure 4. A proposed pathway for the initial degradation of MB by *A. globiformis.*

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