Journal of Hazardous Materials 177 (2010) 281–289

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Partial degradation mechanisms of malachite green and methyl violet B by *Shewanella decolorationis* NTOU1 under anaerobic conditions

C.-H. Chen, C.-F. Chang, S.-M. Liu*

Institute of Marine Biology, National Taiwan Ocean University, 2-Peining Rd., Keelung, Taiwan

ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form 4 December 2009
Accepted 6 December 2009
Available online 8 January 2010

Keywords:
Decolorization
Malachite green
Methyl violet B
Partial degradation mechanisms
Shewanella decolorationis NTOU1

ABSTRACT

This work demonstrated that *Shewanella decolorationis* NTOU1 decolorized 200 mg l^{-1} of crystal violet, malachite green, or methyl violet B within 2–11 h under anaerobic conditions at 35 °C. The initial color removal rate of malachite green was highest, while that of methyl violet was lowest. GC/MS analyses of the intermediate compounds produced during and after decolorization of malachite green and methyl violet B suggested that biodegradation of these dyes involved reduction to leuco form, N-demethylation, and reductive splitting of the triphenyl rings. The number of N-methylated groups of these dyes might have influenced decolorization rates and the reductive splitting of the triphenyl rings of these dyes. Cytotoxicity and antimicrobial test data showed that malachite green and methyl violet B solution (100 mg l^{-1}) were toxic. Toxicity of the dyes decreased after their decolorization, but further incubation resulted in increased toxicity.

1. Introduction

Triphenylmethane dyes are xenobiotic compounds used extensively in many industrial processes, such as textile dyeing, printing, papermaking, and in leather, food, and cosmetic manufacturing [1,2]. These dyes are recalcitrant molecules, toxic to microorganisms and mammalian cells, mutagenic to rodents [3,4]. They also cause reproductive abnormalities in rabbits and fish [5]. Malachite green, in addition to being used in the dyeing process, has widely been used to prevent fungal infections in fish farms [4], methyl violet B has been used as a staining agent in bacteriological and histopathological works [6]. Although, using malachite green has been banned worldwide since 2002 due to its carcinogenic and toxicological effects [7], these dyes may still persist in some environments [8].

Studies have reported several triphenylmethane dyes-decolorizing microorganisms and their characteristics have been reviewed [9–15]. Different groups of microorganisms such as bacteria e.g. *Pseudomonas pseudomallei* [9], *Citrobacter* sp. [14], *Bacillus subtillus* [11]; actinomycetes e.g. *Norcardia corallina* [12]; yeasts e.g. *Rhodotorula* sp. [2], *Rhodotorula rubra* [2]; and fungi e.g. *Phanerochaete chrysosporium* [10], *Cunninghamella elegans* [13] were all found capable of decolorization of triphenyl methane dyes. However, most of these studies were carried out with crystal violet [2,6,12–17]. Few researches have reported the degradation mechanisms or pathways underlying the decolorization of triphenylmethane dyes.

Shewanella decolorationis NTOU1, a facultative iron-reducing bacterium isolated from an oil refinery cooling system, can decolorize crystal violet with high efficiency [15]. Malachite green and methyl violet B are also N-methylated triphenylmethane dyes similar to crystal violet, but with different number of N-methylated groups. Studies of the degradation mechanisms of these two compounds and comparison of their mechanisms with that of crystal violet would help to understand the effect of the number of N-methylated groups on their degradation.

This study, in addition to the potential of the aforementioned bacterial strain to decolorize malachite green and methyl violet B under anaerobic conditions, investigated the probable degradation mechanisms of these two dyes. The current study also tested the potential detoxification of these two dyes by this strain.

2. Materials and methods

2.1. Chemicals

Crystal violet, malachite green, and methyl violet B (about 85% dye content) were purchased from Sigma–Aldrich and were used without further purification. All other chemicals were of reagent grade.

* Corresponding author. Tel.: +886 2 2462 2192x5307; fax: +886 2 2463 3152.
E-mail address: smliu@mail.ntou.edu.tw (S.-M. Liu).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.12.030

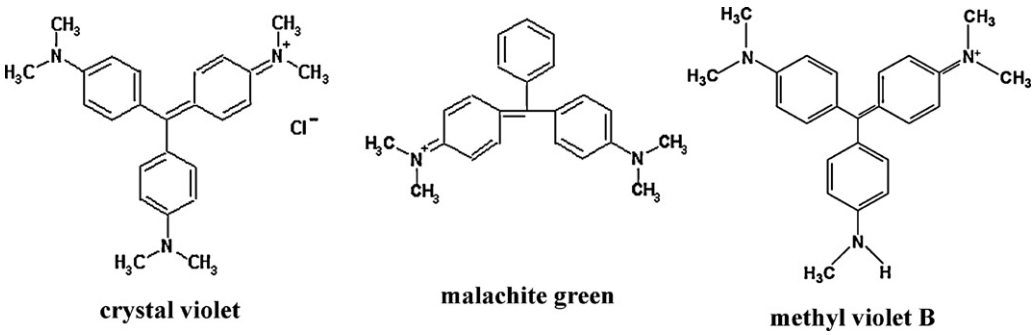


Fig. 1. The structure of the dyes tested in this study.

2.2. Organism

S. decolorationis NTOU1, a facultative bacterium originally isolated from an oil refinery cooling system, was capable of utilizing a variety of electron acceptors including ferric citrate during anaerobic growth [15].

2.3. Decolorization of triphenylmethane dyes under iron-reducing conditions

Decolorization of crystal violet, malachite green, and methyl violet B (Fig. 1) by *S. decolorationis* NTOU1 was carried out in 117 ml serum bottles containing the anoxic phosphate-buffered basal medium (50 ml) [15] supplemented with formate (20 mM) and ferric citrate (20 mM) under an atmosphere of N_2:CO_2 (80:20). Since growth of the strain NTOU1 was much faster under aerobic conditions than under anaerobic conditions (data not shown), cells grown under aerobic conditions were collected and used as the inoculum following the methods used in the previous report [15]. Cell suspensions were inoculated into 117 ml serum bottles containing anoxic medium and a dye (100–1500 mg l^{-1}) to reach an initial cell mass of 0.4–0.5 g l^{-1} (wet weight) and then incubated statically under anaerobic conditions at 35 °C for the dye-decolorization test. Each experiment was conducted in duplicate. Decolorization tests with heat-killed cell suspension or without cell suspension served as controls. Dye concentration in the cell culture was determined by monitoring the absorbance of the filtrate (0.22 μm) of the cell culture at the absorption maximum of each dye, for example 585 nm for crystal violet, 615 nm for malachite green, and 580 nm for methyl violet B.

Decolorization activity (%) was calculated according to the formula: Decolorization activity (%) = $(A - B)/A \times 100$, in which A is the initial absorbance and B is the final absorbance.

2.4. Determination of the degradation products of malachite green and methyl violet B

To determine the intermediate products of malachite green or methyl violet B, 200 ml anaerobic cell suspension supplemented with formate (20 mM), ferric citrate (20 mM) and a dye (100 mg l^{-1}) to an initial cell mass of 0.4–0.5 g l^{-1} (wet weight) were prepared and incubated at 35 °C. At certain times (every 0.4 h in the beginning 2 h) during incubation, this experiment collected 40 ml of the culture.

Intermediate products in each subsample were extracted, dried, and then dissolved in 1 ml of ethylacetate for GC/MS analysis following the methods used in the previous report [15].

Table 1
Effect of initial dye concentration (100–1500 mg l^{-1}) on the initial color removal rate (mg l^{-1} h^{-1}) for the triphenylmethane dyes by the *S. decolorationis* NTOU1 (cell mass inoculated for each test was about 0.4–0.5 g l^{-1}, wet weight).

Triphenylmethane dyes	Dye concentration (mg l^{-1})				
	100	200	500	1000	1500
Crystal violet	24.4	49.3	120	225	316
Malachite green	66.1	156	405	859	1345
Methyl violet B	18.1	34.3	87.4	133	227

2.5. Analytical methods

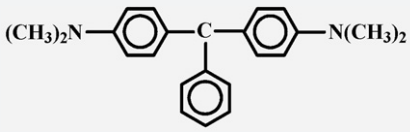
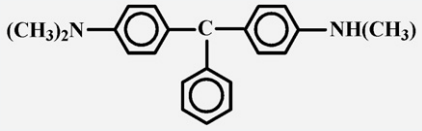
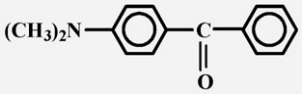
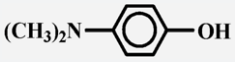
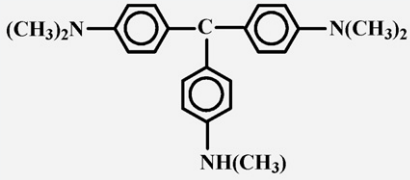
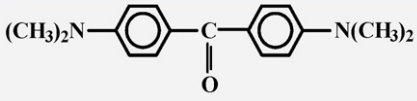
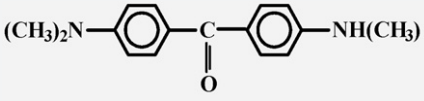
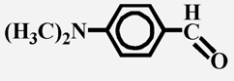
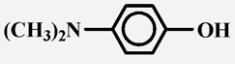
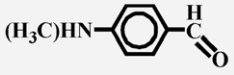
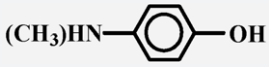
The GC/MS system consists of an Agilent 6890 GC equipped with an Agilent 5973N mass selective detector (GC/MSD) was used to detect the degradation products of malachite green and methyl violet. The mass spectrometer was operated in the electron impact mode with an electron current of 70 eV. Aliquots of 1 μl were injected automatically with an auto sampler (AUC20i) in splitless mode via a GC inlet. An HP-5 MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) was connected directly to the ion source of the mass spectrometer. The oven temperature was kept isothermal for 1 min at 50 °C, was then increased to 270 °C at a rate of 10 °C min^{-1} and held at 270 °C for 7 min. The injector, MS source and MS quad temperature were 250 °C, 230 °C, and 150 °C, respectively. The GC/MS system was operated in full scan (m/z 50–500). Controls and samples were analyzed in duplicate.

2.6. Cytotoxicity and antimicrobial tests

Cytotoxicity of malachite green, methyl violet B, and their decolorization products were determined by measuring their effects on propagating mouse cell NCTC clone L-929 (ATCC CCL-1™) following the methods used in the previous report [15]. Filtrate (0.22 μm) from *S. decolorationis* NTOU1 cell cultures containing malachite green or methyl violet B (100 mg l^{-1}) before and after decolorization (>98% decolorized) were used as the test extracts. The test extracts and the buffer (67 mM phosphate buffer, pH 7.0)-diluted test extracts (20% or 40%) were added to the 4-well tissue culture plates containing mouse cells and then incubated at 37 °C for 48 h. Mouse cells in the confluent monolayer of the 4-well tissue culture plates were counted microscopically. Each test was performed in triplicate. Mouse cell number was expressed as the mean ± standard deviation.

The antimicrobial test was performed by measuring the effect of malachite green (100 mg l^{-1}), methyl violet B (100 mg l^{-1}), or their degradation products on the growth of *Escherichia coli* strain JM 109 (ATCC 53323 and BCRC 51540) following methods used in previous reports [15,18]. Cell number of *E. coli* culture was counted by the most probable number method (MPN). Cell number of *E. coli* culture was expressed as the mean ± standard deviation.

Table 2
Retention times and accurate mass measurements obtained from GC/MS spectra of the malachite green and methyl violet B biodegradation products identified.

Intermediate products	Molecular structure	Retention time (min)	*m*/*z* (Relative intensity of predominant ions in fragmentation pattern; %)
Malachite green			
Leucomalachite green		22.81	330 (97); 253 (100); 239 (17); 210 (18); 165 (26); 126 (18)
N,N,N′-Trimethyl-4,4′-benzylidenedianiline		22.69	316 (100); 272 (8); 239 (98); 223 (9); 207 (10); 194 (16); 165 (15); 119 (20)
[N,N-Dimethylaminophenyl][phenyl]benzophenone		16.85	225 (73); 148 (100); 105 (14); 77 (18)
N,N-Dimethylaminophenol		7.54	136 (100); 121 (21); 94 (7); 65 (7)
Methyl violet B			
Leucomethyl violet B		19.57	359 (100); 253 (38); 239 (54); 223 (14); 126 (7)
Michler's ketone		22.05	268 (100); 251 (21); 224 (33); 148 (81); 120 (16); 77 (7)
[N,N-Dimethylaminophenyl][N′-methylaminophenyl]benzophenone		21.68	254 (100); 237 (16); 224 (11); 210 (18); 148 (55); 134 (38); 113 (15); 77 (9)
N,N-Dimethylaminobenzaldehyde		9.79	148 (100); 132 (10); 105 (6); 77 (11)
N,N-Dimethylaminophenol		7.54	136 (100); 121 (22); 94 (7); 65 (7)
N-Methylaminobenzaldehyde		9.42	134 (100); 106 (20); 77 (17); 65 (7)
N-Methylaminophenol		7.29	123 (100); 108 (21); 94 (17); 81 (12)

3. Results

3.1. Decolorization of triphenylmethane dyes by S. decolorationis NTOU1

The current study demonstrated the decolorization of crystal violet, malachite green, and methyl violet B without a lag phase and at high efficiency under anaerobic conditions by S. *decolorationis* NTOU1. Decolorization rates increased when these dye concentrations increased at least up to 1500 mg l^{-1}. The initial color removal rate of malachite green was highest, while that of methyl violet was lowest (Table 1). These dyes were not decolorized in cultures inoculated with heat-killed cell suspension or without cell suspension. After decolorizing, cell cultures of these dyes were taken and centrifuged (8000 × *g*, 10 min). Findings reveal no color adsorbed into the cell paste. These results suggest that this strain biotransformed or biodegraded these dyes.

3.2. Intermediates formed during and after decolorization of malachite green and methyl violet B

The mass spectra of the intermediates of malachite green were compared to the spectra in the NIST library and identified as leucomalachite green with a molecular weight of 330, N,N,N′-trimethyl-4,4′-benzylidenedianiline with a molecular weight of 316, [N,N-dimethylaminophenyl][phenyl] benzophenone with a molecular weight of 225, N,N-dimethylaminophenol with a molecular weight of 136 (Table 2).

Fig. 2(a) shows malachite green (100 mg l^{-1}; 107.9 μM) decolorized in 2 h, producing leucomalachite green which accumulated to its highest concentration in the first 1.5 h and then started to transform in the next 7 h. N,N,N′-Trimethyl-4,4′-benzylidenedianiline, [N,N-dimethylaminophenyl][phenyl] benzophenone and N,N-dimethylaminophenol produced at the same time with leucomalachite green, however, they accumulated to their highest concentration in the first 0.5 h and then started to transform. Except for leucomalachite green, most intermediate compounds transformed in the extended 10 h after removing the malachite green color.

Based on the time for decolorizing the parent compound and forming intermediate compounds, malachite green split into [N,N-dimethylaminophenyl][phenyl] benzophenone and N,N-dimethylaminophenol. At the time of rings cleavage, malachite green also demethylated to N,N,N′-trimethyl-4,4′-benzylidenediamine. However, whether it was necessary to reduce leucomalachite green before ring cleavage or demethylate malachite green under anaerobic conditions is uncertain.

The current investigation also compared mass spectra of methyl violet B intermediates to spectra in the NIST library and identified leucomethyl violet B with a molecular weight of 359, N,N′-bis[dimethylamino] benzophenone (Michler's ketone) with a molecular weight of 268, [N,N-dimethylaminophenyl] [N′-methylaminophenyl] benzophenone with a molecular weight of 254, N,N-dimethylaminobenzaldehyde with a molecular weight of 148, N,N-dimethylaminophenol with a molecular weight of 136, N-methylaminobenzaldehyde with a molecular weight of 134, and N-methylaminophenol with a molecular weight of 123 (Table 2).

Fig. 2(b) shows methyl violet B (100 mg l^{-1}; 253.8 μM) decolorized within 60 h during incubation. Michler's ketone, N,N-dimethylaminophenol, [N,N-dimethylaminophenyl][N′-methylaminophenyl] benzophenone, and N,N-dimethylaminobenzaldehyde produced immediately after decolorizing methyl violet B. These intermediate compounds accumulated to their highest concentration in 5 h and then started to transform. They decreased at faster rates in the next 10 h, and then at slower rates. Leucomethyl violet B was also detected after 1 h incubation and accumulated to its highest concentration (shown as the peak area) after another 4 h incubation and then transformed to other compound(s). Leucomethyl violet B transformed faster than the other intermediate compounds. This research also detected a small amount of N-methylaminobenzaldehyde and N-methylaminophenol during incubation (not shown in Fig. 2b). These results suggested that methyl violet B might have split into Michler's ketone and N-methylaminophenol or [N,N-dimethylaminophenyl] [N′-methylaminophenyl] benzophenone and N,N-dimethylaminophenol through ring cleavage reaction. Michler's ketone might have further split into N,N-dimethylaminobenzaldehyde and N,N-dimethylaminophenol. Michler's ketone might also have demethylated to [N,N-

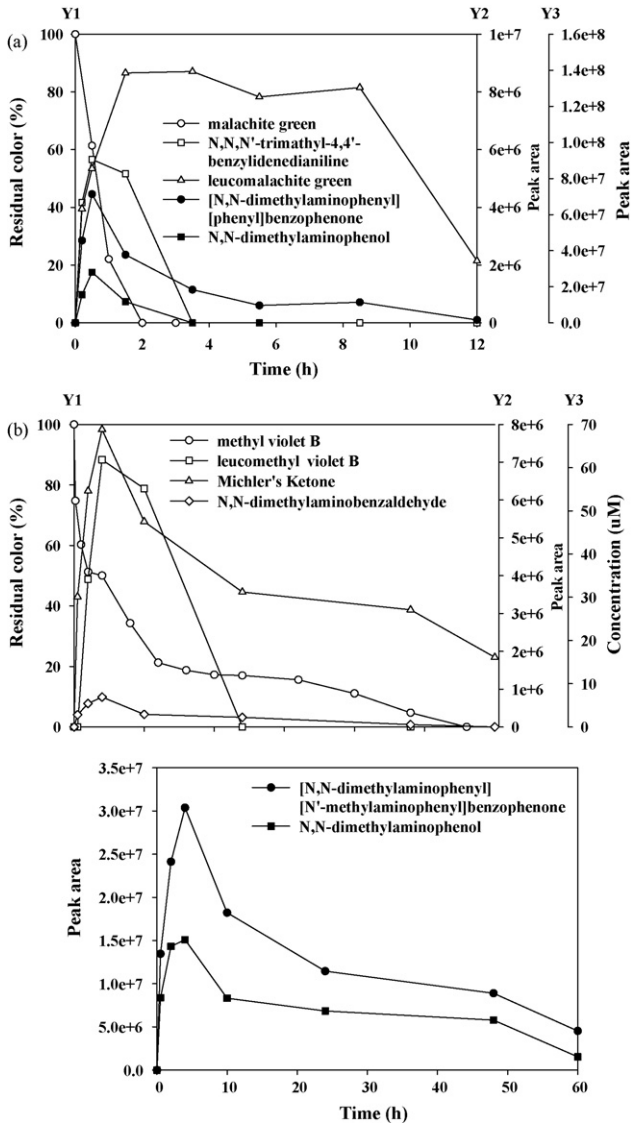


Fig. 2. (a) Anaerobic biodegradation of malachite green by *S. decolorationis*. NTOU1. Concentration of malachite green was expressed as the remaining residual color (%). Concentration of N,N,N′-trimethyl-4,4′-benzylidenedianline, [N,N-dimethylaminophenyl][phenyl] benzophenone and N, N-dimethylaminophenol were expressed in peak area in the GC chromatogram (Y2 axis). Concentration of leucomalachite green was expressed in peak area in the GC chromatogram (Y3 axis). (b) Anaerobic biodegradation of methyl violet B by *S. decolorationis*. NTOU1. Concentration of methyl violet B was expressed as the remaining residual color (%). Concentration of leucomethyl violet B, Michler's ketone, and N,N-dimethylaminobenzaldehyde were expressed in peak area in the GC chromatogram (Y2 axis). Concentration of [N,N-dimethylaminophenyl][N′-methylaminophenyl] benzophenone and N,N-dimethylaminophenol were expressed in peak area in the GC chromatogram (Y3 axis).

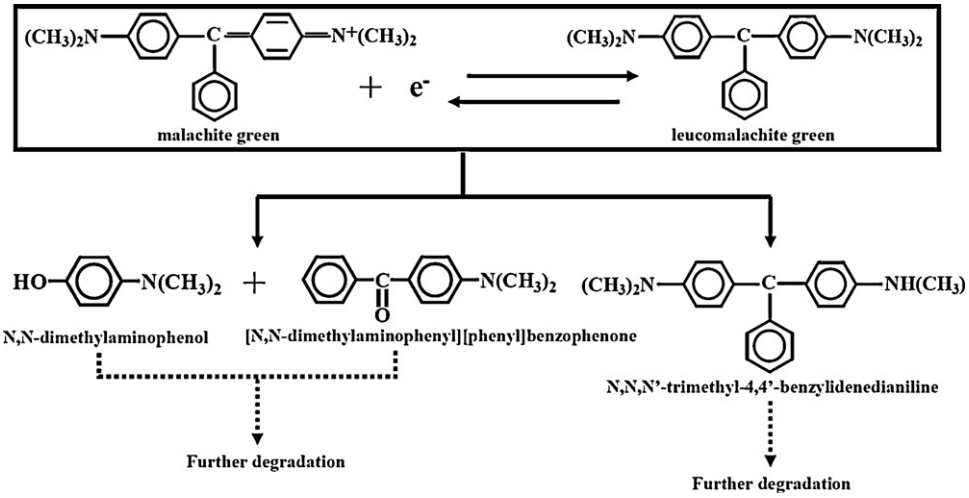


Fig. 3. Proposed decolorization and biodegradation pathways of malachite green by the *Shewanella* sp. NTOU1 under static anaerobic conditions.

Table 3
Cell number of mouse cell clone L-929 in the 4-well plates after incubation with malachite green, methyl violet B, or their degradation products for 48 h.

Group	Mean of cell number (cells ml^{-1})		
	Test extract	Diluted test extract (40%)	Diluted test extract (20%)
Malachite green solution (100 mg l^{-1}) before biodegradation	$5.77 \pm 0.55 \times 10^{5}$*	$6.17 \pm 0.44 \times 10^{5}$*	$7.10 \pm 0.36 \times 10^{5}$*
Malachite green solution after incubation for 3 h (>98% decolorized)	$1.06 \pm 0.05 \times 10^{6}$*	$1.27 \pm 0.04 \times 10^{6}$	$1.28 \pm 0.07 \times 10^{6}$
Malachite green solution after incubation for 51 h	$6.13 \pm 0.46 \times 10^{5}$*	$7.17 \pm 0.35 \times 10^{5}$*	$7.78 \pm 0.48 \times 10^{5}$*
Methyl violet B solution (100 mg l^{-1}) before biodegradation	$4.87 \pm 0.35 \times 10^{5}$*	$5.68 \pm 0.42 \times 10^{5}$*	$7.07 \pm 0.38 \times 10^{5}$*
Methyl violet B solution after incubation for 8 h (>98% decolorized)	$7.20 \pm 0.30 \times 10^{5}$*	$1.25 \pm 0.05 \times 10^{6}$	$1.28 \pm 0.06 \times 10^{6}$
Methyl violet solution after incubation for 56 h	$6.77 \pm 0.15 \times 10^{5}$*	$1.03 \pm 0.04 \times 10^{6}$*	$1.22 \pm 0.05 \times 10^{6}$

Negative control (EMEM medium only): $1.26 \pm 0.07 \times 10^{6}$ cells ml^{-1}. Positive control (EMEM medium with 0.1% phenol): $3.00 \pm 1.00 \times 10^{4}$ cells ml^{-1}.
* The data is significantly different from the data of the negative control ($p < 0.05$).

Table 4
Cell number (cells ml^{-1}) of the *E. coli* strain JM 109 in the test tubes after incubation with malachite green, methyl violet or their degradation products for 1, 12, and 24 h. Cell numbers (cells ml^{-1}) were counted by the most probable number (MPN) method.

Group	Incubation time for antimicrobial test		
	1 h	12 h	24 h
Negative control[a]	$2.01 \pm 0.16 \times 10^{10}$	$1.41 \pm 0.16 \times 10^{10}$	$1.11 \pm 0.26 \times 10^{10}$
Malachite green solution (100 mg l^{-1}) before biodegradation	$5.01 \pm 0.91 \times 10^{9}$*	$2.90 \pm 0.56 \times 10^{7}$*	$2.23 \pm 0.31 \times 10^{7}$*
Malachite green solution after incubation for 3 h (>98% decolorized)	$1.94 \pm 0.23 \times 10^{10}$	$1.33 \pm 0.28 \times 10^{10}$	$3.83 \pm 0.75 \times 10^{9}$*
Malachite green solution after incubation for 51 h	$2.03 \pm 0.03 \times 10^{10}$	$1.48 \pm 0.26 \times 10^{10}$	$2.95 \pm 0.49 \times 10^{9}$*
Methyl violet solution (100 mg l^{-1}) before biodegradation	$1.37 \pm 0.09 \times 10^{10}$*	$1.13 \pm 0.08 \times 10^{9}$*	$2.93 \pm 1.08 \times 10^{7}$*
Methyl violet solution after incubation for 8 h (>98% decolorized)	$1.80 \pm 0.11 \times 10^{10}$	$9.30 \pm 2.40 \times 10^{9}$*	$1.77 \pm 0.23 \times 10^{9}$*
Methyl violet solution after incubation for 56 h	$1.77 \pm 0.08 \times 10^{10}$	$6.63 \pm 1.68 \times 10^{9}$*	$3.23 \pm 0.52 \times 10^{9}$*

[a] Dye solution was replaced by the 67 mM phosphate buffer (pH 7.0).
* The data is significantly different from the data of the negative control ($p < 0.05$).

dimethylaminophenyl] [N′-methylaminophenyl] benzophenone, while [N,N-dimethylaminophenyl] [N′-methylaminophenyl] benzophenone might have further split into N,N-dimethylaminobenzaldehyde and N-methylaminophenol or N-methylaminobenzaldehyde and N,N-dimethylaminophenol. Findings showed no demethylated intermediate compounds of methyl violet B were found. Although this study detected leucomethyl violet B after Michler's ketone and [N,N-dimethylaminophenyl][N′-methylaminophenyl] benzophenone during decolorization time, it is not clear whether leucomethyl violet B reduction was necessary before cleavage reaction.

Results of present study proposes possible pathways for the decolorization of malachite green (Fig. 3) and methyl violet B (Fig. 4a and b) by *S. decolorationis* NTOU1 under iron-reducing conditions.

3.3. Toxicity of triphenylmethane dyes and their degradation products

Table 3 shows that mouse cells in the cell culture added with 100 mg l^{-1} or diluted malachite green solution, were significantly lower than mouse cells in negative controls. Mouse cells in the cell cultures added with degraded intermediate products of malachite green solution, were also significantly lower than mouse cells in negative controls. Mouse cells in the cell cultures added with diluted (20% or 40%) decolorized (>98%) malachite green solution, were not significantly lower than mouse cells in negative controls.

Findings of mouse cell toxicity tests with methyl violet B show similar results.

Table 4 shows that cells of *E. coli* strain JM109 in cultures containing malachite green solution (100 mg l^{-1}) were significantly lower than those in the negative controls during 1 h,

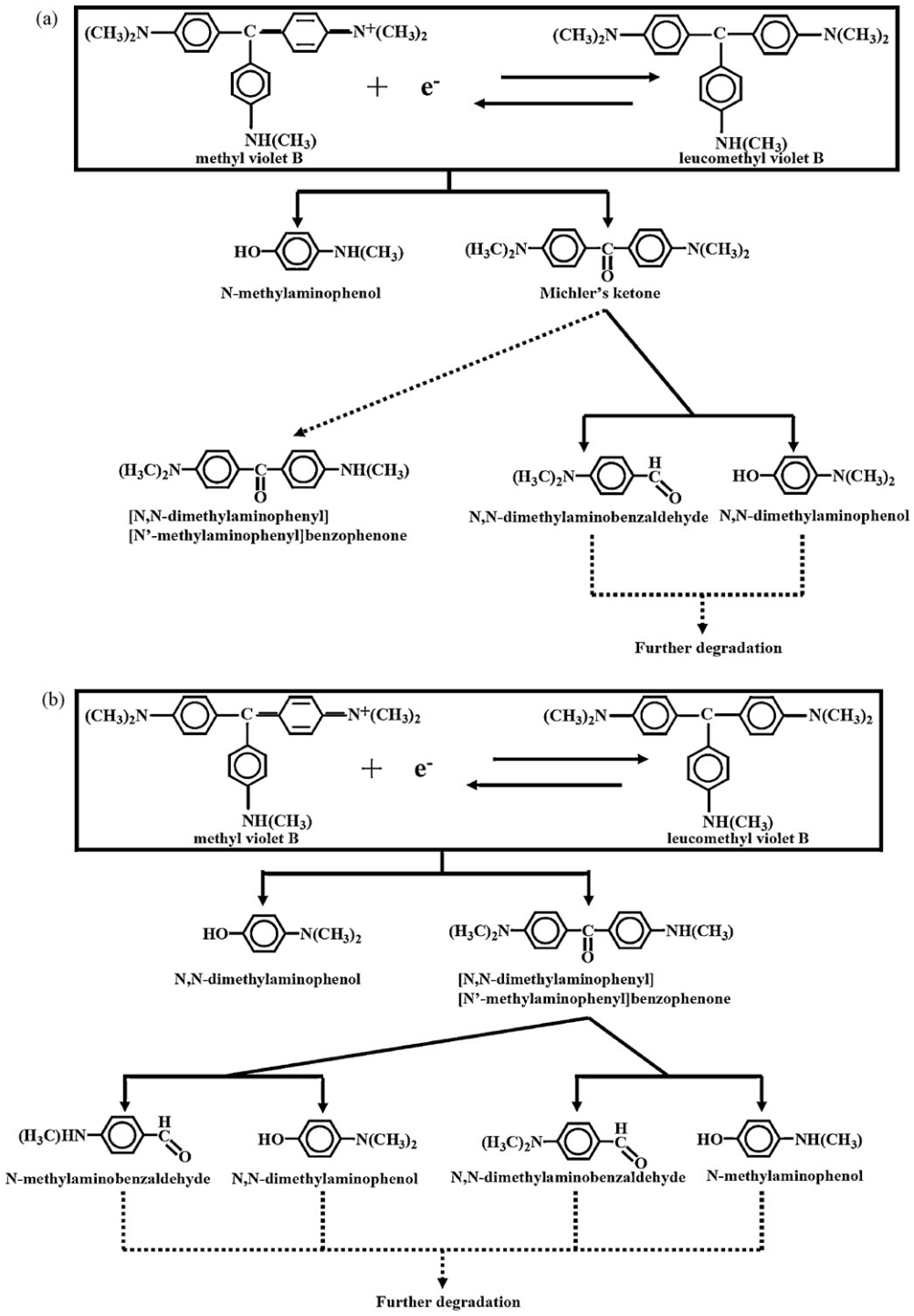


Fig. 4. Two proposed decolorization and biodegradation pathways (a) and (b) for methyl violet B by *Shewanlla* sp. NTOU1 under static anaerobic conditions.

12 h, and 24 h incubation. Cells in cultures containing only decolorized or biodegraded malachite green solutions were not lower than those in negative controls after 1 h incubation, but were significantly lower than those in negative controls after 24 h incubation.

Findings show similar results in antimicrobial tests with methyl violet B. Instead of 24 h, after 12 h incubation the number of *E. coli* strain in cell cultures containing only decolorized or biodegraded methyl violet solutions were significantly lower than those in negative controls.

4. Discussion

This study found that triphenylmethane dyes decolorized at high rates (24.4–1345 mg l^{-1} h^{-1}) using the *Shewanella* strain. The initial color removal rate of malachite green was highest, while that of methyl violet B was lowest. Similar to our results, studies have reported that malachite green (50 mg l^{-1}) completely decolorized within 5 h, but the same concentration of methyl violet B and crystal violet did not decolorize by bacterium *Kocuria rosea* MTCC 1532 under static anoxic conditions [22] (Table 5).

Table 5
Biodegradation of triphenylmethane dyes by different bacteria/fungi.

Dye (concentration)	Bacteria/fungi	Temperature and other conditions	Removal efficiency	Initial decolonization rate	Reference
Malachite green (50 mg l^{-1})	*K. rosea* MTCC 1532	Static anoxic conditions	Complete in 5 h		[22]
Methyl violet (50 mg l^{-1})	*K. rosea* MTCC 1532	Static anoxic conditions	None	–	[22]
Crystal violet (50 mg l^{-1})	*K. rosea* MTCC 1532	Static anoxic conditions	None	–	[22]
Crystal violet (20.4–204 mg l^{-1})	*S. oneidensis* WL-7	Static anoxic conditions, 30–35 °C	None		[20]
Methyl violet (800–980 mg l^{-1})	*P. mendocina* MCM B-402	Aerated culture conditions, 28 ± 2 °C	Remove 98%	2.1 mg l^{-1} h^{-1}	[6]
Crystal violet (40.8 mg l^{-1})	*Citrobacter* sp.	pH 7.0–9.0, 35–40 °C, aerobic conditions	More than 90% in 1 h	36.7–81.7 mg l^{-1} h^{-1}	[14]
Gentian violet (40.8 mg l^{-1}), malachite green (36.5 mg l^{-1}), brilliant green (48.2 mg l^{-1})	*Citrobacter* sp.	pH 7.0–9.0, 35–40 °C, aerobic conditions	More than 80% in 1 h		[14]
Basic fuchsin (33.78 mg l^{-1})	*Citrobacter* sp.	pH 7.0–9.0, 35–40 °C, aerobic conditions	More than 65% in 1 h		[14]
Methyl violet (10–100 mg l^{-1})	*R. radiobacter* MTCC 8161	pH 6.8, 30 °C, static anoxic conditions	Complete	1.2 mg l^{-1} h^{-1}	[21]
Malachite green (20.1 mg l^{-1})	*B. cereus* DC11	pH 6.0–8.0, 20–45 °C, anoxic conditions	96 ± 4% in 4 h	4.6 mg l^{-1} h^{-1}	[16]
Basic violet 1 (19.7 mg l^{-1}), basic violet 3 (20.4 mg l^{-1})	*P. pseudomallei* 13 NA	pH 7.0, 37 °C, static culture	Remove after a certain induced period	0.07–0.08 mg l^{-1} h^{-1}	[9]

Researches have reported that less than 10% of crystal violet (20.4–204 mg l^{-1}) decolorized by *S. putrefaciens* AS96 [19], while this dye did not decolorize by *S. oneidensis* WL-7 under static conditions at 30–35 °C [20] (Table 5). Thus, closely related species of *Shewanella* showed varying decolorization abilities for the same triphenylmethane dye.

Triphenylmethane dyes decolorization rates (24.4–1345 mg l^{-1} h^{-1}) by this *Shewanella* strain were much higher than the other microorganisms such as *Pseudomonas mendocina* MCM B-402 (2.1 mg l^{-1} h^{-1}) [6], *Citrobacter* sp. (36.7–81.7 mg l^{-1} h^{-1}) [14], *Rhizobium radiobacter* MTCC 8161 (1.2 mg l^{-1} h^{-1}) [21], *Bacillus cereus* DC11 (4.6 mg l^{-1} h^{-1}) [16], and the filamentous fungus *C. elegans* ATCC36112 (0.07–0.08 mg l^{-1} h^{-1}) [9] (Table 5).

Some aerobic bacteria could also decolorize triphenylmethane dyes. Among them, *Aeromonas hydrophila* DN322 decolorized malachite green, crystal violet, basic fuchsin, brilliant green, and acid amaranth (50 mg l^{-1}) at similar rates [23]. *Citrobacter* sp. showed higher decolorization capability against crystal violet than malachite green, gentian violet, and brilliant green [14]. However, the same amount of growing cells of *Kurthia* sp. decolorized a higher concentration of malachite green than that of crystal violet [24]. Malachite green did not always decolorize faster than other N-methylated triphenylmethane dyes by aerobic bacteria. Degradation mechanisms of triphenylmethane dyes by the aerobic bacteria are probably different from those by the anaerobic bacteria.

Sarnaik and Kanekar [6] reported that methyl violet B (100 mg l^{-1}) can be mineralized to CO_2 through three intermediate metabolites and phenol by *P. mendocina* MCM B-402 under anaerobic conditions [6]. Since the authors did not detect the structure of these intermediate products, we cannot know the mechanism underline its biodegradation. However, studies have reported that malachite green and crystal violet reduced to their respective leuco derivatives during decolorization by *Clostridium perfringens*, *E. coli*, or *Peptostreptococcus anaerobius* under anaerobic conditions [25,26]. Malachite green demethylated to N,N,N′-trimethyl-4,4′-benzylidenedianiline when it decolorized by *K. rosea* MTCC 1532 under static anoxic conditions [22]. In this study, demethylation reaction and leucomalachite green reduction occurred at the same time as malachite green degradation. Contrary to malachite green, leucomethyl violet B detection occurred after Michler's ketone and [N,N-dimethylaminophenyl][N′-methylaminophenyl] benzophenone detection during methyl violet B biodegradation. At present, we are not clear whether leucomethyl violet B reduction is necessary before cleavage reaction. We are also not clear about what factor controls the preference for reducing to leuco form, or demethylation reaction as the first step during biodegradation of these dyes.

Previous studies detected Michler's ketone as the major degradation product during crystal violet decolorization by the *Shewanella* strain [15], *Bacillus subtilis* IFO 13719[11] or *Nocardia corallina* [12]. Since crystal violet is composed of three N-dimethylphenol rings, the *Shewanella* strain [15] could cleave any crystal violet ring and get the same intermediate compound. This study only detected (N,N-dimethylaminophenyl) (phenyl) benzophenone during malachite green decolorization. But, Michler's ketone and (N,N-dimethylaminophenyl) (N′-methylaminophenyl) benzophenone were detected during methyl violet B decolorization. Thus ring cleavage of malachite green could occur at two sites (Fig. 3), while ring cleavage of methyl violet B could occur at three sites (Fig. 4a and b). Different bond strength between different phenylmethane groups might create a different cleavage pattern and a different cleavage rate. But, the main reason of getting different decolorization (degradation) efficiency of these three dyes are still not clear at present time.

Contrary to our results, other studies detected Michler's ketone and benzophenone as degradation products of malachite green by *B. cereus* DC11 under anaerobic conditions [16]. We cannot explain the reason for different results.

Precious studies have reported N-demethylation reaction during biotransformation of malachite green by *Cumminghamella elegans* [13] and by *Sphingomonas paucimobilis* [27]. Researches have also found N-demethylation reaction during decolorization of methyl violet by *Pseudomonas mendicina* [6] and by *R. radiobacter* (MTCC 8161) [21]. This study found that malachite green could be N-demethylated to N,N,N′-trimethyl-4,4′-benzylidenedianiline, but we did not have any evidence that methyl violet B could be N-demethylated. Previous reports also detected no demethylated crystal violet [11,12,15]. However, in the previous study

[15], we found that N.N-dimethylaminobenzaldehyde, an intermediate of crystal violet, could be demethylated. What control N-demethylation exerts during decolorization of these triphenylmethane dyes is not clear.

Degradation mechanisms of triphenylmethane dyes by eukaryotes were slightly different from the one obtained with different bacteria. Reports have detected mono-, di-, and tri-demethylated derivatives of malachite green and leucomalachite green after malachite green decolorization by the nonligninolytic filamentous fungus *C. elegans* ATCC 36112 [13]. Malachite green can also be decolorized by lignin peroxidase produced by *P. chrysosporium* [10], laccase produced by *Cyathus bulleri* [28], or peroxidase produced by *Pleurotus ostreatus* [29]. In these ligninolytic enzyme system of white-rot fungi, malachite green decolorized via N-demethylation, and the N-demethylated malachite green metabolite further reduced to the corresponding leuco derivative.

This study found that reduction to leuco form derivatives, demethylation, and oxidative cleavage were all involved during decolorization of malachite green and methyl violet, and these corresponding oxidative cleavage products further demethylated or cleaved by *S. decolorationis* NTOU1. As mentioned above, only one or two mechanisms were involved in decolorization of these dyes by the other bacteria or fungi, and that might also confirm that the extent of degradation of these dyes by this *S. decolorationis* NTOU1 was higher than that by other bacteria and fungi. However, CO_2 production was not determined during decolorization of these dyes by the *Shewanella* strain. It is not clear whether these dyes could be mineralized to CO_2.

Myers and Myers [30] reported that localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1 could play a key role in its ability to mediate manganese and iron reduction. Since *S. decolorationis* NTOU1 is capable of both high rates of triphenylmethane dyes decolorization and dissimilatory Fe(III) reduction (15). The dye decolorization was also enhanced by the presence of Fe (III) (15). Thus, the novel cytochromes distribution might contribute to the high rates of triphenylmethane decolorization by *S. decolorationis* NTOU1.

Mouse cell cytotoxicity tests indicated that malachite green solution or methyl violet B solution (100 mg l^{-1}) is toxic, but when the solution was decolorized (>98%) by *S. decolorationis* NTOU1 its toxicity decreased slightly. However, along with further degradation its toxicity increased again. While antimicrobial tests indicated that malachite green solution or methyl violet B solution (100 mg l^{-1}) is toxic, its toxicity decreased slightly after decolorization or extended degradation. Corresponding to our results, the material safety data sheet shows that Michler's ketone (oral-wild bird LD_{50} 100 mg kg^{-1}), N,N-dimethylaminobenzaldehyde (oral-rat LD_{50} 500 mg kg^{-1}), and N-methylaminophenol (oral-mouse LD_{50} 565 mg kg^{-1}) have slightly lower toxicity compared to the malachite green (oral-rat LD_{50} 50 mg kg^{-1}) or methyl violet B (oral-rat LD_{50} 413 mg kg^{-1}). Our results indicated that decolorization or extended degradation of these dyes by *S. decolorationis* NTOU1 does not detoxify these dyes completely. When study degradation of dyes, toxicity of the dyes should be analyzed along with their degradation by certain microbes (bacteria/fungi).

5. Conclusions

The initial color removal rates and the extent of degradation by *S. decolorationis* NTOU1 among crystal violet, malachite green and methyl violet B differ. Results of this study suggest that the number of the N-methylated group in the triphenyl rings might affect on their degradation.

Cytotoxicity tests showed that malachite green and methyl violet B solution (100 mg l^{-1}) were toxic. Toxicity of the dyes decreased after their decolorization by *S. decolorationis* NTOU1, but further incubation resulted in increased toxicity, while antimicrobial tests indicated that toxicity of these dyes slightly decreased. It is evident when study decolorization of dyes, toxicity of the dyes should be analyzed along with their degradation by certain microbes (bacteria/fungi).

Acknowledgments

This work was supported by the National Science Council of Taiwan (grant number NSC 94-2313-B-019-011-).

References

[1] P. Gregory, Dyes and dyes intermediates, in: J.I. Kroschwitz (Ed.), Encyclopedia of Chemical Technology, John Wiley, New York, 1993, pp. 544–545.
[2] W. Azmi, R.K. Sani, U.C. Banerjee, Biodegradation of triphenylmethane dyes, Enzyme Microb. Technol. 22 (1998) 185–191.
[3] K.V.K. Rao, Inhibition of DNA synthesis in primary rat hepatocyte cultures by malachite green: a new liver tumor promoter, Toxicol. Lett. 81 (1995) 107–113.
[4] S.J. Culp, F.A. Beland, Malachite green: a toxicological review, J. Am. Coll. Toxicol. 15 (1996) 219–238.
[5] V. Fessard, T. Godard, S. Huet, A. Mourot, J.M. Poul, Mutagenicity of malachite green and leucomalachite green in *in vitro* tests, J. Appl. Toxicol. 19 (1999) 421–430.
[6] S. Sarnaik, P. Kanekar, Biodegradation of methyl violet by *Pseudomonas mendocina* MCM B-402, Appl. Microbiol. Biotechnol. 52 (1999) 251–254.
[7] P. van West, *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite; new challenges for an old problem, Mycologist 20 (2006) 99–104.
[8] S.B. Turnipseed, J.E. Poybal, H.S. Rupp, J.A. Hurlbut, A.R. Long, Particle beam liquid chromatography-mass spectrometry of triphenylmethane dyes: application to confirmation of malachite green in incurred catfish tissue, J. Chromatogr. B 670 (1995) 55–62.
[9] C. Yatome, T. Ogawa, D. Koga, E. Idaka, Biodegradability of azo and triphenylmethane dyes by *Pseudomonas pseudomallei* 13 NA, J. Soc. Dye Colour. 97 (1981) 166–168.
[10] J.A. Bumpus, B.J. Brock, Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 54 (1988) 1143–1150.
[11] C. Yatome, T. Ogawa, M. Matsui, Degradation of crystal violet by *Bacillus subtilus*, J. Environ. Sci. Health 26 (1991) 75–87.
[12] C. Yatome, S. Yamada, T. Ogawa, M. Matsui, Degradation of crystal violet by *Nocardia corallina*, Appl. Microbiol. Biotechnol. 38 (1993) 565–569.
[13] C.J. Cha, D.R. Doerge, C.E. Cerniglia, Biotransformation of malachite green by the fungus *Cunninghamella elegans*, Appl. Environ. Microbiol. 67 (2001) 4358–4360.
[14] S.Y. An, S.K. Min, I.H. Cha, Y.L. Choi, Y.S. Cho, C.H. Kim, Y.C. Lee, Decolorization of triphenylmethane and azo dyes by *Citrobacter* sp., Biotechnol. Lett. 24 (2002) 1037–1040.
[15] C.H. Chen, C.F. Chang, C.H. Ho, T.L. Tsai, S.M. Liu, Biodegradation of crystal violet by a *Shewanella* sp. NTOU1, Chemosphere 72 (2008) 1712–1720.
[16] D. Deng, J. Guo, G. Zeng, G. Sun, Decolorization of anthraquinone, triphenylmethane and azo dyes by a new isolated *Bacillus cereus* strain DC11, Int. Biodeter. Biodegr. 62 (2008) 263–269.
[17] C.W. Saltikov, A. Cifuentes, K. Venkateswaran, D.K. Newman1, The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3, Appl. Environ. Microbiol. 69 (2003) 2800–2809.
[18] C. Subbalakshmi, R. Nagaraj, N. Sitaram, Biological activities of retro and diastereo analogs of a 13-residue peptide with antimicrobial and hemolytic activities, J. Peptide Res. 57 (2001) 59–67.
[19] A. Khalid, M. Arshad, D.E. Crowley, Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains, Appl. Microbiol. Biotechnol. 78 (2008) 361–369.
[20] J. Wu, K.S. Kim, N.C. Sung, C.H. Kim, Y.C. Lee, Isolation and characterization of *Shewanella oneidensis* WL-7 capable of decolorizing azo dye Reactive Black 5, J. Gen. Appl. Microbiol. 55 (2009) 51–55.
[21] G. Parshetti, G. Saratale, A. Telke, S. Govindwar, Biodegradation of hazardous triphenylmethane dye methyl violet by *Rhizobium radiobacter* (MTCC 8161), J. Basic Microbiol. 49 (2009) 1–7.
[22] G. Parshetti, S. Kalme, G. Saratale, S. Govindwar, Biodegradation of malachite green by *Kocuria rosea* MTCC 1532, Acta. Chim. Slov. 53 (2006) 492–498.
[23] S. Ren, J. Guo, G. Zeng, Decolorization of triphenylmethane, azo, and anthraquinone dyes by a newly isolated *Aeromonas hydrophila* strain, Appl. Microbiol. Biotechnol. 72 (2006) 1316–1321.
[24] R.K. Sani, U.C. Banerjee, Decolorization of triphenylmethane dyes and textile dye-stuff effluent by *Kurthia* sp., Enzyme Microb. Technol. 24 (1999) 433–437.
[25] J.J. McDonald, C.E. Cerniglia, Biotransformation of gentian violet to leucogentian violet by human, rat, and chicken intestinal microflora, Drug Metab. Dispos. 12 (1984) 330–336.

[26] M.S. Jang, Y.M. Lee, C.H. Kim, J.H. Lee, D.W. Kang, S.J. Kim, Y.C. Lee, Triphenylmethane reductase from *Citrobacter* sp. strain KCTC 18061P: purification, characterization, gene cloning, and over expression of a functional protein in *Escherichia coli*, Appl. Environ. Microbiol. 71 (2005) 7955–7960.
[27] L. Ayed, K. Chaieb, A. Cheref, A. Bakhrouf, Biodegradation of triphenylmethane dye malachite green by *Sphingomonas paucimobilis*, World J. Microbiol. Biotechnol. 25 (2009) 705–711.
[28] K. Vasdev, R.C. Kuhad, R.K. Saxena, Decolorization of triphenylmethane dyes by the birds nest fungus *Cyathus bulleri*, Curr. Microbiol. 30 (1995) 269–272.
[29] K.S. Shin, C.J. Kim, Decolorization of artificial dyes by peroxidase from the white-rot fungus *Pleurotus ostreatus*, Biotechnol. Lett. 20 (1998) 569–572.
[30] C.R. Myers, J.M. Myers, Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1, J. Bacteriol. 174 (1992) 3429–3438.