Biotechnol Lett (2007) 29:391–396
DOI 10.1007/s10529-006-9265-6

ORIGINAL RESEARCH PAPER

Biodegradation of crystal violet by *Pseudomonas putida*

Chiing-Chang Chen · Hung-Ju Liao · Chiu-Yu Cheng · Chia-Yuen Yen · Ying-Chien Chung

Received: 25 September 2006 / Revised: 9 November 2006 / Accepted: 22 November 2006 /
Published online: 6 January 2007

Abstract Crystal violet (CV), which has been extensively used as a biological stain and a commercial textile dye, is a recalcitrant molecule. A strain of *Pseudomonas putida* was isolated that effectively degraded CV: up to 80% of 60 μM CV as the sole carbon source, was degraded in liquid media within 1 week. Nine degradation products were isolated and identified. We propose that CV degradation occurs via a stepwise demethylation process to yield mono-, di-, tri-, tetra-, penta- and hexa-demethylated CV species.

Keywords Biodegradation · Crystal violet · *N*-de-methylation · *Pseudomonas putida*

Introduction

The triphenylmethane dye, crystal violet (CV), has been extensively used in human and veterinary medicine as a biological stain and in various commercial textile processes as a dye (Bumpus and Brock 1988). CV has been classified as a recalcitrant molecule, thereby indicating that it is poorly metabolized by microbes and, consequently, is long-lived in a variety or environments. Conventional biological wastewater treatment systems are therefore often incapable of effectively removing CV from the wastewater, resulting in its dispersal into the environment. This was demonstrated by Nelson and Hites (1980) who found CV in soil and river sediments that could be traced back to the dumping of improperly treated chemical waste. An additional worrying factor is that some triphenylmethane dyes, including CV, are potent clastogens, possibly responsible for promoting tumor growth in some species of fish (Cho et al. 2003).

Many alternative processes aimed at removing CV from wastewater have been investigated, including chemical oxidation and reduction, physical precipitation and flocculation, photolysis, adsorption, electrochemical treatment, advanced oxidation, reverse osmosis and biodegradation (Azmi et al. 1998). Of these, biological processes have attracted a great deal of attention because, in general, such systems are regarded as more cost-effective and environmentally friendly than physical and chemical treatment methods, and they produce less sludge.

There have been very few reports of CV biodegradation by bacteria. Azmi et al. (1998)

C.-C. Chen
Department of General Education, National Taichung Nursing College, Taichung 403, Taiwan

H.-J. Liao
Department of Food Science, China Institute of Technology, Taipei 115, Taiwan

C.-Y. Cheng · C.-Y. Yen · Y.-C. Chung (✉)
Department of Biological Science and Technology, China Institute of Technology, 245, Sec 3 Yen-Chu Yuan Rd., Taipei 115, Taiwan
e-mail: ycchung@cc.chit.edu.tw

demonstrated a relatively successful biodegradation of CV using various species of fungi. However, this same group reported the microbial degradation of only low concentrations of CV up to 12.3 μM. Given that typical concentrations of CV in wastewater are at least 40 μM, fungi were considered to be impractical for use in possible commercial biodegradation systems (Azmi et al. 1998). In addition, a lack of knowledge of the pathways of CV biodegradation has limited the development of a successful biological treatment.

The aim of the investigation reported here was to study CV biodegradation by a CV-degrading bacterium, *Pseudomonas putida*, at relatively high concentrations of CV and to determine the demethylation steps involved in CV biodegradation.

Materials and methods

Materials and reagents

CV (purity = 99.8%) was obtained from the Tokyo Kasei Kogyo Co. Reagent-grade ammonium acetate and HPLC-grade methanol were purchased from Merck. Other chemicals were purchased from Sigma unless otherwise specified.

Microorganisms, cultivation and optimal pH for CV degradation

The CV-degrading bacterium was isolated from the wastewater of a dye manufacturing company and subsequently cultured on a medium containing 5.22 g K_2HPO_4 l^{-1}, 4.08 g KH_2PO_4 l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.55 g $CaCl_2$ l^{-1}, 0.4 g NH_4Cl l^{-1} and supplemented with 0.022 mg CV l^{-1} (about 60 μM). Preliminary experiments indicated that this bacterium was able to utilize CV as the sole carbon source and that the optimal temperature for CV degradation was 37°C. To evaluate the effect of pH on CV degradation by this isolate, the pH of the culture medium was carefully adjusted in the range of 5.0 to 8.0. Prior to the experiments, the bacterial cells were pre-cultivated in nutrient broth for 12 h. The cells were then inoculated into sterile CV-containing culture medium at an initial concentration of 6×10^7 c.f.u./ml.

The cells of the isolate were lysed and the DNA extracted. Subsequent PCR amplification and sequencing were according to Sandaa et al. (1999) and resulted in the isolate being identified as *Pseudomonas putida* (GenBank accession number DQ178233; similarity 98.6%).

Instruments and analytical methods

The amounts of residual CV in the culture medium at the end of the various culture periods were determined by HPLC. Organic intermediates were analyzed by coupled HPLC-ESI-MS. LC was carried out on an Atlantis dC18 column. The mobile phase was at 1 ml/min. A linear gradient was run as follows: t = 0, A = 95, B = 5; t = 20, A = 50, B = 50; t = 35–40, A = 10, B = 90; t = 45, A = 95, B = 5 (where A = 25 mM ammonium acetate buffer, pH 6.9, and B = methanol). The column effluent was introduced into the ESI source of the MS.

Results and discussion

CV degradation by *Pseudomonas putida*

Earlier studies of CV biodegradation showed that this triphenylmethane dye is relatively resistant to biodegradation in the environment. As one of the environmental factors attributed to affecting CV biodegradation is the pH (Michaels and Lewis 1986), we examined the effect of pH on CV biodegradation, but only in slightly acid and slightly alkaline conditions in order to avoid possible chemical degradation of this molecule. The optimal pH for CV biodegradation by *P. putida* under our experimental conditions was found to be 7.5 (Fig. 1), although differences in the biodegradation efficiency were insignificant between pH 6.0 and pH 7.5. At a pH higher than 7.5 or lower than 6.0, the capacity of CV biodegradation by *P. putida* was inhibited. The highest CV biodegradation efficiency achieved was 78.5% (pH 7.5), thus demonstrating that *P. putida* is capable of degrading relatively high CV concentrations (60 μM) at a high efficiency relative to the 12.3 μM CV observed by Bumpus and Brock (1988) using

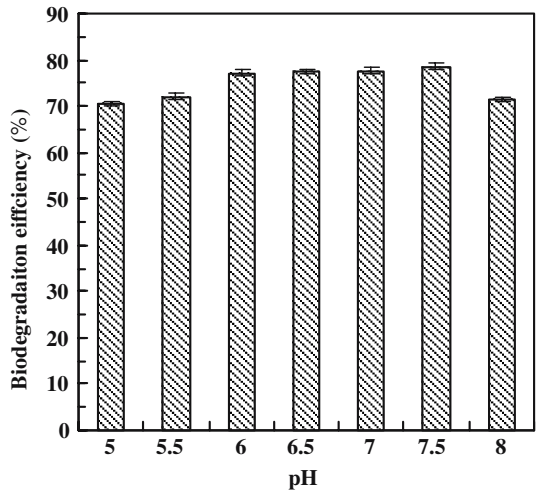

Fig. 1 The effect of pH on crystal violet (CV) degradation *by Pseudomonas putida*. *P. putida* was cultured in liquid medium (initial inoculum: 6×10^7 c.f.u./ml) containing 60 μM CV as the sole carbon source at 37°C. The pH of medium ranged from 5.0 to 8.0. Data represent the means of three independent experiments with standard deviation

Phanerochaete chrysosporium. The inability of many bacteria to degrade CV has been attributed to the dye being toxic to many microorganisms (Michaels and Lewis 1986). However, our results suggest that CV is not toxic to *P. putida* and that this bacterium has the potential to remove CV from the environment without producing toxic byproducts.

Figure 2 shows the changes in the absorption spectra of CV at various culture periods (=biodegradation times). CV concentrations clearly decreased with increasing biodegradation times. The slight shift in the characteristic wavelengths from 588.3 nm to 580.7 nm with increasing biodegradation time suggests that a series of *N*-demethylated intermediates may have formed.

Identification of CV metabolic intermediates

The biodegradation intermediates formed by *P. putida* after a 7-day culture period were analyzed (data not shown). The HPLC peak 1 was identified as CV (retention time: 40.8 min) and peaks 2–10 (retention time: 27.1–39.2 min) as other intermediates. As the polarity of the *N,N*-dimethyl-*N*′,*N*′-dimethylpararosaniline (DDPR), *N,N*-dimethyl-*N*′-methylpararosaniline (DMPR) and *N,N*-dimethylpararosaniline (DPR) species exceeds that of the *N,N*-dimethyl-*N*′-methyl-*N*″-methylpararosaniline (DMMPR), *N*-methyl-*N*′-methyl-*N*″-methylpararosaniline (MMMPR) and *N*-methyl-*N*′-methylpararosaniline (MMPR) intermediates, we expected the latter group to be eluted after the former. Additionally, to the extent that two *N*-methyl groups are stronger auxochromic moieties than the *N,N*-dimethyl or amino groups, we predicted that the maximal absorption of the DDPR, DMPR and DPR intermediates would occur at wavelengths shorter than the band position of the DMMPR, MMMPR and MMPR species. Similar phenomena have been reported for the photodegradation of sulforhodamine-B (Chen et al. 2002) under visible irradiation.

The absorption spectra of each intermediate in the visible spectral region were also measured (data not shown). The absorption spectral bands were observed to shift hypsochromically from 589.4 nm to 542.9 nm, suggesting a step-wise formation of a series of *N*-demethylated intermediates. For example, λ_{max} of CV, DDMPR, DMMPR, DDPR, MMMPR, DMPR, MMPR, DPR, MPR and PR are 589.4, 581.2, 573.5, 579.4, 566.7, 569.9, 560.9, 566.1, 554.3 and 542.9 nm, respectively. This result is in agreement with the results presented in Fig. 2. Other research groups have also reported similar phenomena during the photodegradation of other dyes (Chen et al. 2002; Wu et al. 1998) under visible irradiation.

CV degradation pathway by *Pseudomonas putida*

A sequential identification of the primary and secondary metabolites enabled us to elucidate the metabolic pathway of CV degradation in *P. putida*. MS analysis confirmed that components 1 (m/z = 372.18), 2 (m/z = 358.07), 3 (m/z = 344.09), 4 (m/z = 344.15), 5 (m/z = 330.10), 6 (m/z = 330.16), 7 (m/z = 316.18), 8 (m/z = 316.11), 9 (m/z = 302.13) and 10 (m/z = 288.01) are indeed CV, DDMPR, DMMPR, DDPR, MMMPR, DMPR, MMPR, DPR, MPR, and PR, respectively (data not shown). Each of the three side chains of the CV dye molecules terminates in two dimethyl groups (compound 1, Fig. 3). DDMPR (compound

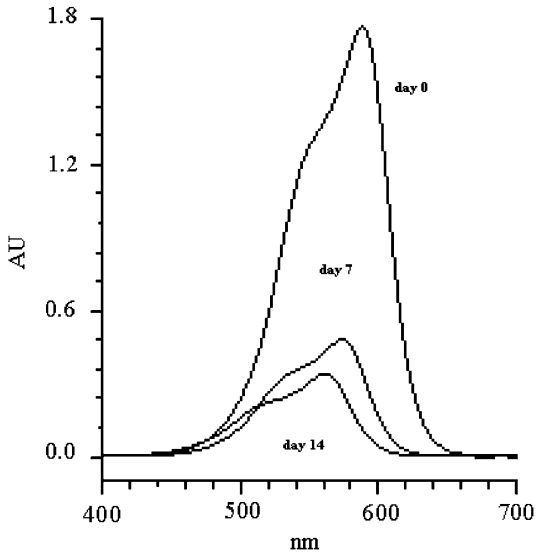

Fig. 2 Changes in the CV absorption spectra over culture time. Spectra from top to bottom correspond to biodegradation times of 0, 7, 14 days, respectively. Initial CV concentration: 60 μM; initial medium pH: 7.5

2, Fig. 3) is obtained by the removal of one methyl group from the CV molecule; DMMPR, DDPR, MMMPR, DMPR, MMPR and DPR (compounds 3–8, Fig. 3) correspond to three pairs of isomeric molecules with two to four fewer methyl groups than the CV dye molecule. DMMPR (compound 3, Fig. 3) is formed through the removal of a methyl group from two different sides of the CV molecule, whereas the other corresponding isomer in this pair, DDPR (compound 4, Fig. 3), is produced by the removal of two methyl groups from the same side of the CV structure. In the second pair of isomers, MMMPR (compound 5, Fig. 3) is formed by the removal of three methyl groups from each side of the CV molecule, whereas DMPR (compound 6, Fig. 3) is produced by the removal of two methyl groups from one side of the CV structure while an additional methyl group is removed from the other side of the CV structure. In the third pair of isomers, MMPR (compound 7, Fig. 3) is produced through the removal of two methyl groups from one side of the CV structure and one methyl group from each of the other two sides of the CV structure, whereas DPR (compound 8, Fig. 3) is formed through the removal of two methyl groups from two different sides of the CV molecule. Compounds 9 (MPR) and 10 (PR) have one and zero methyl groups, respectively.

The results of the HPLC chromatograms, UV–visible spectra and HPLC-ESI mass spectra are summarized in Table 1 and clearly reveal that CV degradation by *P. putida* is a stepwise demethylation process in which the dominating mechanism of the initial step of CV biodegradation is demethylation. Figure 3 presents the proposed degradation pathway.

Based on our analysis of the intermediate products, the degradation pathway of CV in *P. putida* is different from those of *Bacillus subtilis* and *Nocardia coralline*, whose major biodegradation products are 4,4′-*bis*-dimethylamine benzophenone (Michler's ketone) and α-dimethylaminophenol, although no additional metabolites have yet been identified (Yatome

Table 1 The *N*-demethylation intermediates[a] of crystal violet degradation by *Pseudomonas putida*

HPLC peaks	*N*-demethylation intermediates	Abbreviation	ESI-MS peaks	Absorption maximum (nm)
1	*N*,*N*,*N*′,*N*′,*N*″,*N*″-hexamethyl pararosaniline	CV	372.18	589.4
2	*N*,*N*-dimethyl-*N*′,*N*′-dimethyl-*N*″-methyl pararosaniline	DDMPR	358.07	581.2
3	*N*,*N*-dimethyl-*N*′-methyl-*N*″-methyl pararosaniline	DMMPR	344.09	573.5
4	*N*,*N*-dimethyl-*N*′,*N*′-dimethyl pararosaniline	DDPR	344.15	579.4
5	*N*-methyl-*N*′-methyl-*N*″-methyl pararosaniline	MMMPR	330.10	566.7
6	*N*,*N*-dimethyl-*N*′-methylpararosaniline	DMPR	330.16	569.9
7	*N*-methyl-*N*′-methylpararosaniline	MMPR	316.18	560.9
8	*N*,*N*-dimethylpararosaniline	DPR	316.11	566.1
9	*N*-methylpararosaniline	MPR	302.13	554.3
10	Pararosaniline	PR	288.01	542.9

[a] The biodegradation intermediates of crystal violet formed by *P. putida* after 7 days at 37°C were isolated and then analyzed by HPLC-ESI-MS. The initial CV concentration and pH of medium were 60 μM and pH 7.5, respectively

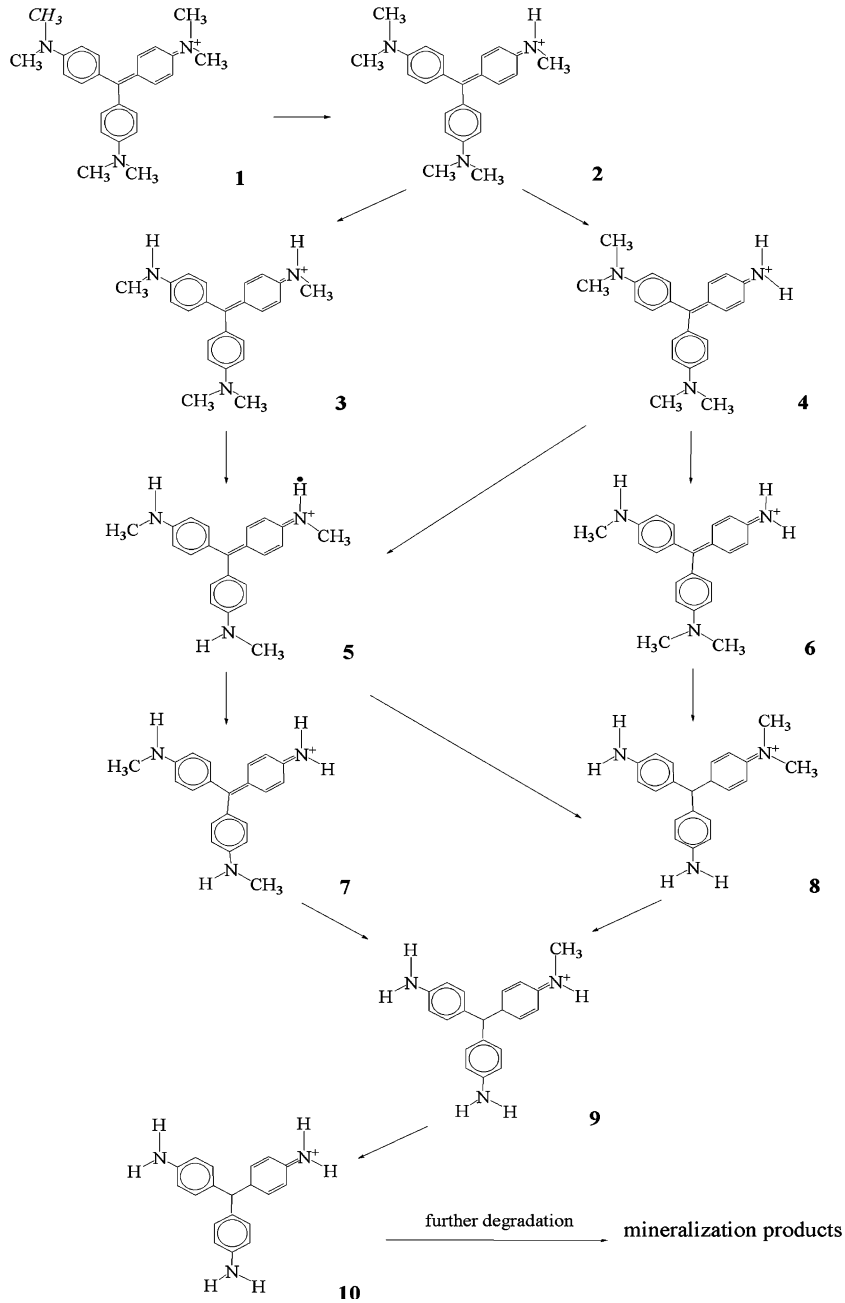

Fig. 3 Proposed CV demethylation pathways in *P. putida*, with the identification of several intermediates by HPLC-ESI-MS

et al. 1991; 1993). *Phanerochaete chrysosporium* apparently has a similar demethylation mechanism but the pathway has only been partially elucidate; to date, only three initial biodegradation products, *N,N,N′,N′,N″*-penta-, *N,N,N′,N″*-tetra- and *N,N′,N″*-trimethylpararosaniline, have been identified (Bumpus and Brock 1988). However, it would be reasonable to infer that *P. putida* and *Phanerochaete chrysosporium* should possess the same or similar degradation enzymes.

To summarize, we have shown here that *P. putida* is capable of degrading industrial concentrations of CV and subsequently determined the complete demethylation degradation pathway of CV in *P. putida*. Future studies by our laboratory will focus on enzyme analyses in order

to determine the potential of this bacterial isolate in the industrial degradation of CV in wastewater systems.

References

Azmi W, Sani RK, Banerjee UC (1998) Biodegradation of triphenylmethane dyes. Enzyme Microb Technol 22:185–191

Bumpus JA, Brock BJ (1988) Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 54:1143–1150

Chen CC, Zhao W, Li JG, Zhao JC, Hidaka H, Serpone N (2002) Formation and identification of intermediates in the visible-light-assisted photodegradation of sulforhodamine-B dye in aqueous TiO_2 dispersion. Environ Sci Technol 36:3604–3611

Cho BP, Yang T, Blankenship LR, Moody JD, Churchwell M, Bebland FA, Culp SC (2003) Synthesis and characterization of *N*-demethylated metabolites of malachite green and leuco malachite green. Chem Res Toxicol 16:285–294

Michaels GB, Lewis DL (1986) Microbial transformation rates of azo and triphenylmethane dyes. Environ Toxicol Chem 5:161–166

Nelson CR, Hites RA (1980) Aromatic amines in and near the Buffalo River. Environ Sci Technol 14:1147–1149

Sandaa RA, Enger O, Torsvik V (1999) Abundance and diversity of *Archaea* in heavy-metal-contaminated soils. Appl Environ Microbiol 65:3293–3297

Wu T, Liu G, Zhao J, Hidaka H, Serpone N (1988) Photoassisted degradation of dye pollutants. V. Self-photooxidative transformation of rhodamine B under visible light irradiation in aqueous TiO_2 dispersions. J Phys Chem B102:5845–5851

Yatome C, Ogawa T, Matsui M (1991) Degradation of crystal violet by *Bacillus subtilis*. J Environ Sci Health A26:75–87

Yatome C, Yamada S, Ogawa T, Matsui M (1993) Degradation of crystal violet by *Nocardia corallina*. Appl Microbiol Biotechnol 38:565–569