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Biodegradation of crystal violet by a *Shewanella* sp. NTOU1

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ABSTRACT

A bacterial isolate, strain NTOU1, originally isolated from the cooling system in an oil refinery could decolorize and detoxify crystal violet under anaerobic conditions. The strain was characterized and identified as a member of *Shewanella decolorationis* based on Gram staining, morphology characters, biochemical tests, the 16S rRNA gene and the gyrase subunit beta gene (*gyrB*). The optimum pH value and temperature for decolorization of crystal violet by this strain under anaerobic conditions were pH 8–9 and 30–40 °C, respectively. Formate (20 mM) was the best electron donor. Addition of ferric citrate did not inhibit decolorization of crystal violet, the addition of thiosulfate, ferric oxide, or manganese oxide slightly decreased decolorization, while addition of nitrite (20 mM) inhibited the decolorization of crystal violet. By supplementing the medium with formate and ferric citrate and cultivating it under optimum pH and temperature, this strain could remove crystal violet, at a concentration of 1500 mg l^{-1}, at the rate of 298 mg l^{-1} h^{-1} (during decolorization the OD_{600} of the cell culture increased from ~0.6 to ~1.2). GC/MS analysis of the degradation products of crystal violet detected the presence of *N,N′*-bis(dimethylamino) benzophenone (Michler's Ketone), [*N,N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, *N,N*-dimethylaminobenzaldehyde, *N,N*-dimethylaminophenol, and 4-methylaminophenol. These results suggest that crystal violet was biotransformed into *N,N*-dimethylaminophenol and Michler's Ketone prior to further degradation of these intermediates. This paper proposes a probable pathway for the degradation of crystal violet by this *Shewanella* sp. Cytotoxicity and antimicrobial tests showed that the process of decolorization also detoxify crystal violet.

1. Introduction

Triphenylmethane dyes are one of the major groups of dyestuffs used for textiles, printing, papermaking, leather, food, and the cosmetics industry (Gregory, 1993; Azmi et al., 1998). These dyes are recalcitrant molecules and can be antimicrobial, toxic to mammalian cells, and mutagenic (Littlefield et al., 1985; Banat et al., 1996; Culp and Beland, 1996). Therefore, the decolorization of wastewaters containing these dyes prior to discharge is mandatory by environmental regulations in most countries.

Although several physicochemical methods, e.g. adsorption, chemical precipitation and flocculation, oxidation by chlorine, hydrogen peroxide and ozone, electrolysis, reduction, electrochemical treatment, and ion-pair extraction have been used to eliminate the color from these wastewaters in the effluent (Reife, 1993; Azmi et al., 1998), they are generally expensive or produce large amounts of sludge. Therefore, these methods have only a limited applicability. As a result, the interest is focused on microbial biodegradation as a better alternative. However, triphenylmethane dyes have complex aromatic molecular structures, and few bacteria have been reported that have the ability to decolorize triphenylmethane dyes (Yatome et al., 1981, 1991, 1993; Kwasniewska, 1985; An et al., 2002). Although several white-rot fungi are known to decolorize triphenylmethane dyes (Bumpus and Brock, 1988; Cha et al., 2001), in practice they are difficult to apply as a wastewater treatment of sewage containing dyes because of their difficulty to survive and adapt in wastewater. Therefore, much work remains to be done to isolate new bacteria that are applicable for the biodegradation of triphenylmethane dyes.

Recently we isolated a facultative bacterium from a cooling system of an oil refinery. It was capable of anaerobic growth utilizing a variety of electron acceptors including ferric citrate. Many pure cultures of iron-reducing bacteria can oxidize a variety of aromatic contaminants such as benzene and naphthalene (Beliaev and Saffarini, 1998). Therefore, in this study, 16S rRNA and *gyr*B gene analysis were used to investigate the phylogenetic placement of this isolate. The potential of this isolate to degrade and to detoxify crystal violet, and its degradation pathways were also investigated.

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2. Materials and methods

2.1. Chemicals

Crystal violet, with around 85% dye content, purchased from Sigma–Aldrich was used without further purification. The chemicals *N*,*N*′-bis(dimethylamino) benzophenone (Michler's Ketone) and *N*,*N*-dimethylaminobenzaldehyde were obtained from Acros Organic Co. (New Jersey, NJ). All other chemicals were of reagent grade.

2.2. Organism

Strain NTOU1 is a bacterium that was originally isolated from a cooling system in an oil refinery. The strain is Gram stain-negative, rod-shaped, pink to orange-pigmented and motile with a single polar flagellum. This strain can grow under both of anaerobic and aerobic conditions. It was not able to ferment glucose, but it was capable of anaerobic growth utilizing a variety of electron acceptors, including Fe(III), Mn(IV), iron oxide, sulfate, sulfite, thiosulfate, nitrate, nitrite, arsenate, selenate, and selenite. Lactate, pyruvate, formate and H_2 were used as carbon and energy sources. This strain has been deposited in the Bioresource Collection and Research Center in Taiwan as BCRC 910321 and in the Japan IAM Culture Collection as JCM 14211.

2.3. Sequence analysis of the16S rRNA gene and the gyrB gene

To further identify this strain, DNA from 50 ml bacterial culture was isolated and purified on hydroxyapatite as described by Cashion et al. (1977). Then 16S rRNA and *gyr*B gene were amplified by using the polymerase chain reaction method. For amplification of 16S rRNA, two universal primers 8F and 1492R were used (Eden et al., 1991). For the amplification of amplicons of 1.2 kbp from the *gyrB* gene (covering positions 274–1525; *Escherichia coli* numbering), two universal primer sets were used (Yamamoto and Harayama, 1995). The sequences amplified from this strain were compared with those in the GenBank nucleotide database by using online basic local alignment search tool (BLAST) searches. Phylogenetic trees were constructed using the neighbor-joining method and nucleotide substitution rates (K_{nuc} values), computed by Kimura's 2-parameter model.

2.4. Media and culture conditions

Luria–Bertani medium (LB medium) (Sambrook et al., 1989) was used for aerobic cultivation at 30 °C. When cultivating under anaerobic conditions, the strain was inoculated into 50 ml or 100 ml serum bottles containing sodium formate (20 mM) and ferric citrate (20 mM) supplemented phosphate-buffered basal medium under an atmosphere of N_2 at 30 °C. The phosphate-buffered basal medium (Saltikov et al., 2003) contains (g l^{-1} of deionized water unless otherwise indicated): K_2HPO_4, 0.225; KH_2PO_4, 0.225; NaCl, 0.46; $(NH_4)_2SO_4$, 0.225; $MgSO_4 \cdot 7H_2O$, 0.117; trace element solution (Skerratt et al., 2002), 1 ml; and vitamin solution (Widdel and Bak, 1992), 1 ml. These serum bottles were capped with butyl rubber stoppers and were aluminum crimp sealed. A modified Hungate technique was used throughout the study for anaerobic cultivation (Ljungdahl and Wiegel, 1986).

2.5. Decolorization of crystal violet under different growth conditions

At first, decolorization of crystal violet (50 mg l^{-1}) by strain NTOU1 under aerobic and anaerobic conditions at 30 °C were tested. LB medium was used for cultivation under aerobic conditions, while formate and ferric citrate supplemented phosphate-buffered basal medium was used for cultivation under anaerobic conditions.

Since growth of this strain was much faster under aerobic conditions (data not shown), cells collected from growth under aerobic conditions were collected and used for decolorization tests under anaerobic conditions. Thus, strain NTOU1 was first cultivated under aerobic conditions at 30 °C overnight in LB medium and then harvested by centrifugation (8000*g*, 10 min). The pellet was washed twice in 10 ml phosphate-buffered basal medium (pH 7.0) and then re-suspended in 10 ml phosphate-buffered basal medium under a gas stream of N_2. Cell suspension was transferred to duplicate 50 ml serum bottles containing fresh anaerobic medium (20 ml) supplemented with a dye (50–1500 mg l^{-1}) to reach an initial cell mass of 0.4–0.5 g l^{-1} (wet weight) and incubated statically under anaerobic conditions at 30 °C for the dye-decolorization test. Decolorization tests with heat killed cell suspension or without cell suspension served as controls. At varies time points (every 1–2 h) during incubation, 1 ml of subsample was taken and centrifuged. The concentration of the dye in the supernatant was determined by monitoring the absorbance at the absorption maximum (λ_{max}) of 585 nm. 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 observed absorbance. To ensure that the pH changes in the dye solution had no influence on decolorization, the visible absorption spectrum was determined between pH 5.0 and 10.0, and it was found that the pH at that range did not affect the spectrum. Decolorization activity (%) was expressed as the average of the duplicate tubes.

2.6. Effect of different electron donors, electron acceptors, pH, temperature and concentration on decolorization of crystal violet

Effect of different electron donors, electron acceptors, pH, temperature and concentration on decolorization of crystal violet was tested sequentially. In order to find the optimum conditions to decolorize crystal violet, after the better substituent or condition was found in one step, it will be included or used in the next step.

When testing the effects of different electron donors on decolorization of crystal violet (50 mg l^{-1}), H_2 (CO_2:H_2 = 4:1, v/v), lactate, formate, pyruvate, arabinose, acetate or butyrate (20 mM) was added to the culture medium containing ferric citrate (20 mM) as the electron acceptor. While testing the effects of different electron acceptors on decolorization, ferric citrate, thiosulfate, nitrite, ferric oxide, iron oxide, or manganese oxide (20 mM) was added to the medium containing formate (20 mM) as the electron donor. When testing the pH range for decolorization, separate batches of medium adjusted to 5–10 with 1 M HCl or 1 M NaOH were used. When testing the temperature range for decolorization, separate batches of medium were incubated at 15–40 °C. When testing the effect of dye concentration on decolorization, separate batches of medium containing 100–1500 mg l^{-1} of crystal violet were used. Each experiment was performed in duplicate. Decolorization activity (%) was expressed as the average of the duplicate tubes.

2.7. Determination of decolorization and transformation products of crystal violet

Aliquots of cell cultures (50 ml) collected at intervals during and after decolorization of crystal violet (100 mg l^{-1}) from a serum bottle containing 800 ml cell culture, were used to study the degradation products of crystal violet. Cell culture (50 ml) was centrifuged at 8000*g* for 10 min, and the supernatant collected was extracted with 100 ml of ethylacetate. The extract was condensed by rotary evaporators at 60 °C. The extract was then dissolved in 1 ml of ethylacetate and passed through a column containing anhydrous