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Environmental Contamination and Toxicology

Biodegradation of Anthraquinone Dyes by *Bacillus subtilis*

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Dyes are released into the environment as industrial effluents from food, cosmetic, drug, textile, and dyestuff factories. With a view towards effective biological treatment of wastewater containing dyes from these factories, the use of microorganisms capable of assimilating or degrading dyes has been proposed. Some azo and triphenylmethane dyes have been efficiently degraded and removed from wastewater by the use of microorganisms. (Bumpus et al. 1988; Horitsu et al. 1977; Meyer et al. 1979; Ogawa et al. 1986, 1990; Yatome et al. 1981, 1991).

Anthraquinone dyes are also widely used in these factories. However, no information is available on the use of microorganisms that are capable of degrading anthraquinone dyes for treatment of wastewaters. Some anthraquinone dyes have been shown to be toxic and carcinogenic to animals and mutagenic to microorganisms. (Sendelbach 1989; Sigman et al. 1985). Microbial degradation of anthraquinone dyes is considered to be a subject of great interest in environmental chemistry.

We screened some strains, and found that Bacillus subtilis decolorized and degraded a few anthraquinone dyes. This paper describes a preliminary study on the microbial degradation of anthraquinone dyes.

MATERIALS AND METHODS

Chemicals: Anthraquinone dyes used are shown in Table 1. PV12 and DV1 were purchased from Tokyo Kasei Kogyo Co., Ltd. DR15 and DO11 were purchased from Aldrich Chemical Co., Inc. PV12 was purified by preparative thin layer chromatography, and recrystallized from glacial acetic acid. DR15 was recrystallized from 50% ethanol. DV1 and DO11 were recrystallized from ethanol. Their purity was checked by thin layer chromatography (TLC).

Microorganism: B.subtilis IFO 13719 (ATCC 6051) was found to be a growable microorganism on the plate of mineral salts agar medium containing anthraquinone dyes (2.5×10^{-5} M/L) as the sole carbon

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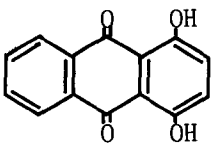
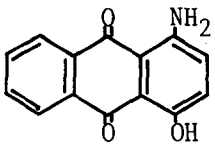
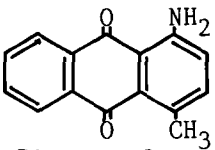
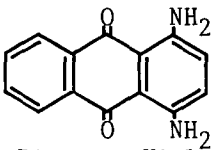
Table 1. Anthraquinone dyes used in the present study.

 C.I. Pigment Violet 12 (PV12)	 C.I. Disperse Red 15 (DR15)
 C.I. Disperse Orange 11 (DO11)	 C.I. Disperse Violet 1 (DV1)

source. This strain, which was supplied by the Institute for Fermentation, Osaka, Japan, is optimally grown at 37°C and pH 7.0 in 802 medium according to the IFO data sheet.

Media: Two media were used for microbial decolorization and degradation experiments of the dyes. The 802 medium contained 10 g of polypeptone, 2 g of yeast extract, and 1 g of $MgSO_4 \cdot 7H_2O$ per liter. The mineral salts medium contained 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 1 g of $(NH_4)_2SO_4$ per liter. The pH of these media was adjusted to 7.0 with 1M NaOH or 1M HCl.

Decolorization reaction of anthraquinone dyes by growing cells: B.subtilis was preincubated at 37°C for 8 hr in a 50 mL Erlenmyer flask containing 20 mL of 802 medium. A 3 mL aliquot of the culture medium was transferred to a 100 mL Erlenmyer flask containing the dye (2.5×10^{-5} M/L) in 50 mL of 802 or mineral salts medium. Then the flask was incubated at 37°C with shaking.

Decolorization reaction of anthraquinone dyes by resting cells: The 20 mL of preincubated culture medium was transferred to a 3 L round-bottom flask containing 2 L of 802 medium. After cultivation for 16 hr with aeration (1 L/min), the cells were harvested by centrifugation (4000g, for 15 min), and washed twice with 0.07 M Sørensen buffer (pH 7.0). The wet weight of the cells resuspended in the same buffer was adjusted to 50 mg/mL. A 25 mL aliquot of the suspension was transferred to a 100 mL Erlenmyer flask containing 25 mL of dye-dispersed water solution (pH 7.0, dye conc.: 5.0×10^{-5} M/L). The flask was incubated as described above.

Measurement of decolorization: Decolorization of PV12, DR15, DO11, and DV1 was measured spectrophotometrically. After a given period of time, the residual dye in the incubated mixture was extracted with 1-butanol, and then the alcohol layer was dehydrated with anhydrous Na_2SO_4. The absorption spectra of the alcohol extracts were measured, and then the value of absorption maximum of the dye

in the visible region was used to estimate decolorization (%). The decolorization (%) was calculated as follows.
Decolorization (%) = 100 x ((initial value of absorption maximum)-(observed value of absorption maximum))/(initial value of absorption maximum)

Isolation and identification of reaction product obtained from PV12: To isolate product formed by the reaction of B.subtilis and PV12, the volume of medium for the decolorization reaction was scaled up. A mixture of 120 mL of preincubated culture medium and 2 L of mineral salts medium containing PV12 ($2.5x10^{-5}$ M/L) was incubated for 20 hr at 37°C with aeration (1 L/min). Then, the mixture was extracted twice with chloroform. The extracts were combined, dehydrated with anhydrous Na_2SO_4, and concentrated. The residue was redissolved in a small quantity of chloroform. And then, the reaction product in the redissolved extract was separated by TLC and detected under UV radiation at 254 nm. The separated reaction product was identified with mass (MS) and nuclear magnetic resonance (NMR) spectrometer.

Quantitative analysis of reaction product: The formation and disappearance of the product with disappearance of PV12 were quantitatively monitored by high performance liquid chromatography (HPLC). A mixture of 6 mL of preincubated culture medium and 100 mL of mineral salts medium containing PV12 ($2.5x10^{-5}$ M/L) was incubated at 37°C with shaking. After a given period time, the reaction product and residual PV12 in the incubated mixture (8 mL) were extracted twice with chloroform (15 mL). The extracts were combined, concentrated, and redissolved in 200 µL of methanol-water (95:5,v/v). A 10 µL aliquot of the redissolved extract was used for HPLC analysis.

Apparatus and conditions: Absorption spectra were obtained with a Hitachi spectrophotometer Model 330. MS spectra were obtained with a Shimadzu GCMS Model QP-1000A spectrometer by a use of direct inlet probe (ionization energy: 70eV). NMR spectrum was obtained with a Hitachi Model R-900A spectrometer. HPLC was performed by using a Jasco Model 800 series (column: Inertsil ODS-2, 4.6 x 150 mm, guard column: Inertsil ODS-2, 4.6 x 50 mm, elution: methanol-water (95:5,v/v), flow rate: 1.0 mL/min, temp.: 30°C, detection: UV/VIS detector).

RESULTS AND DISCUSSION

The decolorization activity of the dyes by growing and resting cells of B.subtilis was expressed by lag time, half decolorization time, and maximum decolorization. The results are summarized in Table 2. The lag time was evaluated as the time required to initiate decolorization of the dyes, the half decolorization time was evaluated as the time required to reach the decolorization of the dye to half of its maximum decolorization, and the maximum decolorization was evaluated as the maximum value of decolorization reached during incubation.

PV12, DR15, and DO11 were decolorized by B.subtilis under these

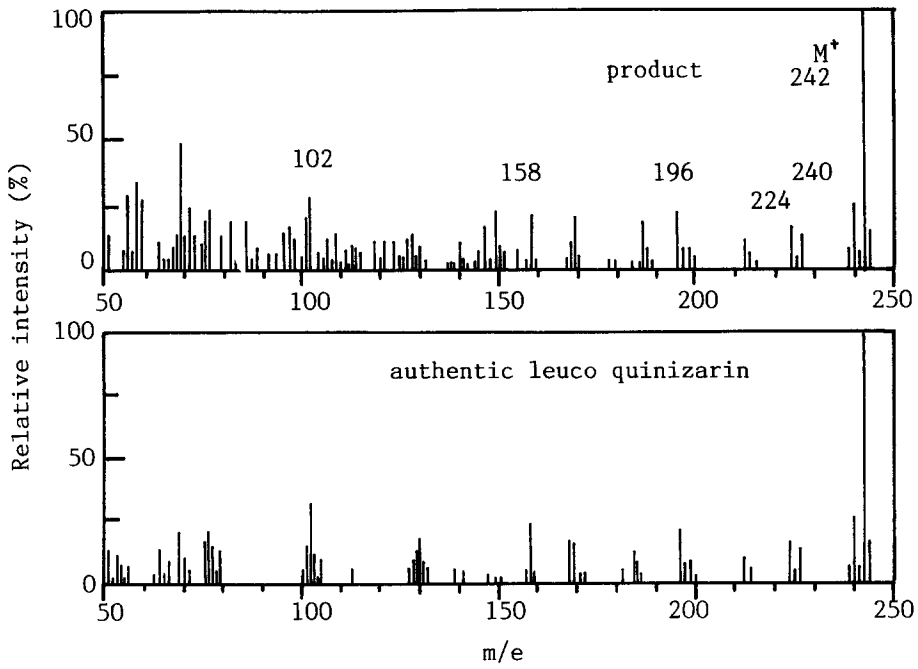


Figure 1. Mass spectra of the product and authentic leuco quinizarin.

Table 2. Decolorization of anthraquinone dyes by B.subtilis.

	Growing cells in 802 medium			Growing cells in mineral salts medium			Resting cells in Sørensen buffer		
Dye	LT	HDT	MD	LT	HDT	MD	LT	HDT	MD
PV12	0	6	73	0	40	78	0	115	79
DR15	10	38	38	50	70	28	30	103	18
DO11	25	50	26	25	73	46	10	76	43
DV1	-	-	0	100	120	9	-	-	0

LT: lag time (hr) HDT: half decolorization time (hr)
MD: maximum decolorization (%)
Incubated time: 144 hr Dye conc.: 2.5×10^{-5} M/L pH: 7.0
Temp.: 37°C

experimental conditions. PV12 with two hydroxyl groups was more readily decolorized than the other dyes by growing and resting cells without a lag time. The half decolorization time of PV12 by growing cells in 802 medium was shorter than that of PV12 by growing cells in mineral salts medium and resting cells in Sørensen buffer. PV12 was quickly decolorized on the initial period of incubation time by growing cells in 802 medium. In addition, the

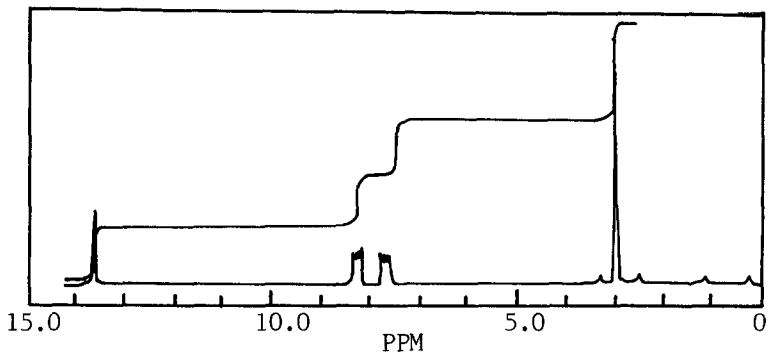


Figure 2. 90-MHz ^{1}H NMR spectrum of the product in $CDCl_3$.

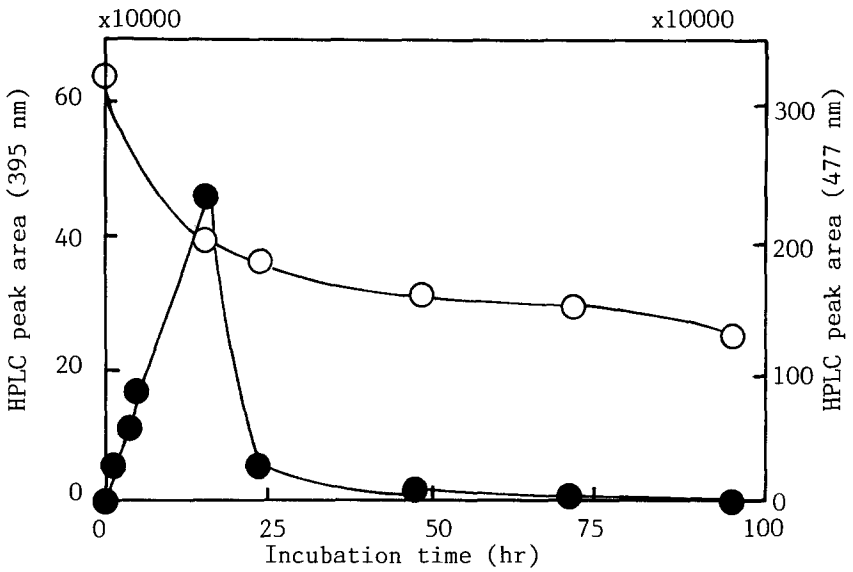


Figure 3. Formation and disappearance of the product (leuco quinizarin), and disappearance of PV12.
product: (●) PV12: (○)
Dye conc.: 2.5×10^{-5} M/L pH: 7.0 Temp.: 37°C
Medium: mineral salts medium

maximum decolorization of PV12 reached nearly 80% in all cases. Whereas, DV1 with two amino groups was hardly decolorized. The maximum decolorization was only 9% after 144 hr in these experimental conditions.

A product (the R_f value 0.51) formed by the reaction of PV12 and B.subtilis was separated by TLC (Merck,Silicagel 60 F_{254}) with a solvent system of hexane-ethyl acetate (3:1,v/v). The mass spectral data of the isolated product exhibited a molecular ion peak at m/e 242. The molecular ion peak was characteristic of an ion on the basis of the leuco form (leuco quinizarin) of PV12. The mass spectra of the product and authentic leuco quinizarin are shown in

Figure 1. The mass numbers of a molecular ion peak and fragment ion peaks of the product coincided with those of authentic leuco quinizarin.

The ^{1}H NMR spectral data determined clearly the structure of the product. The spectral data of the product are shown in Figure 2. The NMR data exhibited methylene resonance at 3.04 ppm, aromatic resonance at 8.09 ppm (the geometric center of symmetrical multiplet), and hydrogen-bonded proton resonance at 13.57 ppm. The NMR data of the product were identical with that of leuco quinizarin (Bloom et al. 1963). Therefore, the initial decolorization step of PV12 resulted in the reductive formation of its leuco form.

The formation and disappearance of the product, as well as disappearance of PV12 with incubation time are shown in Figure 3. Elution of the product (leuco quinizarin; retention time: 3.8 min) and PV12 (retention time: 4.9 min) in extract was monitored at 395 and 477 nm of the absorption maximum, respectively. The amount of the product formed by the biological reduction was gradually increased with increasing incubation time, and which reached the maximum after 16 hr incubation. From that time on, the amount of the product was rapidly decreased. After about 50 hr incubation, the product became scarcely recognizable. This result suggests that the leuco quinizarin was further degraded by B.subtilis in a subsequent step.

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