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Demethylation of Acridine Orange by *Arthrobacter globiformis*

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Biodegradation of dyes by microorganisms, an ecofriendly technology, has been extensively investigated for the treatment of wastewater containing dyes. The microbial degradation of azo, triphenylmethane, and anthraquinone dyes has been documented previously (Yatome *et al.* 1987, 1993; Itoh *et al.* 1993). Although some dyes are biodegradable, most dyes resist to biodegradation (Pagga and Brown 1986).

We focused on microbial degradation of acridine dyes which are an important family of synthetic dyes. Acridine dyes have been used commercially, e.g., lithographic applications, the manufacture process of printing inks, and dyeing leather. The dyes act as mutagens and/or carcinogens in a *Salmonella* /rnicrosom test (Mccan *et al.* 1975; Fenguson and Denny 1991), because acridine dyes can be intercalated between two successive bases in DNA (Brenner *et al.* 1961). The intercalation reduces the biodegradation ability of microorganisms to acridine dyes. Therefore, the most of the dyes are persistent in the natural environment.

In the screening of microorganisms with the ability to degrade acridine dyes, we have found the degradation of Acridine Orange by *Arthrobacter globiformis* (*A. globiformis*) IFO 12137. This paper describes a biodegradation pathway of Acridine Orange involving *N*-demethylation.

MATERIALS AND METHODS

Chemicals: Acridine Orange base (AO; 3,6-bis(dimethylamino)acridine) was purchased from Aldrich Chemical Co. (USA), and purified by column and thin layer chromatography (TLC) eluting with chloroform-methanol-acetic acid (40:10:1, v/v). 3-Dimethylamino-6-methylaminoacridine (DMA) was prepared by the condensation of *N*-methyl-1,3-phenylenediamine, *N,N*-dimethyl-1,3-phenylenediamine, and formaldehyde, followed by oxidation of the *N*-cyclized leuco base (Lubs 1955). 3,6-Bis(methylamino)acridine (BMA) and 3-amino-6-dimethyaminoacridine (ADA) were prepared by the condensation of the corresponding phenylenediamine derivatives and formaldehyde in a similar manner as described above. (DMA: λ_{max} in CH_3OH, 478 nm; mass (MS), m/z 251 (M^+); retention time (R.T.) at high-pressure liquid chromatography (HPLC), 9.3 min; BMA: λ_{max} in CH_3OH, 468 nm; MS, m/z 237 (M^+); R.T. at HPLC, 8.0 min; ADA λ_{max} in CH_3OH, 468 nm; MS, m/z 237 (M^+); R.T. at HPLC, 7.9 min) All other chemicals used were of the highest purity commercially available.

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Microorganism and culture conditions: *A. globiformis* IFO 12137 (ATCC 8010) was kindly supplied by the Institute for Fermentation, Osaka (IFO), Japan, is optimally grown at 30°C and pH 7.0 in 702 medium. The strain was maintained on 702 medium agar slants at 6°C. The composition of the 702 medium was 5 g of polypeptone, 2 g of yeast extract, 1 g of $MgSO_4$. $7H_2O$ per L of distilled water.

Spectral changes of AO: Spectral changes of AO by *A. globiformis* were measured spectrophotometrically. The strain was preincubated at 30°C and pH 7.0 for 16 hr in 702 medium. A culture of the preculture (2 mL) and 702 medium (10 mL) containing the dye (Conc. 2.5 X 10^{-6} M) was incubated with shaking at 30°C. The incubated culture was extracted with 1-butanol (4 mL). The spectra of the extracts were measured at visible region.

Identification of products: The volume of above culture containing AO was scaled up to isolate products. The cultures (1.2 L X 10 = 12 L) were incubated with shaking at 30°C for 144 hr. The incubated cultures were salted out with NaCl, bascified (pH 10) with 5N NaOH, and extracted twice with diethyl ether. The extracts were dried over anhydrous Na_2SO_4 and concentrated. The residue was redissolved in a small amount of methanol and subjected to TLC on Merck Silicagel 60 F_{254S} with chloroform-methanol-acetic acid (40:10:1, v/v). The bands of the products were detected under UV light (254 nm), scraped, and extracted with methanol. After removal of methanol under reduced pressure, the structure of product 1 was identified by comparison of its TLC, HPLC, MS spectrometry, high-resolution and mass (HRMS) spectrometry, and nuclear magnetic resonance (NMR) spectroscopy with those of an authentic sample. The product 2 was identified by comparison of its TLC, HPLC, and MS spectrometry with those of an authentic sample.

HPLC analysis: The consumption of AO and the formation of the product 1 were monitored by HPLC. The incubated culture (12 mL) containing AO was salted out, bascified (pH 10), and extracted as described above. The extracts were dried, concentrated, and redissolved in methanol (1 mL). The solution (10 µL) was used for HPLC analysis. The concentrations of AO, DMA, and BMA were estimated from molar absorptivity of 56000, 53000, and 50000, respectively, on the basis of the reference data (Stone and Bradley 1961) or calculated data.

Apparatus and conditions: Absorption spectra were obtained with a Hitachi spectrophotometer Model 330. MS and HRMS (Ionization energy: 70 eV) spectra were obtained with a Shimadzu Model QP-1000A spectrometer and a JEOL JMS D-300 spectrometer, respectively. NMR spectrum was obtained with a JEOL JNM-EX400 spectrometer in methyl sulfoxide-d_6 with tetramethylsilane as internal standard. HPLC was performed by using a Jasco Model 800 series (column: Inertsil ODS-2, 4.6 X 150 mm, elution: methanol-water-acetic acid (300:100:1, v/v) and 0.01M sodium dodecylsulfate, flow rate: 1.0 mL/min, temp. : 30°C detection: UV/VIS detector). The absorbance was monitored by absorption maximum at 488, 478, and 468 nm.

RESULTS AND DISCUSSION

The spectral changes of 1-butanol extracts from the incubated culture containing AO are shown in Figure 1. As the absorption maximum (λ_{max}) of AO in 1-butanol is 488 nm, the consumption of AO can be monitored by the measurement of

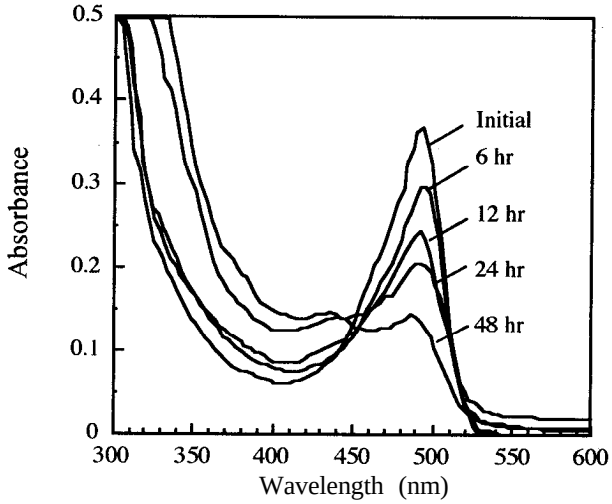

Figure 1. Spectral changes of AO.

Table 1. Rf values on TLC, λ_{max}, M^+ at MS spectra, and R.T. at HPLC of products.

Product	Rf value	λ_{max} (nm)	M^+	R.T.(min)
1	0.56 (main spot)	478, 267	251	9.3
2	0.42 (trace)	468, 266	237	8.0
3	0.38 (trace)	285	–	–
4	0.35 (trace)	280	–	–

absorbance at 488 nm. The absorbance at 488 nm decreased with incubation time, and after incubation for 48 hr, the absorbance was approximately 38 % of the initially present. In addition to the decrease in the absorbance, the absorption maximum shifted from 488 to 478 nm. No signification changes were observed in the control experiments.

The degradation products of AO in the incubated culture were detected by TLC. A main product and three trace products were contained in the ether extract of the culture. The Rf values on TLC, λ_{max}, M^+ at MS spectra, and R.T. at HPLC are shown in Table 1. The product 1 and 2 were detected by individual fluorescent spots, while the product 3 and 4 were traced by individual colorless spots on TLC. The structure of product 1 was assured by HRMS and ^{1}H NMR spectral data (HRMS spectral datum; $C_{16}H_{17}N_3$ Found: M^+ 251.1406, Calculated M: 251.1422 ^{1}H NMR spectral data; δ: 2.80 (3H, d, J=4.9Hz), 3.06 (6H, s), 6.45 (1H), 6.55 (1H, d, J=2.2Hz), 6.81 (1H, d, J=2.7Hz), 6.87 (1H, dd, J=9.0 and 2.2Hz), 7.16 (1H, dd, 5=2.7 and 9.3Hz), 7.74(1H, d, J=9.3Hz), 7.62(1H, d, J=9.0Hz), 8.38 (1H, s)). Product 1 was ultimately confirmed by direct comparison with an authentic sample (DMA) prepared by us. The structure of product 2 was also supported by analytical data as shown in Table 1 and the data of product 2 were coincided with those of prepared BMA. The hypsochromic shift of visible spectra could be explained by the *N*-demethylation of AO, yielding product 1 and product

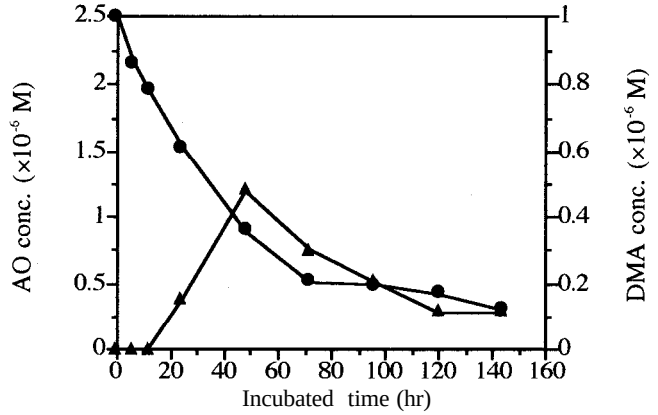

Figure 2. Time course of the consumption of AO and the formation of DMA by *A. globiformis.*

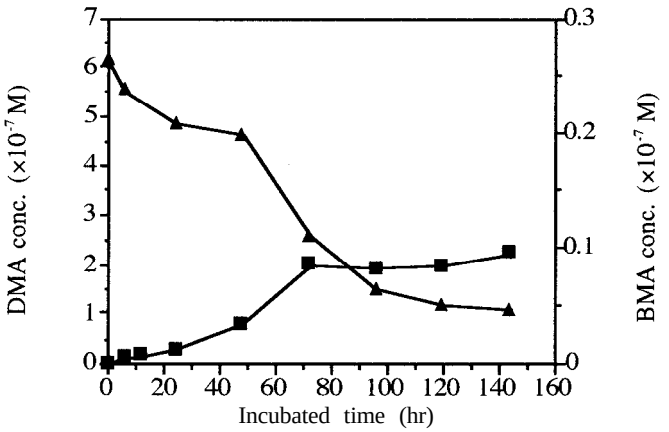

Figure 3. Time course of the consumption of DMA and the formation of BMA by *A. globiformis.*

2. Therefore the *N*-demethylation of AO would regard with the oxidative pathway (The formation of *N*-formyl intermediate (M^+ 279) was concomitantly observed during the MS analysis of product 1). Although the structure of product 3 and 4 were not determined due to their paucity, the decrease of absorbance at the visible region of AO and the appearance of colorless spots on TLC made sure the degradation of the acridine skeleton by the microorganism. This result involves the implication of the presence of the alternative route besides N-demethylation of AO.

The time course for the consumption of AO and the formation of DMA in the incubated culture is shown in Figure 2. The AO (R.T. 10.4 min) and DMA (R.T. 9.3 min) were monitored at 488 nm and 478 nm, respectively. The AO concentration in the culture slowly decreased with incubation time. The DMA concentration was increased with incubation time and reached the maximum after about 48 hr incubation. Further incubation caused the decrease of DMA concentration. Similarly, the time course for the consumption of DMA and the

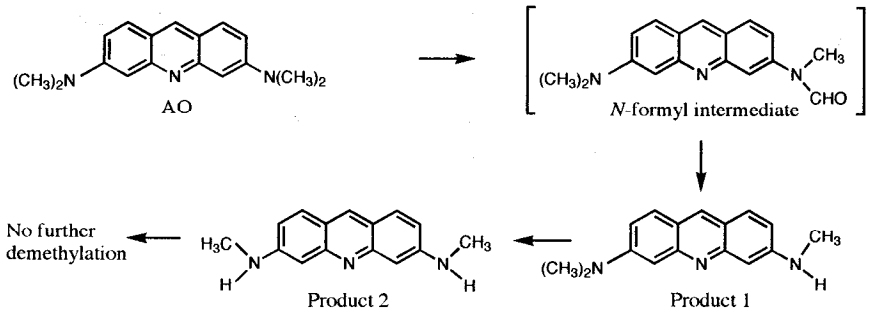

Figure 4. Proposed pathway for AO degradation by *A. globiformis.*

formation of BMA by the incubation of DMA with *A. globiformis* is shown in Figure 3. The BMA concentration also was gradually increased with incubation time to 72 hr, after then BMA concentration reached equilibrium. Further, *N*-demethylation of product 2 by *A. globiformis* was not observed. On the basis of the above results, a proposed pathway for the degradation of AO by *A. globiformis* is shown in Figure 4.

This is the first report for the *N*-demethylation of AO by bacterium except for *N*-demethylation of Crystal Violet by white rot fungus (Bumpus *et al.* 1988). Further investigation is, however, necessary to determine the colorless products by reason of detoxification and/or mineralization of Acridine Orange.

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