Bull. Environ. Contam. Toxicol. (1987) 39:100–107
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Bulletin of Environmental Contamination and Toxicology

Reductive Metabolism of Aminoazobenzenes by *Pseudomonas cepacia*

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The dye composition of the wastewater can change greatly in a short period of time whenever factory dyes are changed. Accordingly, the effects of their acclimation are difficult to obtain. The rate of elimination is low in both the case of the activated sludge plant and the trickling filter plant. Because the dye elimination mechanism chiefly involves bioflocculation, sludge and biomass degrade the eliminability by toxicities of the dyes.

The authors earlier isolated a few strains of microbes in sludge from the sewage of an azo dye factory which had assimilability to azo dye (Ogawa *et al.* 1978). Among them, strain 13NA was identified as *Pseudomonas cepacia* based on Bergey's Manual (Krieg and Holt 1984) and was named *Pseudomonas cepacia* 13NA. A model experiment for continuous treatment of dye waste was also reported (Ogawa *et al.* 1981). Some strain 13NA specificities for aminoazobenzenes and reductive and acetylating pathways are described in the present study.

MATERIALS AND METHODS

p-Acetamidoazobenzene was synthesized by the acetylation of p-aminoazobenzene with a 1:1 acetic anhydride : pyridine mixture: mp, 147-148°C (uncorrected); λ_{max} in EtOH, 350 nm; ε, 17,000; IR, 1704 cm^{-1}; NMR, 2.2 ppm (s,3H); MASS, m/z 239 (M^+). 4,4'-Diaminoazobenzene was prepared by coupling p-aminoacetanilide: mp, 238-241°C (dec.) (Baumgarten 1973). Other chemicals used in this work were commercial products.

Soil samples were gathered from the draining trenches of the dyestuff works in the Hashima district. An enrichment culture was made for strain 13NA and identification was determined using "Bergey's Manual of Systematic Bacteriology, vol 1," (Krieg and Holt 1984). The bacterium belongs to *Pseudomonas cepacia* and was named *Pseudomonas cepacia* 13NA (Ogawa *et al.* 1978).

The culture medium contained 1% glucose,0.5 % polypeptone (Eiken), 0.5 % yeast extract, 0.5 % NaCl and 0.2 % K_2HPO_4. The pH of the

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medium was adjusted to 7.0 with 1 N-NaOH. Three loopful of cells grown overnight at 37 °C on an agar slant were then inoculated in 100 ml of medium in a 500 ml flask and cultivated on a rotary shaker (5 cm stroke, 200 rpm) at 37 °C. Each 100 ml of precultivated broth were used as inoculum for five 10 L flasks (medium, 5 L x 5 = 25 L) and were further cultivated by the same method as the shaking culture.

The cells grown in 10 L flasks were harvested by centrifugation at 10,000 rpm for 15 min. The cells were washed twice with 0.03 M phosphate buffer, pH 6.8. The washed cells were suspended in 0.03 M phosphate buffer, pH 6.8 which contained the test compounds (p-aminoazobebzene, p-acetamidoazobenzene, 2,4-diaminoazobenzene, 4,4'-diaminoazobenzene; 10 ppm). The incubation was carried out at 37 °C under static conditions.

The cultural fluid (25 L) was obtained from the washed cell incubation by centrifugation at 10,000 rpm for 15 min. The cultural fluid was adjusted to pH 12.0 with 1 N-NaOH and extracted 3 times in a separating funnel with CH_2Cl_2 in an amount equal to the cultural fluid by volume. The CH_2Cl_2 layer was washed with water and an aqueous solution saturated with NaCl, respectively, and then dehydrated with anhydrous Na_2SO_4. After being filtered, the solution was concentrated *in vacuo* by a rotary evaporator.

Preliminary identification of metabolites was made by thin layer chromatography (TLC) of authentic compounds. The concentrated samples were applied to a silica gel plate (2.5 x 7.5 cm; Merck's TLC plate, Silica gel 60 F_{254} pre-coated) and developed with MeOH-CH_2Cl_2 (5 : 95, v/v). Spots were also detected under U.V. light at 254 nm wavelength. A high performance liquid chromatograph (HPLC) equipped with a double-scanning detector was used for identification of metabolites. Detailed analysis conditions are described in each test.

The elimination of dye is represented as follows: Elimination of dye(%) = [absorbance of dye (initial)] - [absorbance of dye (observed)] / [absorbance of dye (initial)] x 100. In the case of the cultivation tests, cells were removed by centrifugation at 10,000 rpm for 15 min to exclude absorption by the cell itself.

RESULTS AND DISCUSSION

Strain 13NA was cultivated in either a static or shaking culture. Dye elimination and strain growth were checked with incubation time and the results are shown in Figure 1. In the shaking culture, cell growth was faster and dye elimination was accomplished more quickly. On the other hand, with the static culture, the cell amount was lower and the dye decrease was slow but increased after 24 h. From this result, the static culture was believed superior to the shaking culture concerning activity per cell amount. Therefore, the following study was carried out under static conditions.

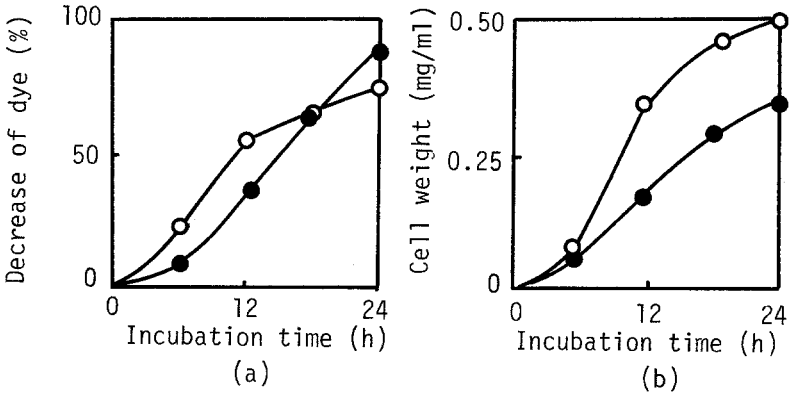

Figure 1. Comparison of the shaking culture with the static culture for the decrease of 2,4-diaminoazobenzene (a) and growth of the strain 13NA (b).
Each culture was performed with three loopfuls of cells inoculated initially at 37 °C. The cell weight was determined after the cells had been washed with water, centrifuged, collected and dried for two hours at 70 °C *in vacuo* [shaking(○) and static culture(●)].

The effects of 4 kinds of dyes on the lag phase, growth constant, generation time and decrease of dye in culture medium, of strain 13NA are shown. As shown in Table 1, the assimilability of dyes was highest with 2,4-diaminoazobenzene, p-acetamidoazobenzene and 4,4'-di-aminoazobenzene. By adding aminoazobenzenes as substrates, the lag phase of strain 13NA was be longer, the growth constant smaller and generation time longer. However, in the case of p-acetamidoazobenzene compared with the control, the lag phase, growth constant and generation time were unaffected. The higher the concentration of dyes, the lower the dye decrease.

Washed cells of strain 13NA that were grown in the shaking culture were incubated in 0.03 M phosphate buffer, pH 6.8 containing 10 ppm p-aminoazobenzene at 37 °C for 24 h under the static conditions. A neutral fraction was extacted from 20 L of the cultural fluid by the same method described in the experimental section. The concentrated fraction was dissolved in CH_2Cl_2 and pre-treated with a short silica gel column for the elimination of contaminants. The eluate was concentrated and then applied to TLC with silica gel or alumina.

On silica gel TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), each R_f of the spots corresponding to p-aminoazobenzene, aniline, acetanilide, p-acetamidoazobenzene, p-phenylenediamine, p-aminoacetanilide and p-phenylenediacetamide coincided perfectly with one of the authentic samples as an inner standard at R_f of 0.95, 0.47, 0.46, 0.32, 0.19 and 0.13, respectively, in the order of the highest R_f value. They were authenticated by the characteristic fluorescence and quenching under U.V. illumination.

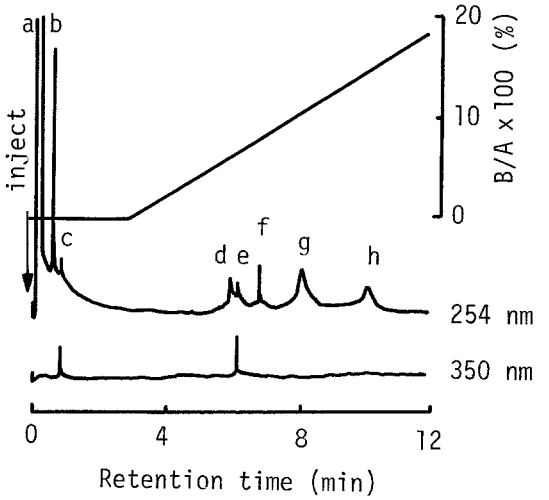

Figure 2. Separation of the metabolites of p-aminoazobenzene:(a) impurity, (b) aniline, (c) p-aminoazobenzene, (d) acetanilide, (e) p-acetamidoazobenzene, (f) p-phenylenediamine, (h) p-aminoacetanilide.
Conditions: column, Permaphase ETH , ϕ 2.1 mm x 500 mm; column temp., 40°C; elution solvent system, A solution: n-hexane; B solution, n-hexane/CH_2Cl_2/EtOH (1 : 1 : 1); gradient rate, 2% (B/A) /min; pressure, 30 kg/cm^2; flow rate, 2.2 ml/min, and detection, U.V. 254 and 350 nm.

Table 1. Influences of dye on the lag phase, growth constant, generation time and dye decrease in strain 13NA[1]

Dye	Conc. (ppm)	T[2] (h)	T'	K (h^{-1})	K'	G (h)	G'	Decrease of dye(%)[3]
p-Aminoazobenzene	0	5.0	-	0.64	-	1.1	-	-
	20	5.7	1.1	0.50	0.8	1.4	1.3	89
	30	6.6	1.3	0.46	0.7	1.5	1.4	83
p-Acetamidoazobenzene	0	7.0	-	1.10	-	0.6	-	-
	7	7.0	1.0	1.10	1.0	0.6	1.0	66
	16	7.0	1.0	1.10	1.0	0.6	1.0	63
2,4-Diaminoazobenzene	0	4.0	-	0.69	-	1.0	-	-
	5	4.6	1.2	0.68	1.0	1.0	1.0	90
	10	4.7	1.2	0.56	0.8	1.2	1.2	91
	20	6.0	1.5	0.56	0.8	1.2	1.2	88
4,4'-Diaminoazobenzene	0	5.4	-	0.69	-	1.0	-	-
	10	6.0	1.1	0.46	0.7	1.5	1.5	66
	20	6.6	1.2	0.49	0.7	1.4	1.4	48
	27	7.0	1.3	0.24	0.4	2.9	2.9	42

[1]Incubation was carried out under static conditions at 37°C.
[2]T: lag phase; T'=T/T(control); K: growth constant; K'=K/K(control); G: generation time; G'=G/G(control). These growth factors are referred to by Tempest (1971).
[3]After incubation for 24 h.

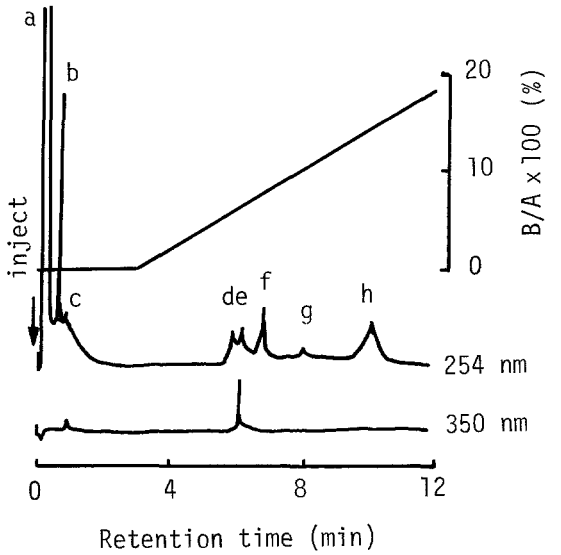

Figure 3. Separation of metabolites of p-acetamidoazobenzene; (a) impurity, (b) aniline, (c) p-aminoazobenzene, (d) acetanilide, (e) p-acetamidoazobenzene, (f) p-phenylenediacetamide, (g) p-phenylenediamine, (h) p-aminoacetanilide. Conditions: column; Permaphase ETH, ϕ 2.1 mm x 500 mm, column temp.; 40 ℃, elution solvent system; A solution, n-hexane; B solution, n-hexane/CH_2Cl_2/EtOH (1 : 1 : 1); gradient rate, 2%(B/A)/min; pressure, 30 kg/cm^2; flow rate, 2.2 ml/min; and detection, U.V. 254 nm and 350 nm.

On alumina TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), each R_f of the spots corresponding to aniline, p-aminoazobenzene, acetanilide, p-acetamidoazobenzene, p-phenylenediamine, p-aminoacetanilide and p-phenylenediacetamide coincided perfectly with the respective R_f of authentic spots with an inner standard at R_f of 0.98, 0.97, 0.93, 0.92, 0.76 0.57 and 0.34, respectively, in the order of the highest R_f value. They were also authenticated by the fluorescence and quenching under U.V. illumination.

The elute was later introduced into HPLC. The conditions of elution and the elution pattern of the sample are shown in Figure 2. Each R_t of peaks corresponding to aniline, p-aminoazobenzene, acetanilide, p-acetamidoazobenzene, p-phenylenediacetamide, p-phenylenediamine and p-aminoacetanilide coincides perfectly with the respective R_f of authentic ones with an inner standard at R_t of 0.8, 0.95, 6.0, 6.2, 6.85, 8.15 and 10.0 (min) at scanning wavelengths of 254 nm and 350 nm, in the order of lowest R_t. Therefore, they were authenticated. Crystallized yields of acetanilide and p-acetamidoazobenzene were 6 % and 2 %, respectively.

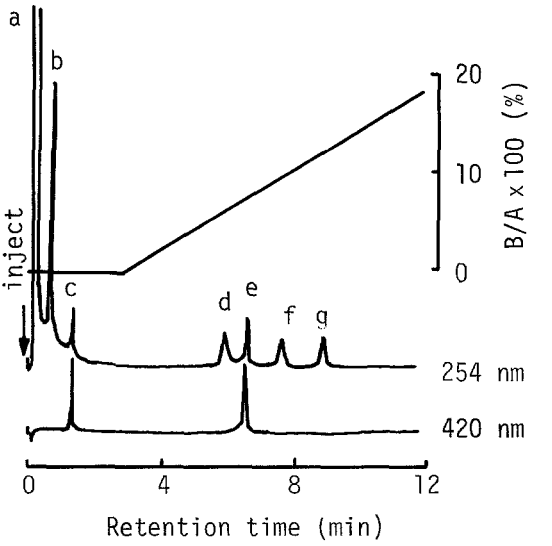

Figure 4. Separation of metabolites of 2,4-diaminoazo benzene: (a) impurity, (b) aniline, (c) 2,4-diaminoazobenzene, (d) acetanilide, (e) 2-amino-4-acetamidoazobenzene, (f) 1,2,4-triaminobenzene, (g) 3,4-diaminoacetanilide.
Conditions: column; Permaphase ETH, ϕ 2.1 mm x 500mm, column temp., 40°C; elution solvent system, A solution: n-hexane; B solution: n-hexane/CH_2Cl_2/EtOH (1 : 1 : 1); gradient rate, 2% (B/A)/min; pressure, 30 kg/cm^2; flow rate, 2.2 ml/min, and detection, U.V. 254 and 420 nm.

From the identification of p-acetamidoazobenzene as one of the metabolites of p-aminoazobenzene, an investigation on the degradability of p-acetamidoazobenzene was also carried out. The incubation and extraction procedure was conducted the same as p-aminoazobenzene. As a result, on silica gel TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), in the order of highest R_f value each of the spots corresponding to p-aminoazobenzene, aniline, acetanilide, p-acetamidoazobenzene, p-phenylenediamine, p-aminoacetanilide and p-phenylenediacetamide coincided perfectly with the respective R_f of authentic samples, and they were also authenticated respectively by the fluorescence and quenching under U.V. illumination.

On alumina TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), in the order of highest R_f value, each R_f of the spots corresponding to aniline, p-aminoazobenzene, acetanilide, p-aminoacetanilide and p-phenylenediacetamide perfectly coincided with those of the authentic ones and was similarly authenticated.

The eluate was then applied to HPLC. The conditions of the elution and the elution pattern of the sample are shown in Figure 3.

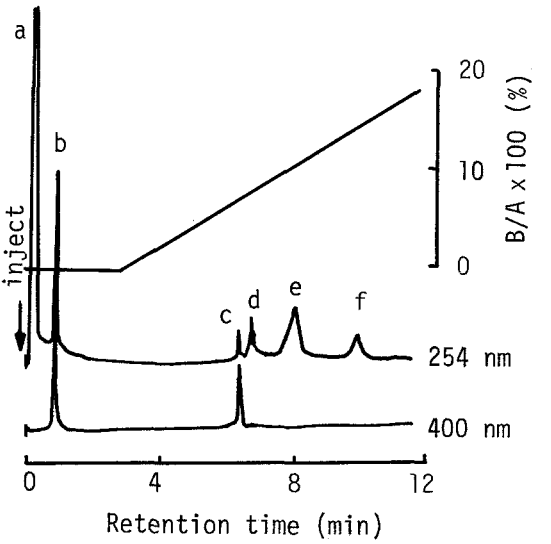

Figure 5. Separation of metabolites of 4,4'-diaminoazobenzene: (a) impurity, (b) 4,4'-diaminoazobenzene, (c) 4-amino-4'-acetamidoazobenzene, (d) p-phenylenediacetamide, (e) p-phenylenediamine, (f) p-aminoacetanilide.
Condidions: column, Permaphase ETH, ϕ2.1 mm x 500 mm; column temp., 40 °C; elution solvent system, A solution: n-hexane; B solution, n-hexane/CH_2Cl_2/EtOH (1:1:1), gradient rate, 2% (B/A) /min; pressure, 30 kg/cm^2; flow rate, 2.2 ml/min, and detection, U.V. 254 nm and 400 nm.

Aniline, acetanilide, p-acetamidoazobenzene, p-phenylenediacetamide and p-aminoacetanilide were identified in the figure. Moreover, trace amounts of p-aminoazobenzene and p-phenylenediamine were identified as respective metabolites.

2,4-Diaminoazobenzene was added as a substrate and metabolic compounds from 2,4-diaminoazobenzene were examined. The cultural fluid was neutralized and extracted with CH_2Cl_2. Then the fluid was passed through a short silica gel column and the eluate was concentrated and applied to silica gel TLC or alumina TLC plates. On silica gel TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), the order of highest R_f value, aniline, 2,4-diaminoazobenzene, acetanilide, 2-amino-4-acetamidoazobenzene, 1,2,4-triaminobenzene and 3,4-diaminoacetanilide were identified with R_f values of 0.90, 0.85, 0.76, 0.60, 0.35 and 0.17, respectively. Similarly on alumina TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), in order of highest R_f value, the same order of metabolites was observed as with silica gel with R_f values of 0.98, 0.92, 0,91, 0.77, 0.65, 0.30 and 0.20, they coincided with standard and were identified with U.V. illumination. The result of the HPLC analysis of the

metabolic mixture of 2,4-diaminoazobenzene is shown in Figure 4. In order of quick retention time, aniline, 2,4-diaminoazobenzene, acetanilide, 2-amino-4-acetamidoazobenzene, 1,2,4-triaminobenzene and 3,4-diaminoacetanilide were identified. But the 2-amino group of 2,4-diaminoazobenzene was not acetylated.

When 4,4'-diaminoazobenzene was used as a substrate, similar results were obtained with TLC or HPLC. On silica gel TLC developed with MeOH/CH_2Cl_2 (5 : 95, v/v), the order of the highest R_f value, 4,4'-diaminoazobenzene, 4-amino-4'-acetamidoazobenzene, p-phenylenediamine, p-aminoacetanilide and p-phenylenediacetamide were identified with R_f values of 0.98, 0.90, 0.32, 0.19 and 0.13. Similarly, on alumina TLC, in the same order as silica gel TLC, they were respectively identified with R_f values of 0.98, 0.95, 0.76, 0.57 and 0.34. The result of HPLC analysis of the metabolic mixture of 4,4'-diaminoazobenzene is shown in Figure 5. In order of quickest R_t, 4,4'-diaminoazobenzene, 4-amino-4'-acetamidoazobenzene, p-phenylenediacetamide, p-phenylenediamine and p-aminoacetanilide were identified.

The metabolism of aminoazobenzenes by seed resting cell culture of *Pseudomonas cepacia* 13NA is supposed to cause reductive cleavage in the azo bond in the beginning and then acetylation of the resulting amine. Moreover, part of the aminoazobenzenes was acetylated and the resulting acetamidoazobenzenes also underwent reductive fission at the azo bond like aminoazobenzenes, the intracellular incorporation is poor, and part of the acetamidoazobenzenes pooled is extracellular. The metabolic pathway of aminoazobenzenes following reduction in the azo bond and acetylation of the amine will be described in the following study.

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Received January 24, 1987; accepted March 17, 1987.