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Transformation of Azo Dye Isomers by *Streptomyces chromofuscus* A11†

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Fourteen mono-azo dyes were used to study the effects of substitution patterns on the biodegradability of dimethyl-hydroxy-azobenzene 4′-sulfonic acids by *Streptomyces chromofuscus* A11. Two substitution patterns were analyzed: (i) all possible substitution patterns of the two methyl and hydroxy substitution groups, 2-hydroxy (3,5; 4,5; 5,6) dimethyl and 4-hydroxy (2,3; 2,5; 2,6; 3,5) dimethyl isomers of azobenzene 4′-sulfonic acid; and (ii) replacement of the sulfonic group with a carboxylic group in these sulfonated azo dyes. The structural pattern of the hydroxy group in *para* position relative to the azo linkage and of two methyl substitution groups in *ortho* position relative to the hydroxy group was the most susceptible to degradation. Replacement of the sulfonic group with a carboxylic group enhanced overall dye degradability by *S. chromofuscus* A11.

It is well-known that under aerobic conditions, sulfonated azo dyes are not easily susceptible to microbial attack (6). In a previous work we showed how to enhance the degradability of azo dyes by linking selected moieties into the dyes' chemical structures (11), without appreciably changing their utility as dyes (4). Lignin-like fragments, specifically guaiacyl (3-methoxy-4-hydroxyphenyl) or syringyl (3,5-dimethoxy-4-hydroxyphenyl) moieties, introduced into the azo dye molecule appeared to provide an access point for microbial oxidative enzymes (11). The modified dye was degraded at least by those actinomycetes shown to be good producers of extracellular peroxidases (9, 11). Peroxidases of *Streptomyces chromofuscus* A11 were then shown to be involved at least in the initial azo transformation process (3, 10). Susceptibility under aerobic conditions was highly influenced by the aromatic substitution pattern (10). Among 22 different aromatic substitution patterns, the sulfonated mono-azo dye with a hydroxy group in the *para* position relative to the azo linkage and two methyl groups in *ortho* position relative to the hydroxy group (3,5-dimethyl-4-hydroxy-azobenzene 4′-sulfonic acid; referred to as azo dye 4s here) was the substrate most susceptible to degradation. Here, we examined how the hydroxy group substitution (*ortho* or *para*) influences the biodegradability of the azo dye by *S. chromofuscus* A11 and further examined the influence of the two-methyl-group substitution pattern on the biodegradability of the dye. This information can lead to a better understanding of the catalytic mechanisms of actinomycete peroxidases. *S. chromofuscus* A11 (ATCC 55184) was selected from 20 strains isolated from higher termites in Kenya (9) because of its superior ability to decolorize dyes (7, 10). We also studied the effect of the replacement of the sulfonic group with a carboxylic group in sulfonated dyes on their biodegradability by *S. chromofuscus* A11, since the replacement of the sulfonic group with a carboxylic group has been reported to significantly alter the degradative pathways of azo dyes Orange I and Orange II by *Pseudomonas* strains (5).

Substrates. Fourteen azo dyes with 2-hydroxy or 4-hydroxy and two methyl groups substituted in azobenzene 4′-sulfonate (dyes s) or 4′-carboxylate (dyes c) were used as substrates in this study (Fig. 1). The substitution patterns were named as follows: 2,3 dimethyl-4-hydroxy-azobenzene (dye 1), 2,5 dimethyl-4-hydroxy-azobenzene (dye 2), 2,6 dimethyl-4-hydroxy-azobenzene (dye 3), 3,5 dimethyl-4-hydroxy-azobenzene (dye 4), 3,5 dimethyl-2-hydroxy-azobenzene (dye 5), and 4,5 dimethyl-2-hydroxy-azobenzene and 5,6 dimethyl-2-hydroxy-azobenzene [dyes (6 + 7)]. Their syntheses have been reported elsewhere (10). High-pressure liquid chromatography (HPLC) analysis of synthesized azo dyes was performed by a slightly modified version of the method of White and Harbin (14) using as mobile phases (degassed) 50% acetonitrile and 50% water, each containing 0.05 M dibasic sodium phosphate and 0.01 M tetrabutylammonium hydrogen sulfate (10). On the basis of the peak purity parameter method previously described (13), HPLC analysis gave purities of 95 to 99%. Isomers 6 and 7 were used as a mixture (6 + 7).

Microorganism and culture maintenance. Stock cultures of *S. chromofuscus* A11 were maintained at 4°C after growth and sporulation at 37°C on sporulation agar (2). The final pH was 7.1. Stock cultures were subcultured every 2 to 10 weeks. A spore suspension was used as the inoculum. The suspension was obtained by suspending spores from an agar slant culture in 10 ml of sterile distilled water containing 0.1% (wt/vol) Tween 80.

Azo dye degradation. *S. chromofuscus* A11 was grown in cotton-plugged 250-ml flasks containing 25 ml of a previously described medium (8). Dye stock solutions (5 mg of dye per ml in 0.09% [wt/vol] NaOH) were filter sterilized and added at 0.005% (wt/vol) final concentration to the autoclaved medium. Three replicate cultures for each dye were incubated in the dark at 37°C for 7 days at 200 rpm in a model G-25 instrument (New Brunswick Scientific Co., Inc., Edison, N.J.). Three replicates of *S. chromofuscus* A11 grown in the same medium without dye were incubated as well. Intracellular protein levels were determined by boiling culture pellets for 20 min in 1 M NaOH prior to protein estimation by a colorimetric procedure

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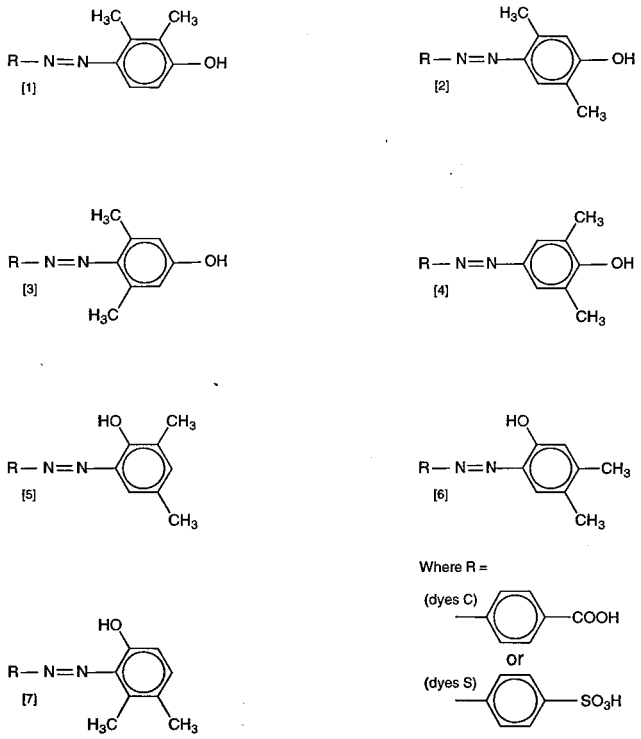

FIG. 1. Structures of the azo dyes tested as substrates in studies of decolorization by *S. chromofuscus* A11.

(catalog no. TPRO-562; Sigma Chemical Co., St. Louis, Mo.). Intracellular protein was used as the index of cellular growth. The amount of extracellular protein was determined by a colorimetric procedure (catalog no. 23225X; BCA protein assay reagent; Pierce, Rockford, Ill.). Samples were taken aseptically over the 7-day growth period, centrifuged, and then diluted 2.5-fold with distilled water. The azo dye substrate present was then quantified spectrophotometrically with a Hewlett-Packard 8452 diode array spectrophotometer operated by a Vectra computer equipped with Hewlett-Packard MS-DOS/UV-VIS software. Since the spectra of dyes were affected by the pH within the physiological pH range, spectrophotometric assays were carried out at their specific isosbestic points. These were 392 nm for dye 1, 394 nm for dye 2, 368 nm for dye 3, 398 nm for dye 4, 432 nm for dye 5, and 430 nm for dyes (6 + 7). Compounds were quantified by using standard curves of absorbance versus concentration.

Table 1 shows the rates of removal of tested substrates from the culture broth by *S. chromofuscus* A11. The sulfonated azo dye 3,5-dimethyl-4-hydroxy-azobenzene 4′-sulfonic acid (dye 4s) was used as a positive control and showed the same values as previously reported (10). Among sulfonated azo dyes, the dyes characterized by a hydroxy group in *para* position relative to the azo linkage (dyes 1s, 2s, 3s, and 4s) were the most susceptible to degradation, while dyes 5s and (6s + 7s) with the hydroxy group in *ortho* position relative to the azo linkage were not degraded. The two methyl substitution groups in *ortho* position relative to the hydroxy group (dye 4) were the most degradable substitution patterns for both sulfonated and carboxylated dyes. The replacement of sulfonic groups by carboxylic groups led to an enhancement of overall biodegradability. In particular, dyes 5 and (6 + 7), which showed no degradability in their sulfonated forms [dyes 5s and (6s + 7s)], were significantly degraded when a carboxylic group replaced the sulfonic group. Figure 2 shows the decolorization patterns for sulfonated (Fig. 2A) and carboxylated (Fig. 2B) dyes over time for cultures of *S. chromofuscus* A11. The greatest decolorization occurred between the first and second days for carboxylated dyes and 24 h later for sulfonated dyes among those dyes that were decolorized within the 7-day growth period. The decolorization values from spectrophotometric data were confirmed by HPLC analyses, which showed the disappearance of these compounds. Since it had been previously reported that color removal from a dye industry effluent by actinomycete strains was likely the result of absorption of the dyes rather than the result of degradation (15), water, methanol, and acetone extracts of the 7-day-old mycelial mats were spectrophotometrically analyzed as well. No significant absorbance was found in these extracts, demonstrating that the removal of dye from the culture broth was due to biotransformation rather than to dye absorption to microbial cells.

Cultures on solid plates, with and without dyes, were also made. The medium used in this case was YCED agar (1). The final pH was 7.1, and the incubation period was 30 days. Dyes were added at 0.005% (wt/vol). Cultures on solid plates confirmed the results obtained with liquid cultures. Both forms of azo dyes 1, 2, and 4, sulfonated and carboxylated, and azo dye 3c were decolorized. Plates containing azo dyes (6c + 7c) showed some decolorization, while plates containing dyes 3s, 5 (sulfonated and carboxylated), and (6s + 7s) showed no visible change with respect to noninoculated plates. Dye decolorization on plates was much slower than that in liquid cultures, becoming noticeable only after 20 to 30 days.

Overall, significant degradation of sulfonated azo dyes by *S. chromofuscus* A11 occurred only when the hydroxy group was in *para* position relative to the azo linkage (azo dyes 1s through 4s [Fig. 2A and Table 1]). No degradation was observed when the hydroxy group was in the *ortho* position, regardless of the substitution pattern of methyl groups. This phenomenon can be explained by considering that the enhanced degradability of sulfonated azo dyes was obtained by modifying the chemical structures of commercial dyes to provide an access point for enzymatic attack on substrates having aromatic rings with substitution patterns similar to those found in lignin. In lignin, the OH group is never in the *ortho* position but in the *para* position. Therefore, it is easily understood why degradability is enhanced when the hydroxy group is in the *para* position rather than in the *ortho* position relative to the azo linkage. The poor

TABLE 1. Azo dye decolorization by *S. chromofuscus* A11 after a growth period of 7 days

Azo dye[a]	% Decolorization
4s*	83
1s*	78
2s*	75
3s*	12
5s**	0
(6s + 7s)**	0
4c*	98
1c*	96
2c*	92
3c*	70
(6c + 7c)**	44
5c**	18

[a] s*, sulfonated dye with auxochrome —OH in *para* or 1,4 position; s**, sulfonated dye with auxochrome —OH in *ortho* or 1,2 position; c*, carboxylated dye with auxochrome —OH in *para* or 1,4 position; c**, carboxylated dye with auxochrome —OH in *ortho* or 1,2 position.

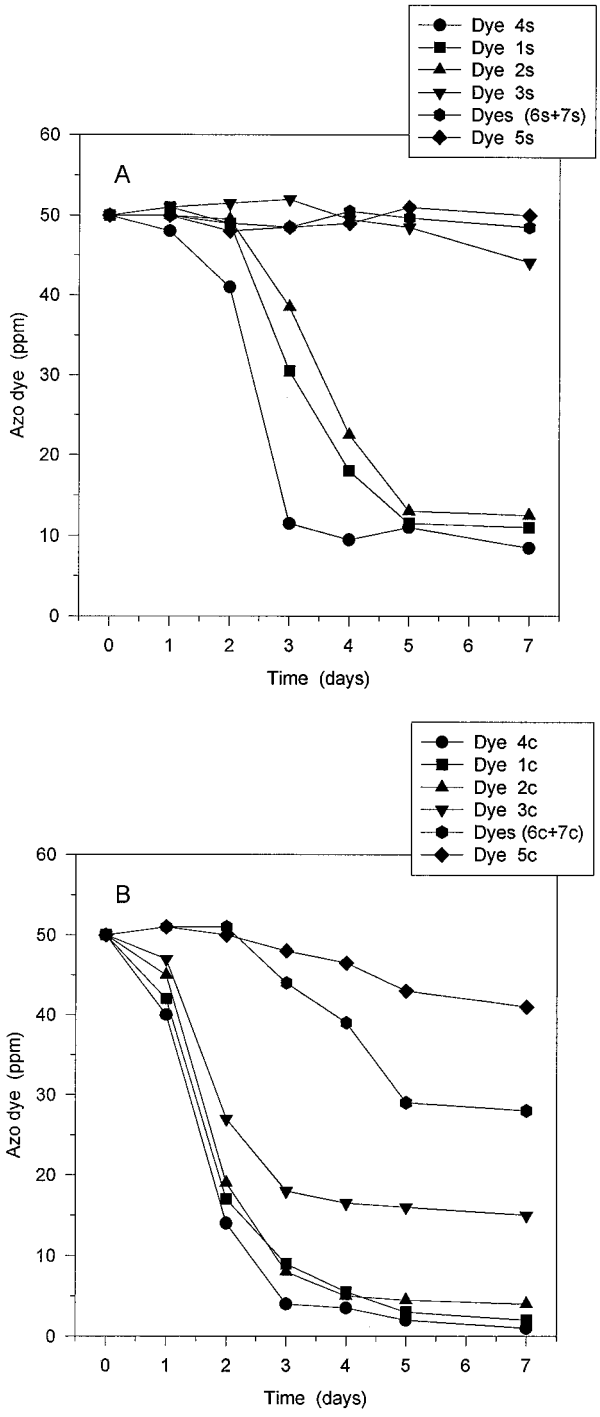

FIG. 2. Decolorization of sulfonated (A) and carboxylated (B) dyes by *S. chromofuscus* A11.

degradability of dyes with an *ortho* substitution of the hydroxy group in relation to the azo linkage can probably be explained by steric hindrance or the possible formation of an internal hydrogen bridge (12). The spatial orientation could prevent some sort of interaction between the active site of the microbial enzyme and the OH group in the substrate. Among the sulfonated azo dyes with the *para* substitution of the OH group in relation to the azo linkage, the substrate most susceptible to degradation was dye 4. We have previously shown that the methoxyl group (particularly in a syringyl pattern) may be changed to a methyl group without changing the enzymatic vulnerability of the molecule (10). This leads to the conclusion that spatial influence is more important than are electronic effects. Among dyes 1, 2, 3, and 4, the substitution pattern of dye 4 is the most similar to that of the syringyl group of lignin, thus making dye 4 the most accessible to the enzymatic system of *S. chromofuscus* A11. Dye 3, characterized by a 2,6 dimethyl-4-hydroxy substitution pattern, is significantly different from the syringyl substitution present in the lignin structure.

Since the replacement of the sulfonic group with a carboxylic group has been reported to significantly alter the degradation pathways of azo dyes Orange I and Orange II by *Pseudomonas* strains (5), we investigated this effect on seven sulfonated azo dye isomers. The replacement of the sulfonic group with a carboxylic group led to an overall enhancement of dye degradability by *S. chromofuscus* A11 (Table 1). All seven dyes [azo dyes 1c through (6c + 7c)], including dyes 5c and (6c + 7c), whose sulfonated forms were not susceptible to microbial attack, were transformed. The time required for the removal of dye from the culture broth confirmed the higher degradability of carboxylated forms with respect to sulfonated forms (Fig. 2). These results are similar to the findings of Kulla et al. (5), who reported an analogous pattern when they grew dye-degrading *Pseudomonas* strains on the carboxylated and sulfonated azo dyes Orange I and Orange II. They explained the recalcitrance of sulfonated dyes by showing that the sulfonated aromatic intermediates formed reacted in the microbial cell with non-sulfonated intermediates of dye degradation, interrupting the degradative pathways. We suspect that the electron-withdrawing properties of the sulfonic group also may play an important role in interfering with the biodegradability of sulfonated dyes. The influences of the substitution positions of sulfonic groups and modified sulfonic groups, such as amides, on azo dye biodegradability are currently under study in our laboratory.

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