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# A Pathway for Biodegradation of an Anthraquinone Dye, C. I. Disperse Red 15, by a Yeast Strain *Pichia anomala*

K. Itoh,[1] Y. Kitade,[2] C. Yatome[2]

[1]Nagoya Municipal Industrial Research Institute, Rokuban 3-4-41, Atsuta-ku, Nagoya 456, Japan
[2]Department of Chemistry, Faculty of Engineering, Gifu University, Yanagido 1-1, Gifu 501-11, Japan

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Colored waste water from dye, food, and textile industries is not easily removed by biological methods due to its resistance against biodegradation. Several researchers have reported that the use of dye assimilating or degrading microorganisms is effective for the biological treatment of such waste water, and showed the pathways of conversion and degradation of dyes in liquid media by microorganisms (Cripps et al. 1990; Horitsu et al. 1977; Meyer *et al.* 1979; Ogawa *et al.* 1986, 1990). However, degradation pathways of dyes by microorganisms are little known except for azo dyes (Bumpus *et al.* 1988; Yatome *et al.* 1991, 1993).

Our previous article has described that several anthraquinone dyes were decolorized by *Bacillus subtilis* (Itoh *et al.* 1993). Especially, C. I. Pigment Violet 12 (PV12: 1,4-dihydroxyanthraquinone) was readily decolorized and degraded. Anthraquinones bearing amino group, however, were hardly decolorized.

The commercially available C. I. Disperse Red 15 (DR15: 1-amino-4-hydroxyanthraquinone), is an important dye used in industry (Gwinn *et al.* 1984). DR15 is a mutagen in a *salmonella*/microsomal test (Brown *et al.* 1976; Venturini *et al.* 1979) and causes DNA damage in repair deficient mutants of *B. subtilis* in rec assay (Brown 1980). Therefore, the toxicity of DR15 in microorganisms causes difficulty in biodegrading the dyes.

In the course of our study on the biodegradation of anthraquinones, we have found that *Pichia anomala* IAM 12210 (ATCC 8168) and IAM 4967 are capable of degrading DR15 and the related anthraquinones. To the best of our knowledge, no other microorganism is reported to biodegrade DR15. This paper describes the degradation of DR15 by *P. anomala* IAM 12210 and shows a plausible initial pathway for the biodegradation of DR15.

## MATERIALS AND METHODS

Chemicals: DR15, PV12, and 1-hydroxyanthraquinone (1-HAQ) were purchased

*Correspondence to:* K. Itoh

from Tokyo chemical industry Co.,Ltd. Leucoquinizarin (LQ; 2,3-Dihydro-9,10-dihydroxy-1,4-anthracenedione) was purchased from Aldrich chemical Co. inc. DR15 was purified by thin layer chromatography (TLC) using with toluene-acetone (9:1, v/v) as eluent and recrystallized twice from 50% ethanol. PV12 was purified by TLC using with hexane-ethyl acetate (3:1, v/v) as eluent and recrystallized twice from glacial acetic acid. 1-HAQ and LQ were recrystallized twice from methanol.

Microorganism and medium: The organism used in this study was *P. anomala* IAM 12210 which was obtained from the Institute of Applied Microbiology, University of Tokyo, Japan, and is optimally grown at 30° C and pH 6.2 in YM medium (10% glucose, 5% peptone, 3% yeast extract and 3% malt extract). This medium was adjusted to pH 6.2 with 1N HCl and autoclaved at 121° C for 15 min and used for microbial degradation experiments of DR15.

Reaction of DR15 and *P. anomala:* One loop of the stock culture was inoculated into 15 mL of YM medium in a 50 mL Erlenmeyer flask and preincubated for 16 hr at 30° C. The culture medium was inoculated into 250 mL of YM medium in a 500 mL Erlenmeyer flask containing DR15 (conc.: $2.5 \times 10^{-5}$ M/L). Then the flask was incubated at 30° C with rotary shaking at 120 rpm. Aliquots (8 mL) of the incubated mixture were removed at selected intervals and were extracted with 5 mL of 1-butanol. The alcohol layer was dried with anhydrous $Na_2SO_4$. The absorption spectra of the alcohol extracts were recorded, and the disappearance of DR15 was monitored by the decrease of the absorption maximum of the dye in the visible region.

Detection and identification of reaction products formed from DR1 5: Each preincubated 120 mL culture media were inoculated into 2 L of YM medium in five 3 L Erlenmeyer flasks containing DRI 5 (conc.: $2.5 \times 10^{-5}$ M/L), and incubated as described above. After incubation for 72 hr, cells from the incubated mixture were removed by centrifugation (3000 rpm, 15 min). The resulting supernatants were collected, and extracted twice with an equal volume of chloroform. The extracts were combined, dried with anhydrous $Na_2SO_4$, filtered, and concentrated in a rotary vacuum evaporator at 40° C. The residue was redissolved in a small amount of chloroform, and subjected to TLC on Merck RP-18 $F_{254s}$ with methanol-water (19:1, v/v). Then, a main band was separated by preparative TLC on Merck Silica gel 60 $F_{254}$ with chloroform-methanol (10:1, v/v). The band was scraped off and extracted with methanol. The extract was dried in a rotary vacuum evaporator at 40° C, and a main product was identified by mass (MS) and nuclear magnetic resonance (NMR) spectroscopy. The other products were identified by analytical TLC and gas chromatography-mass (GC-MS) spectrometry.

Apparatus: Absorption spectra were obtained with a Hitachi spectrophotometer Model 330. MS spectra (El) were obtained with a Shimadzu GC-MS Model QP-1000A spectrometer (Column: id 3 mm 4 $\phi$ x1.6 m, Silicon SE-30 3% on

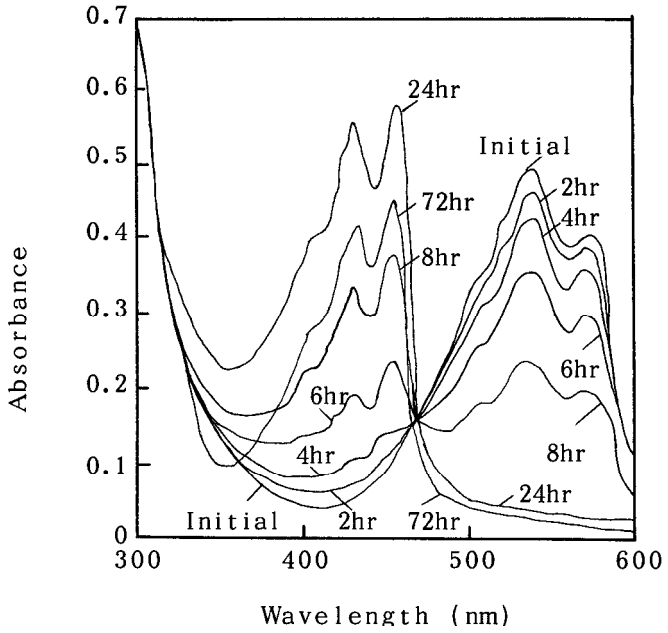


**Figure 1.** Change in absorption spectra of DR15 by *P. anomala* of various incubation time.

Gaschrome Q 80/100, Injection temperature: 200° C, Column temperature: 200° C, Carrier gas: He 40 mL/min, Ionization energy: 70 eV). NMR spectra (400 MHz) were obtained with a JOEL JNM-EX400 spectrometer in $CDCl_3$ with tetramethylsilane as internal standard.

## RESULTS AND DISCUSSION

Disappearance of DR15 by *P. anomala* was monitored spectrophotometrically. Figure 1 shows the change of the visible spectra of 1-butanol extracts of incubated mixture with increasing incubation time. The absorption spectra of DR15 in 1-butanol can be characterized by maxima at 530 nm. The absorbance maxima at 530 nm decreased with increasing incubation time, and the peak at 530 nm gradually shifted to 453 nm with an increase in absorbance. After incubation for 24 hr, the peak at 530 nm almost completely disappeared. Further, the peak at 453 nm also slightly decreased after 72 hr incubation. The control experiment without *P. anomala* was conducted in a similar manner. No reaction has occurred without cells was evident by unchanged absorbance of maxima at 530 nm. Therefore, the changes of the absorption spectra of DR15 were dependent upon biological reaction brought by *P. anomala.*

The reaction products from DR15 were detected by analytical TLC. The chloroform extract of incubated mixture contained a major product and three minor products. The Rf values of the reaction products on TLC are shown in Table 1. In the control experiment without *P. anomala,* no products were detected.

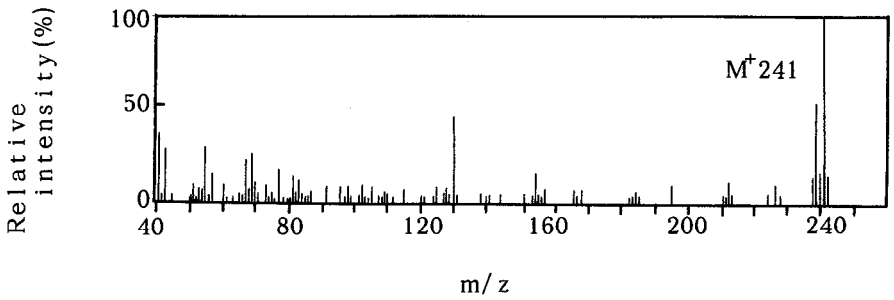


**Figure 2.** MS spectrum of product 1.

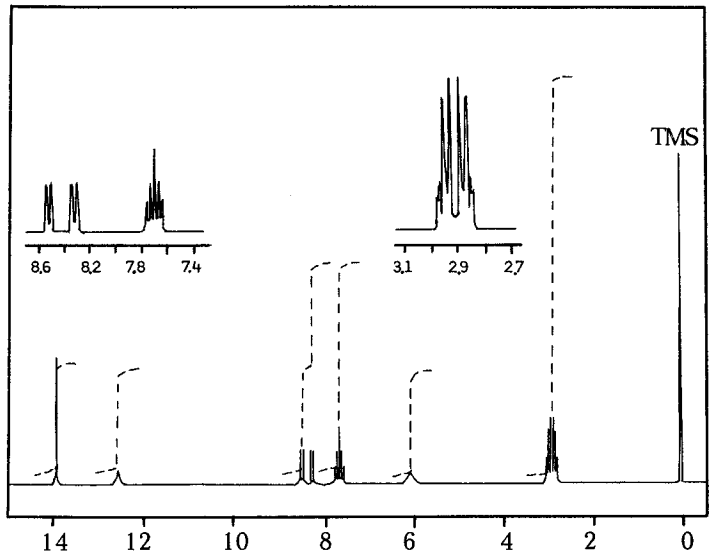


**Figure 3.** $^{1}$H NMR spectrum of product 1.

**Table 1.** Rf values of the reaction products on TLC.

| Product | Rf value |
|---|---|
| 1 | 0.53 (major band) |
| 2 | 0.49 (trace) |
| 3 | 0.45 (trace) |
| 4 | 0.38 (trace) |

The major band (product 1) was isolated and purified by TLC (the Rf value 0.50). The mass spectral datum of the purified product 1 exhibited a molecular ion peak at m/z 241 as shown in Figure 2. The molecular ion peak was characteristic of a

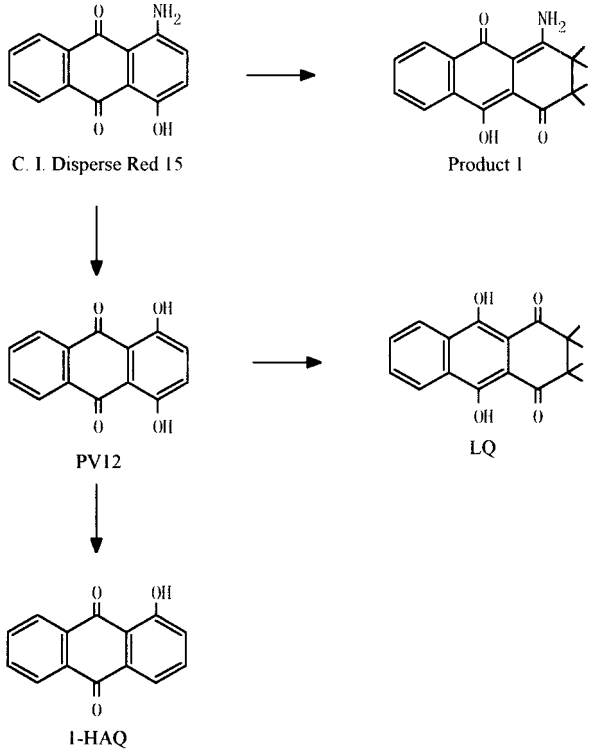


**Figure 4.** A plausible pathway for the biodegradation of DR15 by *P. anomala.*

reduction of DR15. The structure of product 1 further supported by its $^{1}H$ NMR spectral data as shown in Figure 3. The NMR data exhibited methylene resonance at 2.8-3.0 ppm, aromatic resonance at 7.6-7.8 and 8.3- 8.6 ppm, hydrogen-bonded proton resonance at 13.9 ppm, and amino proton resonance at 6.1 and 12.5 ppm. Product 1 of Figure 4 shows the structure proposed from these data.

The Rf values of the products 2, 3, and 4 were identical with those of authentic LQ (the Rf value 0.49), 1-HAQ (the Rf value 0.45) and PV12 (the Rf value 0.38), respectively. These products were further identified by GC-MS. The retention time (r. t.) of the total ion monitor, the mass number of molecular ion peak, and mass spectrum was also similar to authentic LQ (r. t. 14.6 min, m/z 242 ($M^{+}$)), 1-HAQ (r. t. 8.0 min, m/z 224 ($M^{+}$)), and PV12 (r. t. 11.7 min, m/z 240 ($M^{+}$), respectively.

On the basis of above facts, a plausible pathway for the biodegradation of DR15 by *P. anomala* is shown in Figure 4. The formation of product 1, PV12, LQ, and 1-HAQ from DR15 by *P.anomala* will be interpreted as follows. Reduction of DR15 affords product 1 as a major product. Deamination from DR15 involving displacement of a hydroxy group also results in the formation of PV12. Further,

reduction and reductive dehydration of PV12 probably afford LQ and 1-HAQ, respectively.

Though the detailed study is necessary to clarify the further biodegradation of DR15, this work could show the possibility of the biodegradation of anthraquinones bearing amino group, which are difficult be degrade.

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