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Oxidative Biodegradation of an Anthraquinone Dye, Pigment Violet 12, by *Coriolus versicolor*

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Synthetic dyes are mainly used in textile and dyeing industries. About 20% of all dyes are directly lost to wastewater in the dyeing process (Clarke and Anliker 1980). Thus, the wastewater must be treated before release into the natural environment. For biological treatment of the wastewater containing dyes, the microbial decolorization and degradation of dyes has been of considerable interest. Although much attention has been focused on microbial degradation (detoxification and mineralization) of dyes, the biodegradation pathway of dyes is scarcely known in detail (Yatome *et al.* 1993a,b). The pathways involving the initial reduction of anthraquinone dyes using a bacterium (*Bacillus subtilis*) and a yeast (*Pichia anomala*) have been already reported (Itoh *et al.*, 1993,1996).

White rot fungi are known to oxidize polycyclic aromatic compounds, and are often used to degrade organic pollutants (Young and Cerniglia 1995). Polymeric dyes (Glenn and Gold 1983), triphenylmethane dyes (Bumpus and Brock 1988) azo (Goszczynski *et al.* 1994), and heterocyclic dyes (Cripps *et al.* 1990) are also degraded by these fungi. The oxidative biodegradation of anthraquinone dyes by these fungi, however, has not been investigated.

In the course of our study on the biodegradation of anthraquinone dyes by microorganisms, we have found an oxidative biodegradation of an anthraquinone dye, Pigment Violet 12 (PV12), by a white rot fungus *Coriolus versicolor* (*C. versicolor*). This paper describes an oxidative biodegradation pathway of PV 12 by *C. versicolor.*

MATERIALS AND METHODS

Chemicals: PV12 (1,4-dihydroxyanthraquinone) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Japan). The purification of PV12 has been described elsewhere (Itoh *et al.* 1993). Phthalic acid was purchased from Aldrich Chemical Co. (USA). Phthalic anhydride and benzoic acid were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

Microorganism and culture conditions: *C. versicolor* IFO30388 which formed clear halos on potato-dextrose (Difco Laboratories) agar plates containing several anthraquinone dyes (Mordant Red 3, Nuclear Fast Red, Acid Blue 25, Acid Green 25, and Acid Blue 41; Conc. 1 X 10^{-4} M) was found as anthraquinone dyes-decolorizing fungus. This fungus was supplied by the Institute for Fermentation, Osaka (IFO), Japan, is optimally grown at 24°C and pH 5.6, and was maintained on potato-dextrose agar slants at 6°C. It was transferred to potato-dextrose agar

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plates and incubated at 24°C for 7 to 10 days before use in this experiment.

Identification of metabolites from PV12: A potato-dextrose agar gel (5 X 5 mm) cutted from the above culture plates was inoculated in sterilized potato-dextrose liquid culture (250 mL, pH 5.6) containing PV12 (1 X 10^{-4} M) in a 500 mL Erlenmeyer flask. The culture was incubated for 10 days at 24°C on a rotary shaker (120 rpm). The incubated culture was adjusted to pH 7 with 1N NaOH and extracted twice with chloroform (500 mL). The remaining aqueous layer was acidified with 1N HCl to pH 2 and extracted twice with diethyl ether (500 mL). The ether extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure at 30°C. The residue was dissolved in a small amount of methanol. The resulting solution was applied for the analysis by thin layer chromatography (TLC) and gas chromatograph-mass (GC-MS) spectrometry. TLC analysis was carried out on a Silicagel 60 F_{254} plate (5 X 10 cm; E. Merck) or a DC-Fertigplatten plate (RP-18F_{254s}, 5 X 10 cm; E. Merck). Spots were detected under UV light (254 nm). GC-MS analysis was performed with a Hewlett Packard mass selective detector model 5971 equipped gas chromatography model 5890 and using DB-1 fused- silica capillary column, 30 m long with id of 0.25 mm (J&W Science). The oven temperature was programmed from 60 to 200°C at 10°C/min.

Quantification of PV12 and metabolite: A potato-dextrose agar gel (5 X 5 mm) cutted from the above culture plates was inoculated in sterilized potato-dextrose liquid culture (50 mL) containing PV12 (1 X 10^{-4} M) in 100 mL Erlenmeyer flask. The culture was incubated at 24°C on rotary shaker (120 rpm). After an appropriate time, the incubated culture was extracted as described above. The neutral and acidified extracts were dried and evaporated. These residues were dissolved in methanol (0.5 mL). The resulting solutions were applied for quantitative analysis of PV12 and metabolite by GC. GC analysis was performed with a Shimadzu model 14A gas chromatograph equipped with a flame ionization detector and using a SPB-5 fused-silica capillary column, 15m long with id of 0.53 mm (SUPELCO, Inc.). The oven temperature was programmed similarly to that descried above. Quantification was achieved with calibration curves of PV12, phthalic acid, and benzoic acid. The GC retention time of PV12, phthalic acid (detection as anhydride), and benzoic acid were 6.9, 4.8, and 4.1 min, respectively.

RESULTS AND DISCUSSION

The metabolites (metabolite 1 and 2) were present in the acidified extract from the incubated culture. The Rf value of metabolite 1 on TLC identified with that of authentic benzoic acid (Silicagel 60 F_{254}; Rf 0.76, chloroform-methanol (5:1, v/v)) and that of metabolite 2 identified with that of authentic phthalic acid (DC-Fertigplatten RP- 18 F_{254S}; Rf 0.77, methanol-water (1:1, v/v)). The metabolites were also identified by GC-MS. The GC-MS retention time of metabolite 1 was 7.9 min and a molecular ion M^+ was 122, which were consistent with those of authentic benzoic acid (Figure 1. A). The metabolite 2 had GC retention time of 8.6 min and a molecular ion M^+ at 148, which were consistent with those of authentic phthalic anhydride (Figure 1. B). Under the operating conditions of GC-MS, phthalic acid was easily converted into the corresponding anhydride: the mass number (148) of M^+ peak was equivalent to the molecular weight of phthalic acid with loss of H_2O. On the basis of TLC and GC-MS analyses, the metabolites were confirmed as benzoic acid and phthalic acid. No metabolites were observed

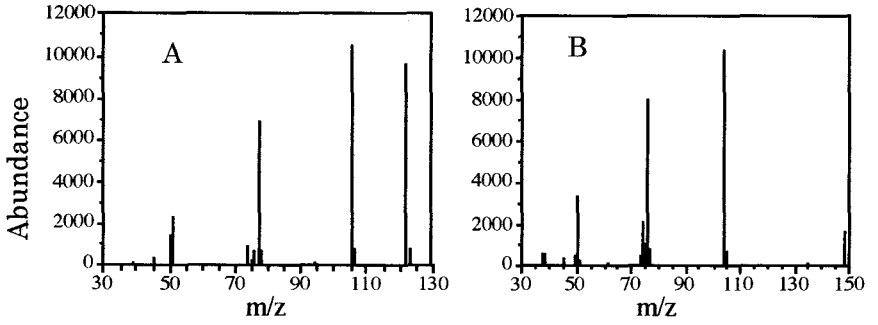

Figure 1. Mass spectra obtained from the GC-MS analysis of the acidified extract.
A: metabolite 1, benzoic acid B: metabolite 2, phthalic anhydride

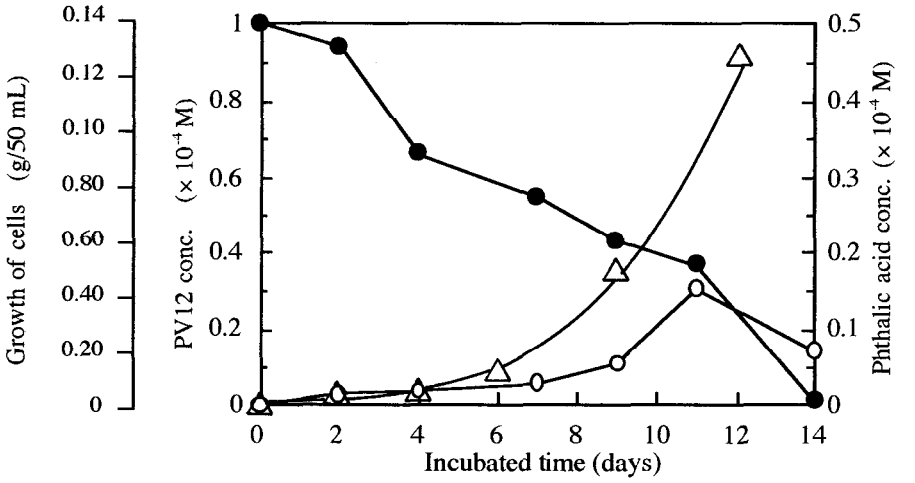

Figure 2. Time course of growth of cells and formation of phthalic acid with consumption of PV12 by *C. versicolor*.
PV12 phthalic acid
growth of cells

in the control experiments.

The time course of growth of cells and formation of phthalic acid with consumption of PV12 under the analogous conditions described above is shown in Figure 2. The growth of cells was monitored by harvesting the whole fungal cell mass in the incubated culture and drying to constant weight (dry weight). The fungus exhibited significant growth in the culture containing PV12 at the experiment conditions. The consumption of PV12 by growing cells of the fungus slowly proceeded dependent on the incubation time. After 14 d incubation, PV12 was no longer detected. On the other hand, the formation of phthalic acid was followed by consumption of PV12. The concentration of phthalic acid was slowly increased with incubation time, reached the maximum after 11 d incubation, and decreased at the end of incubation time. The metabolite, phthalic acid, will be further degraded to benzoic acid. No decrease of PV12 and no formation of

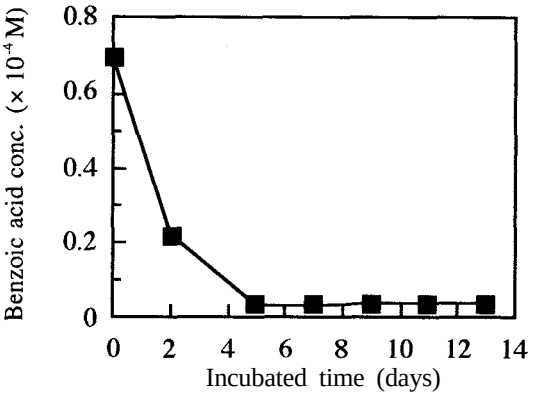

Figure 3. Time course of the consumption of benzoic acid by *C. versicolor.*

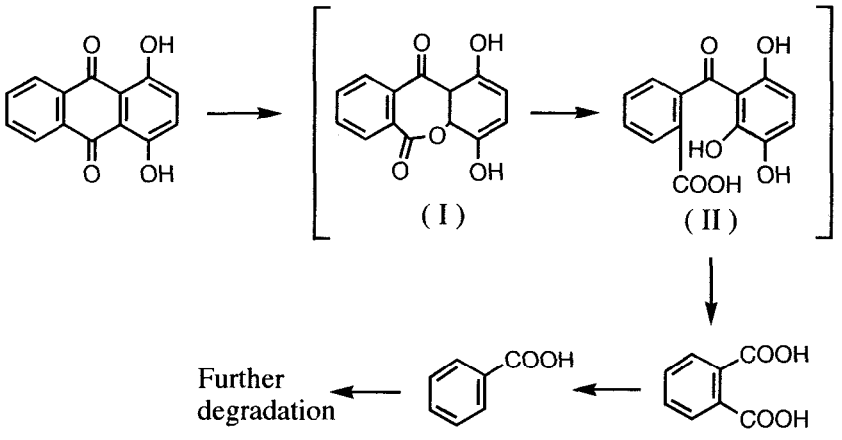

Figure 4. Proposed pathway for the biodegradation of PV12 by *C. versicolor.*

phthalic acid were observed in the control experiments. Similarly, the time course of the consumption of benzoic acid by *C. versicolor* is shown in Figure 3. The concentration of benzoic acid was rapidly decreased, after 5 d incubation, benzoic acid became scarcely recognizable in the incubated culture. Further metabolites of benzoic acid were not detected by GC-MS.

Enzymatic degradation of an anthraquinone derivative (questin) by oxygenase from *Aspergillus terreus* has been reported (Fujii *et al.,* 1988). *C. versicolor* is also known to produce extracelluraly several oxygenases, e.g., ligninase, lactase, peroxidase, and Mn peroxidase (Iimura *et al.* 1996; Paice *et* al. 1993). From these reports and our results, we propose the oxidative biodegradation pathway of PV12 by growing cells of *C. versicolor* as shown in Figure 4. The plausible pathway for the degradation of PV12 by *C. versicolor* and formation of phthalic acid would be as follows: Attack of oxygenase from *C. versicolor* on PV12 leads to the lacton intermediate (I). The formed intermediate produces phthalic acid via hydrolytic cleavage of the carbon-oxygen bond. The presence of the intermediate (II) in the reaction is assumed by analogy with Baeyer-Villiger type reaction (Fujii

et al., 1988). Subsequently, benzoic acid is formed by decarboxylation of phthalic acid.

To the best of our knowledge, this work is the first demonstration of oxidative biodegradation of an anthraquinone dye, PV12, by a single strain. Further studies await the characterization of enzymes involved in this process.

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