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Degradation of 2,4-dichlorophenoxyacetic acid by mixed cultures of bacteria

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SUMMARY

We explored the feasibility of using mixed cultures for herbicide degradation, with the ultimate aim of application for effluent treatment. The present study reports on mixed cultures which were developed to grow aerobically with 2,4-dichlorophenoxyacetic acid (2,4-D) as the sole carbon substrate. Degradation of 2,4-D was verified by HPLC and UV-spectroscopic analysis of the residual 2,4-D concentration in the test cultures. Cultures that were initially developed with 2,4-D also grew readily with glucose, but the degradation of 2,4-D was effectively prevented under mixed substrate conditions. Major intermediates or metabolites resulting from 2,4-D degradation were not detected with the HPLC methodology except 2,4-dichlorophenol which appeared to accumulate transiently in the growth medium.

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide which has a limited half-life ($t_{1/2}$ = 1–6 weeks) in soil primarily because of its susceptibility to biological degradation [17]. Several degradative pathways have been presented for this herbicide [13–15]. The degradation of 2,4-D typically involves oxygen-requiring enzyme-mediated reactions. The mechanism of 2,4-D degradation has been elucidated in several bacteria, including *Pseudomonas* spp. [6,8]. In addition, some species in the genera *Arthrobacter* [2,16] and *Alcaligenes* [5,7] can degrade 2,4-D for energy and carbon. Enzymes involved in the degradative metabolism of 2,4-D are commonly plasmid borne in *Pseudomonas* [12] and *Alcaligenes* spp. [5,7]. For *Arthrobacter* spp., the role of plasmid DNA has not been elucidated. Previous laboratory studies have focused on pure culture approaches necessary to characterize the genetics and underlying biochemical features of the degradative pathways. A number of studies have also been published on the occurrence and distribution of 2,4-D degrading bacteria in soil and other natural habitats [4,10,11].

Although a wealth of information exists on the biochemistry and genetics of 2,4-D degradation by bacteria, the utility of this information has not been evaluated for possible treatment processes of industrial waste streams where 2,4-D may represent a serious disposal problem. This paper reports on an initial phase of a study in which the feasibility of bacterial degradation of 2,4-D was explored for an industrial setting. Because of the ultimate aim of application for a biological treatment process, emphasis was placed in the present work on the development of mixed cultures capable of degrading 2,4-D as the sole source of electrons and carbon. In studies of biodegradation and environmental distribution, 2,4-D has usually been quantified by gas chromatography, UV spectrometry, and radioisotope techniques [18]. In the present study, HPLC methodology is also presented that conveniently avails itself to bacterial growth studies with 2,4-D as the substrate.

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MATERIALS AND METHODS

Bacteria capable of degrading 2,4-D were enriched from samples of soil, a waste pond, and a bioreactor located at a fertilizer manufacturing plant. A reference sample was collected from an activated sludge basin at a municipal sewage treatment plant. Samples (5 g of soil or 10 ml of water) were added to 100 ml of mineral salts medium in 250-ml shake flasks containing 250 to 1000 mg of 2,4-D per liter as the sole carbon and energy source. Both analytical grade and technical grade 2,4-D were used in the experiments. The mineral salts medium contained (per liter): K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 10 mg;

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$CaCl_2 \cdot 2H_2O$, 10 mg; $MnCl_2$, 0.1 mg; $ZnSO_4$, 0.01 mg. The medium was adjusted to pH 7.4 with NaOH before autoclaving it (121 °C, 15 min). Subcultures were maintained with 10% inocula. When desired, the media were solidified with 20 g of agar/liter. Culture flasks were incubated on a shaker at 156 rpm and at 21 °C. Growth was monitored by following turbidity at 550 nm and by microscopic examination.

2,4-D was analyzed by its characteristic UV absorption at 283 nm with a Varian 2200/2300 spectrophotometer. Samples of bacterial cultures were centrifuged at 3500 × *g* for 10 min, followed by alkaline pH adjustment with 0.5 N NaOH and filtration (Gelman ARCO LC25 syringe filter) to remove the turbidity before UV analysis of the supernatant medium. Standard solutions were made of analytical grade 2,4-D in the range of 2 to 100 mg/l.

The concentration of 2,4-D was also determined by reverse-phase high pressure liquid chromatography (HPLC). HPLC was chosen because of its convenience and speediness when compared with the GC methodology used in previously published studies. Samples (10 ml) of bacterial cultures were centrifuged at 3500 × *g* for 10 min to remove bacterial cells and debris. An aliquot of the supernatant medium (1 ml) was mixed with 2 ml each of 0.5 N NaOH and distilled water. Where required, samples were first diluted with distilled water before addition of the base. Samples were filtered (Gelman ARCO LC25 syringe filter) and analyzed for 2,4-D with an Altex model 100A HPLC equipped with a stainless steel column (Spherisorb ODS, 150 mm × 4.6 mm, particle size 5 μm), a UV detector set at 229 nm, and an integrator. The mobile phase was acetonitrile-phosphate buffer solution (40 parts acetonitrile: 60 parts 131.8 mM phosphate, pH 2.8) pumped at a flow rate of 2.0 ml/min which generated a pressure of 15 215 kPa. Analytical grade 2,4-D was used for HPLC standards. A standard curve (peak area vs. concentration) was generated in the range of 2 to 100 mg of 2,4-D per liter. The retention time of 2,4-D under these HPLC conditions was about 3.14 min.

RESULTS AND DISCUSSION

Enrichment cultures derived from samples of a holding pond, bioreactor, and three soils obtained from the fertilizer manufacturing plant site (designated as OMS, BR, S1, S2, and S3, respectively) were able to utilize 2,4-D as the sole carbon and energy source under aerobic conditions. The reference sample from the activated sludge basin did not yield 2,4-D degrading bacteria under these experimental conditions. Twenty isolates, each capable of utilizing 2,4-D as its sole carbon source, were derived from the OMS and BR cultures with solid media containing 2,4-D, thereby suggesting that these test cultures were mixtures of 2,4-D utilizing bacteria. Colonies developing on 2,4-D-containing agar media remained too small for quantitative count. Some isolates also grew in mineral salts agar media without 2,4-D. Microscopic examination of the 2,4-D-grown isolates revealed that all were Gram-negative, rod-shaped cells. The isolates varied in pigmentation.

No further effort was made to characterize the pure cultures and their potential interactions in the present work. It was beyond the scope of the study to differentiate between the primary 2,4-D degraders and those that may have survived by using metabolites excreted by others. The possibility should be recognized that the test cultures may have contained also bacteria that were present owing to their ability to utilize specific downstream metabolites of 2,4-D degradation. It is clear that bacterial interactions, including exchange of genetic information, in mixed cultures may be very complex and warrant further studies at all levels of information. In particular, a better understanding of dynamic interactions and stability of desired traits in mixed cultures are important areas for future developments in the use of mixed cultures for the treatment of hazardous waste materials.

Both the OMS and BR cultures were able to grow with up to 1000 mg 2,4-D/l which was the highest concentration tested. Degradation of 2,4-D is an acid-yielding reaction due to the release of the chloride atoms. Without a pH control, the initial pH 7.4 decreased to pH 3.6 in cultures with 750 mg 2,4-D/l and effectively prevented further biodegradation. A near-neutral pH optimum has been reported for several pure cultures of 2,4-D degrading bacteria, whereas the degradation of 2,4-D in environmental materials has been observed at pH values as low as pH < 5.3 [3,9].

Growth of these batch cultures typically displayed a lag period of 4 to 6 days before the onset of growth, as

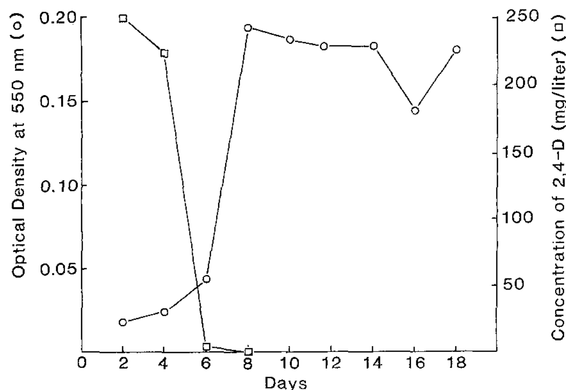

Fig. 1. Growth curve of the OMS culture with 2,4-D as the sole substrate. Symbols: ○, turbidity; □, 2,4-D concentration.

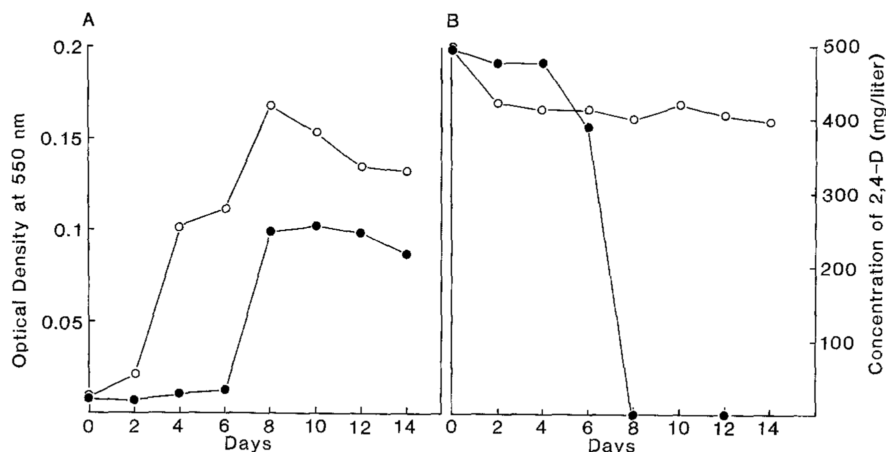

Fig. 2. Growth curve of the OMS culture with 2,4-D and glucose. (A) Culture turbidity with 2,4-D only (●) and with 2,4-D and glucose (○). (B) Concentration of 2,4-D in the culture solution in the presence (○) and absence (●) of glucose.

shown in Fig. 1 for the OMS culture. In general, increases in culture turbidity coincided with decreases in 2,4-D concentration. Excluding the lag phases, the rates of 2,4-D degradation were in the range of 100–140 mg of 2,4-D/l/day in actively growing batch cultures in shake flasks. A close examination of the time course data revealed that the 2,4-D degradation commenced before an increase in turbidity was apparent. This effect may be attributed to the initial events of 2,4-D degradation which do not yield metabolically useful energy for the growth.

The addition of glucose in the range of 500 to 1000 mg/l to the OMS culture essentially prevented the biological degradation of 2,4-D but the bacteria grew to a higher cell density when compared with 2,4-D as the substrate in the absence of glucose (Fig. 2).

The microbiological degradation of 2,4-D occurs via two major pathways [1]: (i) one pathway forms 2,4-DCP and glyoxylate by removal of the two-carbon side chain from the ring structure of 2,4-D; (ii) the other pathway forms 6-hydroxy-2,4-dichlorophenol (6-H-2,4-DCP) by hydroxylation of C-6 position of the ring, and then 3,5-dichlorocatechol by the removal of acetate from 6-H-2,4-DCP. The HPLC chromatograms of culture filtrates displayed no additional major peaks for the OMS culture, whereas the BR culture produced a metabolite that appeared as an additional peak in the chromatogram (Fig. 3). Further diagnostic work suggested that the metabolite was 2,4-dichlorophenol (2,4-DCP) because a reference sample of 2,4-DCP displayed an identical retention time to that of the unknown peak. The identification of 2,4-D and 2,4-DCP was confirmed by HPLC analysis of mixture of analytical grade 2,4-D and 2,4-DCP. Linearity was achieved in the range of 2–100 mg of each l and the ratio of peak positions (2,4-DCP/2,4-D) varied between 1.23 and 1.27 depending on the HPLC conditions.

Analytical agreement was good for 2,4-D determination by the HPLC technique and by UV spectrometry. The peak of maximum absorption was 283 nm for 2,4-D. With both methods, the detection range was linear between 2 and 100 mg of 2,4-D/l. While both methods are relatively fast and sufficiently sensitive for applied mixed culture work, UV-spectrometry is not suitable to quanti-

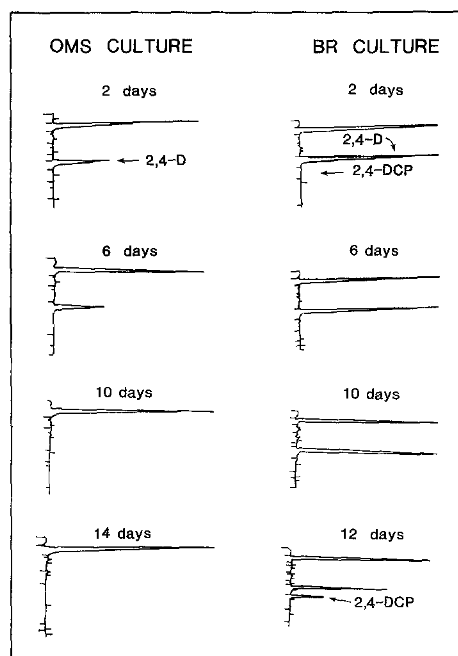

Fig. 3. HPLC chromatograms of filtered culture samples. The OMS culture samples display no additional major peaks; the BR culture samples display an additional peak at about 4.60 min which represents the formation of 2,4-DCP after 12 days of incubation.

tate 2,4-D if UV-absorbing intermediates accumulate. HPLC methodology developed for 2,4-D is based on reverse-phase and will not therefore resolve polar intermediates.

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