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 spp. [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.

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.

For analytical grade and technical grade 2,4-D were used in the experiments. The mineral salts medium contained (per liter): K$_2$HPO$_4$, 0.5 g; (NH$_4$)$_2$SO$_4$, 0.5 g; MgSO$_4$·7H$_2$O, 0.5 g; FeCl$_3$·6H$_2$O, 10 mg;
CaCl₂·2H₂O, 10 mg; MnCl₂, 0.1 mg; ZnSO₄, 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...