Biodegradation of the Phenoxy Herbicides 2,4-D and MCPP

Kye-Heon Oh

Department of Genetic Engineering, Soonchunhyang University

INTRODUCTION

Chlorine-substituted phenoxy herbicides were introduced as aquatic and terrestrial herbicides in the late 1940's (Sinton *et al.*, 1986). The phenoxy herbicides are growth-regulating compounds, designated as auxins which are selectively toxic to most annual and perennial dicotyledonous species and relatively nontoxic to monocotyledonous plants (Loos, 1975; Kirby, 1980; Sandmann *et al.*, 1988). These compounds have found applications in a wide variety of situations, including weed control in lawns, gardens, cereal crops and pastures as well as defoliants in forestry and warfare. The U.S. production of 2,4-dichlorophenoxyacetic acid (2,4-D) alone was estimated at 24 to 30 million kg for 1982 (USEPA, 1988). It is applied directly or in the form of various salts and esters. Although the use of 2,4-D and related herbicides provides many benefits in the world, the wide and sometimes indiscriminate use of these compounds has resulted in environmental problems to man. Environmental pollution problems resulting from use of these compounds have confronted most countries in the world. It is, therefore, important to develop effective means of treating waste streams and to understand the fate of these compounds in the environment. Some commonly used phenoxy herbicides are listed in Table 1.

The mechanism of 2,4-D degradation has been elucidated in several bacteria including *Pseudomonas* spp. (Evans *et al.*, 1971; Kilpi *et al.*, 1980). Enzymes involved in the degradative metabolism of 2,4-D are commonly plasmid borne in *Pseudomonas* (Pemberton and Fisher, 1977) and *Alcaligenes* (Fisher *et al.*, 1978; Don and Pemberton, 1981). A number of studies have been published on the occurence and distribution of 2,4-D degrading bacteria in soil and other natural habitats (Cullimore, 1981; Ou, 1984; Ditzelmuller *et al.*, 1989). Although a wealth of information exists in the biochemistry and genetics of 2,4-D degradation by bacteria, the utility of the information has not been evaluated for possible treatment processes of industrial waste streams where 2,4-D may represent a serious disposal problems.

Little information is available on the microbial degradation of MCPP. Until 1990, there were no reports in the literature on the metabolism of MCPP by a pure culture. Lappin *et al.* (1985) reported that a microbial community containing five bacterial members isolated from a plant root degraded MCPP. Horvath *et al.* (1990) mentioned that a *Flavobacterium* sp. isolated from soil were able to degrade MCPP but no further information in this isolate is presently available. Lindholm *et al.* (1982) proposed two mechanisms for the bacterial degradation of MCPP; (i) the formation on MCPA from MCPP; and (ii) the cleavage of the side chain to form 2-methyl-4-chlorophenol (2,4-MCP). Smith (1985) identified 2,4-MCP as an intermediate in bacterial degradation of MCPP using radiolabelled-MCPP and thin layer chromatography. The definite pathway of MCPP degradation has yet to be elucidated. These proposed pathways are presented in Fig. 1.

Phenoxy herbicides occur in liquid and solid waste stream at those fertilizer manufacturing plants that use these compounds for commercial formulations. 2,4-D is a regulating compound due

Table 1. Common and chemical names of phenoxy herbicides.

Common name	Chemical name
2,4-D	2,4-Dichlorophenoxyacetic acid
MCPA	(4-Chloro-2-methylphenoxy) acetic acid
MCPP	2-(2-methyl-4-chlorophenoxy) propionic acid
MCPB	4-(4-Chloro-2-methylphenoxy) butyric acid
2,4-DB	4-(2,4-Dichlorophenoxy) butyric acid
Dichloroprop	2-(2,4-Dichlorophenoxy) propionic acid
Fenoprop	2-(2,4,5-Trichlorophenoxy) propionic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid

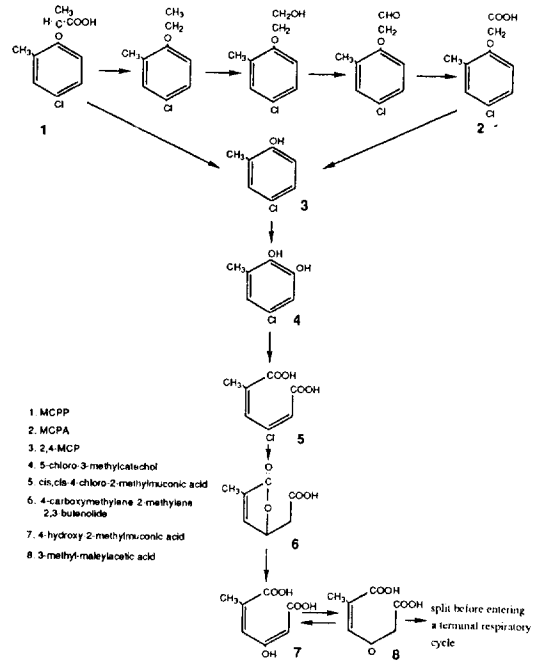

Fig. 1. Hypothetical metabolic pathways for MCPP.

to its toxicity; solids containing 2,4-D in excess of 1000 ppm are classified as hazardous. MCPP displays lower toxicity and a threshold level to regulate environmental discharge has not been defined MCPP.

The present study was undertaken to characterize the biodegradation of phenoxy herbicides, 2,4-D and MCPP, under aerobic conditions. Specifically, the work had the following objectives; (i) to develop and characterize bacterial mixed cultures for the 2,4-D and MCPP degradation, (ii) to evaluate the degradation of 2,4-D and MCPP in shake-flasks and stirred tank reactors; and (iii) to evaluate the treatment of industrial fertilizer solid waste material containing 2,4-D and MCPP.

MATERIALS AND METHODS

Bacteria and growth conditions

The mixed cultures were originally enriched from samples of soils collected from a fertilizer manufacturing plant site (S1 and S2). The cultures were enriched with 2,4-D and MCPP separately as the sole source of carbon and energy. The cultures were maintained separately on technical grade 2,4-D and MCPP (1g/liter) in a mineral salts solution (Oh and Tuovinen, 1990). The media were adjusted to pH 7.4 with NaOH before autoclaving. Cultures were grown in shake flasks (156 rpm) at 21℃. The test cultures comprised Gram-negative rods which were tentatively identified as *Pseudomonas* spp., *Alcaligenes* spp., *Achromobacter* spp., and *Flavobacterium* spp. using Rapid NFT API strips and fatty acid profiles.

Subsequent experiments on mixed substrate utilization were performed using 2-liter stirred tank reactor which was fitted with a water condenser (5℃) and operated with a stir rate of 156 rpm and airflow rate of 1.5 liter/min. A 10% (vol/vol) inoculum if the test cultures initially grown in shake flasks were used. Growth was determined by absorbance at 550 nm using Varian 2200 spectrophotometer and by protein determination (Lowry *et al.*, 1951).

Analytical methods

The HPLC system consisted of a pump (Altex model 100A), an injector fitted with 100 μl loop,

UV detector (Hitachi 100-40), and an integrator (Hewlett-Packard H3396A). A Phenomenex ODS column (150 mm×4.6 mm, particle size 5 μm) was eluted with a mobile phase which contained 40% (vol/vol) acetonitrile and 60% (vol/vol) phosphate buffer (6g K_2HPO_4 and 3 m*l* concentrated H_3PO_4 per liter). The flow rate of the mobile phase was 1.8 m*l*/min. The analytical methodology has been previously described in detail (OH and Tuovinen 1991).

For GC-MS analyses, 30 m*l* of centrifuged culture sample (8,000×g, 20 min.) was acidified to pH 3 with 6N HCl, and was extracted twice with equal volume of ethyl acetate. The solvent was removed under vacuum and the residues were redissolved in dichloromethane. MS data were obtained with a Hewlett-Packard 5970 mass selective detector equipped with a Hewlett-Packard 5890 gas chromatograph. A DB-1 capillary column (30 m by 0.25 mm; J & W Scientific) was used. The analytical methodology for GC-MS has been previously described (Oh and Tuovinen, 1991).

UV-spectrometry was used to monitore the degradation of herbicides. Samples of bacterial cultures were centrifuged at 3,500×g for 10 min to remove the turbidity before UV analyses of the supernatant medium. Standard solutions were made of analytical grade 2,4-D and MCPP in the range of 2 to 100 mg/*l*. The adsorption spectra of centrifuged culture media were recorded from 340 to 220 nm with a Varian 2200 spectrophotometer.

Inorganic chloride was assayed coulometrically by using a chloridometer (Haake Buchler Instruments, Inc., Saddle Brook, NJ).

Industrial fertilizer solid waste

Industrial solid waste material was obtained from a fertilizer manufacturing plant. The waste material was granular. The main bulking agent was vermiculite. The sample contained (wt/wt) 27.4% N, 3.3% P_2O_5, 2.8% K_2O, 1.5% 2,4-D and 1.4% MCPP. The sample was construed to represent waste material from spillage at the baggy plant and dust collected at air filters within the plant.

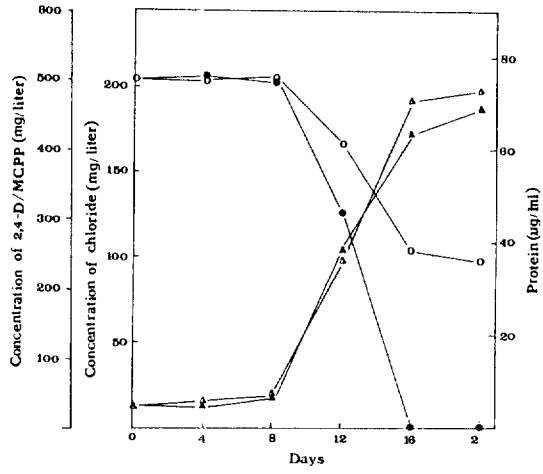

Fig. 2. Concurrent degradation of 500 mg/liter each of 2,4-D (●) and MCPP (○) by culture S1 and the associated changes in protein (▲) and inorganic chloride (△) concentrations. Culture S1 was adjusted to pH 7.4 every four days.

The precise chemical form of nitrogen was not analyzed but it was known to include urea-type fertilizer-N. Phosphate was the exclusive from of phosphorous in P_2O_5. The chemical and physical characterization of fertilizer solid waste material has been previously presented (Oh and Tuovinen, 1989).

RESULTS

In initial experiments, the degradation of 2,4-D and MCPP was studied with two test cultures previously grown with the respective single substrate. Cultures previously grown with 2,4-D completely degraded this substrate but could not degrade MCPP. Cultures initially enriched with MCPP were able to partially degrade this substrate whereas 2,4-D was degraded to completion.

Changes in biomass and inorganic chloride concentration upon degradation of 2,4-D and MCPP with pH adjustment are shown in Fig. 2 for culture S1. Complete degradation of 2,4-D was achieved in this experiment within 16 days during which time only about 50% of the co-substrate, MCPP, was degraded. The concentration of inor-

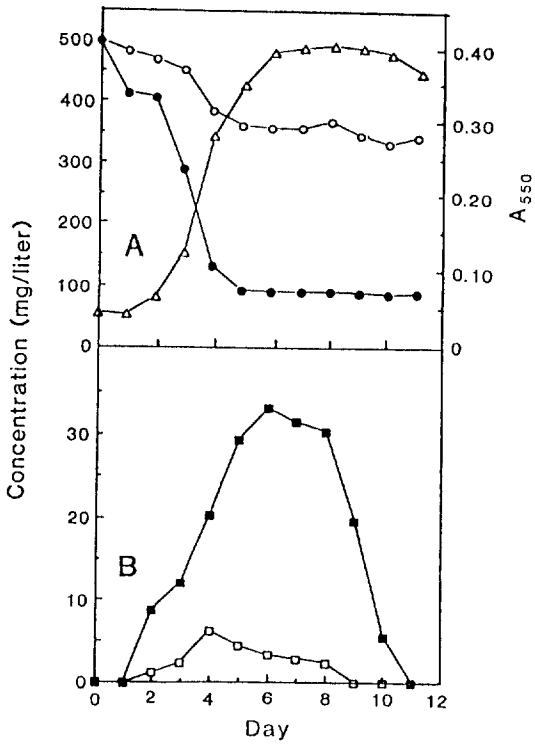

Fig. 3. Growth of test culture S1, measured as cell density (△), associated with concurrent degradation of 0.5g/liter each of MCPP (○) and 2,4-D (●) (A), and the parallel formation of 2,4-MCP (■) and 2,4-DCP (□) (B).

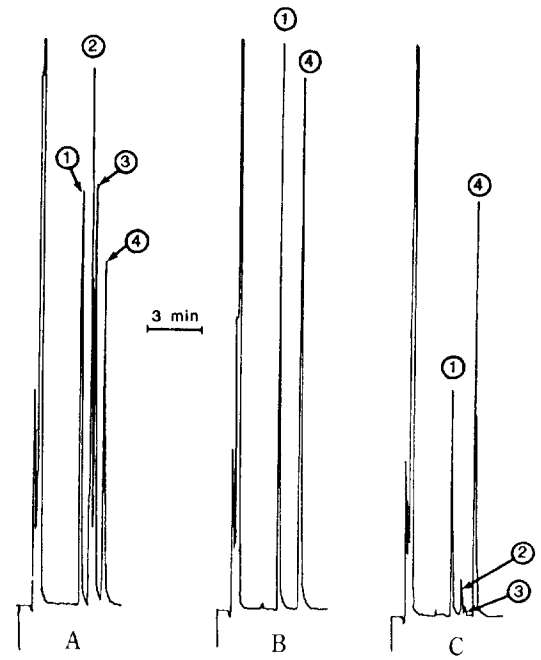

Fig. 4. HPLC chromatograms of standards (A) and of culture samples initially (B) and after 4 days of incubation (C).

ganic chloride as a measure of dechlorination was in good agreement with the residual substrate concentration, based on molar ratios of 2Cl per 2,4-D and 1Cl per MCPP. The inorganic chloride concentration was within 94.7% agreement of the value calculated from the 2,4-D and MCPP degradation data.

When grown in shake flasks with a mixture of MCPP and 2,4-D, the test culture completely utilized 2,4-D whereas only about 50% of MCPP was degraded. The residual MCPP persisted upon prolonged incubation. The time course of mixed substrate utilization in a stirred tank reactor is shown in Fig. 3A. The substrates, MCPP and 2,4-D, were degraded within the first six days of incubation but the degradation did not proceed to completion. 32% of MCPP and 83% of 2,4-D were utilized under these conditions. 2,4-MCP and 2,4-dichlorophenol (2,4-DCP) were detected as the major intermediates in the growth medium in this experiment (Fig. 3). Their concentration were transient, reaching levels as high as 33 mg of 2,4-MCP and 6 mg of 2,4-DCP per liter.

The detection of the intermediates was based on HPLC methodology. The chromatograms shown in Fig. 4 demonstrate that both parent herbicides, MCPP and 2,4-D, and respective intermediates, 2,4-MCP and 2,4-DCP, can be successfully resolved under these analytical conditions. The retention times for the four compounds were 3.23 min for 2,4-D (peak 1), 3.68 min for 2,4-MCP (peak 2), 3.80 min for 2,4-DCP (peak 3), and 4.51 min for MCPP (peak 4). The peaks obtained with culture samples were in complete agreement with those of authentic standards. Plots of concentration vs. peak area for each of the four compounds displayed linearity within 2-200 mg per liter range.

GC-MS data are shown in Fig. 5 for a culture sample analyzed after 4 days of incubation. The total ion chromatogram (TIC) of this sample disp-

Table 2. Spectral changes in culture media containing 2,4-D and MCPP. The maximum absorption is underlined.

Incubation (days)	Absorbance				
	283 nm	282 nm	281 nm	280 nm	279 nm
0	1.4456	1.4562	1.4499	1.4250	1.3851
1	1.4108	1.4239	1.4168	1.3904	1.3580
2	1.3745	1.3881	1.3827	1.3573	1.3251
3	1.2603	1.2728	1.2707	1.2513	1.2197
4	0.9097	0.9233	0.9312	0.9262	0.9115
5	0.7806	0.7973	0.8057	0.8053	0.7967
6	0.7743	0.7919	0.8011	0.8017	0.7933
7	0.7761	0.7912	0.8018	0.8021	0.7925
8	0.7784	0.7944	0.8043	0.8053	0.7962
9	0.7735	0.7926	0.8022	0.8047	0.7938
10	0.7741	0.7933	0.8030	0.8044	0.7931
11	0.7728	0.7929	0.8035	0.8039	0.7951

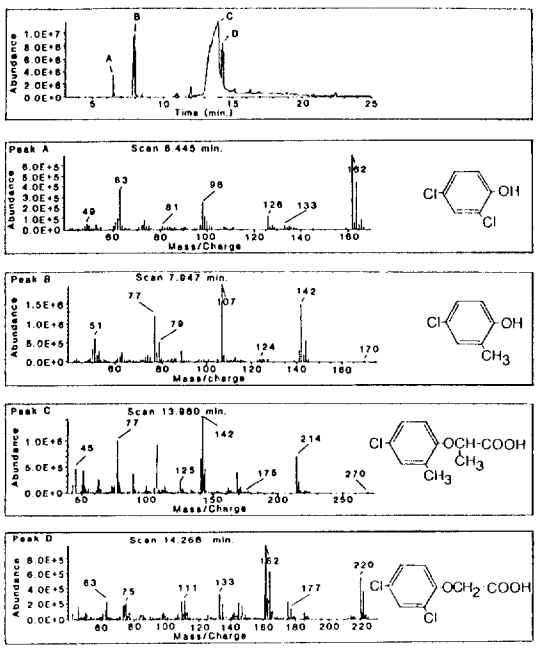

Fig. 5. GC-MS data for a culture sample analyzed after 4 days of incubation. MS fragmentation patterns of the compounds representing the four peaks (A-D) in the TIC are indicated and the respective chemical structures of the compounds are also given.

layed four major as well as a few minor peaks (Fig. 5). The major TIC peaks yielded positive identification based on mass/charge by MS for 2, 4-DCP (TIC peak A at 6.445 min), 2,4-MCP (TIC peak B at 7.947 min), MCPP (TIC peak C at 13.980 min), and 2,4-D (TIC peak D at 14.286 min) (Fig. 5). These data are in keeping with the respective data shown in Fig. 4C for this particular sample.

Biodegradation of 2,4-D and MCPP in 2% (wt/vol) solid waste suspension was studied in a stirred tank reactor with mineral salts (Fig. 6). Complete 2,4-D degradation and partial MCPP degradation (46%) by culture S2 occurred in the mineral salts solution.

The peaks of maximum absorption was observed in the test culture at 279 nm for MCPP, 283 nm for 2,4-D, and 282 nm for a mixture of MCPP and 2,4-D. In samples of culture media, the wavelength of maximum absorption shifted from 282 nm to 280 nm toward the end of the incubation. The shift was gradual (Table 2), reflecting a change in the relative proportion of the two herbicides remaining in the culture solution after 11 days of incubation. The spectral shift of the wavelength of maximum absorption from 282 to 279, characteristic of MCPP, is attributed to the complete degradation of 2,4-D.

DISCUSSION

The reason for the incomplete degradation of

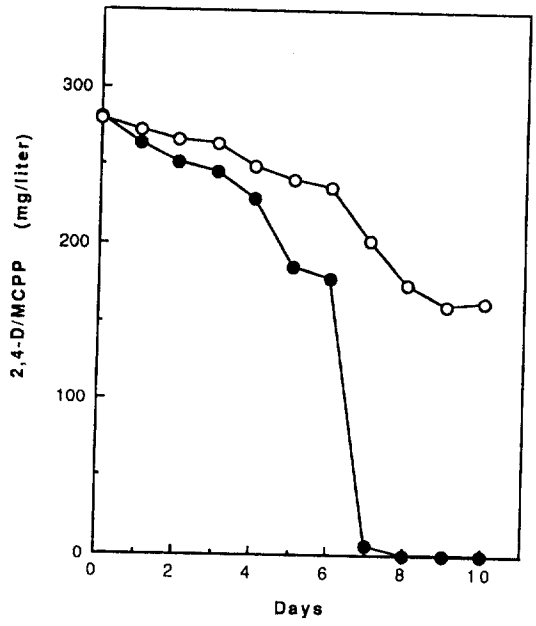

Fig. 6. Concurrent degradation of 2,4-D (●) and MCPP (○) by culture S2 in a stirred tank reactor with mineral salts. The pulp density was 2% (wt/vol).

the parent herbicides in the present work is not known. One possible explanation is that it may be related to the increased toxicity of the corresponding phenolic intermediates. 2,4-DCP is a recognized intermediate associated with the microbiological degradation of 2,4-D (Loos *et al.*, 1967; Greer *et al.*, 1990). 2,4-DCP in cultures grown with 2,4-D as the sole substrate has previously demonstrated (Oh and Tuovinen, 1989). Intermediates of MCPP degradation have not been elucidated. The detection of 2,4-MCP as an intermediate of MCPP degradation has been reported only in one study which was based on incubating [ring-^{14}C] MCPP in a soil environment; the resolution of 2,4-MCP was achieved by thin-layer chromatography (Smith, 1985). Lindholm *et al.* (1982) did not find any intermediates with GC methodology in experiments involving incubation of MCPP- amended soil samples. 2,4-MCP, identified by HPLC and verified by GC-MS in the present work, is concluded to be the first corresponding phenol intermediate during the bacterial degradation of MCPP. Its further metabolism has yet to be elucidated. Kilpi (1980) proposed MCPA as an intermediate of MCPP degradation but no evidence was obtained for MCPA in the present work.

The incomplete degradation of MCPP may be a result of several factors, including the following: (i) the test cultures were sensitive to intermediates of MCPP degradation, (ii) the cultures displayed stereospecificity for one isomer in the recemic mixture (about 50% each) of MCPP; and (iii) the degradation of MCPP proceeds via a pathway that does not yield metabolically useful products for intermediate carbon or energy metabolism. Because similar incomplete degradation also occurred in a single substrate experiments, it can be concluded that 2,4-D neither inhibited nor stimulated the degradation of MCPP. The incomplete removal of MCPP was not related to the industrial solid waste material because a residual of MCPP also persisted in cultures grown with analytical grade MCPP in the absence of the solids. These questions warrant further work on resolving intermediates and on elucidating degradative pathway (s) with other experimental approaches such as enzyme assay, isotope techniques, and separation of racemic forms by chiral HPLC column.

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