

# Microbiological Degradation of the Phenoxy Herbicide MCPP [2-(2-Methyl-4-Chlorophenoxy) Propionic Acid]

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The microbiological degradation of 2-(2-methyl-4-chloro-phenoxy)propionic acid (MCPP) was evaluated using mixed cultures of soil bacteria. The mixed cultures comprised *Pseudomonas* species, *Flavobacterium* species, and *Achromobacter* species. The bacteria used MCPP as the sole source of carbon and energy but only a partial degradation of the parent compound occurred. MCPP degradation proceeded via the formation of 2-methyl-4-chlorophenol (2,4-MCP) which was detected by high pressure liquid chromatography (HPLC) and confirmed by gas chromatography-mass spectrometry. This intermediate occurred only transiently and no evidence was seen for the presence of other intermediates detectable by the reverse-phase HPLC or UV absorbance.

**KEY WORDS** □ MCPP; 2,4-MCP; Biodegradation; Chlorinated phenols; Herbicide degradation; Phenoxy herbicides

2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP) is mostly used to enhance herbicidal properties of other structurally related phenoxyalkanoic acids such as 2,4-dichlorophenoxyacetic acid (2,4-D), which are active against broad-leaf weeds. MCPP is chemically relatively stable but its susceptibility to the microbiological degradation has been demonstrated in soil incubation studies (4, 7, 11, 12). Mixed bacterial cultures have been previously isolated that can actively grow with MCPP as the sole source of carbon and energy (3, 5, 6). Horvath et al. (1) isolated a *Flavobacterium* sp. from a mixed culture which was initially enriched in a 2-(2,4-dichlorophenoxy) propionic acid (2,4-DP)-amended soil column. This strain utilized 2,4-DP and MCPP as the sole source of carbon and energy, but no other information on this microorganism is presently available to evaluate its environmental significance and degradative mechanism.

The biodegradative pathway of MCPP has not been elucidated. Lindholm *et al.* (7) proposed two mechanisms for the initial biodegradation of MCPP: (i) the formation of 4-chloro-2-methylphenoxyacetic acid (MCPA) from MCPP which is then transformed to 2-methyl-4-chlorophenol (2,4-MCP); (ii) the cleavage of the propionate side

chain to directly form 2,4-MCP. Intermediate MCPA has yet to be detected during biodegradation of the MCPP parent molecule. Smith (11) identified 2,4-MCP as an intermediate during the microbiological degradation of [ring-<sup>14</sup>C]-MCPP in soil. We have been previously described the characteristics of enriched cultures which utilized MCPP as well as 2,4-D as the source of carbon and energy (10). Results obtained with other cultures derived from the same source have suggested the formation of trace levels of phenolic intermediates from MCPP (2). For the present study, MCPP was used as the sole substrate for bacterial cultures under aerobic conditions with a view to establishing the initial intermediate of MCPP degradation.

## MATERIALS AND METHODS

### Bacteria and growth conditions

Initially, three mixed bacterial enrichment cultures capable of utilizing MCPP as the sole source of carbon and energy were derived from soil samples collected from a fertilizer manufacturing plant site. The identification of the mixed cultures was performed using Rapid NFT-API System (Analytical Products, Ayerst Laboratories, Inc.,

Plainview, NY). For enrichment, soil samples (5 g) were used to inoculate 100 ml of liquid media in 250 ml shake flasks (156 rpm) at 22°C. The cultures were maintained in a mineral salts medium previously described (9). The concentration of MCPP used in this study was 0.25-1 g (1.16-4.66 mM). The pH of medium was adjusted to 7.4 with NaOH before autoclaving (121°C, 15 min).

The biodegradation experiments were carried out using a 2-liter fermentor stirred at 156 rev/min and aerated at 1.5 l/min. The reactor was maintained at 22°C.

#### Analytical methods

Growth was monitored by measuring the optic density ( $A_{550}$ ) using a Varian 2200 UV/Vis spectrophotometer. Protein content in the culture solutions was determined by the method described by Lowry *et al.*, (8) using bovine serum albumin (Sigma Chemical Co., St Louis, MO) as the standard.

Inorganic chloride was assayed coulometrically by using a chloridometer (Haake Buchler Instruments Inc., Saddle Brook, NJ). MCPP and metabolites were analyzed by reverse phase HPLC. Samples for HPLC were centrifuged; if necessary, the supernatants were diluted with 0.5 N NaOH and HPLC grade water before filtration through a 0.45  $\mu$ m Gelman Arco LC25 disposable syringe filter. An equal volume of 1 N acetic acid was added to filtered solution before injection (20  $\mu$ l) into the HPLC system. Standard stock solution of MCPP and 2,4-MCP (1-200 mg/liter) for HPLC were prepared in 0.0125 N NaOH which contained 40 ml of glacial acetic acid per liter. HPLC conditions have previously described (9).

For GC-MS analyses, 30 ml aliquots of centrifuged culture samples (8,000 $\times$ g, 20 min) were acidified to pH 3 with 6 N HCl, followed by extraction twice with equal volume of ethyl acetate. The solvent was removed under vacuum and the residue was redissolved in dichromethane. 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.

Technical grade MCPP (Dow Chemical Co.) was used all media formulations. The formulation contained 98% MCPP, determined by HPLC analysis. For HPLC and GC-MS analyses, analytical grade MCPP and 2,4-MCP were obtained from Dow Chemical Co. and Aldrich Chemical Co., respectively.

## RESULTS AND DISCUSSION

Initially, three cultures (designated as S1, S2, and S3) were enriched from soil samples, with 0.25 g of MCPP/liter as the sole carbon and energy source under aerobic conditions. The cultures

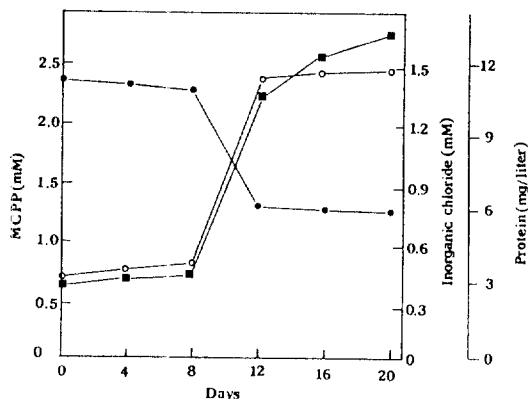


Fig. 1. Growth of test culture S1 in shake flasks, measured as the concentration of residual MCPP (●), chloride (○), and cell protein (■).

were subsequently maintained as mixed cultures in a mineral salt solution containing 1 g of technical grade MCPP/liter.

Changes in inorganic chloride and protein concentration upon degradation of 2.3 mM MCPP are shown Fig. 1 for the test culture S1. The release of inorganic chloride amounted to 0.9 mM concentration and agreed with the observed utilization of 0.9 mM MCPP. The release of inorganic chloride was thus stoichiometric (1 Cl / 1 MCPP) with respect to the degradation of MCPP. A linear relationship ( $r=0.99$ ) was established between the degradation of MCPP and release of inorganic chloride. Both the dechlorination reaction and HPLC data indicated that MCPP was only partially (ca. 50%) degraded in the test culture S1. Cultures S2 and S3 also displayed partial degradation of the substrate, confirmed by HPLC and inorganic chloride data. The concentration of MCPP remained constant in uninoculated control flasks.

In culture S1, the increase in inorganic chloride concentration paralleled protein concentration (Fig. 1). The dechlorination and degradation of 0.9 mmol MCPP was accompanied with a net increase of approximately 10 mg of protein/liter. Similar ratios were determined for test cultures S2 and S3.

At 1 g of MCPP/liter (4.7 mM), the degradation was again incomplete, with about 50% parent substrate utilized within eight days, and it was associated with a pH decrease from 7.4 to 6.3. Periodic neutralization of the pH did not increase the total amount of MCPP degraded. When the medium was supplemented with yeast extract (tested in the range of 0.2-1 g/liter), it enhanced the initial degradation but the incomplete degradation (46-47%) of MCPP persisted.

The degradation of MCPP was also evaluated in a stirred tank reactor. The test culture S3 unde-

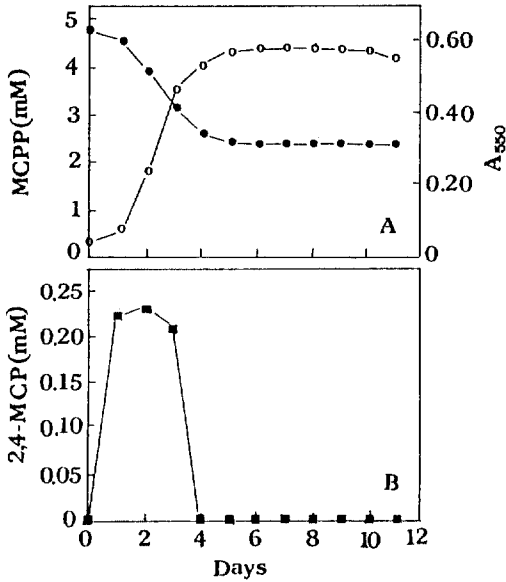


Fig. 2. Growth of test culture S3 in a stirred tank reactor, measured as (A) cell density (○) and degradation of MCPP (●); and (B) the formation of 2,4-MCP (■).

went a rapid phase of MCPP degradation (Fig. 2). During the first five days of incubation, the rate of MCPP degradation was approximately 100 mg (0.46 mmol)/day. The HPLC data indicated that approximately 50% of MCPP was removed after five days of incubation. 2,4-MCP was detected as the major intermediate in the growth medium in this experiment (Fig. 2B). The concentration was transient, reaching levels as high as 32.7 mg of 2,4-MCP/liter (0.23 mM). No 2,4-MCP was detected in chemical control flasks.

The chromatograms shown in Fig. 3 demonstrate that the parent herbicide, MCPP, and the respective intermediate, 2,4-MCP, can be successfully resolved under the analytical HPLC conditions used in the present work. The retention times for two compounds were 4.05 min for 2,4-MCP and 4.85 min for MCPP. The peaks obtained with culture samples were in complete agreement with those of authentic standards. Plots of concentration vs. Peak area each of 2,4-MCP and MCPP displayed linearity within 1-200 mg per liter range.

GC-MS data are shown in Fig. 4 for a culture sample analyzed after three days of incubation. The total ion chromatogram (TIC) of this sample displayed two peaks with retention times 7.67 min for 2,4-MCP and 13.15 min for MCPP (Fig. 4). The fragmentation pattern of the peak and molecular ion for 2,4-MCP ( $R_t=7.67$ ,  $m/z=142$ ) and MCPP ( $R_t=13.15$ ,  $m/z=214$ ) were consistent with those of authentic MCPP and 2,4-MCP. The

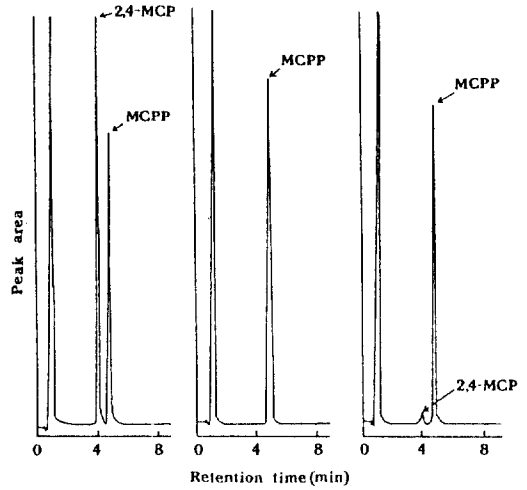


Fig. 3. HPLC chromatograms of (A) authentic standards of MCPP and 2,4-MCP; (B) a culture sample initially; (C) culture sample after three days of incubation.

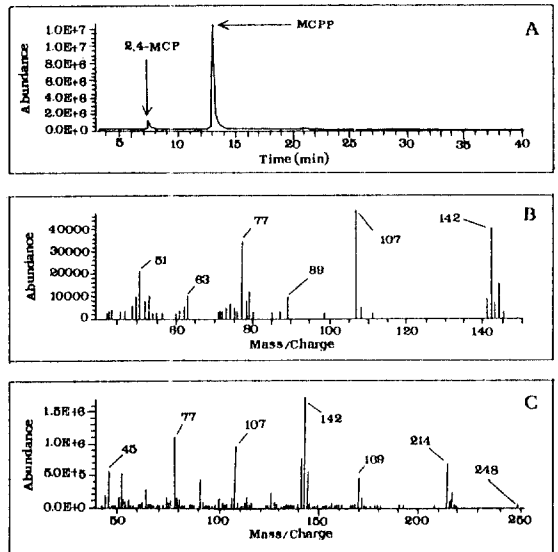


Fig. 4. GC-MS data for a culture sample analyzed after three days of incubation. A, TIC of the sample (2,4-MCP and MCPP peaks indicated with arrows); B, MS fragmentation pattern of the small TIC peak at 7.67 min corresponding to  $m/z$  142 of 2,4-MCP; C, MS fragmentation pattern of TIC peak at 13.15 min corresponding to  $m/z$  214 of MCPP.

presence of MCPP and 2,4-MCP are in keeping with the respective HPLC data shown in Fig. 3C for this particular sample.

The formation of a brown color in culture solu-

tion was occasionally observed in the test cultures grown with MCPP, both in shake flasks and in stirred tank reactor runs. Under the analytical conditions employed, these samples yielded an additional peak in the HPLC chromatogram. It is possible that the brown color is representative of an intermediate such as 3-methyl-4-chlorophenol, formed by repositioning of the methyl group in 2,4-MCP, which was previously detected by thin layer chromatography in radiolabel studies by Smith (11). Analytical grade 2,4-MCP solutions used in the present work for standardizing the HPLC methodology displayed brown discoloration after a few days of storage at room temperature and this coincided with the appearance of multiple peaks in the respective HPLC chromatogram.

The mixed cultures S1, S2, and S3 were also plated with MCPP containing media. Fifteen isolates were initially obtained as small colonies on the primary plates but they all failed to grow in pure culture upon subculture in MCPP liquid media. All isolates grew on trypticase soy agar (TSA) and were gram negative. Radid NFT API tests of TSA-grown isolates indicated that the bacteria could be assigned to *Pseudomonas*, *Flavobacterium*, and *Achromobacter* spp.

While the present work demonstrates 2,4-MCP formation from the parent molecule MCPP, the enzyme activity responsible for the initial removal of the propionate group from MCPP has not been characterized. MCPA was suggested as an intermediate of an alternative, initial pathway in the biodegradation of MCPP, but its formation was not established (7). In the present work, only intermediate 2,4-MCP was detected. If the pathway is via MCPA formation, this would indicate that the reaction of MCPA to 2,4-MCP is faster than the conversion of MCPP to MCPA.

The incomplete degradation of MCPP was consistently observed in the shake flask and stirred tank reactor cultures. MCPP in herbicide formulation is used as a racemic mixture and it is not known how the isomers vary in their biodegradability. The resolution of the (+) and (-) isomers of MCPP may provide further insight into incomplete biodegradation.

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초 록: 페녹시계 제초제 MCPP [2-(2-Methyl-4-Chlorophenoxy) Propionic Acid]의 미생물학적 분해

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토양세균의 혼합배양에 의한 MCPP[2-(2-methyl-4-chlorophenoxypropionic acid)]의 미생물학적 분해를 조사하였다. 혼합배양은 *Pseudomonas*종, *Flavobacterium*종, 그리고 *Achromobacter*종을 포함하였다. 세균들은 유일한 탄소 및 에너지원으로서 MCPP를 이용하였으나, 부분적인 분해만을 보여주었다. MCPP의 분해는 2-methyl-4-chlorophenol의 형성에 의하여 진행되었으며, 이는 HPLC에 의하여 발견되었고, GC-MS에 의하여 확인되었다. 이 중간대사물질은 일시적으로 생성되었으며, reverse-phase HPLC와 UV absorbance에 의하여 다른 대사산물의 존재는 관찰되지 않았다.