한국생물공학회지 제 9 권 제 5 호
Korean J. Biotechnol. Bioeng.
Vol. 9. No. 5. 469-474(1994)

미생물 컨소시엄에 의한 시판 페녹시계 제초제 2, 4-D의 생물분해

*오 계 헌 · 김 용 석
*순천향대학교 공과대학 유전공학과
순천향대학교 자연과학대학 생물학과

Biodegradation of the Commercial Phenoxy Herbicide 2, 4-D by Microbial Consortium

*Kye-Heon Oh and Yong-Seok Kim
*Department of Genetic Engineering, Department of Biology
Soonchunhyang University, Onyang 337-880, Korea

ABSTRACT

The purpose of the work was to evaluate the feasibility of a biological treatment process for the phenoxy alkanoic herbicide 2,4-D(2,4-dichlorophenoxyacetic acid) as a commercial pesticide. The phenoxy herbicide was 2,4-D amine salts which contained 40%(vol/vol) 2,4-D and 60%(vol/vol) solvent. A microbial consortium has been derived by enrichment with 2,4-D. The consortium utilized 2,4-D as the sole source of carbon and energy. Optimal pH on the 2,4-D degradation was 7.0 in this experiment. As concentrations of 2,4-D were increased, the degradation by microbial consontium became inhibited. The amendment with yeast extract and ascorbic acid accelerated the degradation of 2,4-D. High performance liquid chromatography methodology was used to measure 2,4-D and it also resolved 2,4-DCP(2,4-dichlorophenol), the corresponding phenol as intermediate. Gas chromatography-mass spectrometry was used for preliminary identification of the intermediate 2,4-DCP. UV scans of spent cultures showed that the maximum absorption of 2,4-D at the wavelength of 283 nm was decreased toward the end of incubation, but the consortium displayed no detectable spectral changes or peak shifts in the UV absorbance.

INTRODUCTION

The phenoxy alkanoic compound 2,4-dichlorophenoxyacetic acid(2,4-D) is growth-regulating herbicide(1). 2,4-D is a regulated compound due to its toxicity; solids containing 2,4-D in excess of 1,000 ppm are classified as hazardous(2). 2,4-D amine salts or alkali or esters are used as agricultural herbicides against broad leaf weeds in cereal crops as well as on pastures and lawns, in parks, and on golf courses(3). In commercial formulation processes involving phenoxyalkanoic acid herbicide, the level of 2,4-D in liquid and solid waste streams may be excessive and constitute a disposal problem(4, 5). Disposal of unused 2,4-D and washing equipment may result in localized land pollution and also pollution of water supplies through direct contamination or leaching

from soil(3).

2,4-D is readily susceptible to the microbiological degradation. The biodegradative pathways and the underlying genetics and biochemistry of 2,4-D degradation have been elucidated in many studies, and 2,4-D-degrading bacteria are relatively ubiquitous in soils with histories of previous application(1, 2, 4, 6). Repeated 2,4-D treatments promote the establishment of phenoxy herbicide-degrading microorganisms(4, 7). Several bacteria (e.g., *Pseudomonas*, *Alcali-genes*, *Arthrobacter*, *Azotobacter* spp.) have been shown to be capable of utilizing 2,4-D as the sole source of carbon and electrons(8-11).

Some of the environmental conditions that influence the degradation of 2,4-D by bacterial cultures have previously sought to define(12, 13). The biological degradation of phenoxy herbicides is an alternative to non-biological treatment methods such as chemical treatment and thermal destruction, which is the present method of choice of disposal of solid and liquid waste with excessive 2,4-D levels. Biological degradation processes for treating aqueous phenoxy herbicide have been successfully tested (14, 15). In the present feasibility study, a microbiological process for the treatment of phenoxy herbicide 2,4-D as a commercial pesticide was tested in laboratory-scale.

MATERIALS AND METHODS

Bacterial Enrichment and Growth

The microbial consortium, designated as SM1, capable of utilizing 2,4-D as the sole source of carbon and energy was enriched from soil sample collected from rice field near Ye-dang reservoir, Chung-Nam. The consortium was maintained in liquid medium which contained mineral salts (12) supplemented with 2,4-D. The media were adjusted to pH 7.0 with NaOH before sterilization. Biodegradation experiments of commerically available 2,4-D amine salts were carried out using shake flasks (156 rev/min) at 22℃.

Growth was monitored by optical density at 550 nm. Protein content in the culture solutions was determined by the method described by Lowry et al. (16) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard.

Analytical Methods

The HPLC system consisted of a pump (LC1100, ICI), an integrator fitted with 100 μl loop, UV detector (LC1200, ICI), and an integrator (SP4270, ICI). A commercial 5 μl Lichrosorb ODS column was eluted with a mixture of acetonitrile and phosphate buffer (40:60, vol/vol) at a flow rate of 1.5 ml/min. The phosphate buffer contained 6 g of K_2HPO_4 and 3 ml of concentrated phosphoric acid per liter of HPLC grade water. The detection signal was monitored at 229 nm.

For GC-MS analyses, 30 ml of a centrifuged culture sample (8,000 x g, 20 min) was 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 dichloromethane. MS data was 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) was used, and programmed from 70 to 250℃ at 19℃/min. The injector temperature was 250℃. The carrier was helium gas at 1.0 ml/min. Reverse-phase HPLC and GC-MS analysis of 2,4-D have been previously described(14).

UV-spectrometry was also used to monitor the degradation of 2,4-D in the cultures. Bacterial cultures were centrifuged at 6,000 x g for 15 min (4℃) before dilution with double-distilled water. The absorption spectra of centrifuged culture media were recorded from 310 to 230 nm with an UV/vis spectrophotometer (V-500, Jasco Co., Japan).

Chemicals

2,4-D amine salts used for this study as a target substrate was purchased from Sam-Kong

Chemical Co. The 2,4-D amine salt contained (v/v) 2,4-D(40%) and solvent(60%). Technical- and analytical-grade 2,4-D and 2,4-DCP were obtained from Sigma Chemical Co (St. Louis, MO), and HPLC-grade acetonitrile and water from J.B. Baker Chemical Co. (Phillipsburg, NJ).

RESULTS AND DISCUSSION

Biodegradation of 2, 4-D in Shake Flasks

Enriched microbial consortium, designated as SM1, was able to utilize 2,4-D as the sole source of carbon and energy under aerobic conditions. The consortium was able to grow within up to 216 mg 2,4-D/l which was the highest to concentration tested. Changes in protein concentration and pH upon degradation of 2,4-D are shown in Fig. 1. Complete degradation of 2,4-D at the initial concentration of 108 mg 2,4-D/l was achieved in this experiment within 10 days. Degradation of 2,4-D is an acid-yielding reaction due to the release of the chloride atoms(17). A 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(2). In this experiment, the initial pH 7.0 decreased to pH 3.8 during the culture periods. pH change was well correlated with the residual 2,4-D concentration.

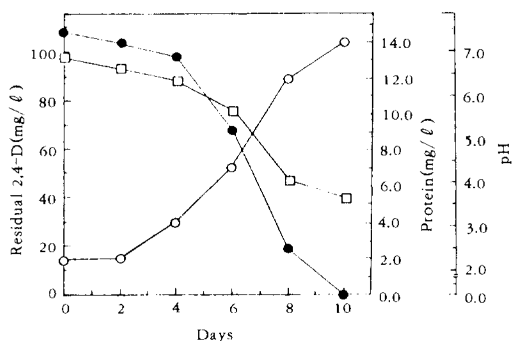


Fig. 1. Degradation of 2,4-D(●) by culture SM1 and the associated changes in concentration of cell protein(○) and pH(□).

Effect of pH

The effect of pH on the degradation of 2,4-D by microbial consortium is shown in Fig. 2. The optimal pHs for the degradation of 2,4-D seem to be 6.0 and 7.0 during the incubation period. In particular, the consortium showed complete degradation of 2,4-D during 10 days of incubation. The consortium also degraded 2,4-D at pH 8.0 and 5.0, but showed partial degradation of 2,4-D. No 2,4-D degradation was observed at pH 9.0 in this study. These results suggest that microorganisms enriched from soil were adapted to the pH because the initial pH of soil collected from environment was around 6.5

Effect of 2, 4-D Concentration

To evaluate the effect of different 2,4-D concentrations, the microbial consortium was cultivated in the mineral salts medium with 216, 108, and 54 mg/l of 2,4-D as the source of carbon. The consortia grown at the concentrations of 54 mg and 108 mg 2,4-D/l completely degraded this substrate within 10 days of incubation. The consortium was able to grow at 216 mg 2,4-D/l which was the highest concentration tested in the

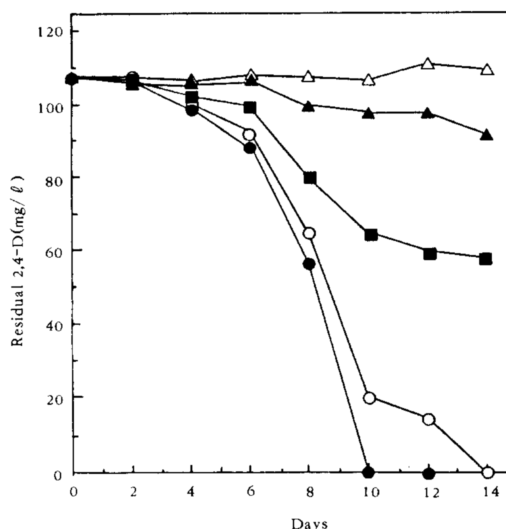


Fig. 2. Degradation of 2,4-D at the initial pH 5.0 (▲), 6.0(○), 7.0(●), 8.0(■), and 9.0(△) in the test culture.

present study, but showed partial degradation of 2,4-D(~80%) during the incubation period. In this study, the degradation of 2,4-D was retarded with higher 2,4-D concentrations in the microbial consortium (Fig. 3). These results indicate that 2,4-D is the substrate for the growth of microorganisms, but increasing 2,4-D concentrations give the inhibitory effect because of its toxicity.

Effect of Additional Nutrients

Yeast extract and ascorbic acid were tested as additional source of nutrients. Supplemental nutrients frequently stimulate the degradation of 2,4-D(1, 14). The amendment of yeast extract provides growth factors for the growth of microorganisms. Also ascorbic acid plays in a role as a reducing agent. Increasing concentations of yeast extract and ascorbic acid resulted in higher growth yields, based on turbidity measurement, and complete degradation of 2,4-D within the 8 days of incubation (Fig. 4).

Detection of Intermediates by HPLC

The residual 2,4-D concentration was determined by reverse-phase HPLC in shake flask experiments with mineral salts. The HPLC chromatograms showed that degradation of 2,4-D took place under the conditions described. The test culture SM1 produced a metabolite that appeared as a minor, distinct peak in chromatogram. The microbiological degradation of 2,4-D occurs via two major pathways(6): (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

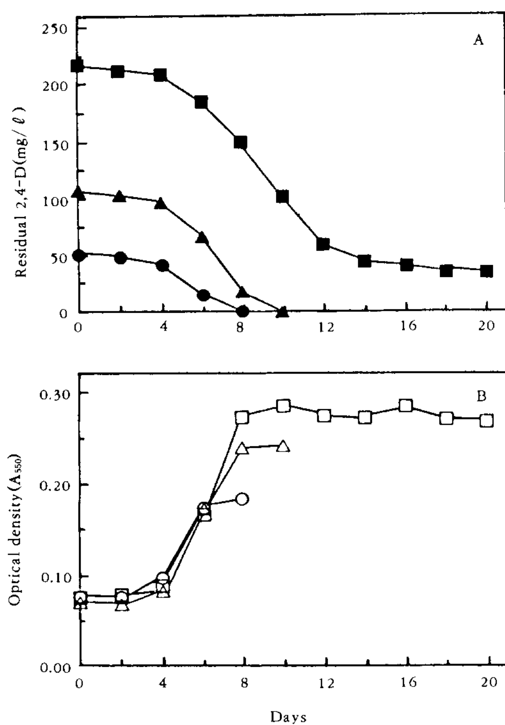


Fig. 3. 2,4-D degradation curve(A) and growth curve(B) of culture SM1 in 54(●, ○), 108 (▲, △), and 216(■, □) mg of initial 2,4-D per liter, repectively.

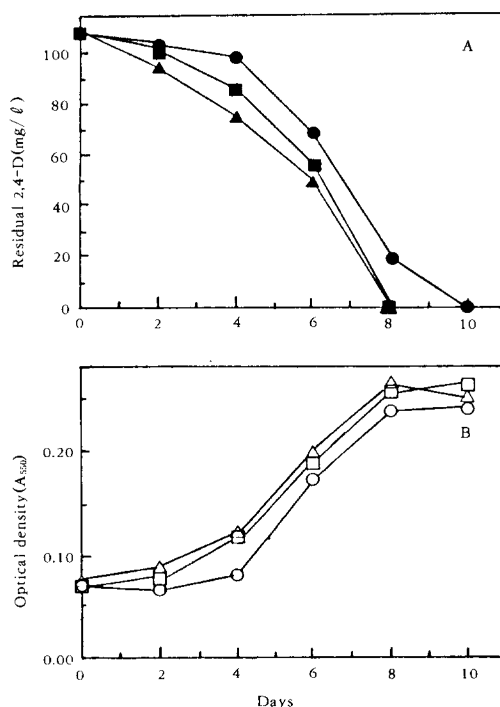


Fig. 4. Degradation of 2,4-D(A) and growth(B) in the absence of additional nutrients(●, ○); in the presence of 200 mg of yeast extract (▲, △) and 100 mg of ascorbic acid(■, □) per liter.

of C-6 position of the ring, and then 3,5-dichlorocatechol by the removal of acetate from 6-H-2,4-DCP. An authentic standard mixture had retention time (Rt) of 4.88 min for 2,4-D and 6.33 min for 2,4-DCP in the HPLC chromatogram (Fig. 5). In samples of culture supernatants, the peak was tentatively identified as 2,4-DCP.

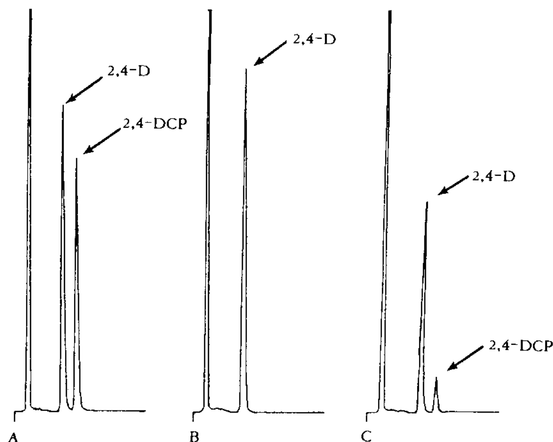


Fig. 5. HPLC chromatograms of standard(A) and of centrifuged culture sample initially(B) and after 6 days of incubation (C).

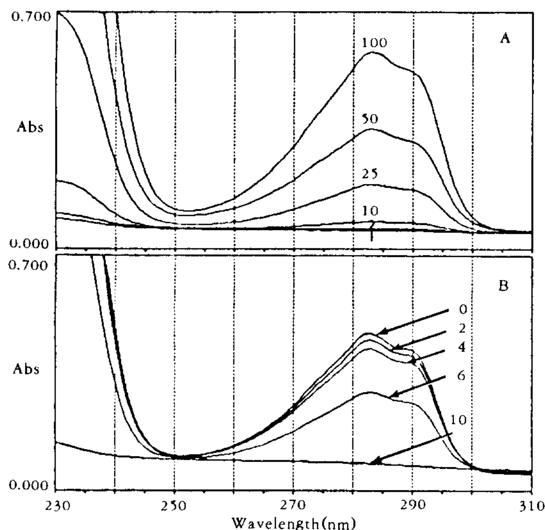


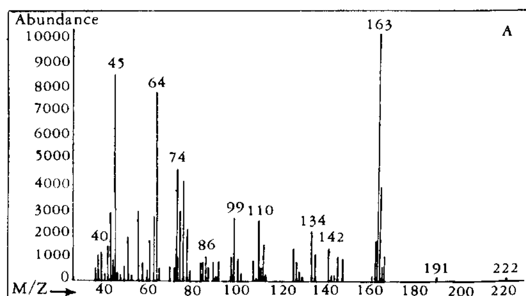
Fig. 6. UV spectral scans of a standard solution containing different concentrations of 2,4-D (A). The concentration of 2,4-D(mg/l) is indicated on the Fig. 6A.; and supernatants of culture SM1 samples (B). The length of incubation preceding the scans is indicated in days in Fig. 6B.

Changes in UV Spectral Characteristics

UV-spectrometry of the authentic standards of 2,4-D showed a maximum peak of absorption of 283 nm. The test culture displayed no detectable spectral changes or peak shifts in the UV-absorbance. The peaks of maximum absorption are shown in Fig. 6A for 2,4-D (283 nm). In culture media, the maximum absorption of 2,4-D was decreased during the incubation period as shown in Fig. 6B.

Verification of Intermediate 2,4-DCP with GC-MS

GC-MS analysis of an ethyl acetate extract of acidified culture supernatant produced multiple peaks in the total ion chromatogram. The fragmentation pattern of a major peak (Rt = 11.38 min) and molecular ion (m/z = 163) were con-



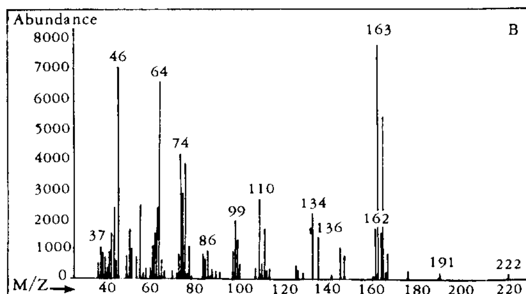


Fig. 7. GC/MS analysis of authentic 2,4-DCP (A) and a microbial culture sample after 6 days of incubation (B).

sistent with those of an authentic 2,4-DCP (Fig. 7).

The present study demonstrated that biological treatment is feasible for degradative removal of phenoxy herbicide from commercially available 2, 4-D amine salts. Eleven isolates from the consortium SM1 were initially obtained as small colonies on the primary plates containing 2,4-D. Microscopic examination of the 2,4-D grown isolates revealed that all were Gram-negative and rod-shaped cells. The isolates varied in pigmentation. Rapid NFT API tests of TSA-grown isoaltes indicated that the bacteria could be assigned to *Pseudomonas*, *Flavobacterium*, and *Arthrobacter* spp. However, a microbial consortium approach was essential for this objective because a pure culture approach would be questionable relevance for environmental treatment applications where aseptic conditions and proper selection pressure for pure cultures cannot be maintained. In particular, a better desired traits in microbial consortia are important areas for future developments of microbial consortia.

ACKNOWLEDGEMENTS

This research was supported by Non-Directed Research Fund, Korea Research Foundation, 1993.

요 약

본 연구의 목적은 시판 페녹시계 제초제 2,4-D의 생물학적 처리의 가능성을 평가하기 위한 것이다. 시판 페녹시계 제초제는 2,4-D 아민염으로서 2,4-D(40%)와 용제(60%)로 구성되었다. 2,4-D에서 농화배양에 의해 얻어진 미생물 컨소시엄은 탄소원 및 에너지원으로서 2,4-D를 이용하였다. 이 실험에서 2,4-D분해의 최적 pH와 기질농도는 각각 7.0과 54mg/ℓ 였다. Yeast extract와 ascorbic acid의 첨가는 2,4-D의 분해와 미생물의 생장을 촉진시켰다. 2,4-D를 정량하기 위해 HPLC가 사용되었으며 그 과정에서 중간대사물질로서 2,4-DCP가 분리되었다. GC-MS는 2,4-DCP를 입증하기 위하여 사용되었다. 배양중의 UV scans 결과, 2,4-D의 최대흡광치는 배양이 진행되는 동안 감소되었으나, spectral 및 peak 변화는 보여주지 않았다.

REFERENCES

1. G. L. Sinton, L. T. Fan, L. E. Erickson and S. M. Lee(1986), *Enz. Microb. Technol.*, **8**, 395.
2. E. R. I. C. Sandmann, M. A. Loos and L. P. van Dyk(1988), *Rev. Environ. Contam. Toxicol.*, **101**, 1.
3. USEPA(1988) *Rev. Environ. Contam. Toxicol.*, **104**, 63.
4. A. E. Smith and G. P. LaFond(1990), Enhanced biodegradation of pesticides in the environment(K. D. Racke and J. R. Coats, eds.), p. 14, American Chemical Society, Washington, D. C.
5. L.-T. Ou(1984), *Soil Sci.*, **137**, 100.
6. P. S. Amy, J. W. Schulke, L. M. Frazier and R. J. Seidler(1985), *Appl. Environ. Microbiol.*, **49**, 1237.
7. A. E. Smith, L. Hume, G. P. LaFond and V. O. Biederbeck(1991), *Can. J. Soil Sci.*, **71**, 73.
8. S. Balajee and A. Mahadevan(1990), *Xenobiotica*, **20**, 607.
9. G. Ditzelmuller, M. Loidl and F. Streichsbier (1989), *Appl. Microbiol. Biotechnol.*, **31**, 93.
10. E. R. I. C. Sandmann and M. A. Loos(1988), *Can. J. Microbiol.*, **34**, 125.
11. D. H. Pieper, W. Reineke, K.-H. Engesser and H.-J. Knackmuss(1988), *Arch. Microbiol.*, **150**, 95.
12. K.-H. Oh and O. H. Tuovinen(1990), *J. Ind. Microbiol.*, **6**, 275.
13. C. W. Greer, J. Hawari, and R. Samson (1990) *Arch. Microbiol.*, **154**, 317.
14. K.-H. Oh and O. H. Tuovinen(1991), *FEMS Microbiol. Lett.*, **79**, 141.
15. K.-H. Oh and O. H. Tuovinen(1994), *Intern. Biodeter. Biodegr.*, **33**, 93.
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall(1951), *J. Biol. Chem.*, **193**, 265.
17. M. P. Kelly, K. B. Hallberg and O. H. Tuovinen(1988), *Appl. Environ. Microbiol.*, **55**, 2717.