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2,4-Dichlorophenoxyacetic Acid Detection Using 2,4-Dichlorophenoxyacetic Acid α-Ketoglutarate Dioxygenase

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The 2,4-dichlorophenoxy acid (2,4-D) α-ketoglutarate dioxygenase gene *tfdA* of *Alcaligenes eutrophus* pJP4 derivative plasmid pRO101 was cloned in pBluescript SK+ and expressed in *Escherichia coli* DH5α at a high level. Cell-free extract of the recombinant organism could convert 2,4-dichlorophenoxyacetic acid into 2,4-dichlorophenol (DCP). Production of DCP was indicated by a color reaction using 4-aminoantipyrine and could be used as an assay for the presence of 2,4-D. After storage at room temperature for 3 months, cell-free extracts prepared from the recombinant organism and air-dried in filter paper bags still converted 2,4-D to DCP. Reaction conditions for conversion of 2,4-D to DCP were optimized, and the enzymatic reaction kinetics were determined. It appears feasible to use the recombinant-produced enzyme for an inexpensive 2,4-D assay.

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is possibly the most widely used herbicide. Although 2,4-D is not very toxic, cleanup of inadvertantly spilled 2,4-D is still necessary (*1*–*5*). This phenoxy herbicide is degraded by several microorganisms, and 2,4-D bioremediation processes have been proposed. Analytical support for 2,4-D cleanup techniques usually involves gas chromatography or immunoassays, which are moderately (ELISA assays) to very (GC/MS) expensive. Therefore, it is important to develop a quick, inexpensive, and easy-to-use 2,4-D detection method for use in the field by nonspecialists.

A well-known color reaction for detecting phenolic compounds uses 4-aminoantipyrine as a coupling reagent (*6*). Color changes in solutions with approximately 2 mg/L of a phenol can easily be seen by unaided eyes. Therefore, this color reaction can be used to indicate the presence of 2,4-D, if 2,4-D can first be converted to 2,4-dichlorophenol (DCP). An enzyme capable of transforming 2,4-D into 2,4-dichlorophenol (DCP) is found in all 2,4-D-degrading microorganisms examined to date.

The best-understood degradation pathway of 2,4-D is encoded on plasmid pJP4 from *Alcaligenes eutrophus* JMP134 (*7*–*9*). 2,4-D is first converted by an α-ketoglutarate-dependent dioxygenase to 2,4-dichlorophenol and then by a DCP hydroxylase to 3,5-dichlorocathechol (DCCAT). Cleavage of DCCAT by a dioxygenase yields 2,4-dichloromuconic acid. A lactonizing enzyme then forms *cis*-2-chlorodiene lactone, and chloromaleylacetic acid is finally produced by a lactonase (*8, 10*). The genes encoding these enzymes, *tfdA*, *tfdB*, and *tfdCDEF*, have been located on plasmid pJP4 (*10*–*13*). The expression of *tfdB* is regulated by the product of gene *tfdS* using 2,4-D and DCP as effectors (*10*). Since both 2,4-D and DCP can induce the expression of *tfdB*, DCP is further transformed by microorganisms bearing pJP4 or its derivatives, pRO101 or pRO103 (*14*). No DCP can be detected by the color reaction in the culture media of these microorganisms when 2,4-D is supplied. In order to detect 2,4-D through the formation of DCP, an organism that accumulates DCP in stoichiometric ratio to 2,4-D must be constructed. Here, we report the construction of such a plasmid, its introduction into *Escherichia coli* DH5α, and the application of cell-free extracts of this organism to highly specific 2,4-D detection.

Materials and Methods

Strains and Plasmids. *E. coli* (pRO101), which was a gift from Dr. R. H. Olsen at the University of Michigan, was used to obtain a DNA fragment containing the gene *tfdA*. Plasmid pRO101 is a derivative of plasmid pJP4 containing Tn*1721* inserted into a nonessential region (*14*). The vector used for *tfd*A expression was pBluescript SK+, which was also maintained and produced in *E. coli* DH5α. Both strains were stored at −20 °C in 50% glycerol solutions. The hybrid plasmid constructed from pBluescript SK+ and the DNA fragment containing gene *tfdA* was designated as pYG3. *E.*

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coli DH5α [φ80dkacZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1] was used as the host cell for the expression of gene *tfdA* from pYG3. The recombinant organism, *E. coli* DH5α (pYG3), was maintained on LB agar plates with 75 mg/L ampicillin and stored at 4 °C.

Media and Cultivation Conditions. LB medium was used for all cultures. Fifty mg/L tetracycline was supplied as selective pressure for the culture of *E. coli* (pRO101), while 75 mg/L ampicillin was supplied for all other cultures.

The cultures for plasmid preparations were grown in 5 mL of media in an orbital incubator-shaker at 37 °C and 200 rpm. The cultures for enzyme production were also grown in an incubator-shaker at 200 rpm but at 30 °C with 500 mL of media inoculated with a 5-mL culture grown overnight at 37 °C.

Plasmid Isolation. Plasmid pRO101 was isolated from *E. coli* (pRO101) by the procedure of Hansen and Olsen (*15*). A 50-mL cell culture was used in each isolation. The DNA sample was further purified by (1) incubation with 10 mg/L RNase for 15 min; (2) phenol extraction; and (3) ethanol precipitation (twice). All these operations were performed according to protocols in Ausubel et al. (*16*).

Wizard Minipreps DNA purification system provided by Promega (Madison, WI) was used to isolate plasmid pBluescript SK$^+$ and pYG3. All operations were carried out according to the manufacturer's instructions, and a 3-mL overnight culture was used in each isolation.

Cloning of *tfdA*. The DNA fragment containing gene *tfdA*, which is about 1.4 kb, was obtained by digesting pRO101 with *Xba*I and *Sal*I restriction endonucleases sequentially at 37 °C. The pRO101 DNA sample was first digested by *Xba*I in medium salt buffer (USB, Cleveland, OH) for 2 h, and then after the salt concentration was adjusted to 120 mM, it was digested by *Sal*I for another 2 h. The digested sample was separated by 1.5% agarose gel electrophoresis. The 1.4-kb fragment containing *tfdA* was cut out from the gel and then purified by QIAEX (QIAGEN), which is a kit for DNA purification from agarose gel. The vector, pBluescript SK$^+$, was also digested by *Xba*I and by *Sal*I and purified by gel separation and QIAEX.

Ligation was performed using T4 DNA ligase (0.3 unit) in a 10-μL volume at 16 °C for 11 h according to standard methods (*17*). All enzymes used for cloning were provided by USB.

The ligation solution was diluted by 5-fold before transformation. Diluted ligation solution (5 μL) was used for the transformation of 100 μL of competent *E. coli* DH5α by standard methods (*17*). Transformants were selected and screened on LB plates with ampicillin (75 mg/L), X-Gal, and isopropyl β-D-thiogalactopyranoside (IPTG).

Gel Electrophoresis. Agarose gel electrophoresis was used for DNA analysis and fragment separation. Restriction digests were routinely analyzed on 1.5% or 0.8% agarose gels, followed by staining with ethidium bromide. DNA fragment sizes were determined using 1 kb DNA ladder and high molecular weight DNA markers (Gibco BRL).

Preparation of Cell-Free Extracts. *E. coli* DH5α (pYG3) cultures (500 mL) were grown in 1-L flasks. When OD_{560} reached approximately 2.5, 200 μL of 115 mg/mL IPTG (46 μg/L final concentration) was added to induce the expression of gene *tfdA*. One hour after adding IPTG, cells were harvested by centrifugation (8000*g*) for 10 min at 4 °C and then washed with 25 mL of 100 mM sodium phosphate buffer (pH 8.0). After the wash, the cells were again resuspended in 25 mL of the phosphate buffer. Cell-free extracts were obtained by passing the cells through a prechilled French Press at 1500 psi once and by clarifying the debris by centrifugation (16000*g*) for 20 min at 4 °C.

Protein Concentration Determination. Protein concentrations were determined by the BCA method (*18*), using the dye reagents supplied by Bio-Rad (Richmond, CA). Bovine serum albumin (BSA) was used as a standard for calibration.

Enzyme Activity Assay. The enzyme activities of cell-free extracts were determined from DCP concentration measurements after incubation at room temperature (20 °C) for 5 min in 125 mg/L 2,4-D and 10 mM sodium phosphate buffer (pH 8.0) containing 100 μM ferrous ammonium sulfate and 1 mM α-ketoglutarate. DCP concentration was determined by the 4-aminoantipyrine method (*6*), in which condensation of 4-aminoantipyrine with a phenol in the presence of alkaline oxidizing agents produces a red antipyrine dye (AAPPC) in what is known as the Emerson reaction (*6*). The assay was similar to the one used by Fukumori and Hausinger (*19*). Three replicates were used for each assay.

Kinetic Model for the Enzymatic Reaction. There are three substrates, 2,4-D, α-ketoglutarate, and oxygen, in the enzymatic reaction in which 2,4-D is oxidized to DCP (*19*). Without considering the effect of oxygen on reaction rate, the reaction is a ping-pong bi-bi reaction. The reaction rate can be expressed as (*20*)

$$\frac{1}{v_o} = \frac{K_M^{2,4\text{-D}}}{V_{max}[2,4\text{-D}]} + \frac{K_M^{\alpha\text{-KG}}}{V_{max}[\alpha\text{-KG}]} + \frac{1}{V_{max}} \qquad (1)$$

where v_o is the reaction rate, [2,4-D] is 2,4-D concentration, [α-KG] is α-ketoglutarate concentration, and V_{max}, $K_M^{2,4\text{-D}}$, and $K_M^{\alpha\text{-KG}}$ are model parameters. The values of model parameters were determined from double reciprocal plots.

Results and Discussion

Cloning of *tfdA*. The construction of pYG3 from an expression vector, pBluescript SK$^+$, and a 1.4-kb *Sal*I−*Xba*I fragment of pRO101 is schematically shown in Figure 1. There are more than 10 *Sal*I restriction sites and at least two *Xba*I restriction sites on pRO101. Although the restriction map of pRO101 by *Sal*I and *Xba*I is unpublished, the nucleotide sequence of a 2.06-kb *Bam*HI−*Sal*I fragment of pJP4 encoding the 2,4-D α-ketoglutarate dioxygenase gene *tfdA* has been published (*12*). There is a *Xba*I site at 110 bp before the *tfdA* start codon and a *Sal*I site 440 bp after the *tfdA* stop codon. Also, there is an *Eco*RI site within *tfdA*. Based on the restriction map of pBluescript SK$^+$ and the sequence of the *Sal*I−*Xba*I fragment, the restriction map of pYG3 was determined and is also shown in Figure 1.

During the construction of pYG3, pRO101 was digested by *Xba*I and *Sal*I, and fragments were separated by gel electrophoresis. A 1.4-kb fragment was cut from the gel, purified, and ligated with pBluescript SK$^+$. A restriction site analysis for this hybrid plasmid is shown in Figure 2. The size of fragments in Figure 2 matched the size calculated from published data (Figure 1), indicating that a correct DNA fragment was cloned.

Expression of *tfdA* in *E. coli*. The *Xba*I restriction site of pYG3 is 110 bases before the *tfdA* start codon. A previous study showed that the promoter of gene *tfdA* was in the vicinity of the *Xba*I site (*12*). Cloning of this 1.4-kb fragment

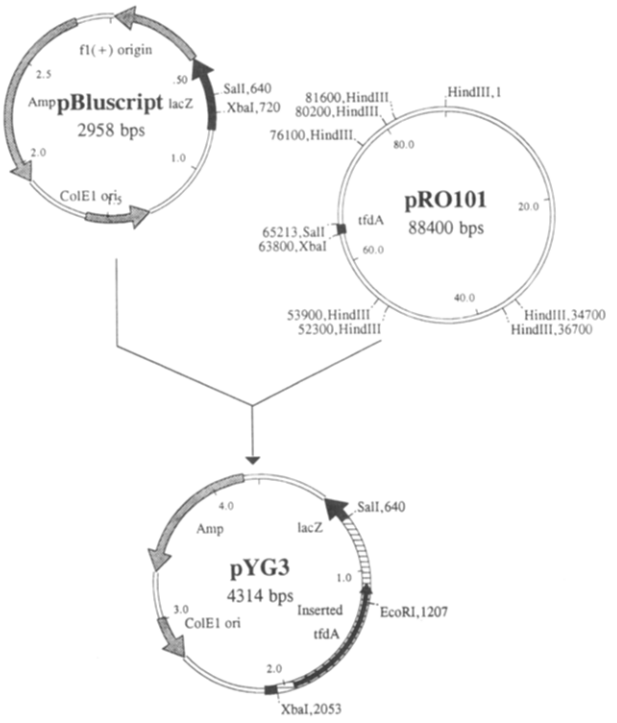

FIGURE 1. Construction of pYG3 from pBluescript SK$^+$ and pRO101.

placed *tfdA* under the control of the *lacZ* promoter. *E. coli* DH5α was used as the host cell for the expression of *tfdA* on pYG3. While *tfdA* was not expressed in the medium with glucose alone, it was expressed in LB medium when IPTG was added as an inducer.

Effect of pH on Enzyme Activity. Since the color reaction is almost instantaneous, the conversion rate of 2,4-D into DCP determines the response time for 2,4-D detection. Higher reaction rates result in smaller response times or less enzyme needed for 2,4-D detection. Figure 3 shows that pH has a strong effect on enzyme activity. The optimum pH for the enzymatic reaction was 6.5. However, the preferred pH for the color reaction is in the range of pH 8.0−12.0. The color is also more stable in this pH range. More importantly, 4-aminoantipyrine can react with potassium ferricyanide at pH 6.5 producing a slight color change that is similar to having 2 mg/L DCP in solutions. This is not acceptable for 2,4-D detection. Although pH 6.5 can be used for the enzymatic reaction and then the solution pH adjusted for the color reaction, this is not convenient for 2,4-D detection. Therefore, a compromise pH 8.0 was selected for the 2,4-D detection system.

Effect of Fe^{2+} on Enzyme Activity. 2,4-D α-ketoglutarate dioxygenase requires ferrous ion as a cofactor (*19*). The effect of ferrous ion concentration on enzyme activity for the cell-free extract is shown in Figure 4. Enzyme activity increased approximately five times by adding ferrous ions up to 50 μM. This saturation concentration should be a function of enzyme concentration used. In order to maximize enzyme activity, a higher ferrous ion concentration, such as 100 μM, should be used for 2,4-D detection.

Effects of α-Ketoglutarate and 2,4-D Concentration on DCP Production Rates. Previously, the conversion of 2,4-D into DCP was thought to be catalyzed by a monooxygenase (*13*). However, recent study has shown that this reaction is catalyzed by an α-ketoglutarate-dependent dioxygenase with oxygen and α-ketoglutarate required as cosubstrates (*19*). Although it is not practical to control

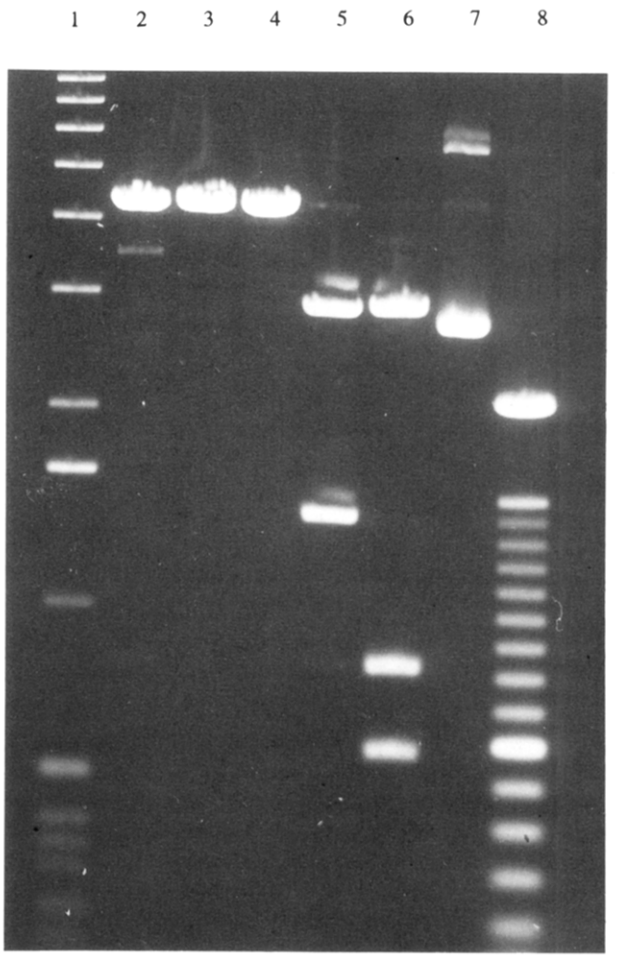

FIGURE 2. Gel picture for restriction site analysis of pYG3. Lane 1, 1 kb ladder; lane 2, by *Sal*I; lane 3, by *Xba*I; lane 4, by *Eco*RI; lane 5, by *Xba*I and *Sal*I; lane 6, by *Xba*I, *Sal*I, and *Eco*RI; lane 7, undigested; lane 8, 100 bp ladder. There was only one restriction of *Xba*I, *Sal*I, and *Eco*RI in pYG3. The size of pYG3 was 4.36 kb from the gel (lanes 2−4). The size of inserted DNA fragments was 1.43 kb, and the size of pBluescript was 2.93 kb (lane 5). The inserted DNA fragments could be cut by *Eco*RI into 0.84 and 0.57 kb fragments (lane 6).

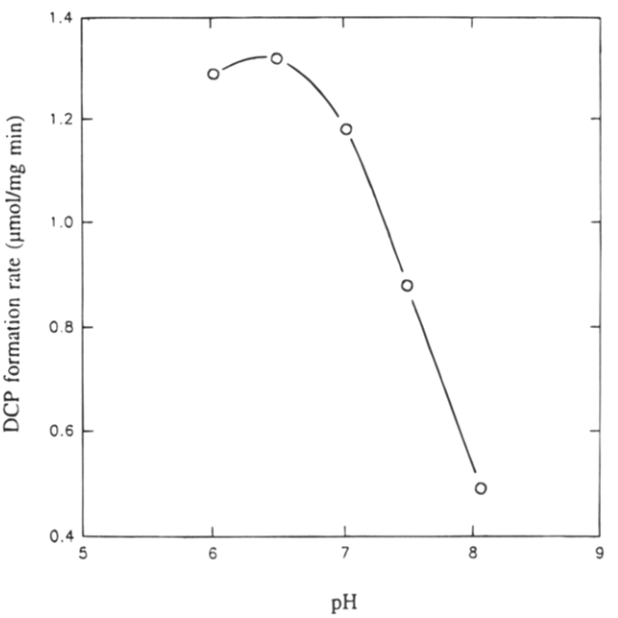

FIGURE 3. Effect of pH on enzyme activity.

oxygen concentration for 2,4-D detection, α-ketoglutarate concentration can be optimized. The effects of α-keto-

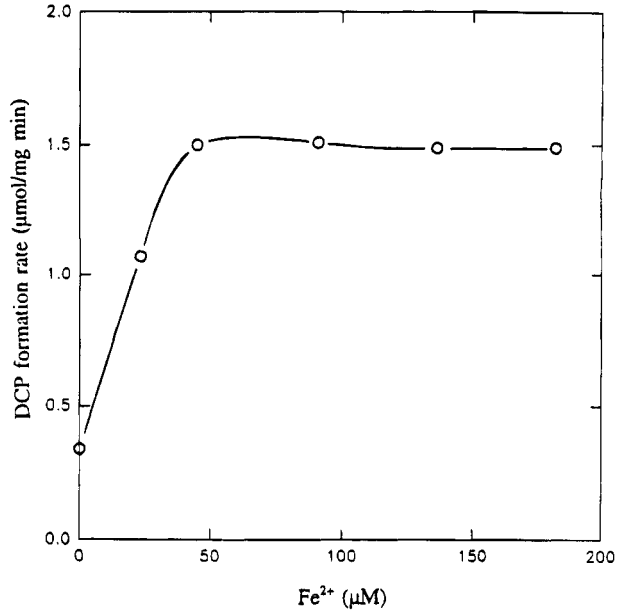

FIGURE 4. Effect of ferrous ion concentration on enzyme activity (pH 6.5).

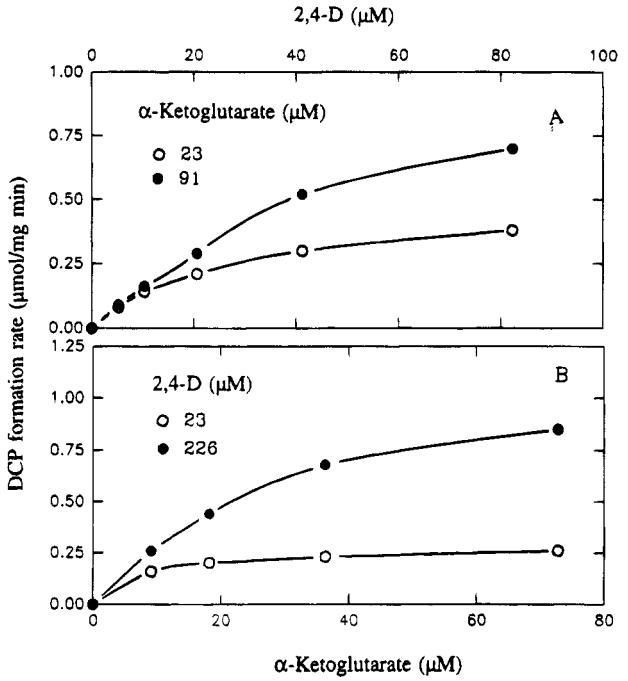

FIGURE 5. Effects of 2,4-D (A) and α-ketoglutarate (B) on DCP production rate.

glutarate and 2,4-D concentrations on DCP production rate are shown in Figure 5. The saturation concentration of α-ketoglutarate for 23 μM 2,4-D was approximately 10 μM, while the saturation concentration for 226 μM 2,4-D was greater than 70 μM. Since 10 mg/L DCP (equivalent to 77 μM 2,4-D) is sufficient for 2,4-D detection, 1 mM α-ketoglutarate is sufficient for 2,4-D detection.

Kinetics of Enzymatic Reaction. Since oxygen cannot be controlled in the process of 2,4-D detection, oxygen was not considered in the model. Kinetic data were measured at pH 8.0, 100 mM Fe^{2+}, and room temperature (20 °C). Two Lineweaver–Burk plots are shown in Figure 6. Clearly, the enzymatic reaction has a ping-pong bi-bi mechanism with respect to 2,4-D and α-ketoglutarate. Kinetic parameters obtained from the rate data are listed in Table 1. The kinetic model can be directly applied to the quantitative

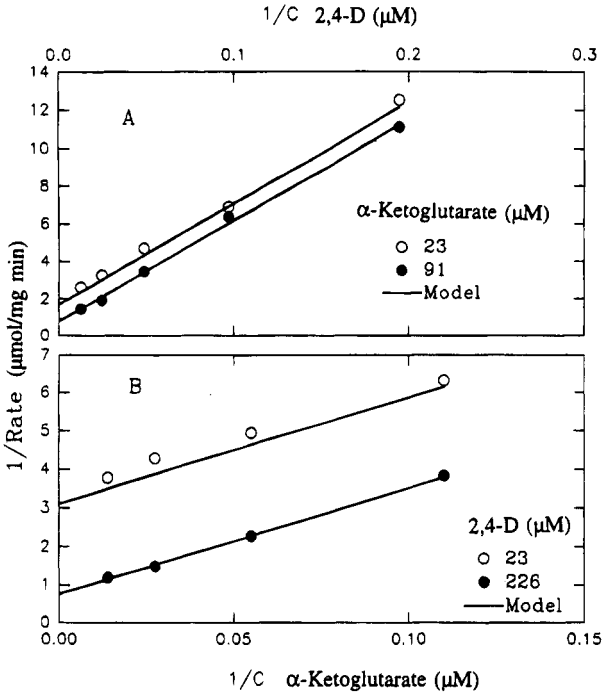

FIGURE 6. Lineweaver–Burk plots for DCP production at different 2,4-D (A) or α-ketoglutarate (B) concentrations.

TABLE 1

Enzyme Parameters Determined from Kinetic Models[a]

parameters	value
V_{max} (μmol min^{-1} mg^{-1})	2.0
$K_M^{2,4\text{-}D}$ (μM)	107
$K_M^{\alpha\text{-}KG}$ (μM)	55

[a] At 20 °C, pH 8.0, 50 μM Fe^{2+}.

design of a 2,4-D detection system using the cell-free extract.

Stability of Dried Enzyme. For practical application of 2,4-D detection, enzyme stability is also very important. Since the dried enzyme is intended to be used in 2,4-D detection, only the stability of the dried enzyme was examined. Samples were prepared by adding cell-free extracts into small filter bags (0.7 × 8 cm) and drying under vacuum at room temperature for 3 h. Cell-free extract protein (50 μg) was used for each sample. The dried samples of cell-free extract were stored at different temperatures, and activity was measured periodically. The results are shown in Figure 7. After a period of activity decrease, the activity of dried cell-free extract in the filter bag reached a stable value.

2,4-D Detection System. The 2,4-D detection system includes the following items: (1) 5 mM $Fe(NH_4)_2(SO_4)_2$ solution; (2) 100 mM α-ketoglutarate solution; (3) 40 mM 4-aminoantipyrine solution; (4) 8 wt % potassium ferricyanide solution; and (5) dried 2,4-D α-ketoglutarate dioxygenase in filter bags. 2,4-D α-ketoglutarate dioxygenase is the enzyme that converts 2,4-D into DCP. Fe^{2+} is used as a cofactor by the enzyme, and α-ketoglutarate is a cosubstrate for the 2,4-D conversion reaction. 4-Aminoantipyrine and potassium ferricyanide are reagents for the color reaction. The reaction conditions and amounts of the solutions used for each detection are summarized in Table 2. If the pH of water samples is around 7, pH 8.0 can be automatically achieved by the buffer ingredients in the

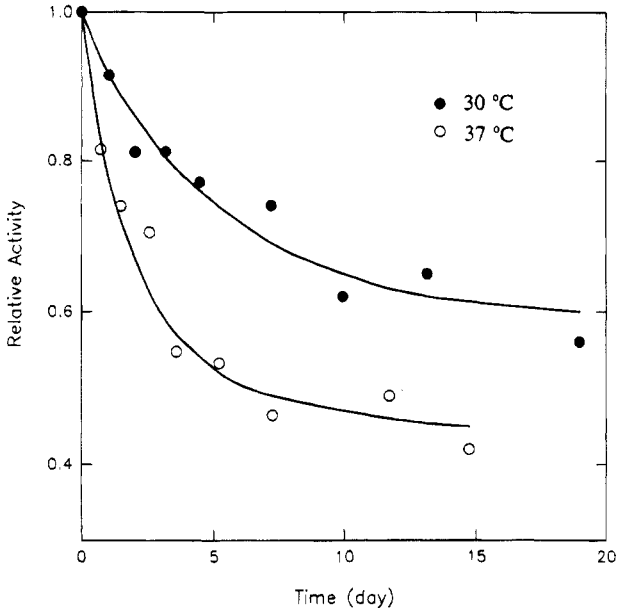

FIGURE 7. Stability of dried enzyme samples at 37 and 30 °C.

TABLE 2

Summary of Recommended Conditions and Procedure for 2,4-D Detection[a]

reaction conditions	recommended value
volume (water sample containing 2,4-D) (mL)	1
reaction temp	room temp (≈20 °C)
pH	8.0
$Fe(NH_4)_2(SO_4)_2$ (μM)	50
α-ketoglutarate (mM)	1
reaction time (min)	≥10
40 mM 4-aminoantipyrine (μL)	10
8 wt % potassium ferricyanide (μL)	5
Protein amount for each sample (dried) (μg)	100
enzyme storage temp	4 °C or at room temp

[a] Procedure: To 1 mL of 2,4-D containing sample (adjust pH if necessary), add 10 μL of 5 mM $Fe(NH_4)_2(SO_4)_2$, 10 μL of 100 mM α-ketoglutarate, and enzyme (dried in a filter paper bag). Incubate at room temperature (~20 °C) for 10 min. Add 10 μL of 40 mM 4-aminoantipyrine and 5 μL of 8 wt % potassium ferricyanide. Score + (≥2 ppm 2,4-D) if red; score−if yellow (<2 ppm 2,4-D).

cell-free extract. Otherwise, a pH adjustment of water samples is required before using this system. In order to improve the stability of the detection system, the amount of enzyme used for each sample was adjusted to twice that used in enzyme stability measurements (100 μg of protein for each sample).

This 2,4-D detection system was designed to determine the presence of 2,4-D with unaided eyes. After adding 4-aminoantipyrine and potassium ferricyanide solution, the strength of red color formed from a DCP solution was proportional to the DCP concentration in the range 0−10 mg/L. However, only when DCP concentration was higher than 2 mg/L, could the color change be easily distinguished from a control by unaided eyes. The color formed from a 2 mg/L DCP solution was slightly red, while the color of a control is bright yellow. The color formed from a 10 mg/L DCP solution was dark red. For DCP concentrations between 2 and 10 mg/L, the DCP concentration could be estimated according to color intensity by unaided eyes. However, it was difficult to distinguish a 10 mg/L DCP solution from a higher concentration of DCP solution by unaided eyes. The concentration of DCP converted from 2,4-D in this detection system was affected by 2,4-D concentration, enzyme activity and amount, α-ketoglutarate and Fe^{2+} concentrations, and reaction time. With the conditions recommended in Table 2, almost all 2,4-D was converted into DCP. A longer reaction time can be used to compensate enzyme activity loss during storage to maintain 2,4-D detection sensitivity.

TABLE 3

Stability of 2,4-D Detection System[a]

time (week)	OD_{510}
0	0.56
1	0.52
2	0.54
3	0.67
4	0.61
5	0.68
6	0.64
8	0.69
10	0.63
13	0.48
15	0.35

[a] Solutions and enzyme were stored at room temperature (20 °C); 10 mg/L of 2,4-D was used for all assays.

In addition to the enzyme, some solutions used in the detection system are not very stable. Therefore, the stability of this 2,4-D detection system was further examined experimentally. A solution of 10 mg/L 2,4-D and a reaction time of 10 min were used to examine the stability of this 2,4-D detection system. All solutions and the enzyme were stored at room temperature (approximately 20 °C). 4-Aminoantipyrine was stored in a brown bottle because it is light sensitive. The stability of this system is shown in Table 3. A similar DCP concentration was produced by this detection system throughout the 13-week test period. It is shown in Figure 7 that the enzyme was more stable at lower temperatures. Therefore, it is expected that this 2,4-D detection system will be more stable when it is stored at 4 °C in a refrigerator or in a cool dark place.

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