A Potentiometric Microbial Biosensor for Direct Determination of Organophosphate Nerve Agents

Ashok Mulchandani, Priti Mulchandani, Samir Chauhan, Irina Kaneva,* and *Wilfred Chen*

Department of Chemical and Environmental Engineering, University of California, Riverside, Riverside, CA 92521, USA

Received: September 18, 1997
Final version: June 15, 1998

Abstract
An easy to construct and inexpensive potentiometric microbial biosensor for the direct measurement of organophosphate (OP) nerve agents was developed. The biological sensing element of this biosensor was recombinant *Escherichia coli* cells containing the plasmid pJK33 that expressed organophosphorus hydrolase (OPH) intracellularly. The cells were immobilized by entrapment behind a microporus polycarbonate membrane on the top of the hydrogen ion sensing glass membrane pH electrode. OPH catalyzes the hydrolysis of organophosphorus pesticides to release protons, the concentration of which is proportional to the amount of hydrolyzed substrate. The sensor signal and response time were optimized with respect to the buffer pH, ionic concentration of buffer and temperature, using paraoxon as substrate. The best sensitivity and response time were obtained using a sensor operating in pH 8.5, 1 mM HEPES buffer and 37 °C. The biosensor was applied for measurement of paraoxon, ethyl parathion, methyl parathion and diazinon.

Keywords: Organophosphate nerve agents, Potentiometric microbial biosensor, Paraoxon, Ethyl parathion, Methyl parathion, Diazinon

1. Introduction

Organophosphate (OP) compounds are widely used as pesticides, insecticides and chemical warfare agents [1, 2]. A large volume of wastewater contaminated with these acutely toxic compounds is generated at both the producer- and consumer-levels [3]. Increased public concerns and regulatory mandates for the way OP contaminated wastewaters are managed has stimulated the development of technologies for effective treatment (detoxification/disposal) of these wastes [4–8]. Additionally, the recently ratified Chemical Weapons Treaty requires the United States to destroy all of its chemical weapons arsenal, including the organophosphorus-based nerve gases, within ten years [9, 10]. The successful use of currently researched technologies for detoxification of OPs will require sensors for monitoring and control of the process.

Gas, liquid and thin-layer chromatography coupled with different detectors and different types of spectroscopy, immunoassays and biosensors based on inhibition of cholinesterase (AChE) activity are commonly used methods for OP determination [11, 12]. Although sensitive and useful for environmental monitoring, these techniques are unsuitable for on-line monitoring of detoxification processes. Chromatography techniques are time consuming, expensive, require highly trained personnel and are available only in sophisticated laboratories [13]. Immunoassays are time consuming (1–2 h), labor intensive and require extensive sample handling, (large number of washing steps) [13]. AChE-based [14–34] biosensing devices measure OP concentration indirectly (by measuring the inhibition) and are nonselective, laborious, time consuming and unstable due to incomplete regeneration of the enzyme activity as a result of strong irreversible binding of certain inhibitors [14, 17, 26].

Soil microorganisms, *Pseudomonas diminuta* MG and *Flavobacterium* sp., possess the capability of hydrolyzing organophosphorus pesticides (P-O and P-S bond hydrolysis) and nerve gases (P-F or P-CN bond cleavage) [35-37]. These bacterial strains possess high activity of the constitutively expressed organophosphorus hydrolase (OPH) which in both *P. diminuta* MG and *Flavobacterium* is encoded by the *opd* genes on large plasmids (40–64 kilobases). The *opd* gene has been cloned into *E. coli* [38], insect cell (fall armyworm) [39], *Streptomyces* [40], and soil fungus [41] for overexpression of OPH. The catalytic hydrolysis of each molecule of these compounds releases two protons, the measurement and correlation of which to the OP concentration, forms the basis of a potentiometric enzyme electrode. Unlike the AChE inhibition based detection, which is nonselective, indirect and involve multiple steps, detection scheme based on monitoring the OPH-catalyzed hydrolysis products of OPs is selective, direct, and requires a single step [13, 42].

Recently, we reported on the development of an OPH-based potentiometric enzyme electrode for OP determination [42]. This new analytical tool provides direct, rapid, precise and accurate measurement of OP. Although elegant, a drawback of the enzyme electrode is the time, effort and cost of isolating and purifying the enzyme. Immobilized microorganisms can be employed as an alternate sensing element of biosensors to alleviate these problems. Many examples of microbial-based biosensors for a variety of applications have been reported [43]. Two such potentiometric biosensor systems were based on recombinant *Escherichia coli* cells expressing OPH, although they were not biosensors in a ‘true’ sense [13]. These sensor systems comprised of *E. coli* cells cryoimmobilized by entrapment in poly(vinyl)alcohol gel that were either suspended in a reactor with a pH electrode or packed in a column reactor placed upstream of a flow-cell. The need of a special equipment for cryoimmobilization of the cells and the slow response were the limitations of the reported systems. The latter is attributable to the various mass transfer resistances, in particular the transport of substrate (OPs) and product (protons) through the poly(vinyl)alcohol gel used for cell immobilization, present in the system.

The objective of this study was to develop a cheap/inexpensive, simple and easy to construct potentiometric microbial electrode using OPH expressing recombinant *E. coli* immobilized behind a microporus membrane on the surface of a pH electrode for the direct, rapid, selective, precise and accurate determination of organophosphate nerve agents that can potentially be useful for on-line process monitoring.

2. Materials and Methods

2.1. Reagents

HEPES, yeast extract, tryptone, potassium monobasic phosphate, potassium dibasic phosphate, cobalt chloride, and glycerol were

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purchased from Fisher Scientific (Tustin, CA, USA). Paraoxon, methyl parathion, sevin, sutan, atrazine, simazine and diazinon were acquired from Supelco Inc. (Bellefonte, PA, USA). 0.05 μm pore size Nucleopore polycarbonate membrane was purchased from Corning Costar Corp., (Cambridge, MA). All the solutions were made in distilled deionized water.

2.2. Bacteria Strains, Media, and Growth Conditions

The recombinant *E. coli* strain JM105 [F$'$ *traD36 lacI^q* Δ(*lacZ*)*M15 proA$^+$B$^+$ rpsL* (Str^r) *endA sbcB15 sbcC hsdR4*($r_k^- m_k^+$) Δ(*lac-proAB*)] carrying plasmid pJK33 (obtained from Dr. Jeffrey Karns, USDA, Beltsville, MD) was used in this study for the production of native OPH in the cytoplasm.

Cells were grown in 50 mL of culture broth at 30 °C containing 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 0.4 % (v/v) glycerol, 80 mM K_2HPO_4 and 20 mM KH_2PO_4. After the culture reached stationary phase (35–38 h), cells were harvested by centrifugation at 8000 × g for 10 min at 4 °C, washed twice with buffer A (pH 8.5, 50 mM HEPES buffer + 50 μM $CoCl_2$), resuspended in 2 mL of buffer A and stored at 4 °C. In order to ensure good electrode-to-electrode performance reproducibility, the cells were always harvested at the same time (35–38 h from the start of culture). As an additional control, the OPH activity in each batch of cultured cells was measured before using them for biosensor construction. OPH activity was measured by measuring μmoles of *p*-nitrophenol formed per min per OD_{600} during the hydrolysis of 1 mM paraoxon in pH 8.5 buffer at 20 °C; *p*-nitrophenol formation was measured spectrophotometrically at 400 nm ($\varepsilon_{400} = 17\,000\ M^{-1}\ cm^{-1}$).

2.3. Microbial Biosensor Construction

The microbial-based potentiometric electrode was constructed by immobilizing the recombinant *E. coli* cells directly on the hydrogen ion sensing glass membrane of the pH electrode (Accumet, Model 13-620-289, Fisher Scientific, Tustin, CA, USA). An appropriate volume of the cell suspension containing 1.5 mg dry weight of cells was dropped slowly at the center of the 0.05 μm polycarbonate membrane with slight suction. The cell retaining membrane was then attached to the hydrogen ion sensing glass surface of the pH electrode and held in place by an O-ring.

2.4. Experimental Setup and Measurement

All measurements were made in 5 mL of an appropriate buffer, thermostated to the desired temperature, in a 10 mL working volume jacketed glass cell, equipped with magnetic stirrer. The temperature of the liquid in the cell was controlled by circulating water in the cell jacket using a circulating water bath (Model 1160, VWR Scientific, San Francisco, CA, USA). 5–10 μL of OP nerve agent, dissolved in pure methanol, was added to the cell and the change in potential, i.e., pH, recorded with a pH/ion analyzer (Model 255, Corning Science Products, Corning, NY, USA) connected to a flat bed chart recorder (Model BD112, Kipp and Zonen, Holland).

3. Results and Discussion

3.1. Optimization of Sensor Operating Conditions

Experiments were performed to investigate the effect of buffer concentration, starting pH of buffer and temperature on the rate of change of the initial response (determined by drawing a tangent to the response curve) of the electrode to 100 μM paraoxon.

3.1.1. Effect of Buffer Concentration

The buffer concentration has a marked influence on the rate of potential change, which was an inverse function of the buffer concentration (Fig. 1). The inverse relationship is due to the fact that a higher concentration buffer counteracts the pH change resulting from protons released during the OPH-catalyzed hydrolysis of organophosphate nerve agents better than a lower concentration buffer. Although the magnitude, the lower detection limit and the response time of the electrode was better in the weak buffer, the linear dynamic concentration range was better in the stronger buffer (data not shown). Since an objective of this work was to develop a rapid and sensitive biosensor for organophosphate nerve agents, 1 mM buffer was selected for subsequent investigations. In the above experiments the total salts concentration of the buffers were adjusted to 150 mM by adding sodium chloride, to provide an isotonic environment for the cells so that they will not lyse due to osmotic shock. The use of this neutral salt was also helpful in stabilizing the weaker buffers, especially at 1 mM.

3.1.2. Effect of Starting pH

The pH profile for the microbial biosensor is shown in Figure 2. The profile is similar to that for the free and immobilized enzyme [42]. This observation in conjunction with the fact that there was no potential drop when the cells were absent, indicate that the observed pH dependence of the microbial biosensor response is due to the pH dependence of the OPH activity. Based on the maximum sensitivity, lowest response time and largest dynamic range, a starting pH of 8.5 was selected for subsequent use.

3.1.3. Effect of Temperature

Figure 3 shows the effect of temperature on the response of the potentiometric microbial biosensor. As shown, the sensor response increased with temperature up to 37 °C and then decreased when the temperature increased to 45 °C. The initial increase in the rate is

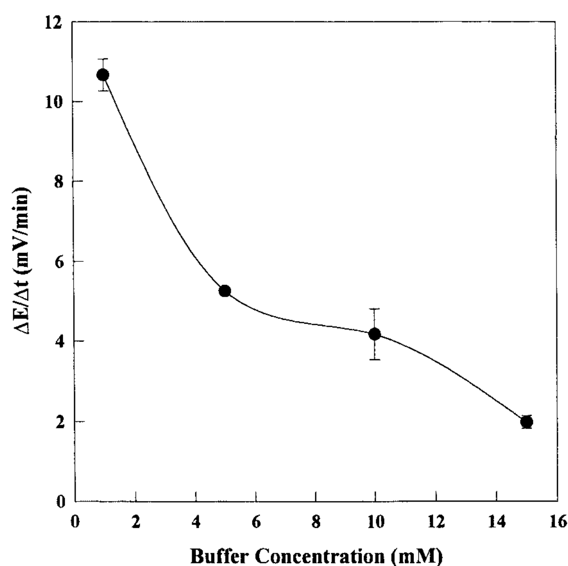

Fig. 1. Effect of buffer concentration on the response of the microbial biosensor to 0.1 mM paraoxon in pH 8.5 HEPES buffer with 0.05 mM $CoCl_2$ at 20 °C. Cell loading: 1.5 mg dry weight. Each point represents the average of three measurements and the error bar represents ±1 standard deviation.

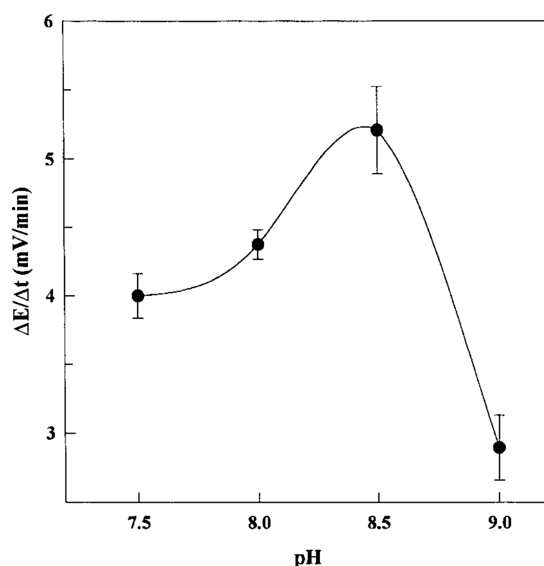

Fig. 2. Effect of buffer starting pH on the response of the microbial biosensor to 0.1 mM paraoxon in 1 mM HEPES + 150 mM NaCl + 0.05 mM $CoCl_2$ buffer at 20 °C. Cell loading: 1.5 mg dry weight. Each point represents the average of three measurements and the error bar represents ±1 standard deviation.

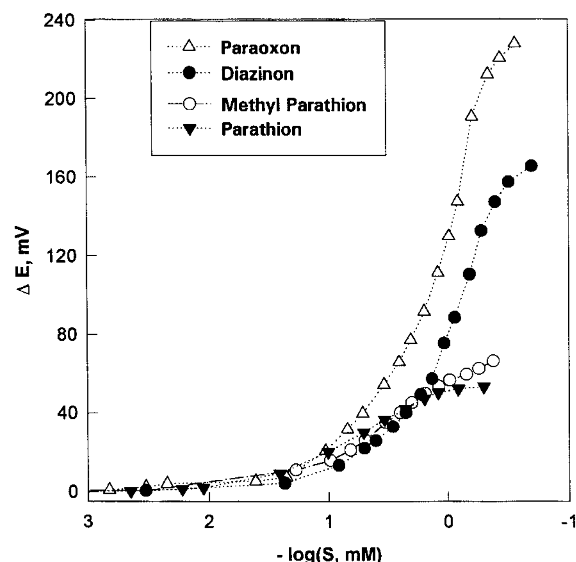

Fig. 4. Calibration plots for organophosphates. Conditions: 1 mM HEPES + 150 mM NaCl + 0.05 mM $CoCl_2$, pH 8.5, 20 °C; 1.5 mg dry weight.

attributed to the increase of both the enzyme reaction and mass transport rates. The decrease in the rate at higher temperatures is due to enzyme denaturation and disruption of the cell wall membrane. Although 37 °C was determined to be the optimum temperature for the enzyme electrode operation, subsequent experiments were still performed at room temperature, 20 °C. This was done in order to prevent evaporative losses during the course of the experiment and ease of operations.

3.2. Analytical Characteristics of Microbial Biosensor

3.2.1. Calibration Plots for Organophosphates

The calibration plots for paraoxon, parathion, methyl parathion and diazinon using the potentiometric microbial biosensor (these plots were prepared from the steady-state response data) are shown in Figure 4. As is generally observed with potentiometric biosensors [44], the calibration plots were not linear. This nonlinearity can be easily handled with computer support. The sensor operating range for the studied analytes spanned two orders of magnitude. The lower detection limit (defined as three times the standard deviation of the response obtained for a blank) of the electrode for all four OPs studied was 3 μM. This value is comparable to that reported for the OPH-based enzyme electrode [42] and microbial biosensor system [13]. It is, however, 1 to 3 orders of magnitude higher than for AChE-based biosensors [14–34]. The high lower detection limit will restrict the use of this microbial biosensor for environmental monitoring. For any such application, sample preparation and concentration prior to analysis will be necessary.

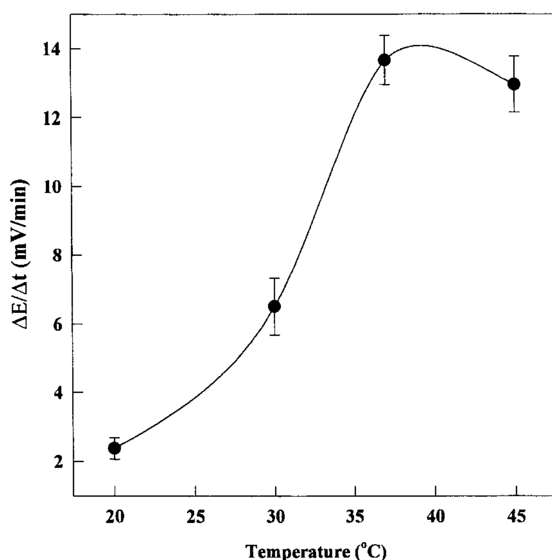

Fig. 3. Effect of temperature on the response of the microbial biosensor to 0.1 mM paraoxon in pH 8.5, 1 mM HEPES + 150 mM NaCl + 0.05 mM $CoCl_2$. Cell loading: 1.5 mg dry weight. Each point represents the average of three measurements and the error bar represents ±1 standard deviation.

3.2.2. Selectivity

Unlike the AChE-based biosensors that cannot distinguish between OPs and other neurotoxins [14–34], the present microbial biosensor was very selective for OPs. Other commonly used pesticides such as simazine, atrazine, sutan and sevin at concentrations 20-fold higher than the minimum paraoxon concentration did not interfere.

Nonspecific cellular responses generally limit the selectivity of microbial biosensors. Since *E. coli* can metabolize a variety of sugars to produce acidic products that can cause pH drop, sugars can interfere in quantification. The response of microbial biosensor prepared with freshly grown OPH-expressing *E. coli* cells, was not interfered by sucrose, fructose or galactose at 20 fold (5 mM) higher concentrations than paraoxon (25 μM). However, there was a significant (approximately 300 %) interference in the response of the biosensor by 5 mM glucose, which disappeared after 4 days. While the results for the nonspecific responses to sucrose, fructose and galactose agree with the biosensor system based on cryo-immobilized OPH-expressing *E. coli* cells the interference by glucose was not observed previously [13]. In order to investigate whether the cell age or cell immobilization method was responsible for the observed difference in the response to glucose, nonspecific cellular responses of a series of microbial biosensors prepared with cells that were grown, harvested and stored in the buffer under starved conditions for different time periods were evaluated. The glucose interference trend for these electrodes was similar to that

seen earlier, i.e. interference gradually declined from a relatively high value to zero as the cells aged. We attribute this phenomenon to the weakening of the transport machinery responsible for pumping substrate(s) across the cell membrane and therefore hypothesize that the degree of non-specific response to glucose is governed by the cell age and not the method of cell immobilization. Since Rainina et al. [13] did not report the cell age at the time of carbohydrate interference investigations with cryoimmobilized cells, it is a speculation that a similar high glucose interference would be present at the start.

3.2.3. Precision and Reproducibility

The relative standard deviation of the microbial electrode for paraoxon, methyl parathion and diazinon were 2.1 % ($n = 5$), 5.38 % ($n = 5$) and 7.18 % ($n = 5$), respectively. This low relative standard deviation demonstrates a good precision of analysis. Similarly, a very low relative standard deviation of 2.57 % ($n = 3$) in the response of three different microbial electrodes demonstrates an excellent electrode-to-electrode reproducibility.

3.2.4. Stability and Analysis Time

The long-term storage lifetime stability of the microbial biosensor was investigated by evaluating the response of the sensor to paraoxon and storing back at 4 °C in pH 8.5, 1 mM HEPES + 150 mM sodium chloride + 0.05 mM $CoCl_2$ buffer. The biosensor response was fairly stable, only a 6 % decline from the original response, up to three days. The response subsequently decreased rapidly to 58 % of the original response by the end of 24 days (Fig. 5). A similar decline in the OPH activity with time has been reported for *E. coli* cells expressing OPH intracellularly [45]. The observed decrease of the electrode response, in conjunction with the observation of a gradual decline of glucose interference with time, lead us to hypothesize that the decline in the sensor response is a result of the weakened transport machinery of the cells. Such a phenomenon would suggest that all types of microbial biosensors based on cells expressing OPH intracellularly should be unstable. This, however, was not observed for the biosensor system based on cryoimmobilized OPH expressing *E. coli* cells [13]. The stability of the microbial biosensor system based on cryoimmobilized cells [13] was similar to the enzyme electrode [42]. Based on the fact that this contradicts reported progressive decline in the OP uptake rate by the cells [45], lead us to speculate that the *E. coli* cells in the cryogel might be lysed and not intact. The absence of the cell wall enveloping the enzyme will make the cryoimmobilized cells essentially perform like cryoimmobilized enzyme and therefore exhibit a stability similar to the enzyme electrode.

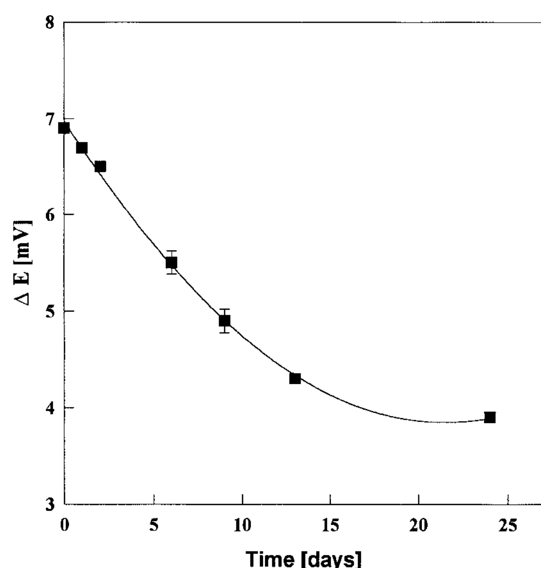

Fig. 5. Stability of the potentiometric microbial biosensor. Response of the sensor to 0.025 mM paraoxon in 1 mM HEPES + 150 mM NaCl + 0.05 mM $CoCl_2$, pH 8.5 at 20 °C. Cell loading: 1.5 mg dry weight. Each point represents the average of three measurements and the error bar represents ±1 standard deviation.

The problem of membrane transport of cell substrate can be reduced by treating cells with permeabilizing agents such as EDTA, DMSO, tributyl phosphate etc. [46] or by UV irradiation [47]. However, not all enzymes are amenable to such treatments, and viable cells cannot be subject to permeabilization. One potential solution is to anchor and display the enzyme responsible for catalyzing the reaction onto the cell surface, thereby eliminating transport limitation. Recently, we have successfully anchored and displayed OPH onto the surface of *E. coli* [48]. Cultures with surface-expressed OPH hydrolyzed parathion and paraoxon very effectively without the transport limitation observed in cells expressing OPH intracellularly. Whole cells with surface-expressed OPH retained 100 % activity over a period of one month when incubated at 37 °C [45]. Using the cells expressing OPH on their cell surface instead of the one expressing OPH intracellularly can potentially improve the biosensor stability significantly.

The analysis times of the microbial biosensor in steady-state (determined from the time required to achieve 90 % of maximum response) and kinetic modes (to operate the sensor in kinetic mode, it will have to be interfaced to a computer with appropriate support software) of operations were 10 min and 2 min, respectively. These analysis times are comparable to the other OPH-based biosensors [13, 42] and the disposable AChE-based biosensors, where the final enzyme regeneration/reactivation step is omitted, [31, 32]. On-the-other hand, the analysis times for the present microbial biosensor are far superior than the 1 to 5 h necessary for reusable type AChE-based biosensors [14–30, 33, 34].

4. Conclusions

In conclusion, an inexpensive and easy to construct potentiometric microbial biosensor for the direct, rapid and selective measurement of organophosphate nerve agents was developed. The sensor had short response time, wide operational span and was stable up to three days. These features will make it a potentially useful analytical tool for monitoring chemical or biological detoxification processes [2–10]. The high lower detection limit, however, will limit the applicability of the present sensor for environmental monitoring, to off-line analysis. For any such application off-line sample preparation involving solvent extraction and concentration will be necessary. The sensitivity and detection limit necessary for environmental monitoring applications can be potentially improved by 1) measuring the pH differential between two pH electrodes, one modified with the cells and the other unmodified [49] and/or 2) using *E. coli* mutants expressing OPH variant with a higher V_m/K_M than the present. Additionally, the long-term stability of the microbial biosensor can be improved by replacing the present microbial cells with the ones that express OPH on the cell surface [48].

5. Acknowledgements

This work was supported by a grant from the U.S. EPA (R8236663-01-0). We thank Dr. J.S. Karns of the USDA for providing *E. coli* strain carrying plasmid pJK33.

6. References

[1] Food and Agricultural Organization of the United Nations, Rome, *FAO Prod. Yearb.* **1989**, *43*, 320.
[2] J.A. Compton, in *Military Chemical and Biological Agents*, Telford Press, NJ **1988**, p. 135.
[3] D.M. Munnecke, *J. Agric. Food Chem.* **1980**, *28*, 10.
[4] W.M. Mulbury, P.L. Del Valle, J.S. Karns, *Pestic. Sci.* **1996**, *48*, 149.
[5] S.J. Coppella, N. DelaCruz, G.F. Payne, B.M. Pogell, M.K. Speedie, J.S. Karns, E.M. Sybert, M.A. Connor, *Biotechnol. Prog.* **1990**, *6*, 76.
[6] K.J. Grice, G.F. Payne, J.S. Karns, *J. Agric. Food Chem.* **1996**, *44*, 351.
[7] K.E. LeJeune, A.J Mesiano,. S.B. Bower, J.K. Grimsley, J.R. Wild, A.J. Russell, *Biotechnol. Bioeng.* **1997**, *54*, 105.
[8] S.R. Caldwell, F.M. Raushel, *Biotechnol. Bioeng.* **1991**, *37*, 103.
[9] J.E. Kolakowski, J.J. DeFrank, S.P. Harvey, L.L. Szafraniec, *Biocat. Biotransform.* **1997**, *15*, 297.
[10] V.K. Rastogi, J.J. DeFrank, T.C. Cheng, J.R. Wild, *Biochem. Biophys. Res. Commun.* **1997**, *241*, 294.
[11] J. Sherma, *Anal. Chem.* **1993**, *65*, R40.
[12] S. Yao, A. Meyer, G. Henze, *Fresnius J. Anal. Chem.* **1991**, *339*, 207.
[13] E. Rainina, E. Efremenco, S. Varfolomeyev, A.L. Simonian, J. Wild, *Biosens. Bioelectron.* **1996**, *11*, 991.
[14] C. La Rosa, F. Pariente, L. Hernandez, E. Lorenzo, *Anal. Chim. Acta* **1994**, *295*, 273.
[15] P. Skladal, *Anal. Chim. Acta* **1991,** *252*, 11.
[16] P. Skladal, M. Mascini, *Biosens. Bioelectron.* **1992**, *7*, 335.
[17] J-L. Marty, K. Sode, I. Karube, *Electroanalysis* **1992**, *4*, 249.
[18] D. Martorell, F. Céspedes, E. Martínez-Fàregas, S. Alegret, *Anal. Chim. Acta* **1994**, *290*, 343.
[19] N. Mionetto, J-L. Marty, I. Karube, *Biosens. Bioelectron.* **1994**, *9*, 463.
[20] G. Palleschi, M. Bernabei, C. Cremisini, M. Mascini, *Sens. Actuators B* **1992**, *B 7*, 513.
[21] M. Trojanowicz, M.L. Hitchman, *Trends Anal. Chem.* **1996**, *15*, 38.
[22] S. Kumaran, M. Morita, *Talanta* **1995**, *42*, 649.
[23] S. Kumaran, C. Tranh-Minh, *Anal. Biochem.* **1992**, *200*, 187.
[24] C. Tran-Minh, P.C. Pandey, S. Kumaran, *Biosens. Bioelectron.* **1990**, *5*, 461.
[25] V.L.F. Chuna Bastos, J. Chuna Bastos, J.S. Lima, M.V. Castro Faria, *Wat. Res.* **1991**, *25*, 835.
[26] S.V. Dzyadevich, A.P. Soldatkin, A.A. Shul'ga, V.I. Strikha, A.V. El'skaya, *J. Anal. Chem.* **1994**, *49*, 874.
[27] C. Garcia de Maria, T.M. Munoz, A. Townshend, *Anal. Chim. Acta* **1994**, *295*, 287.
[28] W. Hobel, J. Polster, *Fresenius J. Anal. Chem.* **1992**, *343*, 101.
[29] P. Morris, I. Alexandre, M. Roger, J. Remacle, *Anal. Chim. Acta* **1995**, *302*, 53.
[30] K.R. Rogers, C.J. Cao, J.J. Valdes, A.T. Eldefrawi, M.E. Eldefrawi, *Fund. Appl. Toxicol.* **1991**, *16*, 810.
[31] I. Palchetti, A. Cagnini, M. Del Carlo, C. Coppi, M. Mascini, A.P.F. Turner, *Anal. Chim. Acta.* **1997**, *337*, 315.
[32] J. Diehl-Faxon, A.L. Ghindilis, P. Atanasov, E. Wilkins, *Sens. Actuators B* **1996**, *35-36***,** 448.
[33] R.T. Andres, R. Narayanaswamy, *Talanta.* **1997**, *44*, 1335.
[34] W. Trettnak, F. Reininger, E. Zinterl, O.S. Wolfbeis, *Sens. Actuators B* **1993**, *11*, 87.
[35] H. Attaway, J.O. Nelson, A.M. Baya, M.J. Voll, W.E. White, D.J. Grimes, R.R. Colwell, *Appl. Environ. Microbiol.* **1987**, *53*, 1685.
[36] J.S. Karns, M.T. Muldoon, W.W. Mulbury, M.K. Derbyshire, P.C. Kearney, in *Biotechnology in Agricultural Chemistry. ACS Symposium Series. 334*, (Eds: H.M. LeBaron, R.O. Mumma, R.C. Honeycutt, J.H. Duesing). ACS, Washington, DC **1987**, pp. 156–170.
[37] D.M. Munnecke, D.P.H. Hsieh, *Appl. Environ. Microbiol.* **1976**, *31*, 63.
[38] K.I. Dave, C.E. Miller, J.R. Wild, *Chem.-Biol. Interactions*. **1993**, *87*, 55.
[39] J.P. Phillips, J.H. Xin, K. Kirby, C.P. Milne, P. Krell, J.R. Wild, *Proc. Natl. Acad. Sci. (USA)* **1990**, *87*, 8155.
[40] J.G. Steiert, B.M. Pogell, M.K. Speedie, J. Laredo, *Bio. Technol.* **1989**, *7*, 65.
[41] B. Xu, J.R. Wild, C.M. Kenerley, *J. Ferment. Bioeng*. **1996**, *81*, 473.
[42] P. Mulchandani, A. Mulchandani, I. Kaneva, W. Chen, *Biosens. Bioelectron*. **1998**, in press.
[43] I. Karube, in *Biosensors Fundamentals and Applications* (Eds: A.P.F. Turner, I. Karube, G.S. Wilson), Oxford University Press, Oxford, UK, **1987**, pp 13–29.
[44] U. Brand, T. Scheper, K. Schügerl, *Anal. Chim. Acta* **1989**, *226*, 87.
[45] W. Chen, A. Mulchandani, *TIBTECH* **1998**, *16*, 71.
[46] H.J.P. Marvin, M.B.A. ter Beest, B. Witholt, *J. Bacteriol*. **1989**, *179*, 5262
[47] .S-H. Hung, J.C. Liao, *Appl. Biochem. Biotechnol*. **1996**, *56*, 37.
[48] R. Richins, I. Kaneva, A. Mulchandani, W. Chen, *Nat. Biotechnol*. **1997**, *15*, 984.
[49] M. Luzzana, G. Dossi, A. Mosca, A. Granelli, D, Berger, E. Rovida, M. Ripamonti, A. Musetti, L. Rossi-Bernardi, *Clin. Chem.* **1983**, *29*, 80.