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Microbial Biosensors for Organophosphate Pesticides

Ashok Mulchandani · Rajesh

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Abstract Organophosphates, amongst the most toxic substance known, are used widely in agriculture around the world. Their extensive use, however, has resulted in their occurrence in the water and food supply threatening humans and animals. Therefore, there is a need for determination of these neurotoxic compounds sensitively, selectively, and rapidly in the field. The present work is a brief review on the recent advancements in amperometric, potentiometric, and optical biosensors using genetically engineered microorganisms expressing organophosphate hydrolyzing enzyme intracellularly or anchored on the cell surface for the detection of organophosphate pesticides. The benefits and limitations associated with such microbial biosensors are delineated.

Keywords Biosensor · Microbial · Organophosphate · Nerve agents · Pesticides

Introduction

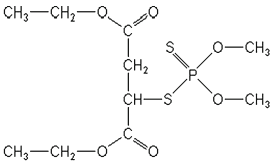
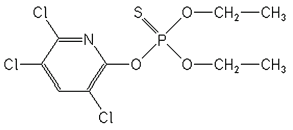
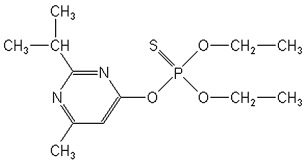
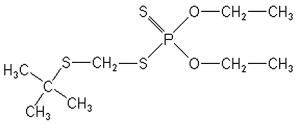
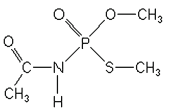
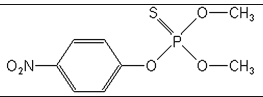
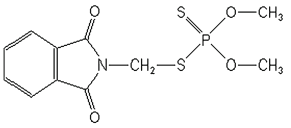
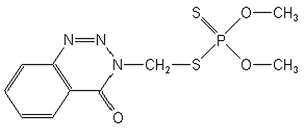
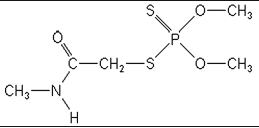
Organophosphates (OP) are dual-use compounds that have found use both as chemical warfare agents (nerve gases) that are weapons of mass destruction and pesticides that have been able to increase the agriculture production around the globe to help feed the increasing global population. These extremely toxic compounds (Table 1) are inhibitors of cholinesterase enzymes that are involved in the central nervous system of humans and insects leading to neurotransmitter acetylcholine buildup which interferes with muscular response, functioning of vital organs, and eventually death [1, 2].

In 2000, the year for which the latest data is available, approximately 33 million kg of OP pesticides, accounting for 70% of all the pesticides, were used in the USA alone

A. Mulchandani (✉) · Rajesh
Department of Chemical and Environmental Engineering, University of California,
Riverside, CA 92521, USA
e-mail: adani@engr.ucr.edu

Rajesh
National Physical Laboratory (Council of Scientific & Industrial Research), New Delhi, India

Table 1 OP pesticides name, structure, quantity used in USA in 2000, and lethal dose

Ingredient	Formula/Structure	Amount used in US, million kg	Mammalian LD_{50} (mg kg^{-1})
Malathion		10.45–15.5	1000–1375
Chlorpyrifos		5–7.27	135–163
Diazinon		1.8–3.18	1250
Terbufos		1.36–2.27	1.3–9.2
Acephate		0.9–1.36	1,030–1,447
Methyl parathion		0.45–1.36	0–25
Phosmet		0.45–0.9	147–316
Azinphos-Methyl		0.45–0.9	4
Dimethoate		0.45–0.9	160–387

(Table 1) [3]. The widespread use of OP in rural and urban theater as pesticides and insecticides has resulted in the observance of trace amount of these extremely toxic compounds in ground and surface waters [4] and on fresh and processed fruits and vegetables [5]. Therefore, sensitive, selective, rapid, reliable, and cost-effective methods/tools to detect these compounds are necessary to protect humans and animals.

Conventional analytical methods for monitoring OP pesticides are primarily based on gas and liquid chromatography with different detectors. While very sensitive and selective, the inability to deploy them in the field, expensive instrumentation, and need of highly trained technician for operation are a few limitations of these techniques. Biological methods, such as immunoassays, have been developed as an alternative to chromatography for several important OP pesticides. Although very sensitive and selective, they are time consuming, require extensive sample handling, and like chromatographic methods are not field-deployable [6]. Biosensors, on the other hand, have the potential for miniaturization, portability, and simple operation without compromise of sensitivity and selectivity.

Biosensors

Biosensing devices for OP pesticides can be classified into two groups: inhibition-based and catalytic-based. The basic principle of operation of the former biosensors is the ability of pesticides to inhibit cholinesterases, acetylcholinesterase, or butyrylcholinesterase and the ability to hydrolyze acetylcholine or butyrylcholine, respectively [7–9]. On the other hand, the catalytic-based biosensors use OPs, pesticides, and nerve gases as substrate and hydrolyze them to generate an acid and alcohol [10].

Inhibition-Based Biosensors

Acetylcholinesterase's (AChE) biological role is the termination of impulse transmissions at cholinergic synapses within the nervous system of the insects and mammals by rapid hydrolysis of the neurotransmitter acetylcholine. Pesticides block the catalytic activity of the active center, thus acting as inhibitors of AChE. This results in the accumulation of acetylcholine in the synaptic membrane, which blocks the nerves to process the signals properly. A number of enzyme-electrodes using the cholinesterase alone or in combination with choline oxidase based on potentiometric, amperometric, and optical transducers have been developed. Excellent review articles documenting the development and application of cholinesterase inhibition-based biosensors have been published [11, 12].

Despite their high sensitivity, biosensors based on enzyme inhibition suffer from major drawbacks including a long and tedious protocol for sample preparation, poor selectivity (since cholinesterases are inhibited by not only OP pesticides but also by carbamate pesticides and many other compounds), and are limited to single use unless regenerated by treatment with pyridine 2-aldoxime methiodide or 1,1 V-trimethylene-bis-4-formylpyridinium bromide dioxime, which in many cases is only partial.

Catalytic-Based Biosensors

A preferred biosensing route for OP pesticides detection involves the biocatalytic activity of organophosphotriester hydrolyzing enzymes. Besides being more specific for OPs,

catalytic-based biosensors are simple, single-step, fast, and can be used multiple times. Several enzymes are capable of hydrolyzing OP compounds. These include paraoxanase (PON), a serum high-density lipoprotein-associated esterase which among other things provides some protection against OP poisoning and diisopropyl fluorophosphate (DFPase) found in squid nervous tissues, organophosphorus acid anhydrolase (OPAA), and organophosphorus hydrolase (OPH). The latter two are bacterial enzymes with the widest substrate range, stability in various environments, and are relatively easy to express in bacterial species. OPAA has high specificity for hydrolysis of P–F and a P–CN bond present in nerve gases sarin and tabun [13] but is not capable of hydrolyzing OPs with P–O and P–S bonds present in widely used pesticides and insecticides, such as parathion, malathion, and demeton-S [14]. On the other hand, OPH catalyzes hydrolysis of the P–O, P–F, P–S, or P–CN bonds of various OP compounds, although to different extent as shown in Table 2, making it attractive for much broader spectrum of OPs [15]. OPH has been studied extensively over the last decade, and several genetically engineered variants have been produced in an effort to improve its catalytic ability [16, 17].

OPH hydrolyzes OPs generating an acid and an alcohol (Fig. 1). While the acid production can be monitored by hydrogen ion-sensing potentiometric transducer or pH-sensitive dye, the alcohol, which in many cases is chromophoric, fluorogenic, and/or electroactive, can be monitored optically and/or electrochemically. Because of the many advantages offered by catalytic-based biosensor over the inhibition-based biosensor, several OPH- and OPAA-based potentiometric, optical, amperometric, microcantilever, conductometric, and lab-on-a-chip biosensors have been developed to monitor OP pesticides paraoxon, methyl parathion, diazinon, coumaphos, fenitrothion, and ethyl parathion [18–27] and simulants of nerve gases sarin and VX [28]. Although these OP hydrolase-based biosensors provide simple, rapid, accurate, and direct monitoring means for OP compounds, the lower sensitivity of potentiometric and optical biosensors and poor selectivity of amperometric biosensor over phenolic compounds limit their applications in environmental monitoring. Moreover, these enzyme electrodes required purified enzyme, which is a laborious, time-consuming, and costly effort.

Table 2 Kinetic parameters of OPH-hydrolyzed organophosphates [15]

Substrate	Bond type	K_{cat}/s^{-1}	Km/mmol L^{-1}	K_{cat}/Km/mol^{-1} L s^{-1}
Paraoxon	P–O	3,170	0.058	5.5×10^{7}
Parathion	P–O	630	0.24	2.6×10^{6}
Methyl parathion	P–O	76	0.84	0.5×10^{9}
Coumaphos	P–O	600	0.39	1.5×10^{6}
Malathion	P–S	6.7×10^{-3}	0.14	47.8
Acephate	P–S	2.8	160	17.5
Demeton-S	P–S	1.2	0.78	1.5×10^{3}
VX	P–S	0.3	0.44	$6.\times10^{2}$
Diazinon	P–S	176	0.45	3.9×10^{6}
Diflufenican (DFF)	P–F	74.7	0.96	7.8×10^{4}
Diisopropyl fluorophosphates (DFP)	P–F	465	0.048	9.7×10^{4}
Sarin	P–F	56	0.7	8.0×10^{4}
Soman	P–F	5	0.5	1.0×10^{4}

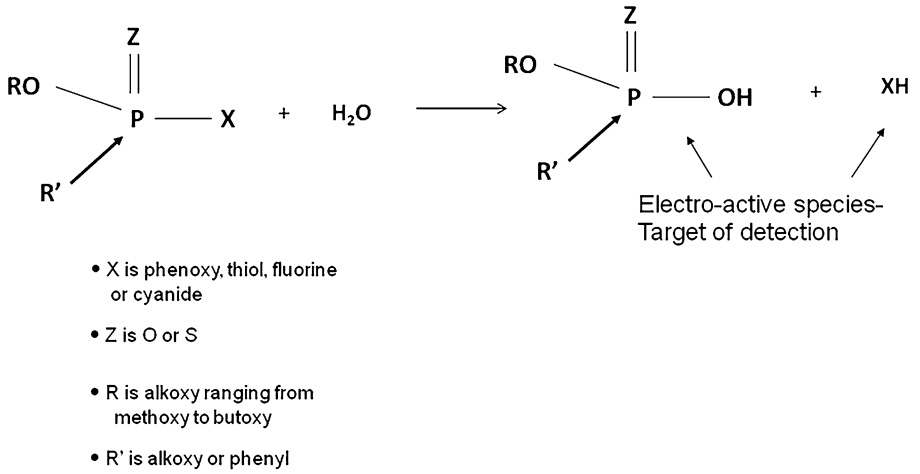


Fig. 1 Reaction scheme for the OPH-catalyzed hydrolysis of OPs

Microbial Biosensors

A whole cell is a complex biological recognition element that holds the promise of presenting the intact sub-cellular machinery for the identification of physiologically relevant events, and providing functional and analytical information [29]. The internal amplification cascades of cells can be used to increase the sensitivity of the devices. In principle, the microbial sensors mainly involve changes in respiratory activity, or the production of electrochemically active metabolites. The progress in molecular biology/recombinant DNA technologies provides avenues for tailoring the microorganisms to improve the existing enzyme activity or express a foreign protein/enzyme in the host cell. The former can be categorized into two groups: activation of microbial respiration by assimilation of organic compounds and inactivation of the respiration by inhibitory substances. These changes can be monitored by using a dissolved oxygen electrode. On the other hand, electrochemically active metabolites produced by the cells can be monitored amperometrically. Biosensor based on whole cell sensing systems carrying a genetically engineered reporter gene that is inserted into the cell under study has attracted a lot of interest since the inserted genes expressed only upon exposure to a monitored toxicant, which can be quantitatively measured. The use of whole cells has been explored as an alternative biological catalyst without the high cost of purifying enzymes. Over the past decades, different types of cells have been incorporated into the transducers for making microbial biosensors for the detection of organophosphoric acid [30, 31]. Thus, using microorganisms as biorecognition element provides an ideal alternative to purified enzyme. In this article, we review the different types of microbial biosensors for detection of OP pesticides.

The simplest microbial biosensors for OP pesticides are based on genetically engineered *Escherichia coli* harboring the OP degradation gene of *Pseudomonas putida* MG [32] or *Flavobacterium* sp. expressing OPH in cytoplasm in conjunction with hydrogen ion selective, i.e., pH, electrode transducer. The pH electrode measured the protons released in the OPH-catalyzed hydrolysis of OP pesticides. Cells were either cryoimmobilized by entrapment in poly(vinyl alcohol) gel and packed in a column reactor upstream of the pH sensor [33] or immobilized by entrapment behind a microporus

polycarbonate membrane on the top of the pH electrode (Fig. 2). While these biosensors provided simple and direct monitoring of OP compounds, the slow response and low sensitivity, essentially due to mass transport resistance of OPs and product across the periplasmic and cytoplasmic membranes, and in case of cryoimmobilized cells also across the gel, are major limitations of these biosensors. Additionally, the biosensor had a limited storage life as evidenced by a small decline of 6% in the original response over the first 3 days, which decreased rapidly to 58% by the end of day 24. The rapid decline after day 3 was attributed to the weakening of the cell transport machinery [34]. Further, the biosensor had to be used in a weak buffer to achieve the reported limit of detection, which can be problematic with environmental samples.

Microbial Biosensors with OPH Displayed on Cell Surface of *E. coli*

The diffusion resistance to mass transport by the cell membranes can be reduced by treating cells with permeabilizing agents such as EDTA, but all the enzymes are not amenable to this treatment. However, the problem of mass transport resistance can be overcome by genetically engineering microorganisms to anchor and display active OPH onto the cell surface. We have used the lipoprotein (Lpp) and outer membrane protein A (OmpA) fusion anchor and ice nucleation protein (INP) systems to display functional OPH on *E. coli* surface. The whole cell expressing OPH on the cell surface degraded parathion and paraoxon very effectively without any diffusion limitation, resulting in seven-fold higher rate of parathion degradation compared with the whole cell with similar level of intracellular OPH [35]. Similar to the above intracellular enzyme expressing *E. coli*, cells expressing OPH on the cell surface were integrated with potentiometric transducer (pH electrode) to construct a simple microbial biosensor that was able to detect as low as 2 μM of paraoxon, methyl parathion, and diazinon rapidly. The biosensor had comparable limit of detection but improved response time and stability to the potentiometric microbial biosensor based on intracellularly expressed OPH [36].

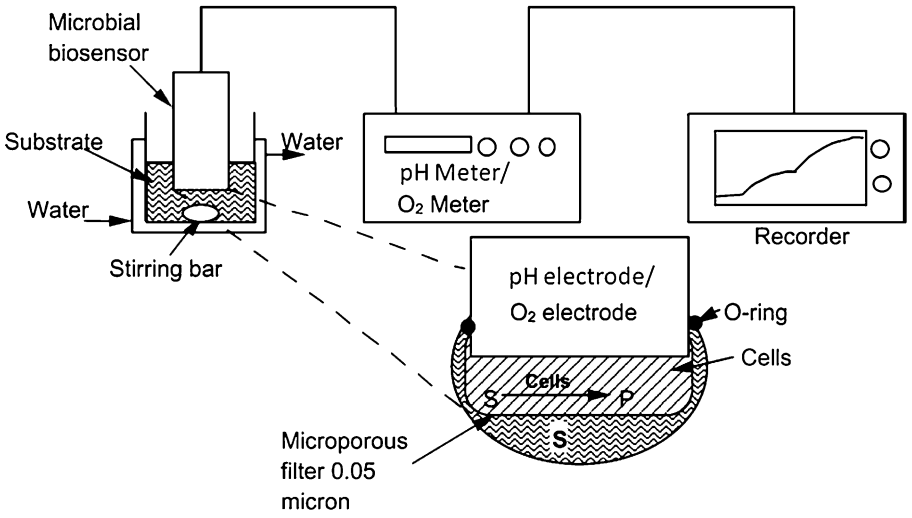


Fig. 2 Schematic of O_2/potentiometric OPH-based biosensor [36]

E. coli with surface anchored and displayed OPH has also been integrated with optical transducer [37]. This allowed the detection of different OPs in a mixture by monitoring the absorbance of the chromophoric products of OPH-catalyzed hydrolysis with distinct maximum absorbance wavelength, which is presently not feasible with cholinesterase-based biosensors, and in higher concentration buffer. The biosensor was fabricated by immobilizing *E. coli* cells in a low melting temperature agarose on a nylon membrane and attached to the common end of a bifurcated fiber-optic bundle. The backscattered radiation of the specific wavelength incident light was measured using a photomultiplier detector and correlated to the OP concentration (Fig. 3). At optimized conditions, the biosensor measured paraoxon, parathion, and coumaphos pesticides with high selectivity against triazine and carbamate pesticides in approximately 10 min. The lower detection limits were 3 μM for paraoxon and parathion and 5 μM for coumaphos, and the biosensor was stable for over a month and showed no decline in response for over 75 repeated usages [37].

While elegant, the micro-molar limit of detection is a major limitation of the above potentiometric and optical microbial biosensors based on *E. coli* with surface anchored OPH for use in environmental monitoring. Additionally, the low ionic strength buffer required for analysis using potentiometric microbial biosensor poses a problem for untreated environmental samples.

Microbial Biosensors with OPH Displayed in Cell Surface of PNP-Degrading Bacteria

When compared to optical and potentiometric transducers, amperometric transducers are more sensitive. As stated previously, alcohols produced by OPH-catalyzed hydrolysis of OPs are in many case electroactive and thus can be potentially detected amperometrically. For example, *p*-nitrophenol, the product of hydrolysis of paraoxon, ethyl parathion, methyl parathion, fenitrothion, and EPN, can be analyzed amperometrically. Taking advantage of this property, facile, sensitive, and selective amperometric detection of PNP-substituted OP pesticides was demonstrated. Carbon paste electrodes were modified with genetically engineered *Moraxella* sp. expressing OPH on the cell surface (using the ice nucleation protein from *Pseudomonas syringae* INA5) and used as a working electrode for a three-electrode electrochemical cell. OPH catalyzed the hydrolysis of PNP-

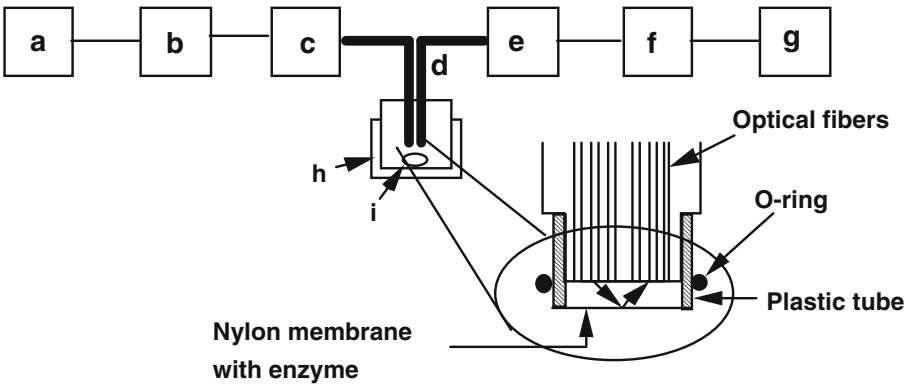


Fig. 3 Schematic of OPH-modified fiber optic microbial biosensor [37]. (a) Power supply; (b) Xenon-arc lamp; (c) monochromator; (d) bifurcated fiber bundle; (e) monochromator; (f) photomultiplier detection system; (g) chart recorder/computer; (h) thermostated vessel; (i) magnetic stirring bar

substituted OP pesticides to produce PNP which was detected anodically at the working electrode at +0.9 V vs. Ag/AgCl reference with the oxidation current proportional to the OP concentration (Fig. 4). Using this simple biosensor, as low as 0.2 μM paraoxon and 1 μM methyl parathion were detected with good sensitivity, excellent selectivity against carbohydrates, non-PNP OP, carbamates and triazine pesticides, and reproducibility. The sensor was stable for over a 45-day period when stored at 4 °C. However, the biosensor suffered from interferences from phenolic compounds, which are also oxidized at the biased potential of +0.9 V [38]. This problem can be addressed by measuring and subtracting the current from the cell-free electrode.

An alternative method for alleviating the phenol interference is by genetically engineering OPH in the microorganisms such as sp., *Arthobacter* sp. JS 443, and *P. putida* JS 444 that inherently mineralize PNP and either monitoring the co-substrate oxygen or other metabolic products of PNP (Fig. 5). Combining the Clark dissolved oxygen electrode with *Moraxella* sp. and *P. putida* JS 444 expressing OPH on the cell surface (Fig. 2), two simple yet sensitive, selective, and rapid microbial biosensors for PNP-substituted OP pesticides were reported. Operating under optimized conditions, the microbial biosensors detected as low as 27.5 ppb of paraoxon, 227 ppb of fenitrothion, and 1.6 ppm of EPN without interference from phenolic compounds, s-triazines, carbamates, and non-PNP substituent OP pesticides [39, 40]. A further improvement in the limit of detection of the amperometric microbial biosensor was realized by the detection of electrochemically active intermediates such as benzoquinone and hydroquinone produced during the degradation of PNP and 3-methyl-4-nitrophenol generated by *P. putida* JS444. The microbial biosensor consisted of genetically engineered *P. putida* JS444 endowed with OPH on its cell surface immobilized on a polycarbonate membrane and attached to the carbon paste electrode which acted as the working electrode in a three-electrode

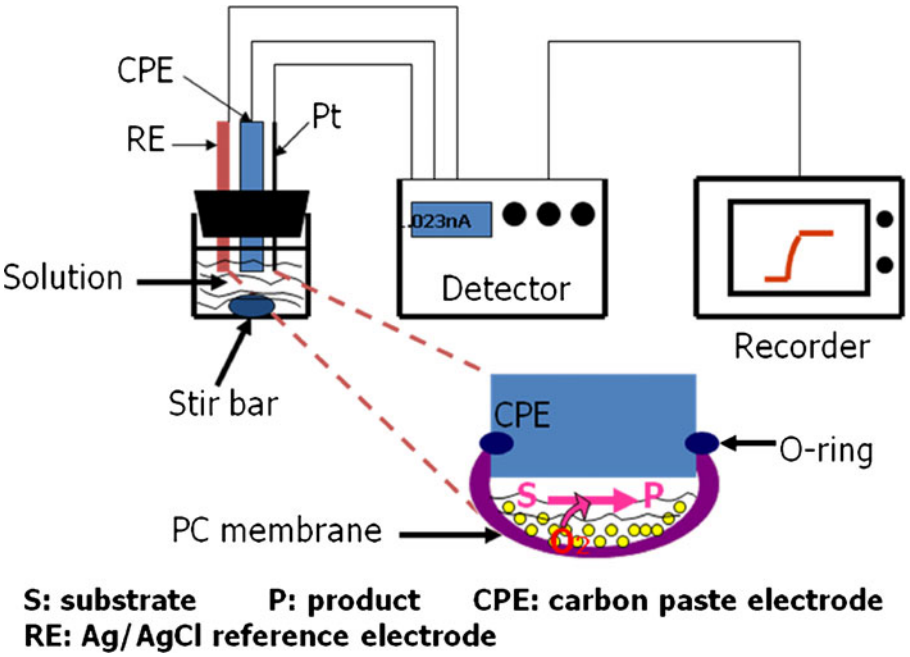


Fig. 4 Schematic of carbon paste electrode-based amperometric biosensor

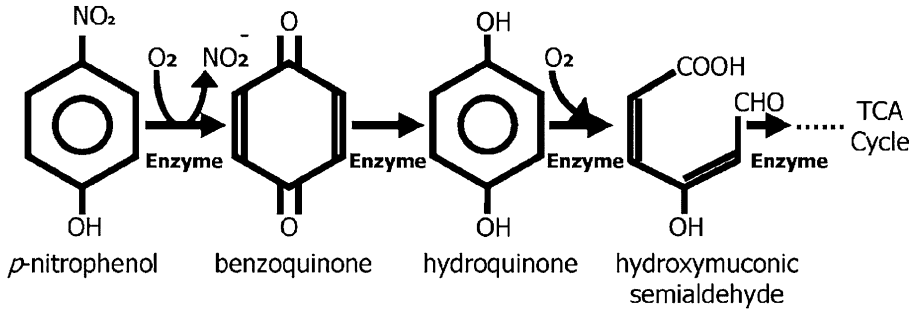


Fig. 5 Proposed pathway for PNP degradation [41]

electrochemical cell. Correlating the oxidation current measured at +600 mV biased potential vs. Ag/AgCl reference electrode; the biosensor measured as low as 0.28 ppb of paraoxon, 0.26 ppb of methyl parathion, 0.29 ppb parathion, 1.4 ppb fenitrothion, and 1.6 ppb EPN without interference from the other commonly used pesticides, such as atrazine, coumaphos, sutan, sevin, and diazinon, and naturally occurring chemicals such as phosphorus, nitrogen, chlorophyll, and metal ions in the water from Lake Elsinore, CA, USA. The service life of the hybrid biosensor was approximately 5 days when stored in the operating buffer at 4 °C [41, 42]. These are the best limit of detection of OPH-based biosensors, both microbial and enzyme, for OP pesticides reported to-date and comparable to cholinesterase inhibition-based enzyme electrodes. More importantly, unlike inhibition-based enzyme electrodes, this sensor had exquisite selectivity for *p*-nitrophenyl containing OPs, has a facile single-step protocol with short response time, does not require any sample pretreatment, and can be used repeatedly several times thus opening the avenue of real-time in situ monitoring. The above biosensors are however limited to nitrophenyl-substituted OPs.

Microbial Biosensor Based on *Saccharomyces cerevisiae*

The common baker's yeast, *S. cerevisiae*, because of its "generally regarded as safe (GRAS)" status, is an ideal microorganism as the biorecognition element for biosensors. *S. cerevisiae* was engineered to hydrolyze paraoxon through the heterologous expression of the *Flavobacterium* sp. *opd* gene [43]. Candidate paraoxon-inducible promoters were cloned and fused to the yeast-enhanced green fluorescent protein (yEGFP), and candidate promoters associated with paraoxon hydrolysis were fused to the red fluorescent protein (yDsRed). The ability of the yeast biosensor to detect paraoxon and its hydrolysis was demonstrated by the specific induction of the fluorescent reporter (yEGFP and yDsRed, respectively). The biosensor was found to have a rapid detection response of 15 to 30 min. Recently, we genetically engineered *S. cerevisiae* to anchor and display OPH and enhanced green fluorescent protein (EGFP) using the Flo 1p anchor system [44, 45]. Taking advantage of the pH dependence of EGFP fluorescence, a yeast microbial bioassay for detection of paraoxon was demonstrated. OP degradation releases protons and causes a change in pH. This pH change results in structural deformation of EGFP, which triggers quenching of its fluorescence, thereby making this cell useful for visual detection of OP. Cells were placed in a cell chip (Muranaka Medical Instruments, Osaka, Japan; Fig. 6), and the change is EGFP fluorescence upon addition of paraoxon was monitored by a fluorescence microscope.

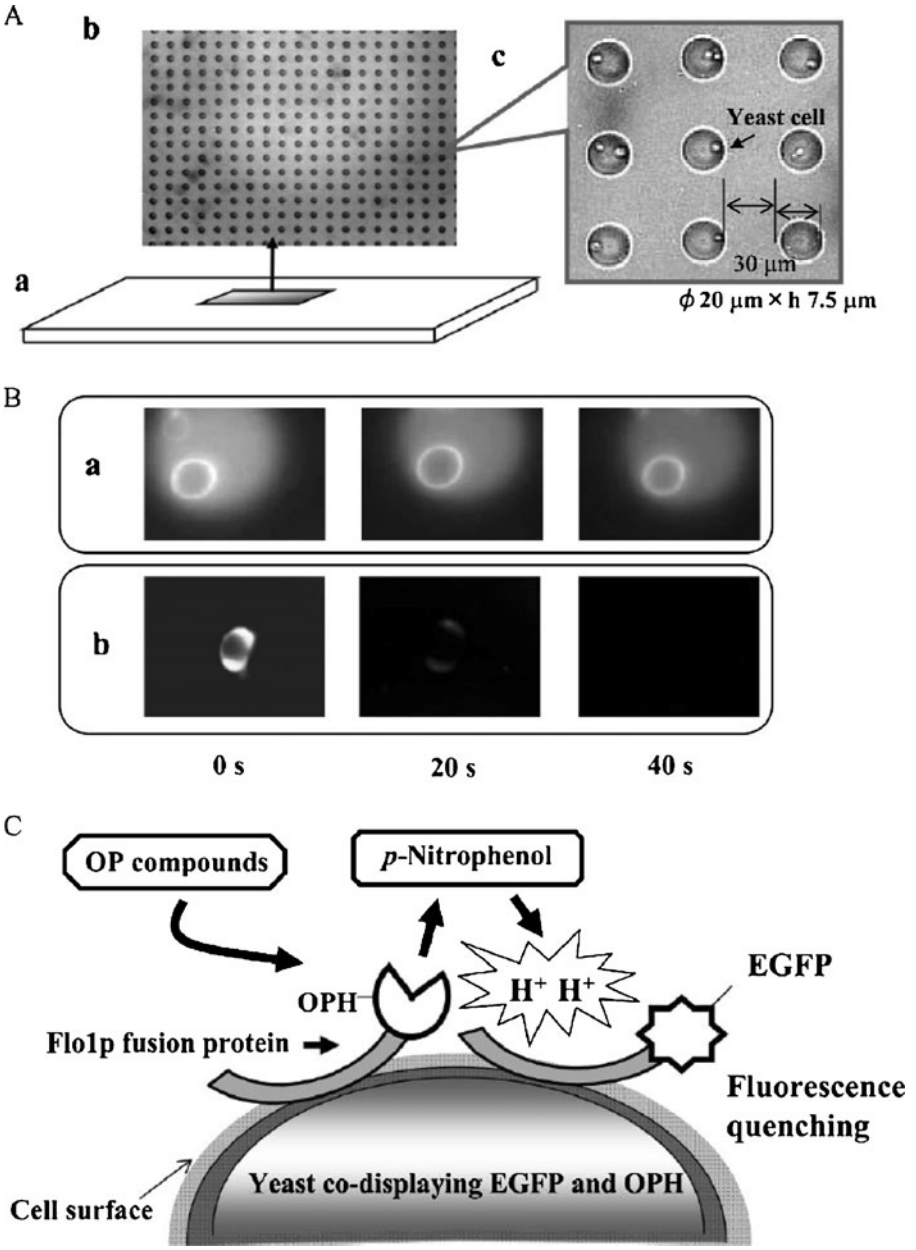


Fig. 6 (*A*) Fluorescence quenching of EGFP on a single yeast cell using a cell chip [45]

Conclusions and Future Directions

In summary, a number of microbial biosensors that detect OP pesticides sensitively, selectively, rapidly, and directly have been developed. The sensors were based on genetically engineered bacteria and yeast expressing organophosphorus hydrolase, an

OP ester-hydrolyzing enzyme, isolated from either *Pseudomonas diminuta* MG or *Flavobacterium* sp., intracellularly or on the cell surface integrated with potentiometric, amperometric, or optical transducer. Because the detection involves catalytic conversion of the analyte to a product as against inhibition-based enzyme, electrodes are single-step, rapid, and reusable. Some of the reported formats are potentially field-deployable for on-site and in situ measurements. Table 3 provides a summary of the performance characteristics of the different microbial biosensors reviewed in this manuscript. Using microorganisms as opposed to purified enzyme is cost-effective and simple. The endowment of OPH activity on the cell surface alleviated mass transport resistance of the cell walls and in the case of PNP degrader immobilization of a second biological transducer.

All the microbial biosensors developed to-date were based on wild-type OPH. As shown in Table 2, the catalytic effectiveness (K_{cat}/K_M) of this enzyme range from a high of 5.5×10^7 for paraoxon to a low of 14.5 for acephate. The sensitivity and detection limit of these biosensors can be improved by increasing the K_{cat} and/or lower the K_M. The advancement in protein/enzyme engineering has now made these goals possible. Examples of the engineering of OPH by site-directed mutagenesis and directed evolution have been reported to improve the rate of hydrolysis of paraoxon, methyl parathion, chlorpyrifos, sarin, and soman [16, 46–50]. The tools of protein engineering can be applied to generate OPH

Table 3 Analytical characteristics of some microbial biosensors for organophosphorus compounds in water

Target analyte	Microorganism	Transducer	Limit of detection	Range	Reference
Paraoxon	Recombinant *E. coli*	Potentiometric	–	0.001–1.0 mM	[33]
Paraoxon	*E. coli* containing plasmid pJK33	Potentiometric	3 μM	–	[34]
Paraoxon, methyl parathion, and diazinon	*E. coli* cells with surface-expressed OPH	Potentiometric	2 μM	0.055–1.8, 0.06–0.91, and 0.46–8.56 mM	[36]
Paraoxon, parathion, and coumaphos	*E. coli* cells with surface-expressed OPH	Optical	3 and 5 μM	0.0–0.6, 0.0–0.03, and 0.0–0.075 mM	[37]
Paraoxon, methyl parathion	*Moraxella* sp. with surface-expressed OPH	Amperometric	0.2 and 1 μM	Up to 40 μM, up to 175 μM	[38]
p-Nitrophenyl (PNP)-substituted organophosphates (OPs)	*Moraxella* sp. with surface-expressed OPH	Oxygen electrode	0.2 μM paraoxon	Up to 0.05 mM	[39]
Fenitrothion and EPN	*P. putida* JS444 with surface-expressed OPH	Oxygen electrode	277 ppb fenitrothion and 1.6 ppb EPN	Up to 0.05 mM	[40]
Paraoxon, methyl parathion, and parathion	*P. putida* JS444 with surface-expressed OPH	Amperometric	1, 1, and 1 nM	Up to 2 μM	[41]
Fenitrothion and EPN	*P. putida* JS444 with surface-expressed OPH	Amperometric	1.4 and 1.6 ppb	Up to 5 μM	[42]

variants with improved catalytic efficiency for other OPs. Additionally, recent reports of isolation of new bacteria with improved catalytic activity for methyl parathion compared to OPH from *Flavobacterium* sp. ATCC 27551, from contaminated sites in different parts of the globe [51], suggest the possibility of finding more bacteria-expressing enzymes that can hydrolyze OPs that are poorly hydrolyzed by OPH.

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