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Microbial biosensor for detection of methyl parathion using screen printed carbon electrode and cyclic voltammetry

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ABSTRACT

Whole cells of recombinant *Escherichia coli* were immobilized on the screen printed carbon electrode (SPCE) using glutaraldehyde. Recombinant *E. coli* was having high periplasmic expression of organophosphorus hydrolase enzyme, which hydrolyzes the methyl parathion into two products, p-nitrophenol and dimethyl thiophosphoric acid. Cells immobilized SPCE was studied under SEM. Cells immobilized SPCE was associated with cyclic voltammetry and cyclic voltammograms were recorded before and after hydrolysis of methyl parathion. Detection was calibrated based on the relationship between the changes in the current observed at +0.1 V potential, because of redox behavior of the hydrolyzed product p-nitrophenol. As concentration of methyl parathion was increased the oxidation current also increased. Only 20 μl volume of the sample was required for analysis. Detection range of biosensor was calibrated between 2 and 80 μM of methyl parathion from the linear range of calibration plot. A single immobilized SPCE was reused for 32 reactions with retention of 80% of its initial enzyme activity.

1. Introduction

Organophosphorus (OP) pesticides have been widely used in the field of agriculture for insect pest control. These pesticides and their degradation products cause environmental pollution and ecological problem. Methyl parathion is a nitro-aromatic organophosphate compound which was widely used as an insecticide although it is toxic to mammals (Melnikov, 1995; Stolyarov, 1998). Microquantities of organophosphorus compounds are being measured using analytical methods such as spectrophotometer, gas–liquid chromatography and thin-layer chromatography. But these existing analytical methods are having certain limitations, such as, it requires specialized laboratory personnel and expensive equipment, required certified standards for each known pesticides for evaluation and cannot be applied for online monitoring (Turner et al., 1987). Recently, there has been an intense research effort to develop biosensor devices for the determination of organophosphorus pesticides (Mulchandani et al., 2001a,b; Kumar et al., 2006). Fundamental requirement of biosensor is that the biological component should bring the physico-chemical changes in close vicinity of a transducer and in this direction immobilization technology has played a key role (Turner et al., 1987; D'Souza, 1999, 2001a,b; Tembe et al., 2006; Kumar and D'Souza, 2008, 2009, 2010). In case when enzymes expressed in periplasm and in cytoplasmic membrane of cells, whole cells directly can be used for immobilization even without permeabilisation (Svitel et al., 1998; D'Souza, 2001a). They can be used for simple biosensor applications, which do not require cofactor regeneration. Passive trapping of cells into the pores or adhesion on the surfaces of glass fibre or other synthetic membrane has been well documented (D'Souza, 1999, 2001a,b; Kumar et al., 2006; Jha et al., 2009). The major advantage of the cells immobilized through adhesion is that they are in direct contact with the liquid phase containing the substrate thus eliminating the mass transfer problems commonly associated with entrapment and other methods of immobilization (D'Souza, 2001a,b).

Screen printed electrode (SPE) has been used for the development of much simpler and portable devices for environmental analyses, which are low cost and can be mass-produced and applied for on-site analysis with low volume of sample (Bello-Rodriguez et al., 2004; Noh et al., 2005). There are many commercial sources of screen printed electrodes in different configurations (e.g., Zensor R&D electrochemical sensor, http://www.zensor.com.tw; PalmSens Electrochemical Sensor Interface, http://www.palmsens.com; BioSens Technology, http://www.bst-biosensor.de) are available which can be used for the immobilization of biological elements for biosensor development (Tudorache and Bala, 2007). Among biological elements mostly enzymes, antibodies and DNA have been immobilized on SPE for biosensors applications. Very few reports are available on microbial cells immobilization on SPE. Timur et al. (2003) have demonstrated the use of bacterially modified SPEs for the detection of phenol.

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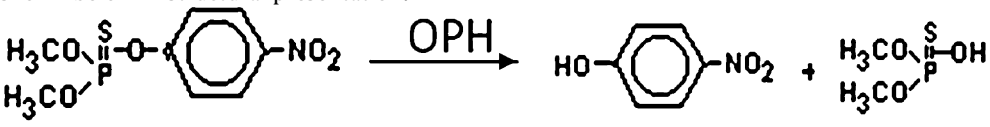
Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolyzing enzyme, first discovered in soil micro-organisms *Pseudomonas diminuta* MG and *Flavobacterium* sp. (Dumas et al., 1989; Munnecke and Hsieh, 1974). In each of these organisms enzyme was coded by *opd* gene (Mulbry et al., 1986; Harper et al., 1988; Somara et al., 2002) and hydrolyzed the methyl parathion into detectable product p-nitrophenol (Kumar et al., 2006) as shown below in structural presentation:



Methyl Parathion **P-Nitrophenol** **DMTP**

PNP can be detected by electrochemical or colorimetric methods and in turn can be exploited to develop a biosensor for detection of organophosphate pesticide. Our laboratory has been working in this field and had described an optical microbial biosensor for detection of methyl parathion pesticide using *Flavobacterium* sp. whole cells adsorbed on glass fiber filters as disposable biocomponent (Kumar et al., 2006). We have also developed a recombinant *Escherichia coli* which has a periplasmic expressed organophosphorus enzyme for methyl parathion hydrolysis (unpublished).

Objective of the present study was to immobilize the recombinant *E. coli* on screen printed carbon electrode (SPCE) using glutaraldehyde. Whole cells immobilized SPCE was studied by scanning electron microscope (SEM). Cyclic voltammograms were recorded for hydrolysis of methyl parathion using immobilized SPCE. Only 20 μl of sample is required for analysis. Analysis was based on the relationship between the hydrolysis of methyl parathion and the redox behavior of the hydrolyzed product, p-nitrophenol which causes change in amount of current at certain potential in cyclic voltammograms.

2. Materials and methods

2.1. Materials

Methyl parathion (*O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothionate) purity, 98.5% analytical grade was purchased from Dr. Ehrenstorfer Schorfers Augsburg, Germany, p-nitrophenol from Central Drug House, New Delhi, India. Recombinant *E. coli* having *opd* gene (accession no. AY766084.1) for periplasmic expression was developed in the laboratory by cloning the gene into the expression vector pET 28a and transforming it into the expression host *E. coli* strain BL21-CodonPlus®-RP (unpublished). Electrophoretic analysis (SDS-PAGE gel) of the periplasmic fractions of cells collected before and after induction was included as supplementary material to prove the overexpression of the OPH enzyme (supplementary Fig. S1). All other analytical grade chemicals were purchased from Sisco Research Laboratory, Mumbai, India.

2.2. Micro-organism and culture condition

Recombinant *E. coli* carrying higher expression plasmid with *opd* gene were grown in 100 ml Luria Bertani media supplemented with Kanamycin to a final concentration of 50 μg/ml in flasks on a shaker with vigorous aeration (150 rpm at 30 °C). *E. coli* bacteria harboring expression vectors were grown upto OD_{600} = 0.5 (attained in 3 h) before induction with 1 mM IPTG. 1 mM $CoCl_2$ was added to the culture after 24 h of induction and allowed it to grow for next 24 h. Cells were harvested and resuspended in 1/10th volume of the phosphate buffer (pH 8.0). Harvested cells were stored at 4 °C.

2.3. Apparatus

Cyclic voltammetry (CV) study was performed using CHI 405 electrochemical workstation (CH Instrument Company, Shanghai, China). The three-electrode system (TE100, Zensor R&D, Taichung, Taiwan) consists of a SPCE as working electrode, an Ag/AgCl pseudo reference electrode, and a platinum auxiliary electrode (Zen et al., 2004). The working, reference and counter electrodes are integrated on the screen printed carbon electrode as shown in supplementary Fig. S2. Diameter of the working electrode is 3 mm and its working area is 0.071 cm^2. Microbial cells were immobilized on SPCE and associated with electrochemical workstation.

2.4. Immobilization of whole cells on SPCE

Microbial cells were immobilized on the working area of SPCE. A 20 μl of whole cells suspension of recombinant *E. coli* was immobilized on the surface of working area by cross-linking with the 2% glutaraldehyde for 20 min. After immobilization microbial SPCE was washed with buffer and stored at 4 °C until and between uses.

2.5. Operating system and experimental procedure

As shown in supplementary Fig. S2 SPCE was directly associated with an electrochemical analyzer which was completely controlled by PC software for all operations including data acquisition and analysis. A 20 μl sample was added on working area of the immobilized SPCE and readings were recorded online using CH Instrument as it is shown in supplementary Fig. S2. First of all cyclic voltammogram was recorded in phosphate buffer (20 μl, pH 8.0) using blank (unmodified) electrode and cells immobilized (modified) electrode between +1.2 and −1.2 V at scan rate of 100 m V s^{-1}. Then cyclic voltammograms were also recorded using immobilized SPCE in buffer as well as in methyl parathion pesticide by providing the same condition. After then, again cyclic voltammograms were recorded before and after the hydrolysis of methyl parathion pesticide using immobilized electrode by providing the same condition. Immobilized electrodes were washed with buffer after every analysis of sample and were reused. All the experiments were carried out at room temperature. Response time of the microbial biosensor in steady-state (determined from the time required to achieve 90% of hydrolysis of methyl parathion) was considered for 5 min because of incubation of reaction and accumulation of product for better performance and no variability was observed in the sensor response.

2.6. SEM study of the whole cells immobilized SPCE

A scanning electron microscope (Model XL30 Philips, Netherlands) was employed to observe the surface structure of the cells immobilized SPCE. For SEM study, cells immobilized working area of the SPCE was mounted on stubs and coated with Au/Pd using a sputter coater. The SEM micrographs of the whole cells immobilized and unimmobilized (blank) SPCE were observed at magnification (5000×).

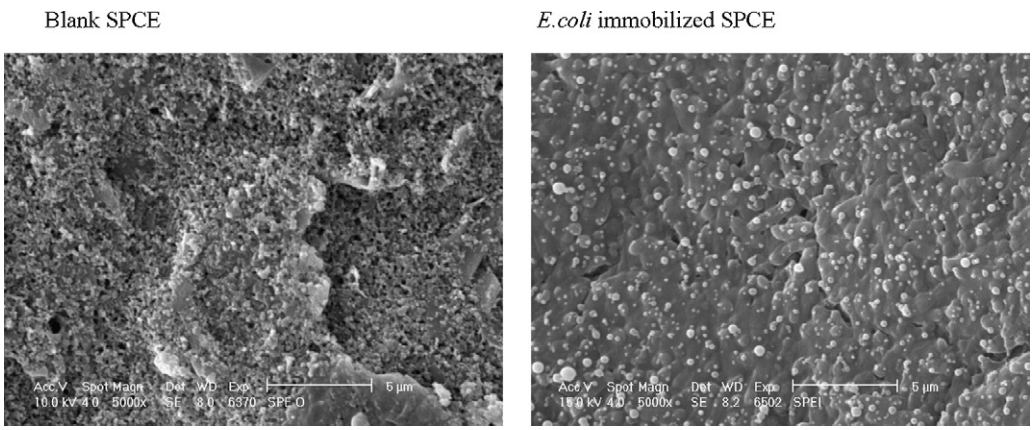


Fig. 1. SEM analysis of immobilized SPCE.

3. Results and discussions

3.1. SEM study of the immobilized SPCE

SEM study was carried out to detect the surface characteristics of the matrices before and after immobilization of the bacterial cells and to confirm the occurrence of biological materials on immobilization platforms. In this part, surface morphologies of the working area of both blank and whole cells immobilized SPCE were studied by SEM. As observed in Fig. 1a, bacterial cells were absent in the micrograph of blank SPCE and were present in immobilized SPCE (Fig. 1b). Presence of bacterial cells (size 0.5–2 μm) on the micrograph of immobilized SPCE confirms the immobilization of recombinant *E. coli* on SPCE.

3.2. Cyclic voltammogram of whole cells immobilized SPCE

Change in cyclic voltammograms between the unimmobilized SPCE and whole cells immobilized SPCE illustrates the immobilization of microbial cells on SPCE (supplementary Fig. S3). A control experiment that characterizes the cyclic voltammetry of methyl parathion and p-nitrophenol at SPCE in the absence of microbial cells was also illustrated (supplementary Fig. S4). Changes in cyclic voltammograms were also observed when cells immobilized SPCE were used for analysis in sodium phosphate buffer (pH 8.0, 50 mM) with and without methyl parathion (Fig. 2). Cyclic voltammograms of whole cells immobilized SPCE were also recorded before and after the hydrolysis of methyl parathion pesticide (Fig. 2). It was observed that two new oxidation peaks appeared after hydrolysis

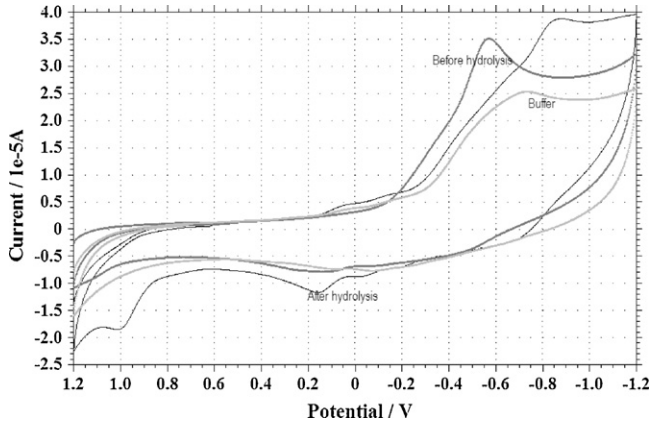


Fig. 2. Microbial cells immobilized SPCE, before (light line) and after hydrolysis of methyl parathion (20 μM) (dark line).

of methyl parathion, first between +0.2 V and +0.05 V and second between +1.05 V and +0.95 V and a reduction peak between −0.5 V and −0.7 V was shifted to above −0.85 V. As concentration of methyl parathion was increased, the oxidation current peak between +0.2 V and +0.05 V also increased. Therefore oxidation current at +0.1 V potential was considered as enzyme activity using immobilized SPCE. There is also an earlier report (Zhaona et al., 2009) which observed the redox behavior of p-nitrophenol at +0.1 V by cyclic voltammetry for the development of electrochemical sensor for detection of p-nitrophenol in wastewater.

3.3. Calibration of the biosensor and detection range

Biosensor was calibrated using standard methyl parathion concentration 2–400 μM and oxidation current was observed at +0.1 V from the cyclic voltammogram as shown in Fig. 3a. A linear response of hydrolyzed methyl parathion was obtained in phosphate buffer when cells immobilized SPCE were used. As shown in Fig. 3b, a linear range 2–80 μM of methyl parathion was estimated from calibration plot with a linear regression equation, $y = 1.0378 + 0.1055x$, $r^2 = 0.99784$. Therefore detection range, 2–80 μM methyl parathion, was estimated from the linear range. Experimental detection limit, 0.5 μM was estimated from signal to noise ratio (S/N = 3) in response to blank sample. Detection range of the present biosensor was equally comparable to the previously reported OPH based biosensor amperometric (1–5 μM) (Wang et al., 1999, 2003; Mulchandani et al., 1999, 2001a,b), potentiometric (2 μM) (Mulchandani et al., 1998a,b,c) and optical (2–8 μM) (Mulchandani et al., 1998d, 1999; Kumar et al., 2006; Kumar and D'Souza, 2010) transducer.

3.4. Study of interference of other compounds for specificity

Specificity is the fundamental component of biosensor. Therefore some compounds glucose, sucrose, endosulfan and phenol were investigated in response to biosensor and results were observed (Table 1). Glucose and sucrose are the carbon and energy source of bacterial cells and therefore both were chosen to know whether these metabolites are interfering with the sensor response or not and it was observed that both were not interfering with the sensor response. Endosulfan was also chosen because it is one another type of pesticide which may interfere but actually it was also not interfering with the sensor response. Phenol had caused some interference which may be because of sensor signal is based on the redox potential of p-nitrophenol. This interference was rectified by measuring and reducing the response of blank SPCE. *p*-Aminophenyl phosphate was also used for the interference study

Table 1
Interference study using cells immobilized SPCE biosensor.

Interference	Response (current μA)	Interferences in percentage (%) against 40 μM methyl parathion)
Glucose (5 mM)	0	0
Sucrose (5 mM)	0	0
Endosulfan (0.01 mM)	0	0
Phenol (0.005 mM)	0.12	2.1
p-Aminophenyl phosphate (1 mM)	0.108	1.9

and observed that it causes very less interference at +0.1 V potential, with the response of present biosensor although in one of earlier report (Stege et al., 2009) it was mentioned that *p*-aminophenyl also have a redox potential between −250 and 400 mV. It was also observed that *p*-aminophenyl phosphate was not hydrolyzed by the immobilized microbial cells of present biosensor and that might be the reason for not causing interference. The values of interferences percentage are insignificant as compared to signal/noise ratio. Study of interference of ambient oxygen has been also carried out by removal of oxygen from samples by purging the nitrogen in the samples and no changes were observed in the peak and shape of signals of p-nitrophenol even after removal of oxygen from sample and it might be because of low sample volume (only 20 μl) which carried insignificant amount of oxygen in ambient condition.

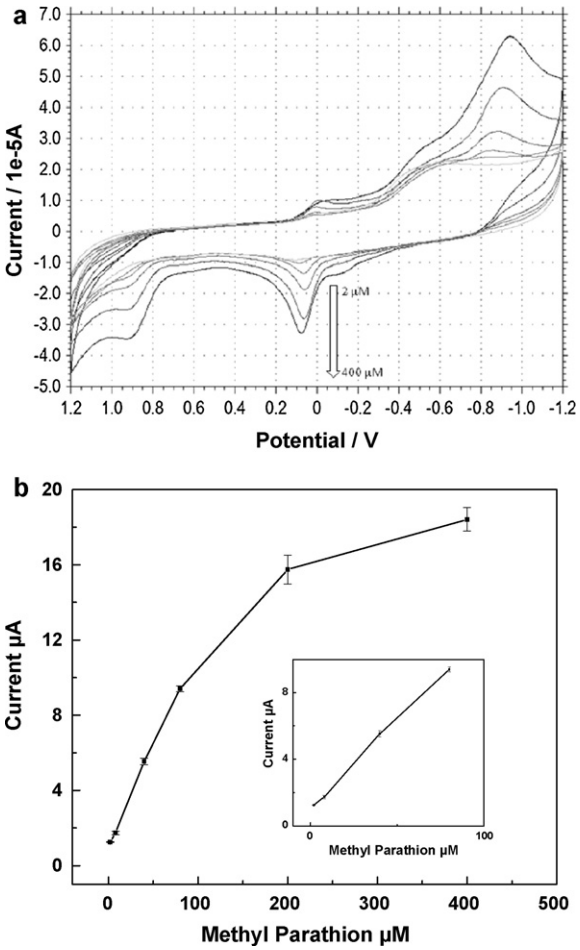


Fig. 3. (a) Cyclic voltammogram of different conc. (2–400 μM) of methyl parathion using microbial cells immobilized SPCE. (b) Enzymatic assay with methyl parathion (2–400 μM) for the calibration of sensor response (inset: linearity between 2 and 80 μM with linear regression equation, $y = 1.0378 + 0.1055x$, $r^2 = 0.99784$ where 'y' represents the current (10^{-6} A) and 'x' the substrate concentration).

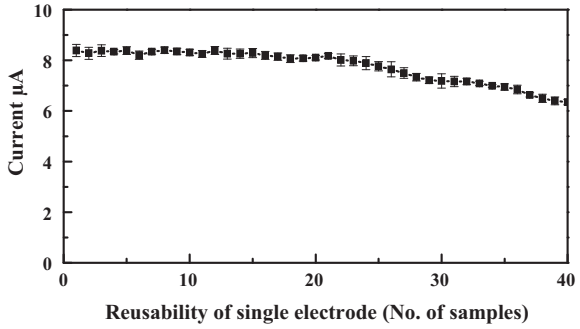


Fig. 4. Reusability of the microbial cells immobilized SPCE.

3.5. Reusability, stability and reproducibility of the whole cells immobilized SPCE

Reusability, stability and reproducibility are desirable characteristics of a good biosensor. The reusability of whole cells immobilized SPCE, prepared in the presence and absence of glutaraldehyde was studied. Whole cells immobilized SPCE prepared in the presence of glutaraldehyde, showed a number of reusability. Cells immobilized SPCE prepared in the absence of glutaraldehyde did not show any reusability and there was sharp decrease in the enzymatic activity. Decrease in hydrolytic activity might be because of leaching of cells in absence of glutaraldehyde. Glutaraldehyde treatment cross-linked the microbial cells onto the screen printed carbon electrode and reduced the leaching of enzyme or whole cells both, thus increasing the reusability of the immobilized microbial cells. It was observed that 80% activity of immobilized whole cells enzyme was retained up to 32 repeated reactions with single, cells immobilized SPCE (Fig. 4). Reusability is one of the desirable factors which are important for the applicability of the immobilized biocomponent in biosensor application. In our earlier report (Kumar et al., 2006) immobilized biological component was disposable while in this report immobilized biocomponent is reusable for 32 reactions. Microbial cells immobilized SPCEs were also stable for 22 days of investigation with retention of 80% activity (supplementary Fig. S6), when stored at 4 °C. Although it was less stable as compare to earlier reports (Mulchandani et al., 2001a,b; Kumar et al., 2006) but it was more reusable, 32 times, which made it more improved biosensor. The low relative standard deviations 0.156 (mean = 8.48×10^{-6} A, when $n = 6$) in the response of microbial cells immobilized SPCE for 80 μM methyl parathion also demonstrated the high reproducibility of analysis.

4. Conclusion

We describe immobilization of recombinant whole cells of *E. coli* on SPCE and its association with cyclic voltammetry for development of biosensor for detection of methyl parathion. Recombinant *E. coli* expressed, organophosphorus hydrolase enzyme in periplasm and hydrolyzed the methyl parathion into two products, p-nitrophenol and dimethyl thiophosphoric acid. Immobilization of microbial cells on SPCE was confirmed by SEM study.

Microbial cells immobilized SPCE was associated with the cyclic voltammetry and cyclic voltammograms were recorded before and after hydrolysis of methyl parathion. Here very low volume of sample, only 20 μl was required for analysis of methyl parathion and was based on the relationship between the concentrations of methyl parathion hydrolyzed and the amount of current changes recorded in cyclic voltammograms. A wide detection range, 2–80 μM of methyl parathion was estimated from the linear range of calibration plot of biosensor readings. Single microbial cells immobilized SPCE was reused for 32 reactions with retention of 80% of its initial enzyme activity and was stable for 22 days (80% activity) when stored at 4 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.027.

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