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An optical microbial biosensor for detection of methyl parathion using *Sphingomonas* sp. immobilized on microplate as a reusable biocomponent

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ABSTRACT

Organophosphorus pesticides such as methyl parathion 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. With a view to monitor these pesticides biosensors are being developed. A bacterium *Sphingomonas sp*. from field soil has been isolated and identified in our laboratory that hydrolyzes the methyl parathion upto a chromophoric product, p-nitrophenol (PNP). PNP can be detected by electrochemical and colorimetric methods, which can be exploited to develop a biosensor for detection of the organophosphate pesticide. Whole cells of *Sphingomonas* bacteria were immobilized directly onto the surface of the wells of polystyrene microplates (96 wells) using glutaraldehyde as the cross-linker. SEM study confirmed the immobilization of *Sphingomonas sp.* Immobilized bacterial microplate was associated directly with the optical transducer, microplate reader. The microplate-based biosensor is having advantages as it has 96 reaction vessels and therefore it provides a convenient system for detecting multiple numbers of samples in a single platform. Detection range of the biosensor from the linear range was determined to be 4–80 μM methyl parathion. Cells-immobilized microplates were having reusability upto 75 reactions. Present study reports an innovative concept where the microplate can be used as immobilizing support for development of reusable microbial biocomponent.

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). 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). 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).

Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolyzing enzyme, first discovered in soil microorganisms *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 hydrolyzes the methyl parathion into detectable product *p*-nitrophenol (PNP) (Kumar et al., 2006). 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, had isolated, and identified a bacterium *Sphingomonas sp.* JK1 from field soil, which hydrolyses methyl parathion pesticide upto a chromophoric product, PNP. We had also 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).

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In the present study, we report an innovative concept where the polystyrene made 96 wells microplate was used as support for immobilization of microbial cells for biosensor application. Microplate technique provides a simpler alternative for the typical two-dimensional micropositioning, enabling to acquire the whole array simultaneously and making the measurement time independent of the number of wells in the plate (Filippini et al., 2003). Whole cells of isolated *Sphingomonas sp.* were immobilized on the bottom surface of the wells of microplate, which was associated with optical transducer of microplate reader for detection of methyl parathion pesticide. The analysis was based on the relationship between the amount of methyl parathion hydrolyzed and the amount of chromophoric product, PNP formed which was quantified by measuring the absorbance at the λ_{max} 410 nm. This microplate-based biosensor is having advantages as it has 96 reaction vessels and therefore it provides a convenient system for detecting multiple numbers of samples in a single platform. Here biocomponent, cells-immobilized microplate was also having reusability which increases the performance of the system.

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. A bacterium *Sphingomonas sp*. JK1 was isolated from field soil for hydrolysing methyl parathion pesticide upto a chromophoric product, PNP using oligonucelotide primers from *opd* gene (Accession No. EU709764) and organophosphorus hydrolase enzyme activity (unpublished). Isolate was identified on the basis of morphological, biochemical and 16S rRNA gene (Accession No. EU616621). All other analytical grade chemicals were purchased from Sisco Research Laboratory, Mumbai, India.

2.2. Microorganism and culture condition

Isolate, *Sphingomonas sp*. JK1 was grown in 100 ml modified Wakimoto Broth media (consist of 15 g sucrose; 5 g peptone; 2 g $Na_2HPO_4{\cdot}12H_2O$; 0.5 g $Ca(NO_3)_2{\cdot}4H_2O$ and 0.5 g $FeSO_4{\cdot}7H_2O$ in 1 l milliQ water) for 24 h at 30 °C.

2.3. Optimization of growth time of Sphingomonas sp. for higher microbial OPH

The growing cells biomass of *Sphingomonas sp*. JK1 was studied by measuring the absorbance λ_{max} 600 nm. Inoculation of Luria broth (200 ml) was carried out with the 1/100th volume from the overnight grown culture (from modified Wakimoto broth) and incubated for 48 h at 30 °C on a rotary shaker at 140 rpm. The growing cells were collected at certain time intervals (4, 6, 8, 12, 16, 24, 36 and 48 h) from the growing culture and whole cells organophosphorus hydrolase (OPH) activity was measured (Kumar et al., 2006). Cells were harvested by centrifugation at 5000 × *g* for 10 min and washed twice with buffer. Finally, the pellet was resuspended in 1/10th volume in phosphate buffers (pH 8.0) and stored at 4 °C.

OPH activity was measured by using 100 μM methyl parathion and 50 μl whole cells suspension in phosphate buffer (pH 8.0) and appearance of PNP was determined by measuring absorbance at λ_{max} 410 nm (ε_{410} = 16,500 M^{-1} cm^{-1} for PNP).

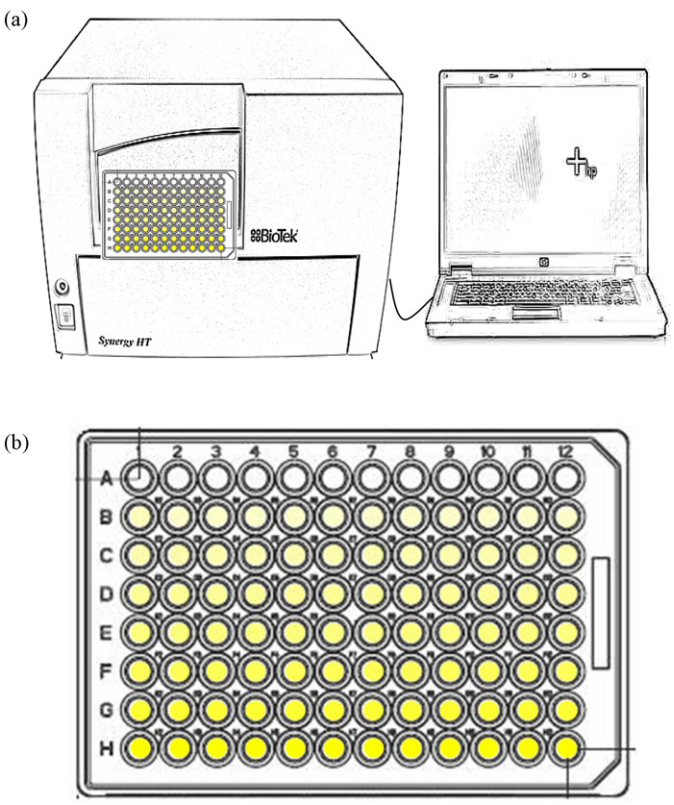

Fig. 1. (a) Block/schematic diagram of experimental components/set up. (b) Block/schematic diagram of microlate as reaction vessels.

2.4. Optimization of cells loading and glutaraldehyde concentration for immobilization

Amount of the harvested cells suspension to be immobilized was optimized because of the limited space of the bottom surface of the wells (diameter = 5.7 mm; area = 25.74 mm^2) of microplate (96 wells). For that different amount of cells suspensions (10 20, 30, 40 and 50 μl) were immobilized on bottom surface of the wells of microplates and hydrolytic activity were observed. Immobilized cells were also cross-linked with different concentrations (0.2, 0.5, 1, 2 and 5%) of glutaraldehyde. The immobilized microbial hydrolytic activity was observed in response to methyl parathion.

2.5. Immobilization of whole cells on microplate

Microplates (96 wells) used for immobilization were made up of polystyrene material and were procured from BD Flacon (USA). A 40 μl cells suspension was immobilized onto the interior surface of the wells of microplate and was air dried for 1 h at room temperature. After then, 8 μl of 2% glutaraldehyde was used for cross-linking and binding the cells to the surface and to each other. After immobilization, microbial microplate was washed with buffer and stored at 4 °C until use.

2.6. Transducer and operating system

The transducer used here is the optical transducer. The Multidetection microplate reader (MDMR) from Biotek (model Synergy™ HT) was used for the detection of absorbance at 410 nm (Fig. 1a). The Synergy Time Resolved (TM) option allows time-resolved measurements by using the xenon flash light source in conjunction with the photomultiplier tubes (PMT) detector. The monochromator provides wavelength selection from 200 to 999 nm in 1 nm

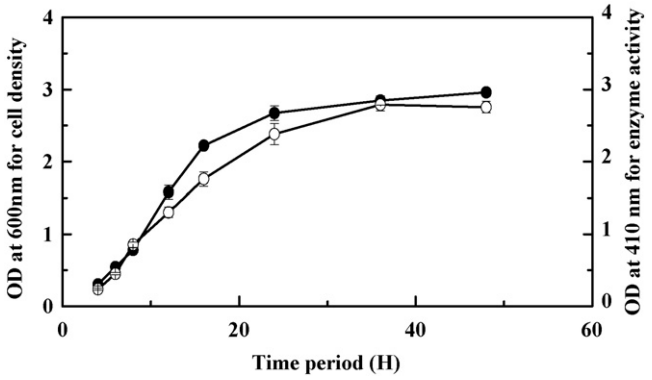

Fig. 2. Optimization of growth time of *Sphingomonas sp.* for higher enzymatic activity.

increments. The reader is completely controlled via KC4^{TM} PC software for all operations including data reduction and analysis. Online monitoring of each well of 96 wells microplate can be carried out using this KC4 software. A 200 μl volume of methyl parathion sample was added into the wells where cells were immobilized and readings were acquired at 410 nm wavelength that corresponds to PNP (Fig. 1). The absorbance difference between the initial and final reading were proportional to the concentration of PNP. Cells-modified microplate was washed with buffer after every analysis of sample and reused. All the experiments were carried out at room temperature for convenience of system even though the optimum temperature for hydrolysis of methyl parathion was 37 °C.

2.7. SEM study of the whole cells-immobilized microplate

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

2.8. Enzymatic assay using cells-immobilized microplate

Optical microplate reader was calibrated using standard concentration (4–400 μM) of PNP by determining the absorbance at λ_{max} 410 nm (ε_{410} = 16,500 M^{-1} cm^{-1} for PNP). Enzymatic assays were made with cells-immobilized microplate using methyl parathion concentration ranging from 4 to 400 μM in phosphate buffers (pH 8.0) at room temperature for 5 min and appearance of PNP was measured on optical microplate reader at λ_{max} 410 nm.

3. Results and discussions

3.1. Optimization of growth time of Sphingomonas sp. for higher enzymatic activity

The relationship between growth time of *Sphingomonas sp.* and hydrolysis of methyl parathion was optimized as shown in Fig. 2. It was observed that cells grown for 36 h were having higher hydrolytic activity. Therefore cells grown under the same provided conditions were used for the subsequent experiments.

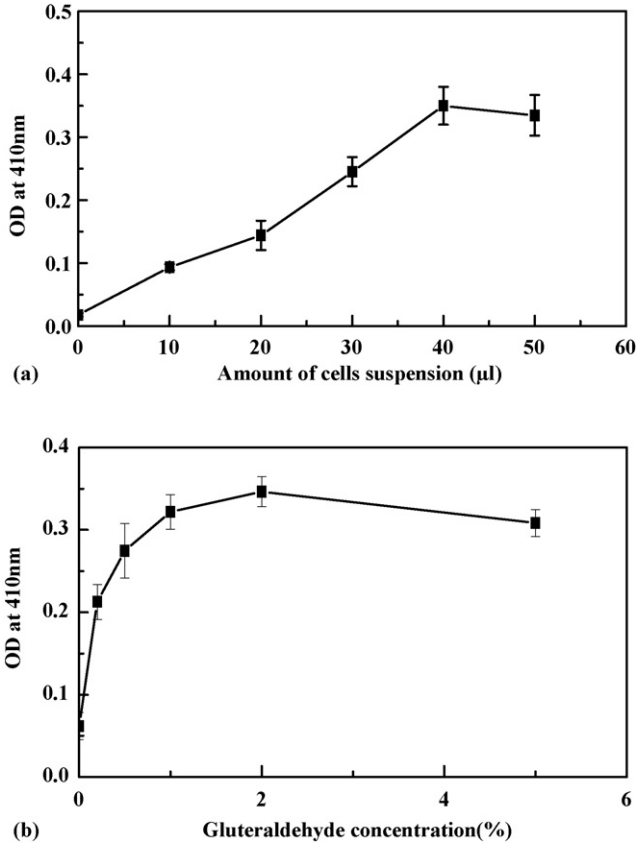

Fig. 3. Optimization of cells loading (a) and glutaraldehyde concentration (b) for immobilization.

3.2. Optimization of cells loading and glutaraldehyde for immobilization

Because of the limited space on bottom surface of the wells (diameter = 5.7 mm; area = 25.74 mm^2) of microplate, there is need to optimize the effect of biomass (cells) loading for immobilization of cells. Therefore, in order to investigate the effect of biomass loading, different amounts of cell suspensions were used for immobilization and hydrolytic activity was observed. As shown in Fig. 3a, 40 μl cell suspensions used for immobilization was having optimum enzyme activity. Glutaraldehyde concentration was also optimized for cross-linking. It was also observed in Fig. 3b that 2% glutaraldehyde was performing better for cross-linking and binding cells to the surface and to each other to prevent the leaching effect and thereby increase the stability and longevity of the biocomponent.

3.3. SEM study of the cells-immobilized microplate

SEM study was carried out to detect the surface characteristics of the whole cells-immobilized and unimmobilized (blank) wells of microplate which confirm the occurrence of biological materials on immobilization platforms. In this part, surface morphologies of the immobilized area of both blank and whole cells-immobilized microplate were imaged by SEM as shown in Fig. 4. SEM micrographs were acquired on interior surface of the wells of microplates. A bunch of bacterial cells (sizes 0.4–0.8 μm) were observed in the micrograph of cells-immobilized surface of well (Fig. 4b) and were absent in the micrograph of unimmobilized surface of well (Fig. 4a). Presence of bacterial cells confirms the immobilization of *Sphingomonas sp.* into the wells of microplate.

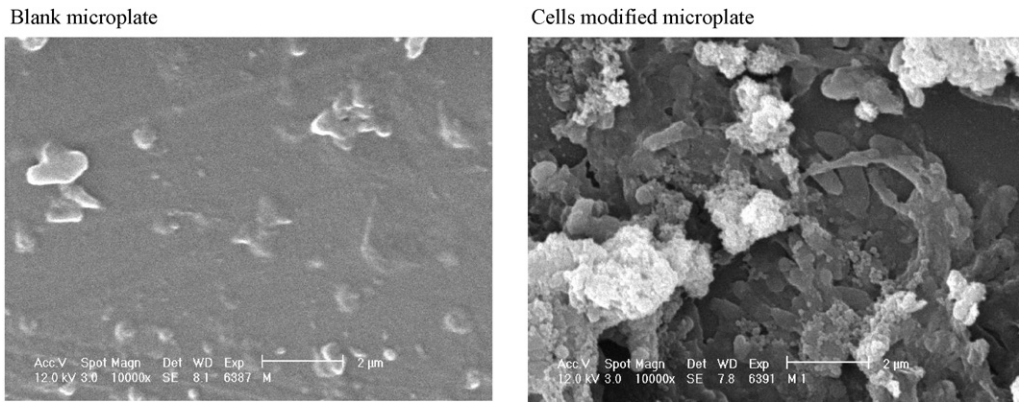

Fig. 4. SEM study of the cells-immobilized microplate.

3.4. Calibration of the biosensor and detection range

Biosensor was calibrated in association with cells-immobilized microplates using different (4–400 μM) concentration of methyl parathion and absorbance was observed. As shown in Fig. 5a linear range 4–80 μM of methyl parathion was estimated using cells-immobilized microplate, therefore the detection range of biosensor was calibrated between 4 and 80 μM methyl parathion. Detection range of present biosensor was equivalent to our earlier reported microbial biosensor (Kumar et al., 2006). Detection range of the present biosensor was higher and less sensitive in comparison to alkaline phosphatase inhibition (50 ppb) based chemiluminescence biosensor (Rekha et al., 2000) and amperometric OPH based biosensor (~0.4 μM) (Wang et al., 1999, 2003; Mulchandani et al., 2001a,b) but it was equally sensitive and comparable to the other previously reported enzymatic and microbial OPH biosensors those were based on potentiometric (2 μM) (Mulchandani et al., 1998a,b,c) and optical (2–8 μM) (Mulchandani et al., 1998d, 1999) transducers.

3.5. Characteristic of the microplate-based biosensor

Microplate technology was utilized to develop an effective biosensor tool for detection of multiple numbers of samples. There are two characteristic of microplate which was used for development of improved biosensor. First characteristic is that the microplate is having 96 wells means it has 96 reaction vessels and therefore multiple number of samples can be handled simultaneously on a single platform (Fig. 1b). A second characteristic is that the microplate technique provides a simpler approach of two-dimensional micropositioning, which enable the acquisition of time independent measurement of the whole plate irrespective of the number of samples present on the plate (Filippini et al., 2003). Cells-modified microplate in association with optical transducer (MDMR) and KC4 software was capable for rapid data acquisition and efficient data handling and therefore this system became capable for online monitoring of multiple samples, even with different concentrations, on a single platform simultaneously. Although present system is less sensitive as compared to the previously reported alkaline phosphatase inhibition and chemiluminescence based optical biosensor (Rekha et al., 2000; Chouhan et al., 2006) and amperometric OPH based biosensor (Wang et al., 1999, 2003; Mulchandani et al., 2001a,b) but it can be used when large number of samples is to be detected in very less time. The response time was only 5 min for measurement of multiple samples, which is better than the any previous reported inhibition and OPH based enzymatic and microbial biosensors (Rekha et al., 2000; Wang et al., 1999, 2003; Mulchandani et al., 1998a,b,c, 2001a,b). This is the first report where cells have been immobilized on the polystyrene microplate and used in biosensor application.

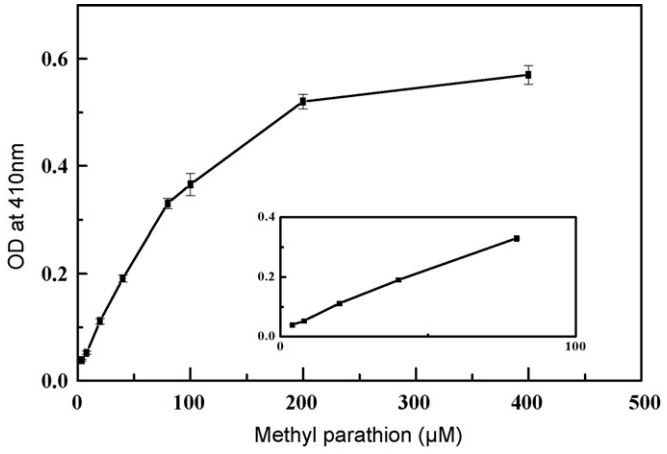

Fig. 5. Calibration of the biosensor using cells-immobilized microplate with methyl parathion (4–400 μM) (inset: linearity between 4 and 80 μM with linear regression equation, $y = 0.02329 + 0.004x$, $r^2 = 0.99369$ where 'y' represents the absorbance at 410 nm and 'x' the substrate concentration) (relative standard deviation 0.00698).

3.6. Reusability of the whole cells-immobilized microplate

Reusability is one of the desirable factors which are important for the applicability of the immobilized biocomponent in biosensor application. The reusability of whole cells-immobilized microplates, prepared in the presence and absence of glutaraldehyde were studied. Whole cells-immobilized microplate, prepared in the presence of glutaraldehyde, showed a number of reusability. Glutaraldehyde treatment cross-linked the microbial cells onto the microplate and to each other and therefore it reduced the leaching of whole cells, thus increasing the reusability of the immobilized microbial cells. It was observed (Fig. 6) that 80% activity of immobilized whole cells enzyme was retained up to 75 repeated reactions with single, cells-immobilized microplate. In our earlier report (Kumar et al., 2006) immobilized biocomponent was disposable while in this report immobilized biocomponent is reusable for 75 reactions.

3.7. Reproducibility and stability of the whole cells-immobilized microplate

Reproducibility and stability are desirable characteristics of a good biosensor. The reproducibility of the measurement in differ-

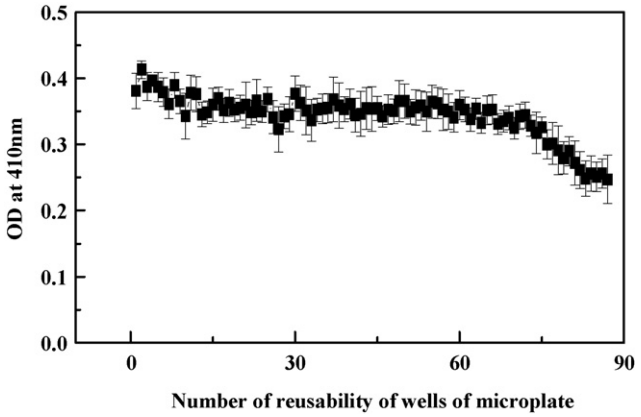

Fig. 6. Reusability of the wells of cells-immobilized microplate.

ent wells across the microplate was quite good. The low relative standard deviations (RSD), 0.156 (mean = 8.48×10^{-6} Å, when $n = 6$) in the response of immobilized microplate for 80 μM methyl parathion also demonstrated the high reproducibility of analysis. Microbial cells-immobilized microplate was also stable for 18 days of investigation with retention of 80% activity, when stored at 4 °C.

4. Conclusion

We describe the use of microplate technology for the immobilization of microbial cells and development of an effective microbial biosensor tool for detection of multiple numbers of samples. A bacterium *Sphingomonas sp*. which hydrolyzes the methyl parathion upto a chromophoric product, p-nitrophenol was isolated from field soil and whole cells suspensions were immobilized directly onto the surface of the wells of polystyrene microplates (96 wells). Immobilization of microbial cells was confirmed by SEM study. Immobilized bacterial microplate was associated directly with the optical transducer, microplate reader. The microplate-based biosensor is having advantages as it has 96 reaction vessels and therefore it provides a convenient system for detecting multiple numbers of samples in a single platform. Detection range of the biosensor was 4–80 μm methyl parathion and cells-modified microplates were having reusability upto 75 reactions. Present study report an innovative concept where the microplate was used as immobilizing support for microbial cells and its application in biosensor development with reusable microbial biocomponent.

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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.2010.07.016.

References

Chouhan, R.S., Babu, K.V., Kumar, M.A., Neeta, N.S., Thakur, M.S., Rani, B.E.A., Pasha, A., Karanth, N.G.K., Karanth, N.G., 2006. Biosens. Bioelectron. 21, 1264–1271.
D'Souza, S.F., 1999. Curr. Sci. 77, 69–79.
D'Souza, S.F., 2001a. Appl. Biochem. Biotechnol. 96, 225–238.
D'Souza, S.F., 2001b. Biosens. Bioelectron. 16, 337–353.
Dumas, D.P., Caldwell, S.R., Wild, J.R., Raushel, F.M., 1989. J. Biol. Chem. 264, 19659–19665.
Filippini, D., Andersson, T.P.M., Svensson, S.P.S., Lundström, I., 2003. Biosens. Bioelectron. 19, 35–41.
Harper, L.L., McDaniel, C.S., Miller, C.E., Wild, J.R., 1988. Appl. Environ. Microbiol. 54, 2586–2589.
Jha, S.K., Kanungo, M., Nath, A., D'Souza, S.F., 2009. Biosens. Bioelectron. 24, 2637–2642.
Kumar, J., D'Souza, S.F., 2008. Talanta 75, 183–188.
Kumar, J., D'Souza, S.F., 2009. Biosens. Bioelectron. 24, 1792–1795.
Kumar, J., Jha, S.K., D'Souza, S.F., 2006. Biosens. Bioelectron. 21, 2100–2105.
Melnikov, N.N., 1995. Pesticides and Plant-growth Regulators: A Handbook. Khimiya Publishers, Moscow (in Russian).
Mulbry, W.W., Karns, J.S., Kearney, P.C., Nelson, J.O., McDaniel, C.S., Wild, J.R., 1986. Appl. Environ. Microbiol. 51, 926–930.
Mulchandani, A., Mulchandani, P., Chauhan, S., Chen, W., 1998a. Field Anal. Chem. Technol. 2 (6), 263–269.
Mulchandani, A., Mulchandani, P., Chauhan, S., Kaneva, I., Chen, W., 1998b. Electroanalysis 10 (11), 733–737.
Mulchandani, A., Mulchandani, P., Kaneva, I., Chen, W., 1998c. Anal. Chem. 70, 4140–4145.
Mulchandani, A., Kaneva, I., Chen, W., 1998d. Anal. Chem. 70, 5042–5046.
Mulchandani, A., Pan, S., Chen, W., 1999. Biotechnol. Prog. 15, 130–134.
Munnecke, D.M., Hsieh, D.P.H., 1974. Appl. Microbiol. 28, 212–217.
Mulchandani, A., Chen, W., Mulchandani, P., Wang, J., Rogers, K.R., 2001a. Biosens. Bioelectron. 16, 225–230.
Mulchandani, P., Chen, W., Mulchandani, A., 2001b. Environ. Sci. Technol. 35, 2562–2565.
Rekha, K., Thakur, M.S., Karanth, N.G., 2000. Crit. Rev. Biotechnol. 20, 213–235.
Somara, S., Manavathi, B., Tebbe, C., Siddavattam, D., 2002. Indian J. Biochem. Biophys. 39, 82–86.
Stolyarov, M.V., 1998. Locust in southern Russia. Zaschita I Karanthin Rastenii 3, 16–17.
Svitel, J., Curilla, O., Tkac, J., 1998. Biotechnol. Appl. Biochem. 27, 153–158.
Tembe, S., Karve, M., Inamdar, S., Haram, S., Melo, J., D'Souza, S.F., 2006. Anal. Biochem. 349, 72–77.
Turner, A.P.F., Karube, I., Wilson, G.S. (Eds.), 1987. Biosensors Fundamentals and Applications. Oxford University Press, Oxford, UK.
Wang, J., Chen, L., Mulchandani, A., Mulchandani, P., Chen, W., 1999. Electroanalysis 11, 866–869.
Wang, J., Krause, R., Block, K., Musameh, M., Mulchandani, A., Schoning, M.J., 2003. Biosens. Bioelectron. 18, 255–260.