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Optical microbial biosensor for detection of methyl parathion pesticide using *Flavobacterium* sp. whole cells adsorbed on glass fiber filters as disposable biocomponent

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Abstract

An optical microbial biosensor was described for the detection of methyl parathion pesticide. Whole cells of *Flavobacterium* sp. were immobilized by trapping in glass fiber filter and were used as biocomponent along with optic fiber system. *Flavobacterium* sp. has the organophosphorus hydrolase enzyme, which hydrolyzes the methyl parathion into detectable product *p*-nitrophenol. The immobilized microbial biocomponent was disposable, cost-effective and showed high reproducibility and uniformity. The detection of methyl parathion by the use of disposable microbial biocomponent with optical biosensor was simple, single step and direct measurement of very low quantity of the sample. The home made reaction vessel was small and needed only 75 μl of sample. A lower detection limit 0.3 μM methyl parathion was estimated from the linear range (4–80 μM) of calibration plot of organophosphorus hydrolase enzymatic assay. The applicability to synthetic methyl parathion spiked samples was demonstrated.

Keywords: Microbial biosensor; Disposable biocomponent; Organophosphorus hydrolase; Methyl parathion; Glass fiber filter; Optical fiber

1. Introduction

Environmental pollution caused by pesticides and their degradation products is a major ecological problem. An important group of pesticides used currently in insect-pest control is organophosphorus (OPs) nitro-aromatic insecticides. One of these is metaphos (synonym: methyl parathion) (Stolyarov, 1998). Being highly active, organophosphorus insecticides are used; however, they are toxic to mammals (Melnikov, 1995). Microquantities of organophosphorus compounds are being measured using analytical methods such as spectrophotometer, gas–liquid chromatography and thin-layer chromatography. Recently, there has been an intense research effort to develop biosensor devices for the determination of organophosphorus pesticides.

Biosensor for organophosphorus insecticides based on the acetyl cholinesterase (AChE) inhibition test, using AChE modified amperometric (Palchetti et al., 1997), potentiometric (Danzer and Schwedt, 1996), conductometric (Dzyadevich et al., 1994) and fiber optic (Rogers et al., 1991; Andres and Narayanaswamy, 1997) transducers have been reported. Biosensors based on AChE inhibition, although sensitive, have limitations: since AChE is inhibited by neurotoxins, which include not only organophosphorus pesticides but also carbamate pesticides and many other compounds. It also requires multiple steps for measurement of the uninhibited activity of AChE, followed by incubation of the sensor with the analyte sample for 15–20 min and measurement of the AChE again to determine the degree of inhibition.

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). It has broad substrate specificity and is able to hydrolyze a number of organophosphate pesticide; such as methyl parathion, parathion, diazinon and chemical warfare reagents like sarin and soman (Munnecke, 1980; Mulbry and Karns, 1989a,b). Organophosphorus hydrolase is a cytoplasmic membrane enzyme; hydrolyzes the parathion into *p*-nitrophenol (PNP) and diethyl thiophosphoric acid (Gaberlin et al., 2000). PNP can be detected by electrochemical and colorimetric methods, which can be exploited to develop a biosensor for detection of

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organophosphate pesticide. Several types OPH enzyme based biosensors have been introduced including potentiometric (Mulchandani et al., 1998a), amperometric (Wang et al., 1999, 2003; Chough et al., 2002) and optical (Roger et al., 1999; Mulchandani et al., 1999) transducer. Microbial biosensors have also been introduced using a combination of recombinant *E. coli* cells expressing OPH intracellularly with potentiometric transducer (Rainina et al., 1996; Mulchandani et al., 1998b). Recombinant micro-organisms with surface expressed OPH have also been used to develop potentiometric (Mulchandani et al., 1998c), amperometric (Mulchandani et al., 2001a,b) and optical (Mulchandani et al., 1998d) biosensors.

The basic requirement of a biosensor is that the biological material should bring the physico–chemical changes in close proximity of a transducer (Turner et al., 1987). In this direction, immobilization technology has played a major role. Biomaterials can be immobilized either through adsorption, entrapment, covalent binding, cross-linking or as a combination of all these techniques (D'Souza, 1999, 2001a,b; Bickerstaff, 1997). In the case of periplasmic and cytoplasmic membrane enzymes, whole cells can be used for immobilization even without permeabilisation (Svitel et al., 1998). 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 cellulose or other synthetic membrane has been well documented (D'Souza, 1999, 2001a,b; Mulchandani and Rogers, 1998e). 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).

An optical biosensor was designed for determination of herbicides with immobilized *Chlorella vulgaris* entrapped on a quartz microfiber filter through filtered off algae (Vedrine et al., 2003). A disposable microbial biosensor has been developed to detect urea in milk, using filtered off microbial cell on Whatman No. 1 filter paper as disposable biocomponent (Verma and Singh, 2003).

Objective of the present study was to develop a microbial optical biosensor containing a disposable microbial membrane. The biocomponent was developed using whole cell *Flavobacterium* sp. by adsorption of cell suspension on a glass fiber filter paper. It was used as disposable biocomponent in the home made reaction vessel, carrying 75 μl sample and associated with optical fiber transducer for the detection of methyl parathion. The analysis was based on the relationship between the amount methyl parathion hydrolyzed and the amount of chromophoric product, PNP formed. It was quantified by measuring the absorbance at the λ_{max} 410 nm of the product by the enzyme-catalyzed hydrolysis.

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. Glass fiber filter were purchased from Schleicher & Schuell Dassel, Germany. All other analytical grade chemicals were purchased from Sisco Research Laboratory, Mumbai, India.

2.2. Micro-organism and culture medium

Flavobacterium sp. MTCC 2495 was obtained from Microbial Type Collection Center, Institute of Microbial Technology, Chandigarh, India, in lyophilized form. Lyophilized *Flavobacterium* sp. was first cultivated in modified Wakimoto 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 milli Q water) for 24 h at 30 °C.

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

The growing cell biomass of *Flavobacterium* sp. was studied by measuring the absorbance λ_{max} 600 nm. Inoculation of Luria broth (200 ml) was done 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 (0, 1, 2, 4, 6, 8, 10, 16, 24 and 48 h) from the growing culture and the cell organophosphorus hydrolase (OPH) activity was measured. Cells were harvested by centrifugation at 5000 × *g* for 10 min and washed twice with 0.2 M bicarbonate buffer (pH 8.5). Finally, the pellet was resuspended in 1/10th volume in bicarbonate–carbonate buffers (pH 8.5) and stored at 4 °C.

Microbial OPH activity was measured as follows: a 50 μl aliquot from whole cell suspension was added to 5.0 ml of 0.2 M bicarbonate–carbonate buffers (pH 8.5) containing 200 μM methyl parathion per ml of buffer. The production of *p*-nitrophenol was measured on spectrophotometer at λ_{max} 410 nm.

2.4. Immobilization

For immobilization, the *Flavobacterium* sp. cells were grown in 200 ml Luria broth up to 2.2 optical density at λ_{max} 600 nm. Cells were harvested and resuspended in 20 ml buffer to increase the cell density by 10-fold than in the natural medium. Glass fiber filter paper was cut into small (5 mm diameter) disc like pieces and 20 μl aliquot of the whole cell suspension were trapped on each disc and was air dried at room temperature for 1 h and stored at 4 °C until use.

2.5. Transducer

The transducer used was a SF2000 miniature optical fiber spectrophotometer from Ocean Optics Inc. Duiven, The Netherlands. The heart of the SF2000 miniature optical fiber spectrophotometer is preconfigured to a 360–1000 nm-wavelength range with a 200 μm entrance and detector collection lens

(for increased light throughput). The data acquisition and visualization software OOIBase32™ was provided with the instrument. The 32-bit PCI analog to digital converter card (ADC) required to interface the equipment with the computer was also supplied along with the instrument (model ADC 2000 PCI).

Absorbance is linearly related to the concentration of the substance. The software calculates absorbance using the following equation:

$$A_\lambda = -\log_{10}\left(\frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda}\right)$$

where S is the sample intensity at wavelength λ, D the dark intensity at wavelength λ, R is the reference intensity at wavelength λ.

2.6. Operating condition

Reaction vessel was designed in the laboratory, on a piece of Teflon block. A groove of 3 mm depth with 6.5 mm diameter at the center of the Teflon block was made so that it can hold 75 μl of sample. Light was incidence at right angle from the LED (Light Emitted Diode) to reaction vessel through a 200 μm diameter bifurcated fiber optic probe (model no. QBIF200-UV–vis, Ocean Optics Inc.) associated with the lid of the reaction vessel and was recollected into the miniature optical fiber spectrophotometer as shown in Fig. 1.

At the start, the dark (background) intensity was recorded, when the reaction vessel was blank and tightly closed with the lid (associated with 200 μm diameter bifurcated fiber optic probe). Reference intensity (75 μl samples) was recorded at λ_{max} 410 nm and subsequently absorbance reading was acquired for 2 min for each sample of methyl parathion using microbial filter disc as disposable biocomponent. All experiments were performed at room temperature.

2.7. Calibration of optical biosensor

The standard solution of *p*-nitrophenol (PNP) (3.5, 7, 35 and 70 μM) was prepared in 0.2 M bicarbonate–carbonate buffer (pH 8.5). Optical fiber biosensor was calibrated using 75 μl of standard PNP and absorbance was recorded at λ_{max} 410 nm. It was compared and correlated with the spectrophotometer (Hitachi 2000, Japan) reading of standard PNP.

2.8. Enzymatic assay of the microbial OPH on optical biosensor and spectrophotometer

Enzymatic assays were made with disposable microbial glass fiber disc using methyl parathion concentration ranging from 4 to 400 μM in 0.2 M bicarbonate–carbonate buffers (pH 8.5) at room temperature and λ_{max} 410 ($\varepsilon_{410} = 16{,}500\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol). The appearance of *p*-nitrophenol was measured on optical fiber biosensor for 2 min and on spectrophotometer for 30 min.

2.9. Analysis of methyl parathion spiked samples with gas chromatograph and optical biosensor

Synthetic methyl parathion spiked samples were prepared by incubating methyl parathion in the soils for 5 days at 30 °C with concentrations of 10, 12, 14, 16, 18 and 20 μg per 20 g of soil. Methyl parathion was extracted from the soil using Soxhlet extraction method and analyzed by a gas chromatograph (GC) (GC-17A, Shimadzu, Japan) equipped with a ^{63}Ni electron capture detector (Luchini et al., 2000). The preincubated soil samples were thoroughly suspended in the 0.2 M bicarbonate–carbonate buffers (pH 8.5) containing 10% methanol. Soils were allowed to settle down and then the aqueous layer was decanted and analyzed by optical biosensor at room temperature.

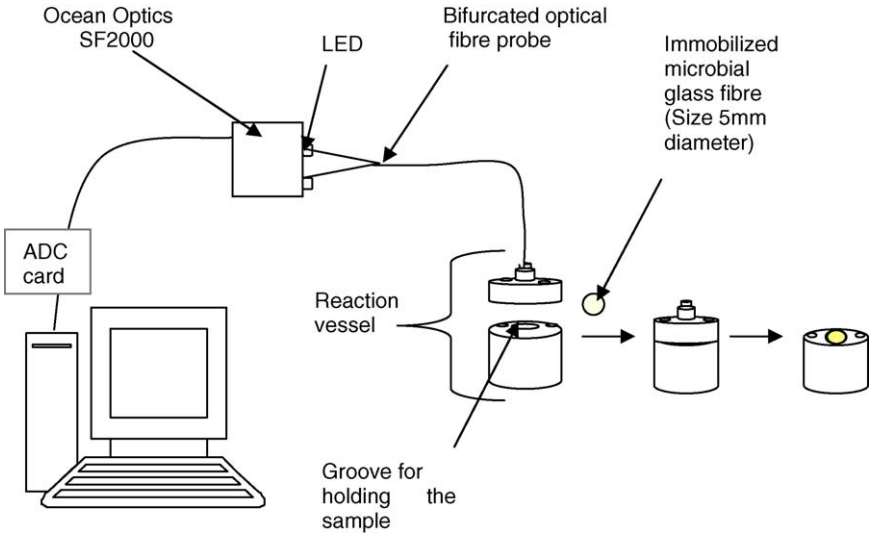

Fig. 1. Schematic diagram of operating system of optical fiber biosensor and reaction vessel.

3. Results and discussion

3.1. Optimization of growth time of Flavobacterium sp. and their OPH activity

The relationship between growth time of *Flavobacterium* sp. and production of organophosphorus hydrolase activity is shown in Fig. 2. It shows that the cells grown for 16 h having absorbance 2.2 at λ_{max} 600 nm have maximum OPH activity. The cells grown under these conditions were used for the subsequent experiments.

3.2. Effect of cell loading

The limited space on the surface of the glass fiber filter disc (diameter 5 mm) did not allow the spreading of more than 20 μl of the whole cell suspension. Hence, in order to investigate the effect of increased biomass loading, cell density was increased 2.5-, 5-, 10-, 15- and 20-fold. The immobilized microbial OPH activity was checked in response to methyl parathion. The result from Fig. 3 shows that a 10-fold increase in cell density has the best relation in terms of wide detection range as well as sensitivity. The sensitivity was poor below this biomass loading. However, there were no significant changes in the sensor sensitivity with higher biomass loading.

3.3. Calibration of optical biosensor

The plot of spectrophotometer data versus corresponding values from optical fiber assembly showed a linear fit ($Y = 0.009 + 0.58X$) with a slope of 0.58 ($R^2 = 0.994$) (figure not shown) for the calibration range with standard solution of *p*-nitrophenol (PNP) (3.5, 7, 35 and 70 μM).

3.4. Comparison of optical biosensor with spectrophotometer

Comparison of the enzymatic assay analysis of immobilized microbial OPH on spectrophotometer and optical biosensor is shown in Fig. 4a and b. Coefficient correlation, linear regression line and goodness of fit were established in linear plots and were observed over a wide range of methyl parathion concentration ranging 4–80 μM in both spectrophotometer and optical biosensor (Fig. 4a and b). There was also saturation at high concentration of methyl parathion. The curve is hyperbolic, following zero order kinetics at high concentration.

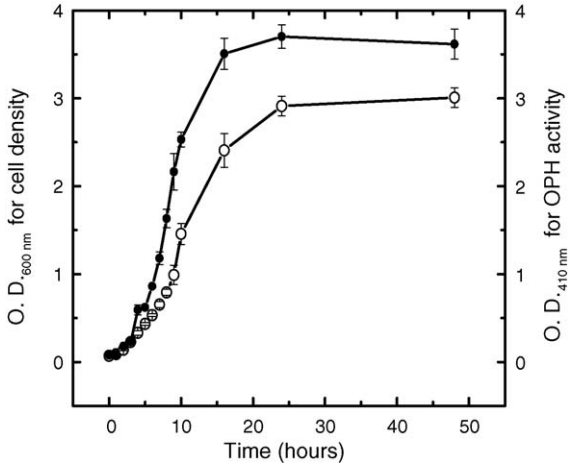

Fig. 2. Optimization of growth time of *Flavobacterium* sp. and OPH activity: (◯) optical density of grown cells at 600 nm, (●) optical density for OPH activity at 410 nm.

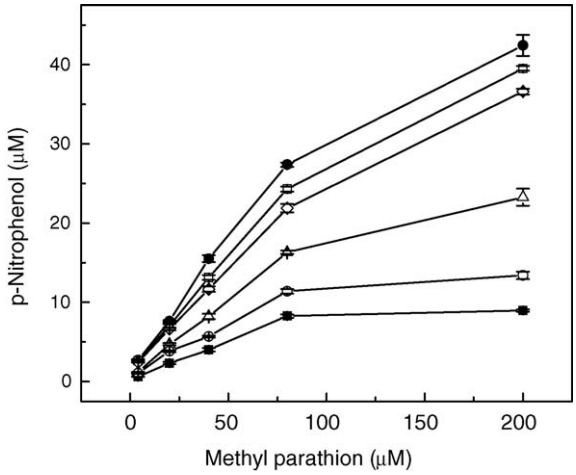

Fig. 3. Effect of loading different cell densities for immobilization: (■) normal harvested cell density; (◯) 2.5-fold; (△) 5-fold; (◊) 10-fold; (♦) 20-fold; (□) 25-fold increase of cell density.

3.5. Reproducibility of immobilization of the microbial cells on glass fiber filter

The low relative standard deviations 0.080 (mean = 0.978 optical density when $n = 6$) in the response of immobilization of 20 μl microbial cells, for 200 μM methyl parathion, demonstrated the high reproducibility and uniformity of analysis. Additionally, a very low relative standard deviation 0.019 (mean = 0.98) of three different experiments done; using the same condition further demonstrated the result.

3.6. Detection limit, response time and storage stability

A biosensor should be simple to operate, having low detection limit, short response time, storage stability, require very less amount of sample and its disposable biocomponent should be cost effective. Detection limit of the biosensor in the present study was compared with the available literature on either enzymatic or microbial OPH based biosensor for the detection of organophosphate pesticide. A lower detection limit 0.3 μM was estimated from the linear range (4–80 μM) of calibration plot of OPH enzymatic assay. It was comparable with the literature on acetylcholine esterase inhibition and amperometric (0.1–0.4 μM) OPH biosensor (Wang et al., 1999, 2003; Mulchandani et al., 2001b) and it was lower and more sensitive than the potentiometric (2 μM) OPH (Mulchandani et al., 1998a,b,c) and optical (2–8 μM) OPH (Mulchandani et al., 1998d, 1999; Roger et al., 1999) biosensor. The detection of methyl parathion by the use of immobilized, disposable, microbial glass fiber disc with optical fiber was a simple, single step

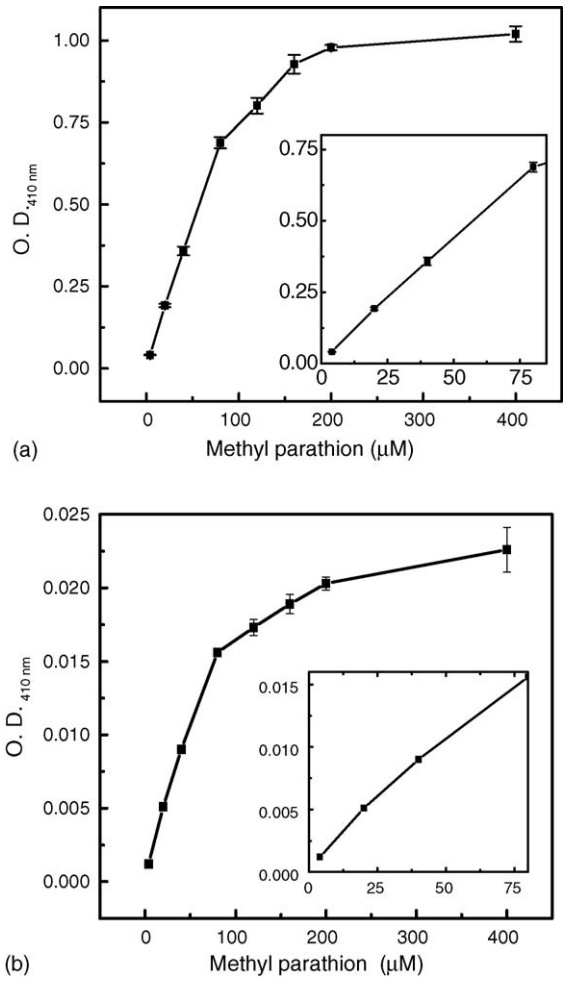

Fig. 4. (a) Enzymatic assay of the immobilized microbial OPH on spectrophotometer (enzymatic assay on spectrophotometer, inset: linearity range on spectrophotometer $Y = 0.0155 + 0.0084X$, $R^2 = 0.998$, $\chi^2 = 8.3964 \times 10^{-6}$). (b) Enzymatic assay of the immobilized microbial OPH on optical fiber biosensor (enzymatic assay on optical fiber, inset: linearity range of detection on optical fiber biosensor $Y = 0.042 + 0.0078X$, $R^2 = 0.997$, $\chi^2 = 2.0353 \times 10^{-8}$).

and direct measurement of very low amount of sample. The home made reaction vessel was small that it needed only 75 μl of sample and its disposable biocomponent was also cost effective for monitoring of methyl parathion. The response time was less than 3 min, comparable or better than the reported OPH based enzymatic and microbial biosensors (Wang et al., 1999, 2003; Mulchandani et al., 1998a,b,c, 2001a,b). In contrast, AchE based biosensors involve multiple steps and require analysis time at least 15 min even for the disposable type. As shown in Fig. 5, the immobilized microbial glass fiber discs were stable for 1 month of investigation, when stored at 4 °C and subsequently response decreased. The stability was also comparable to those OPH based enzymatic and microbial biosensor.

3.7. Correlation of biosensor response on methyl parathion spiked samples with gas chromatography

In order to access the matrix effect due to naturally occurring compound in real world samples, the optical biosensor

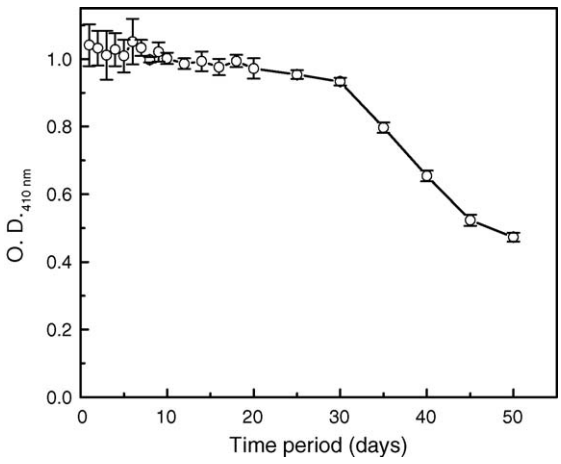

Fig. 5. Storage stability of the immobilized microbial enzyme on glass fiber filter paper. A concentration of 50 μM methyl parathion was used for the study.

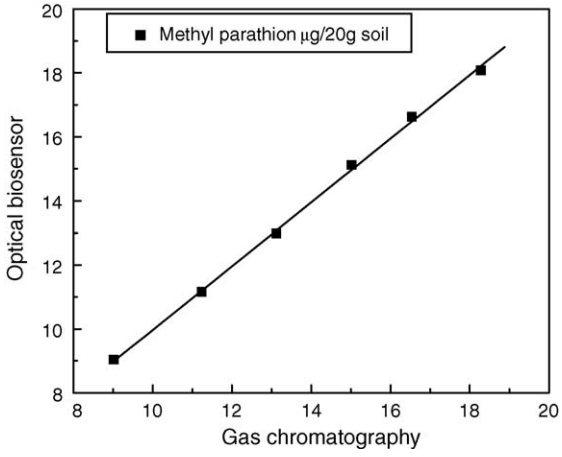

Fig. 6. Correlation of the optical biosensor measurement with the gas chromatography for methyl parathion spiked sample. (■) Methyl parathion with concentrations of 10, 12, 14, 16, 18 and 20 μg per 20 g of soil.

response obtained with methyl parathion spiked samples were compared with results obtained using GC. The plot between the two results is shown in Fig. 6. The straight line fit yielded slope of 0.996 ($R^2 = 0.998$, $\chi^2 = 0.0181$), which demonstrates the feasibility and a good correlation of the biosensor with existing method (GC).

4. Conclusion

We have described an optical based disposable microbial biosensor to determine methyl parathion pesticide. The detection of methyl parathion by the use of immobilized, disposable, microbial glass fiber disc with optical fiber was a simple, single step and direct measurement of very low quantity of sample. Here, the only immobilized biocomponent, trapped whole cell *Flavobacterium* sp. MTCC 2495 on glass fiber filter was disposable. Disposable biocomponent was cost effective for methyl parathion detection. The home made reaction vessel was so small that it needed only 75 μl of sample. A lower detection limit 0.3 μM was estimated from the linear range (4–80 μM) of calibration plot of organophosphorus hydrolase enzymatic assay,

which was not only better to the reported optical biosensor but also comparable to the reported amperometric biosensor, for detection of other organophosphate pesticide. The applicability of the sensor to synthetic methyl parathion spiked samples was also demonstrated and found a good correlation with existing method (GC).

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