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Research Article

DEVELOPMENT OF A NOVEL OPH BASED FIBRE OPTIC BIOSENSOR FOR DETECTION OF CHLORPYRIFOS

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ABSTRACT

In the present communication, we have reported a novel, simple, fast, convenient and sensitive method for determination of Chlorpyrifos, an organophosphorus pesticide based on organophosphorous hydrolase (OPH) activity. *Bacillus flexus*, accession number (MF800923) strain was isolated from chlorpyrifos contaminated soils of Punjab, India exhibiting significant OPH activity. Viable and actively growing bacterial cells were immobilized using Sol-Gel immobilization method. As a result of OPH activity, chlorpyrifos was mineralized into TCP (3-5-6 trichloropyrinidol), an metabolite of CP. A sensitive colorimetric based method was developed for the detection of TCP generated upon CP mineralization. Detection of CP was found to be in the linear concentration range 1 mg/L - 100 mg/L. Accuracy and reliability of developed biosensor was also validated and percent accuracy was calculated in the range of 94-96 %. Monitoring of detection of CP by naked eyes, low cost and easy operation were the key attributes of the developed biosensor. Under the optimized experimental conditions, the biosensor exhibited a lowest detection limit of 0.01 mg/L and response time of fifteen minutes. Developed system was also applied for determination of CP residues in the real soil samples. Hence, a highly sensitive and rapid whole cell based biosensor, holding the promise for detection of CP content in the water and agricultural soils has been developed. Outcome of present work facilitate development of rapid and high-throughput screening of CP.

INTRODUCTION

Chlorpyrifos (CP), O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate), is one of the most widely used Organophosphorous (OP) Pesticide in the world. Owing to its toxicity concerns, extensive use of CP results in accumulation of its residues in various environment samples and impose numerous life threatening effects on human population. CP targets different organ systems and generate neurological disorders, developmental disorders, autoimmune disorders (Ventura *et al.,* 2012; Estevan *et al.*, 2013). Hence, rapid determination as well as reliable quantification of trace levels of CP has become critically important.

Over the past few decades a number of techniques like high-performance liquid chromatography (HPLC), GC (Gas Chromatography), LC (Liquid Chromatography), GC–MS, LC–MS (Mass Spectroscopy) have been adopted for monitoring of CP. Although, these methods are highly sensitive but also associated with various limitations like expensive instrumentation, need of highly trained technician for operation, laborious procedure, undergo cleanup, risk of errors etc. (Jiang *et al.,* 2008). Sample preparation and sample pretreatment in HPLC and GC is an elaborative task thus limits the analysis of number of sample analysis (Meng *et al.,* 2013). Nevertheless, high cost and being complicated to operate making them inappropriate for on-site sample detection (Zhao *et al.,* 2014; Regueiro *et al.,* 2015).

As an alternative, attention has been paid towards biosensors technology for detection of environmental pollutants like pesticides, heavy metals etc. Biosensor technology employs a biological sensing entity connected to a transducer that can convert an observed response into a measurable electrical signal (Verma and Bhardwaj, 2015). Biosensor-based devices are introduced in the analytical biology to improve recognition and simplifying the complication of preparation procedures (Kumar *et al.,* 2015, Hasani *et al.,* 2017). Biosensors exhibits

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attributes of specificity, sensitivity, low cost, miniaturization of sample, fast response time, portability, compact size, user-friendly operation, continuous real time analysis less laborious sample pretreatment etc (Verma and Kaur 2016, Kaur and Verma, 2018).

Mainly two approaches are implemented for the development of biosensor for the detection of CP and are described as AChE (Acetylcholinesterase) enzyme inhibition based and OPH (Organophsphorous Hydrolase) enzyme catalytic based. AChE-based biosensors are quite sensitive but are not easily manageable when operated, exhibits poor specificity and generates jammed signals with multistep protocol (Mulchandani *et al.,* 2001). OPH is one of the typical bacterial enzymes which mineralize OP pesticides b utilizing them as a substrate. Biosensors based on a catalytic reaction are superior to the inhibition mode since they can be potentially reused, much specific and are also suitable for continuous monitoring (Wang *et al.,* 2003). OPH-based biosensors are classified on the basis of types of transducer used as potentiometric, amperometric and optical have been introduced (Tang *et al.,* 2014).

In the present work, authors have reported a OPH based biosensor which can detect CP in both water and soil samples. Bacterial strain (*Bacillus flexus*) exhibiting OPH activity has been used as a bio-component, immobilized in a semi-solid matrix to study the conversion of CP in to 3,5,6-trichloropyrinidol (TCP). Sol-gel along with diazotized aniline (indicator) has been used to detect the generation of TCP as a result of OPH activity of bacterial. To best of our knowledge, this strategy of immobilization of bacterial is within sol-gel matrix along with a fluorescent dye i.e., diazotized aniline has not been experimented yet for CP detection. The aim of study is to fabricate a novel disposable biosensor for CP detection with low detection limits, lower response time and high specificity.

Experimental

Reagents and Solvents

All the chemicals and reagents used in the present work were of utmost purity and analytical grade. The standard samples were used for the development of bioassay principle and were procured from Sigma-Aldrich: Chlorpyrifos (Sigma Aldrich no. 45395), TCP (Sigma Aldrich no. 41252). Growth media chemicals and other chemicals used in the present work were procured from Hi-media Ltd. (Mumbai, India). HPLC grade acetonitrile and methanol were purchased from Merck for the extraction of CP from water and soil samples

Instrumentation: The absorption measurements for bacterial growth were performed on fibre optic spectrophotometer (Maya 2000, Oceanic Optics, USA). Absorption spectra's in the biosensor studies were also recorded on fiber optic spectrophotometer (Maya 2000, Oceanic Optics, USA). For the validation of developed biosensor, samples were simultaneously evaluated by using isocratic HPLC (High Pressure Liquid Chromatography), Waters 51, HPLC pump 2489 (Switzerland) equipped with dual absorbance (2489) U.V. detector and C18 column (Ascentis 3µm, 4.6 mm × 15 cm). Mixture of methanol: water (85:15, v:v), was used as mobile phase which was pumped through the column at a flow rate of 1 ml/min.

Bio-component of the Biosensor

Bacillus flexus, (NCBI accession number MF800923) was isolated from CP contaminated soils of Punjab, India and was screened as a bio-component for biosensor construction. Strain was capable of mineralizing CP in to TCP quite efficiently within shorter time period. Bacterial strain was cultured in the nutrient broth medium containing CP 200 mgL^{-1} pH (7.4±0.2) for bacterial cell growth. Actively growing cells from nutrient broth were cultured in sterilized MSM (Minimal Salt Media) containing K_2HPO_4 1.5, KH_2PO_4 0.5, $(NH_4)_2SO_4$ -1.0, NaCl -0.5, $MgSO_4$ -0.2, $CaCl_2$ -0.05, $FeSO_4$, -0.02 (gL^{-1}) and also

supplemented with CP (500 mgL^{-1}) as the sole carbon source. Culture media was then incubated at 30°C on rotatory shaker (130 rpm) for 36 h and actively growing CP induced cells were then separated by centrifugation (5000 rpm, 8 min at 4°C). Cells were finally suspended in sterilized phosphate buffer saline (7.2±0 .2) and employed for biosensor bioassay development.

Development of Bioassay principle

The bioassay principle developed for the fabrication of biosensor based on OPH activity for CP detection is illustrated in Fig. 1. Chlorpyrifos was hydrolyzed in to TCP by the OPH activity of bacteria. Production of the TCP was then detected by adding diazotized aniline in to sample having TCP. Diazotized aniline was prepared employing methodology reported (Venugopal *et al.,* 2012; Patel *et al.,* 2012) with slight modifications. Diazotized aniline was added to the sample containing different concentrations of CP and observed for color change in liquid medium. Post color development absorbance of the reaction mixture was recorded spectrophotometrically and value of absorption maxima was also determined.

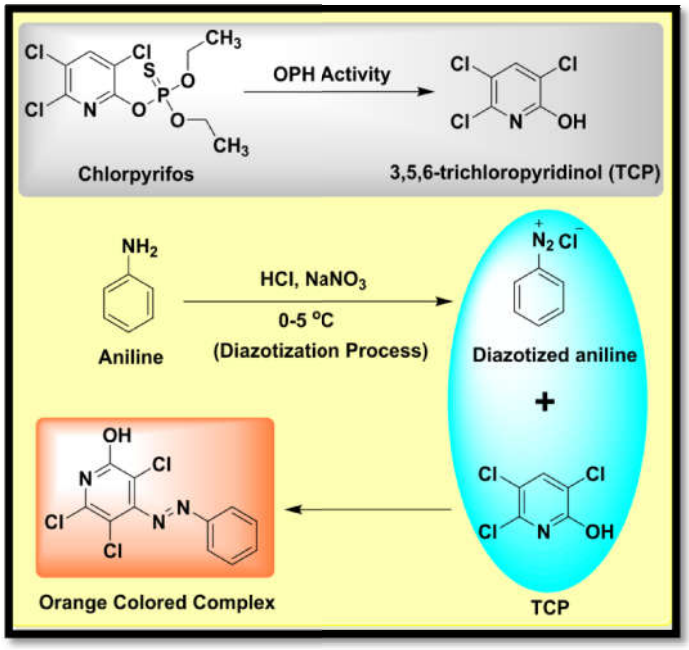

Fig 1 Schematic representation of development of bioassay principle for biosensor construction

Preparation of Bio-sensing Disc for biosensor development

Bacterial cells used for the biosensor construction were initially induced by growing in MSM containing CP (500 mgL^{-1}) as only carbon source for 36 h, harvested and dispensed into Phosphate buffer saline (pH-7±0.2). Viable cell count was adjusted spectrophotometrically $_{(O.D.-\ 600nm)}$, and optimized further for biosensor construction. Bio-sensing disc was constructed according to Sol-Gel method developed (Verma *et al.,* 2011, Verma *et al.,* 2016, Verma *et al.,* 2017) with slight modifications. 10 µL of prepared sol-gel solution along with diazotized aniline and bacterial cells was poured on to the transparent plastic discs and kept for immobilization at 4^0C for 10 minutes. To TMOS-sol gel coated discs 10 µL CP (50 mgL^{-1}) was added and observed for color change manually at room temperature.

CP solution (10 µL) of different concentrations (1 mgL^{-1} -100 mgL^{-1}) was dispensed on to the TMOS-sol gel coated sensing discs. Post sample addition discs were placed on to the the fibre-optic probe. Absorbance of immobilized tip was scanned from 300 nm-700 nm using fiber optic spectra suit (Maya 2000 series) to find out the absorption maxima. Standard reference graph was prepared to find out the CP concentrations in unknown samples.

Optimization of Response time and cell biomass concentration

Volume of sol-gel mixture employed for the preparation of bio-sensing disc was miniaturized to the possible lowest value. Immobilized plastic discs containing CP of different concentrations (1-100 mg^{-L}) were analyzed for color development after every five minutes spectrophotometrically. Optimization of the bacterial cell biomass concentration was also carried out simultaneously. Phosphate buffer saline (pH 7.2) containing different cell concentrations corresponds to optical density $_{(O.D.-600)}$ (0.5, 1.0, 1.2, 1.5) were immobilized in sol-gel matrix. After the immobilization, CP containing sample was added for absorbance.

Application of the developed biosensor:

The standard reference graph was used for quantifying unknown CP concentration in soil samples. Reliability of the developed biosensor was checked with the water samples spiked with known CP concentration and was compared by standard graph. A total ten soil samples were collected from the various regions of the Malwa belt of the Punjab, India. For the analysis of content of CP in soil, 10 gm soil was mixed with 20 ml of HPLC grade acetonitrile and incubated for 30 min on a rotary shaker at 130 rpm for extraction process (Abraham and Silambarasan 2015). After extraction, clear supernatant was obtained containing residues of CP and monitored by developed biosensor.

RESULTS AND DISCUSSION

Development of Bioassay principle

Chlorpyrifos, due to the OPH activity of bacterial strain was hydrolyzed in to TCP under slightly alkaline conditions. Generation of the TCP was confirmed by adding diazotized aniline mixture in to the reaction mixture. Standardization of developed bioassay principle was with standard TCP (10-100 mgL^{-1}) in the liquid medium. Diazotized aniline was prepared and added to the, TCP (50 mgL^{-1}) containing solution. Within 10 seconds orange color complex appeared in the mixture (Fig. 2 B).

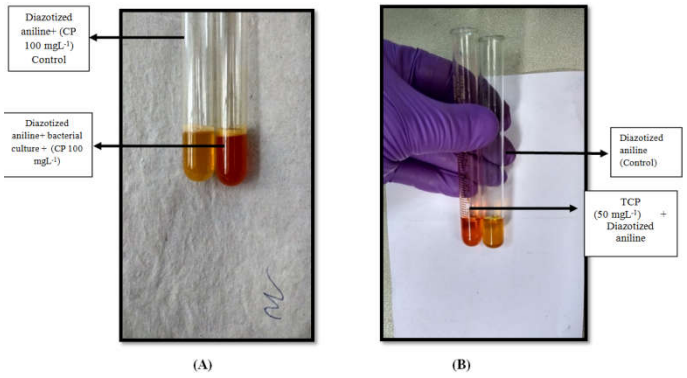

Fig 2 (A) Orange colored complex in the reaction mixture cont aining CP (100 mgL^{-1}) degraded with bacterial culture and diazotized aniline after 15 minutes (B) Orange color complex development in standard TCP added with diazotized aniline

Afterwards method was employed to confirm the OPH activity of bacterial strain for conversion of CP into the TCP in the liquid reaction mixture. Test tube containing 5 ml sample containing CP, (100 mgL^{-1}) as only carbon source was augmented with actively growing and induced bacterial cells (1 ml, O.D.-1) incubated at room temperature for 30 minutes. Diazotized aniline was added every five minutes and it was observed that after 15 minutes strong visible orange color was developed (Fig 2. A). Bacterial strain was found to possess strong OPH activity. This outcome of the study which was in contrast from the previously reported which suggest that bacterial strains possess low to moderate OPH activity for CP (Singh *et al.,* 2003; Lakshmi *et al.,* 2009: Yadav, *et al.,* 2015).

The enormous diversity of bacterial strains with tremendous variation in enzymatic variety has been available in the ecosystem. Low cost and ability to withstand a wide range of pH and temperatures conditions make whole cells potentially suitable candidate/biocatalyst for biosensors development. Enzymatic activity of OPH has been studied extensively and several genetically engineered variants have been discovered having efficient catalytic abilities. (Di Sioudi et al., 1999). It has been suggested that OPH hydrolyzes a wide range of organophosphates, the effectiveness of hydrolysis varies considerably. OPH hydrolyze most widely used insecticides such as methyl parathion, chlorpyrifos, diazinone etc. with a lower rate than its preferred substrate, paraoxon (Singh *et al,* 2003). Bacterial strain used in present work possess strong OPH activity which may be due to continuous exposure of strain to higher concentration of CP.

Diazotized aniline in presence TCP generate an orange red color complex and exhibited absorption maxima at 430 nm (Patel *et al.,* 2012) and 456 nm (Venugopal *et al.,* 2012). The absorption spectrum of the colored complex produced in the present study showed lambda max at 502 nm in liquid medium and diazotized aniline was used as blank. Subsequent increase in absorbance with increase in CP concentration was also observed.

Development Fiber optic biosensor

An optical based biosensor has been developed in the present study. Cell concentration was adjusted spectrophotometrically and optimized. Actively growing and CP induced bacterial cells with a specific cell count were then immobilized by Sol-Gel immobilization method developed and optimized in the laboratory previously. Post immobilization, sample i.e., CP (10 µl) was added in to the immobilized discs, incubated at 30^0C and allowed to proceed the reaction undisturbed until visible color development begins. After color development Lambda max (absorbance) was observed manually and recorded spectrophotometrically by scanning the immobilized tip.

Visual color production was observed manually and it was found that with the increase the concentration of CP, color intensity was also increase. Whole cells immobilized plastics discs along with reaction sample showing in the increase color intensity with increase in CP concentration (Fig. 3).

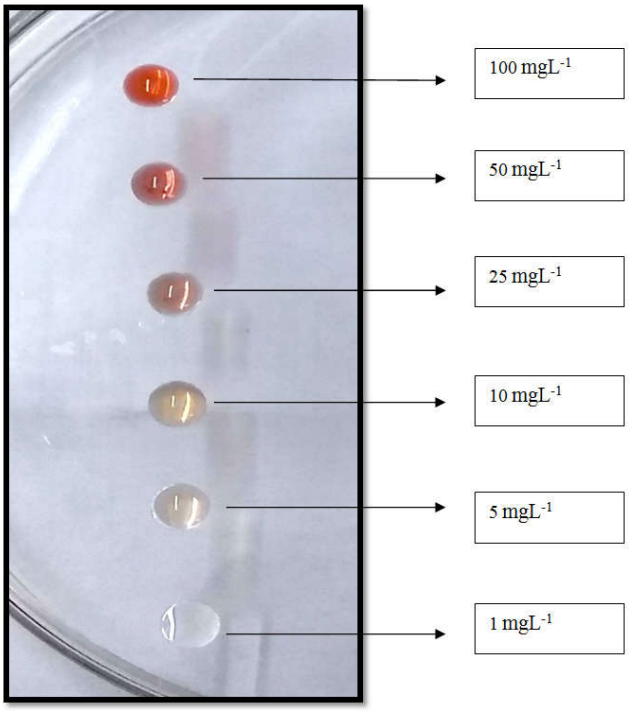

Fig 3 Visual increase in color intensity of the immobilized bio-sensing disc with increase in CP (1 mgL-1 100 mgL-1)

Absorption spectrum of different concentrations of CP with diazotized aniline showed a linear increase in the absorption (Fig. 4).

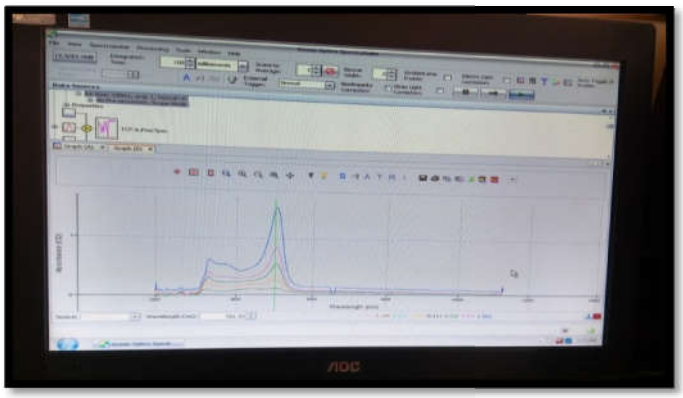

Fig 4 Absorption spectrum of different concentrations of CP (1 mg L^{-1}-100 mgL^{-1}) recorded on immobilized bio-sensing discs after 15 minutes

After 15 minutes of reaction time, absorbance of all the samples were recorded and a good linear co-relation was found between absorption by colored complex and concentration of CP in the reaction samples (Fig 5).

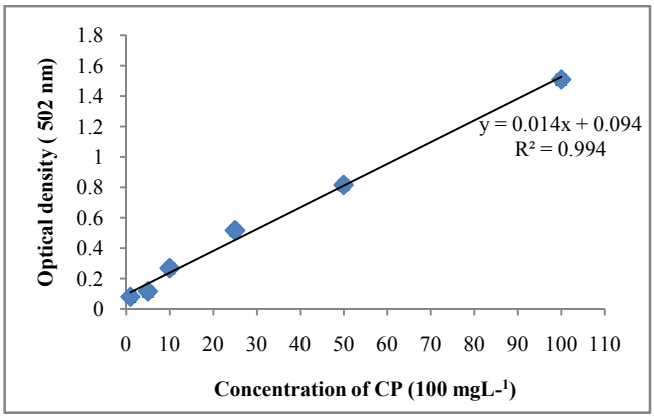

Fig 5 Standard curve showing the linear relationship between OPH activity on the basis of absorption at different concentration of CP (1 mgL^{-1} -100 mgL^{-1})

Miniaturization of sample volume and Optimization of Response time and cell biomass

Sample volume required for the analysis of CP concentration in the reaction sample was miniaturized to a level as low as 10 µL. It was established that miniaturization of sample volume to 10 µL is also among one of the striking outcome of the present study. Lowest limit of detection was found to be 0.01 mgL^{-1}. Linear range of detection of CP using developed biosensor was found from 0.1 mgL^{-1}-100 mgL^{-1}. As discussed earlier absorption of the sensing plastic discs was recorded after every five minutes to find out the optimized response time of. For initial ten minutes of the reaction no significant increase in the absorption was recorded. Although within the 15-20 minutes elevated absorption was observed and beyond 15 minutes, any prominent increase in absorbance was not recorded. Therefore, 15 minutes were considered as optimized response time for the developed biosensor. Procedure was repeated with different concentrations (1 mgL^{-1} -100 mgL^{-1}) of CP and it was analyzed that maximum absorption was observed at 15 min as beyond that no distinct increase in absorption was absorbed (Table 1).

In the course of biosensor development, it has been suggested that response time is one of the main factors that describes the efficiency of a biosensor. OPH activity is reported to be slow for Chlorpyrifos and Methyl parathion in comparison other member OP pesticides (Singh *et al*., 2006) like paraxon and parathion. In contrast to this, there are reports in the literature demonstrating the isolation of bacterial strains having the strong OPH activity. Xu *et al.,* 2007 have isolated *Serratia sp*. from sludge of one of the pesticide manufacturing unit in China and reported 100% of CP (50 mgL^{-1}) conversion with in 18 h of incubation. Another bacterial strain *Ralstonia sp*. T6 isolated from sludge from wastewater treatment system of chemical factory, Nanjing, China. Isolate has demonstrated 100% CP (100 mgL^{-1}) removal in 12 h (Li *et al.,* 2010). Frequent exposure of CP to the bacterial strains generate a possibility of evolution of the novel and efficient metabolic pathways with inclined OPH activities.

Table 1 Absorption of different samples of CP having different concentrations recorded up to 20 min at 502 nm

		Time (min)				
Absorbance at 502 nm	CP Concentration	2	5	10	15	20
	100 mg L^{-1}	0.003±0.042	0.105±0.052	1.023±0.015	1.52±0.036	1.499±0.012
	50 mg L^{-1}	0.007±0.036	0.091±0.062	0.445±0.036	0.814±0.022	0.811±0.015
	25 mg L^{-1}	0.005±0.041	0.072±0.033	0.325±0.042	0.521±0.032	0.518±0.012
	10 mg L^{-1}	0.002±0.029	0.063±0.025	0.156±0.019	0.265±0.62	0.271±0.061
	5 mg L^{-1}	0.002±0.054	0.011 ±0.045	0.088±0.021	0.111±0.052	0.106±0.032
	1 mg L^{-1}	0.003±0.038	0.005±0.018	0.021±0.027	0.081±0.042	0.077±0.52

Furthermore, induction of the bacterial cells prior to the immobilization increase the OPH activity significantly. Un-induced bacterial cells were also checked for their OPH activity. It has been found that OPH activity triggers and visible color was developed on the discs after one hour. Bacterial cultures are induced to increase the OPH activity so that CP breakdown can be achieved easily and quickly (Akabr and Sultan 2016, Bhardwaj and Verma, 2018). Optimization of the bacterial biomass concentration was also done. Different cell concentrations corresponds to optical density (0.5,1.0,1.2,1.5) were immobilized in sol-gel matrix and after immobilization 50 mg L^{-1} CP (10 µl) was added and absorption was recorded after 15 minutes. Maximum absorption 0.89 was observed at absorption maxima with cell concentration (O.D-1.2) (Fig.6). Therefore, further experimentation was carried out with cell concentration corresponds to O.D.-1.2 for immobilization for bacterial cells as bio-sensing element.

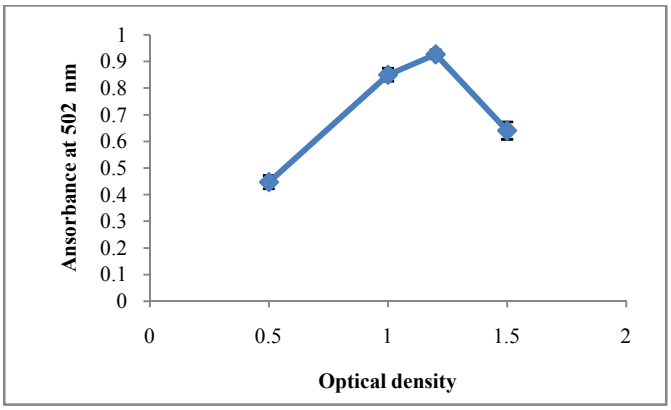

Fig 6 Optimization of cell biomass for biosensor development

Validation of developed biosensor by spiking

Validation of the developed biosensor was carried out by spiking of the known concentrations of CP in the drinking water sample. Two different known CP concentrations were added in water samples and extracted accordingly. Water sample of CP concentration 25 mgL^{-1} exhibited 0.511 absorbance whereas 50 mgL^{-1} showed 0.788 absorbance at 502 nm. When absorption values were compared with the standard graph it has been found that developed biosensor exhibited more than 94- 96 % accuracy which noticeably illustrated its competency (Table 2). Extraction of CP from the water was also found in the range of 96% - 98% throughout experimental studies.

Table 2 Validation of the developed biosensor by spiking CP in the water sample

Sample	CP concentration detected from standard graph mgL^{-1}	Validation
Drinking water	N/A	N/A
Drinking water Spiked with CP- I (25 mg L^{-1})	24.12 ±0.06	96.49 %
Drinking water Spiked with CP- II (50 mg L^{-1})	47.11 ±0.04	94.23 %

Application of Developed Biosensor in real water samples

For the application studies of developed OPH based fiber optic biosensor, detection of the CP in the real test samples was carried out. A total ten soil samples were collected from the various regions of the Punjab, India and processed further. Concentration of CP in the unknown samples was calculated from the standard calibrated curves, considering the dilutions factors as well. From the results it was observed that all samples were detected positive for the CP presence. The mean concentration of chlorpyrifos recorded in soils was higher than the United States (US) maximum residue limit of 0.03 mg/kg for agricultural soils (Fosu-Mensah *et al.*, 2016). Authentication of the results of the developed biosensor by were conceded by HPLC studies. According to a survey it was reported that farmers in this area spraying pesticides 32 times on fields with in period of six months which is almost five times more than the prescribed value mentioned by Punjab Agricultural University, Ludhiana, Punjab, India (Mathur *et al.*, 2005). Farmers and pesticide applicators are not versed with basic education and are unaware to the ill effects imposed by high use of pesticides on the health.

Comparison of developed biosensor with existing biosensors

A comparison of the characteristic features of developed fiber-optic biosensor in the present study with the previously reported (Table 3). From best of our knowledge, development of a novel substrate based chlorpyrifos biosensor has been carried out as most of biosensor constructed for CP detection are inhibition based. In comparison to other biosensors, sample requirement in the present work is minimum i.e., 10 µl. One of the other major attribute of the developed biosensor is its cost effectiveness. Most of the biosensor developed for CP detection are enzyme based. Using enzyme as biocomponent require its purification which increases overall cost of detection process. Response time and time period required for the immobilization of bio-sensing element was also found to be less. Lowest limit of detection was found to be 0.01 mgL^{-1}. Linear range of detection of CP using developed biosensor was found from 0.1 mgL^{-1}-100 mgL^{-1}.

Table 3 Comparison of the performance of biosensor developed with previously reported

S. No.	Transducer type	Inhibition /Substrate based	Detection of Limit (M)	(Whole cell/ Enzyme based)	Immobilization time	Response time (min)	Sample required	Reference
1	Electrochemical	Inhibition	Not given	Enzyme	24 h	20	1 ml	Lin *et al.,* 2003
2	Fiber Optic	Inhibition	10^{-8}	Enzyme	5 days	12	1.5 ml	Kuswandi *et al.,* 2007
3	Amperometric	Inhibition	10^{-8}	Enzyme	24 h	15	Not given	Kuswandi *et al.,* 2008
4	Electrochemical	Inhibition	10^{-12}	Enzyme	30 min	30	100 µl	Viswanathan *et al.,* 2009
5	Electrochemical	Inhibition	Not given	Enzyme	1h	Not Given	25 µl	Cao *et al.,* 2015
6	Fiber Optic	Substrate	10^{-7}	Cell	20 min	15 min	10 µl	Present study

A number of researchers working in Punjab area have reported the presence of pesticides in various environmental samples like soil, water milk etc. as well as in the living systems. Indiscriminate use of pesticides, along with environmental and social factors are the major cause of entry of CP in the food chain (Mittal *et al.,* 2014). The farmers and agricultural workers are inadvertently exposed to pesticides through occupational use at all stages starting from pesticide purchase, transport, storage, dilution of pesticide concentrate, leaking of spray equipment, and inhalation during pesticide spraying (Maroni *et al*. 2000). Almost 17 different types of pesticide constituents have been detected in the region in different samples and according to Government of India, (CIBRC) 2012 most of these have now been categorized into banned or restricted pesticide. Thus there is an urgent need to detect the residual CP concentration in order to get an insight of its presence in various ecosystems. Hence, concerted effort to develop biosensors for detecting residual organophosphates and their degradation products in the contaminated environment is the need of hour.

CONCLUSIONS

Present study related to the development of a novel, prompt, cost effective and reliable absorption based fiber optic biosensor with a lowest detection limit of 0.01mg L^{-1} CP in polluted soil and water samples. Till date biosensors developed for CP detection were based on the AChE enzyme activity. In comparison to the previously reported biosensors for CP detection, present system is novel with respect to the bio-sensing element involved in the detection of CP i.e., OPH activity of the isolated bacterial strain. Other unique features are quick response, cost effectiveness, miniaturization of sample volume to a level of 10µl.

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