International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: 6.614
Available Online at www.journalijcar.org
Volume 11; Issue 01 (A); January 2022; Page No.51-56
DOI: http://dx.doi.org/10.24327/ijcar.2022.56.0011

Research Article

EGGSHELL MEMBRANE BASED ELECTROCHEMICAL BIOSENSOR FOR THE DETECTION OF ORGANO PHOSPHORUS (OP) COMPOUNDS

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A R T I C L E I N F O

Article History:
Received 6th October, 2021
Received in revised form 15th November, 2021
Accepted 12th December, 2021
Published online 28th January, 2022

Key words:
Organophosphorus(OP) compounds, *Pseudomonas diminuta*, Eggshell membrane (ESM), Screen printed carbon electrode (SPCE), electrochemical and Methyl parathion.

A B S T R A C T

Synthetic organophosphorus (OP) compounds are highly poisonous substances, extensively applied in the agriculture field as pesticides and insecticides and used in the military as chemical warfare agents. The aim of the present study is to develop an eggshell membrane (natural polymer) based electrochemical biosensor for the detection of OP compounds. We have selected the eggshell membrane as a natural support for immobilization of microbial cells. The enzyme organophosphorus hydrolase (OPH) enzyme was produced in one of the soil borne bacteria *Pseudomonas diminuta* species, which is capable of hydrolyzing methyl parathion into dimethyl thiophosphoric acid and yellow colour detectable product p-nitrophenol. Whole *Pseudomonas diminuta* bacterial cells were immobilized on the eggshell membrane using glutaraldehyde as cross linking agent and placed on the working area of screen printed carbon electrode (SPCE). ESM with immobilized bacteria was placed on SPCE and it was connected to electrochemical work station and cyclic voltammograms were recorded. The potential +0.1 V was optimized to measure the changes in the current as per redox role of p-nitrophenol. There was increased oxidation current as the concentration of methyl parathion was increased. A single ESM with immobilized bacteria can be reused for 22 reactions and it was stabilized for 32 days at 4°C with 80% of initial enzyme activity.

INTRODUCTION

Organophosphorus (OP) compounds are widely used in the field of agriculture as pesticides, insecticides and in military as chemical warfare agents. The extreme toxicity of these neurotoxic compounds created the concern towards developing quick and simple techniques to protect water supplies and food supplies and also for monitoring terrorist activity and detoxification mechanisms (Mulchandani *et al*., 1999a, b). Various analytical methods have been developed for the detection of organophosphorus compounds such as gas liquid chromatography, high performance liquid chromatography and spectrophotometer. However, these existing methods having their own limitations like time consuming, expensive and difficult to monitor the experiment constantly and also require trained personnel (Jaffrezic-Renault, 2001).

Currently, different type of biosensors has been developed for the detection of OP compounds due to a profound research (Tembe *et al*., 2006; Kumar and D'Souza, 2008, 2009, 2010). Biosensor is an analytical device which consists of a biorecognition element (Whole cells, enzymes, DNA and Antibodies) and a transducer for the detection and quantification of specific analyte. Generally in microbial biosensors, whole bacterial cells were immobilized on a different matrix that is conjugated with a transducer and a specific change was measured. Selection of a suitable matrix for the immobilization of microbial cells is a very crucial step in the development of any type of biosensors; choice of support should be able to maintain the bacterial cells activity along with reusability and storage stability.

Nowadays, various types of natural and synthetic membranes are utilized for the fixation of enzymes, microbial cells and nucleic acids in the construction of biosensors. These natural membranes are highly biocompatible in nature due to their biochemical compositions like proteins, carbohydrates, and lipids which create comfortable tiny surroundings for the ideal working of biomolecules including microbial cells. From last decade, eggshell membrane, inner epidermis of onion membrane and bamboo inner shell membrane are the natural polymers proved to be useful support for enzyme immobilization for biosensor application (Wu *et al*., 2004; Yang *et al*., 2006; Tembe *et al*., 2008). Egg shell membrane is a light pink double layered membrane, having the thickness of around 65-96µm. The membrane contains an inner and outer membrane of different thickness; it mainly consists of highly cross-linked protein fibers, biological molecules and also possesses an excellent gas and water permeability (Liong *et al*., 1997; Takiguchi *et al*., 2006). Egg shell membrane is

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biodegradable, non toxic, ecofriendly, cheap and easily available. These entire qualities make the egg shell membrane as an ideal platform for the immobilization of enzymes and microbial cells in the biosensors preparation. In 2004, Wu *et al*., immobilized glucose oxidase enzyme on the eggshell membrane for the glucose biosensor application. Yeni *et al*., in 2008, first time reported about microbial biosensor, in their work they have immobilized whole bacterial cells on eggshell membrane to measure toxicity and biological oxygen demand. Recently Kumar and D'Souza, 2009; reported that they have immobilized glucose oxidase enzyme on the inner epidermis of the onion bulb scales for biosensor. Eventually, Wang *et al*. (2010) utilised inner epidermis of onion membrane for the immobilization of glucose oxidase and developed a biosensor for the detection of glucose. Onion membrane consists of number of guard cells with elongated tubular cells (Scott *et al*., 1958; Bruce and Hepworth, 2004; Kumar and D'Souza, 2009). Screen-printed carbon electrodes (SPCEs) are extensively used in the development of electrochemical biosensors for the determination of OP compounds. SPCEs based biosensors have several advantages include low cost, simplicity, mass production capabilities, portable, ease of production, small size and they can be applied for onsite detection with less amount of sample (Rao *et al*., 2006; Bello-Rodriguez *et al*., 2004; Noh *et al*., 2005).

The SPCEs of different configurations are commercially available (Palm- Sens Electrochemical Sensor Interface; Zensor R&D electrochemical sensor etc) which can be used to immobilize variety of biological molecules like antibodies, enzymes, proteins, DNA and RNA for biosensor applications (Tudorache and Bala, 2007). Timur *et al*. (2003) have used the modified SPCEs by applying bacteria for the determination of phenol.

In 2011, Kumar and D' Souza, reported that they have immobilized whole cells of recombinant *Escherichia coli* on SPCE and developed an electrochemical biosensor for the detection of methyl parathion.

In the present study, we demonstrate that the natural polymers based egg shell membrane as platform for the immobilization of bacterial cells *Pseudomonas diminuta* for the detection of methyl parathion and it was associated with electrochemical work station. Bacterial cells immobilized on egg shell membrane using glutaraldehyde as cross linker and it was placed on the working electrode, which was associated with an electrochemical analyzer. Cyclic voltammograms were recorded using methyl parathion as substrate on cells immobilized ESM and SPCE. The hydrolyzed product of methyl parathion is p-nitrophenol, by applying specific potential produces change in amount of current due to redox behaviour of p-nitrophenol.

MATERIALS AND METHODS

Materials

Methyl parathion (O,O-Dimethyl O-4-nitrophenyl phosphorothioate) was procured from Sigma-Aldrich, USA and p-nitrophenol from Central Drug House, New Delhi India. Other chemicals were purchased from SRL, India. The deionized water was used for the preparation of solution. The eggs were purchased from a local supermarket (Suguna brand) and stored at 4^{o}C until use.

Micro-organism and culture condition

Microorganism

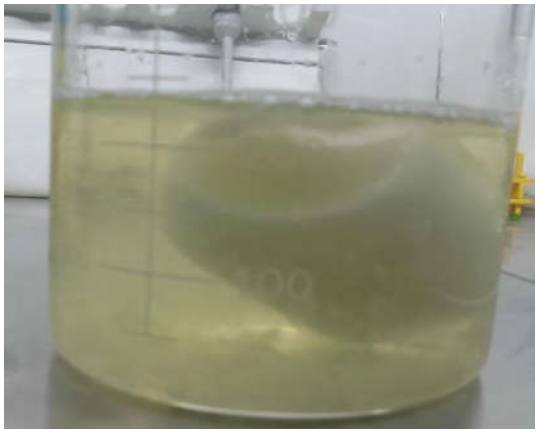
Pseudomonas diminuta strain was obtained from Institute of Microbial Technology, Chandigarh, India.

Revival and Growth of Lyophilised sample of Bacteria

Lyophilised sample of bacteria was taken in a test tube and about 5 ml of nutrient broth (pH-8.0) was added to make a bacterial suspension. For culturing *Pseudomonas diminuta* cells, the revived bacterial suspension was transferred to the conical flask (250 ml each) containing nutrient broth. Incubated the culture medium in a rotator incubator shaker (200 rpm) at 30˚C for 24 hours or overnight and growth of the bacteria was monitored by measuring Optical Density (OD) at 600 nm.

Preparation of eggshell membrane (ESM)

The outer eggshell membrane composed of 94% of calcium carbonate and other 6% includes magnesium carbonate, calcium phosphate and organic matter (Tsai *et al*., 2006). The major component calcium carbonate dissolves completely in acetic acid. The eggs were submerged in 100% acetic acid at room temperature for 18 hrs, and then inner eggshell membranes were easily removed and cleaned thoroughly using distilled water. The separated eggshell membranes were cleaned again with a large amount of deionized water to remove the egg contents albumin and yolk (Zhang *et al*., 2006). The thoroughly washed eggshell membranes were kept in Phosphate Buffer Saline (pH 7.4) until further use as shown in Fig.1 (a) and (b).



(a)

(b)

Fig 1 (a) Egg kept in acetic acid (b) Eggshell membrane

Cell loading and glutaraldehyde concentration optimization for immobilization

An eggshell membrane was cut into small circular membrane (diameter = 3 mm; area = 17.64 sq mm) using a biopsy punch and used for immobilization of bacterial cells. We have optimized the amount of cell titres to be immobilized on the ESM along with glutaraldehyde concentration. Different amounts of cell titres (0.5 X 10^9,1.0 X 10^9 , 1.5 X 10^9, 1.7 X 10^9 , 2.0 X10^9, 2.5 X 10^9 and 3 X 10^9) were prepared and immobilized on eggshell membrane using various concentrations (0.2, 0.5, 1, 2 and 5%) of glutaraldehyde as cross linking agent. The immobilized bacterial cells hydrolytic activity was observed in response to methyl parathion (Kumar and D'Souza, 2011).

Immobilization of microbial cells on eggshell membrane

A 1.7 X 10^9 of bacterial cell titer was adsorbed onto the ESM and was dried in air for 45 minutes at room temperature. Then cross linking was done by adding 2.5 µl of 1% glutaraldehyde and further incubated for another 20 minutes at room temperature for proper binding of bacterial cells to each other and to the surface. Bacterial cells immobilized eggshell membrane was washed with buffer and stored at 4°C until use (Kumar and D'Souza, 2011).

Apparatus, operating system and experimental procedure

Cyclic voltammetry (CV) study was performed using CHI 660C Beta electrochemical workstation (CH Instruments, USA). The SPCEs used in this work were purchased from local vendor (Zensor R&D, USA). SPCEs consist of a three electrode configuration comprises a working electrode, an Ag/AgCl pseudo reference electrode, and a platinum auxiliary electrode (Zen *et al*., 2004). The counter electrode, working and reference electrodes are incorporated on the screen printed carbon electrode and the diameter of the working electrode is 3 mm and its working area is 0.071 cm2. A bacterial cell immobilized ESM was placed on the working electrode, approximately 4µl of substrate is added, incubated for 2 minutes and cyclic voltammograms were recorded. Bacterial cells immobilized ESM was placed on the SPCE and it was directly associated with an electrochemical analyzer which was completely controlled by PC software for all operations including data acquisition and analysis. Primarily cyclic voltammograms were recorded in phosphate buffer (5µl, pH 8.0) using control ESM and cells immobilized ESM between +1.2 and −1.2 V at scan rate of 100 mVs^{-1}. Subsequently cyclic voltammograms were recorded using methyl parathion pesticide on SPCE with bacterial cells immobilized ESM under the same condition. Immobilized ESM were washed separately along with electrode using buffer after every analysis of sample and were reused. All the measurements were carried out at room temperature in steady-state conditions. The response time of a microbial biosensor under steady-state (the time required for maximum percentage of hydrolysis of methyl parathion) was about 5 mins, and it was based on the incubation time for reactants along with accumulation of product for finer performance. There was no significant variability was observed in the sensor response (Kumar and D' Souza, 2011).

Scanning Electron Microscope (SEM) study of bacterial cells immobilized eggshell membrane

In this study we used a Scanning electron microscope (FEI Quanta 200) to observe the surface morphology of control eggshell membrane (ESM) and bacterial cells immobilized eggshell membrane. For SEM study, both control ESM and cells immobilized ESM were mounted on stubs and coated with Au/Pd using a sputter coater. SEM micrographs of cells immobilized ESM and control ESM were taken at different magnification at 500 x.

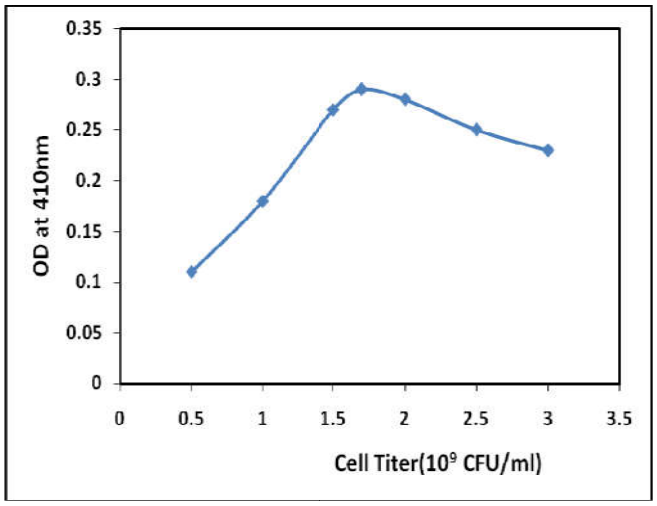
Reusability, stability and reproducibility

The single eggshell membrane with bacterial cells immobilized was tested by repeated reactions using cyclic voltammetry. The cyclic voltammograms were recorded with above mentioned parameters from day1 to day 30 days with 5 days of intervals. Immobilized ESM were washed separately along with electrode using buffer after every analysis of sample and were reused.

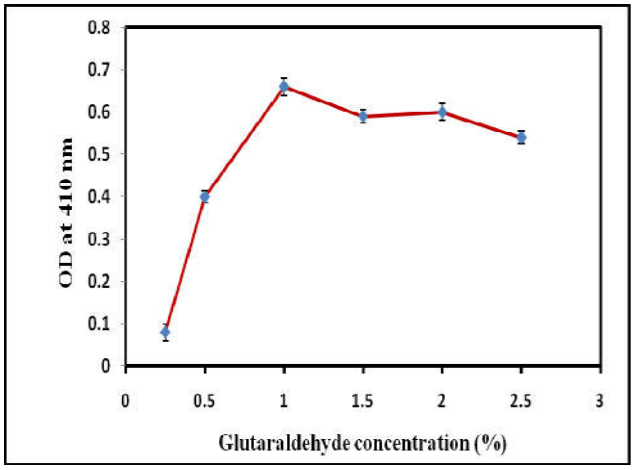
RESULTS AND DISCUSSION

Optimization of cell loading and glutaraldehyde concentration

In order to attain optimum hydrolytic activity, there is need to optimize cell loading on the eggshell membrane. Here various concentrations of cell suspension were used for immobilization on ESM and cyclic voltammograms were observed. As shown in Fig.2, only 10µl of cell suspension generates maximum oxidation current with 1% glutaraldehyde. Glutaraldehyde is an amine reactive bifunctional agent used as cross linker; it was also optimized to increase the stability of cells and also to restrict the leaching effects and thereby increases the endurance of the biomolecules. It was observed that in the presence of 1% of glutaraldehyde concentration the peak intensity of p-nitrophenol was increased and the peak intensity was decreased in the in the absence of cross linking agent optimum for cross linking of cells along with binding to each other and to the surface (Fig.2b). It increases the The optimized glutaraldehyde concentration was matching with earlier report (Kumar and D' Souza, 2011).



(a)

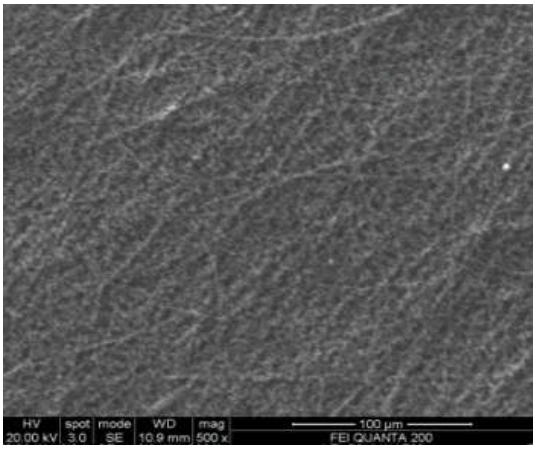


(b)

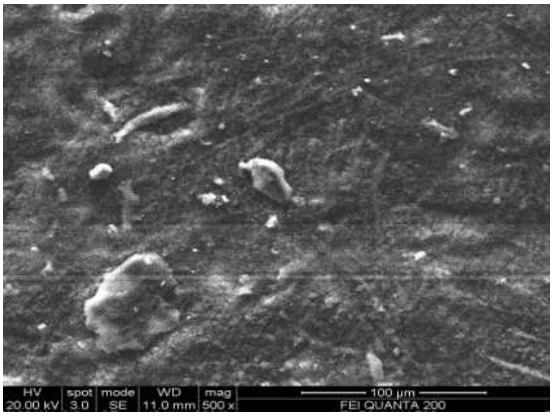
Fig 2 (a) Optimization of cell loading and (b) glutaraldehyde concentration as cross linking agent for immobilization on egg shell membrane (size 3mm diameter).

SEM study of bacterial cells immobilized eggshell membrane

The SEM study of both control and cells immobilized ESM was carried out to detect the surface characteristics and also to confirm the biological components on the immobilization matrix. The control i.e. cells unimmobilized eggshell membrane shows highly cross-linked protein fibers and small cavities which make the ESM to posses excellent gas and water permeability. The bacterial cells immobilized ESM shows accumulated cells at bottom region and individual cells at the top region (Fig.3.A and B).



(a)



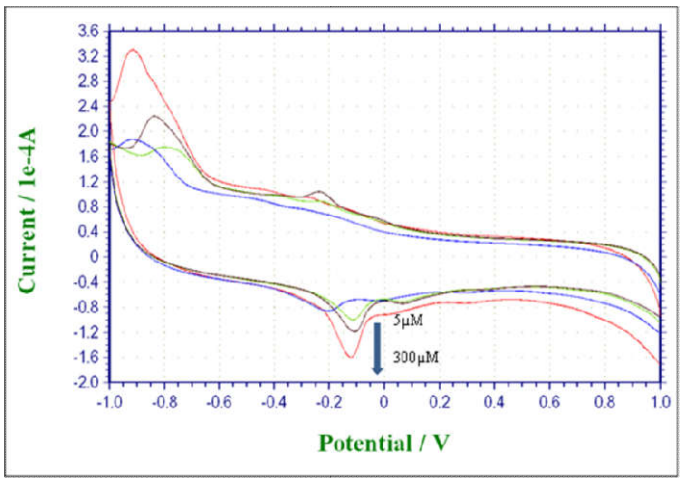
(b)

Fig 3 SEMs of the (a) control eggshell membrane and (b) cells immobilized eggshell membrane

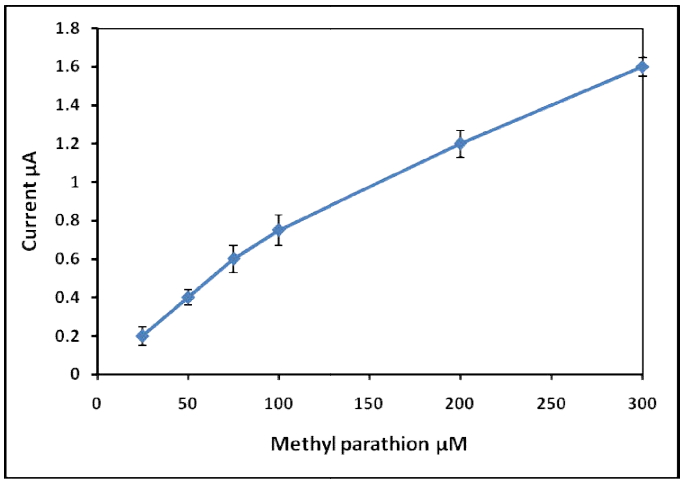
Cyclic voltammogram of bacterial cells immobilized ESM on SPCE

Change in cyclic voltammograms between the control ESM on SPCE and cells immobilized ESM on SPCE were recorded under steady state conditions. The changes in the cyclic voltammetry of methyl parathion and p-nitrophenol were recorded for control i.e. unimmobilized ESM after that difference in the cyclic voltammograms were observed using cells immobilized ESM at SPCE. Changes in cyclic voltammograms were also observed when cells immobilized ESM-SPCE was used for analysis in sodium phosphate buffer (pH 7.5, 50 mM) with and without methyl parathion (Fig. 4 a). Cyclic voltammograms of bacterial cells immobilized ESM SPCE were also recorded before and after the hydrolysis of methyl parathion. It was observed that two new oxidation peaks appeared after hydrolysis.

The oxidation current, measured at a fixed potential using a potentiostat, is directly proportional to the concentration of PNP formed.



(a)



(b)

Fig.4. (a) Cyclic voltammograms of different concentrations of methyl parathion using bacterial cells immobilized ESM on SPCE. (b) Enzymatic assay with methyl parathion (5-300 µM) for the calibration of sensor response (linearity between 5 and 75 µM with linear regression equation, Y = 0.0049 x+0.1752, r^2=0.982.

Calibration of the biosensor and detection range

The prepared biosensor was calibrated along with the eggshell membrane using different concentrations (5-300µM) of standard methyl parathion and cyclic voltammograms shows

the oxidation current at +0.1V. The linear response was observed after methyl parathion hydrolysis, dynamic concentration range from 5 to 75 µM which was estimated from the calibration graph with a linear regression equation, Y = 0.859 x+16.22, r^2 =0.991as shown in Fig. 4b. Detection limit of the biosensor is 5µM of methyl parathion and it was estimated from linear range. Detection range of the developed biosensor method is matching with the earlier reported OPH based amperometric biosensor methods (Mulchandani *et al*., 1999, 2001a, b, Kumar and D' Souza, 2011).

Stability, reusability and reproducibility of the whole cells immobilized ESM on SPCE

Stability, reusability and reproducibility are the most important qualities of an effective biosensor. The bacterial cells immobilized eggshell membrane reusability is tested in the presence and absence of glutaraldehyde. Glutaraldehyde presence increases the reusability of the bacterial cells immobilized ESM by bounding the adsorbed bacterial cells to each other onto the ESM and also maintains the stability by cross linking. Consequently glutaraldehyde treatment reduces the leaching of cells, thus enhances the reusability of cells immobilized ESM. As per the observation a single cells immobilized eggshell membrane can be used for 22 repeated reactions with 90% retention of activity. The reproducibility was tested using different bacterial cells immobilized ESM on SPCE and the response is good with negligible variations. The low relative standard deviations (RSD), with respect to cells immobilized ESM against 70µM methyl parathion also. Bacterial cells immobilized ESM was stable for 32 days.

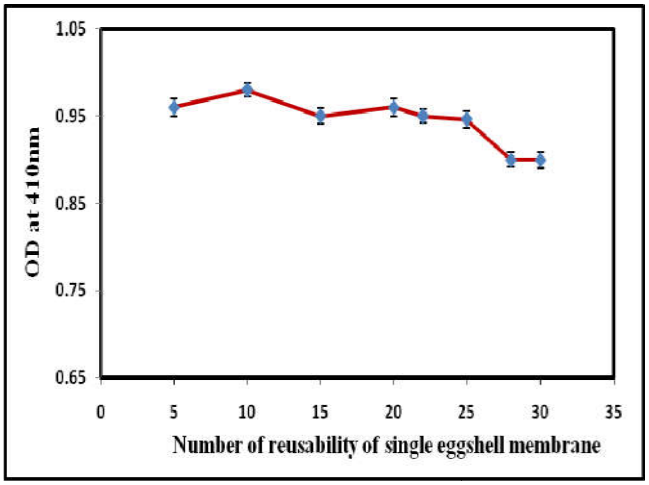


Fig 5 Reusability of cells immobilized on eggshell membrane

Evaluation of biosensor applicabilty using methyl parathion spiked samples

The tap water samples were mixed with different concentrations of methyl parathion and then mixed with phosphate buffer (pH 8.0) in ratio 3:1 incubated as per the standard protocol. The spiked samples were analysed using pseudomonas diminuta immobilized ESM based electrochemical biosensor. As shown in Fig. 6 the straight line fit plot between the spiked concentration of methyl parathion and the biosensor results yielded a slope of 0.961 with a positive correlation (r^2 = 0.9971) which demonstrates the feasibility of the cells immobilized ESM membrane for biosensor application.

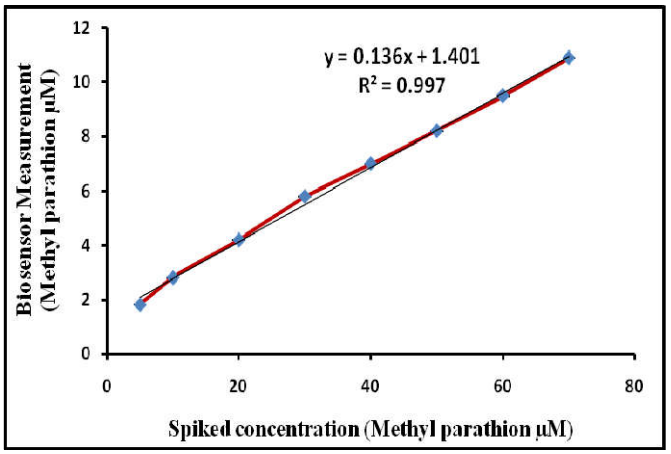


Fig 6 Correlation of the biosensor measurement with actual methyl parathion spiked concentration. Different concentrations of methyl parathion (5, 10, 20, 30, 40, 50, 60 and 70 µM) spiked.

CONCLUSION

In this work, we have developed the microbial electrochemical biosensor using natural polymer for the detection of OP compounds. Here, we exploited the use of Egg shell membrane (ESM) as biocompatible platform for the immobilization of bacterial cells. The developed system shows wide detection range, 5-75µM for methyl parathion and only 15 µl volume of sample is required for the analysis. A single eggshell membrane with cells immobilized bacterial can be reused for 22 reactions with 80% retention of its initial enzyme activity and it was stable for 32 days, at 4°C.

Acknowledgement

Authors are thankful to Director, DEBEL, DRDO Bengaluru for the constant support and encouragement.

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How to cite this article:

Maheshwari D. T and Kumar N. S (2022) 'Eggshell Membrane Based Electrochemical Biosensor For The Detection of Organo Phosphorus (Op) Compounds', *International Journal of Current Advanced Research,* 11(01), pp. 51-56.
DOI: http://dx.doi.org/10.24327/ijcar.2022.56.0011

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