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Easy-to-use and reliable absorbance-based MPH-GST biosensor for the detection of methyl parathion pesticide

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ABSTRACT

Due to high contamination of organophosphate (OP) insecticides in agricultural products and the environment, efficient and convenient devices for their monitoring are necessary. Here, a simple, inexpensive, efficient, and easy-to-use absorbance-based biosensor was fabricated utilizing recombinant methyl parathion hydrolase fused with glutathione-*S*-transferase (MPH-GST), covalently immobilized onto a chitosan film-coated polystyrene microplate, for the detection of methyl parathion (MP) as a representative of OPs. Having been connected to the transducer system designed to work through an Arduino microcontroller, the biosensor could detect MP as efficiently as the conventional methods, with the detection limit of 0.1 μM, the lowest value ever reported for this method. It was stable at 25 °C for 30 days, could function 100 rounds repetitively, and yielded high recovery with real samples. Hence, this simply designed MPH-GST biosensor could be an easy and inexpensive alternative for efficient OP screening at site to help control its contamination.

1. Introduction

Organophosphate (OP) insecticides, originally produced for use as chemical warfare nerve agents around the time of World War II [1], have been extensively used in agricultural areas of many countries. Some of the major OPs that are still in use in many places are methyl parathion (MP), malathion, dimethoate, fenitrothion, profenofos, and chlorpyrifos. The excessive use results in the contamination of OPs and their residues in agricultural products and the environment [2]. OPs are capable of inhibiting acetylcholinesterase (AChE) at nerve endings and central nervous system (CNS) of vertebrates resulting in the accumulation of the neurotransmitter acetylcholine, thus in turn causing neurotoxicity [3,4]. Apart from their neurotoxicity, they also cause other types of toxicity including genotoxicity, carcinogenicity, teratogenicity, reproductive toxicity, developmental toxicity, and immune toxicity [2,5]. OP toxicity can be acute, sub-chronic, and chronic. The typical symptoms can be restlessness, hyperexcitability, tremors, paralysis, diarrhea, convulsions, and respiratory failure, which can even lead to death [2,4,6].

Due to their strong hazardous impacts on human health and the environment, simple, sensitive, and rapid sensing devices with low cost and high efficiency are needed for OP detection in order to screen for their contamination in food products, soil, and water sources. Biosensors, analytical devices based on bio-recognition elements directly conjugated with transducers, are suitable for the determination of OP compounds [3]. They provide several advantages over traditional detection methods such as gas chromatography (GC) [7] and liquid chromatography (LC) [8], since they can offer qualitative and quantitative data with simplicity [9], inexpensiveness [10], minimal sample preparation [10], short-time response [11], high accuracy [12], and ability for onsite detection [3].

There were several enzymes reported capable of degrading OP insecticides and used as biomolecules for biosensor fabrication [13]. OP-degrading hydrolase (OPH) has been studied extensively. It was encoded from the OP degradation (*opd*) gene isolated from OP-degrading soil bacteria, *Flavobacterium* sp. ATCC 27551 [14] and *Pseudomonas diminuta* [15]. Another OP-degrading enzyme highly homologous to OPH, namely OpdA, was isolated from *Agrobacterium radiobacter* [16,17]. OPH and OpdA have similarity both in their sequences and structures [18]; however, the catalytic efficiency of OpdA was reported to be 10-folds more efficient than that of OPH [17]. These enzymes were capable of degrading a broad

Abbreviations: PX, paraoxon; MP, methyl parathion; CP, chlopyrifos; OPH, organophosphate hydrolase; AChE, acetylcholinesterase; MPH, methyl parathion hydroloase; MPH-GST, methyl parathion hydrolase fused with glutathione-*S*-transferase.

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range of OP compounds, such as paraoxon, and OP nerve agents including VX, tabun, soman, and sarin [13], via the hydrolysis of P—O, P—CN, P—F, and P—S bonds [19]. The enhancement of the OP degradation activity was achieved through the mutation of these OP-degrading enzymes [20–22].

Methyl parathion hydrolase (MPH), another type of OP degrading enzymes, was initially isolated from MP-contaminated soil bacteria, *Plesiomonas* sp. strain M6 [23]. The nucleotide sequence of its methyl parathion-degrading (*mpd*) gene showed no homology to that of *opd* gene. Another MPH was isolated from *Pseudomonas* sp. strain WBC-3 [24], with the nucleotide sequence of its gene highly homologous to that of *mpd* gene. MPH is a metal ion-dependent enzyme capable of hydrolyzing a wide range of OP substrates [25,26]. Its structure has been studied extensively and concluded to be a member of the metallo-β-lactamase family with a binuclear metal center [27,28]. The catalytic efficiency, K_{cat}/K_M, of MPH ($\sim 10^6$ M^{-1} s^{-1}) was reported to be 100 and 1000 folds higher than that of OpdA and OPH, respectively [19,27,28]. MPH substrate specificity and reactivity could be further improved by directed evolution. Via site saturation mutagenesis and DNA shuffling, an MPH mutant was demonstrated to have approximately 100-fold increase in catalytic efficiency for ethyl paraoxon [29]. Random mutagenesis was also adopted to enhance the reactivity of MPH towords a poorly-hydrolyzed OP substrate, chlorpyrifos, giving rise to a variant with a 5-fold increase in K_{cat} value [30]. With the possibility that its substrate specificity and reactivity can be improved by protein engineering, MPH could therefore be utilized as an enzyme of choice for practical use in industrial applications and bioremediation in a broader range.

In Thailand, an MPH was isolated from the MP-degrading *Burkholderia cepacia* [31], and was capable of degrading MP and other phenyl-substituted OPs to the yellow-colored *p*-nitrophenol (PNP) and dimethylthiophosphoric acid (DMPA) [25]. It was found to be constitutively expressed as a membrane-bound protein. In our previous study, the enzyme was purified to homogeneity and found to have the molecular mass of about 35 kDa and the pI of 8.5. Its gene, designated as *mpdB*, coding for 331 amino acid residues, was almost identical to *mpd* gene reported by Cui et al., 2001 [23], but different from *opd* gene [25,32]. The *mpdB* gene was cloned and transformed into *Escherichia coli* yielding a recombinant clone BpGP. The recombinant MPH, expressed as the fusion protein of MPH with glutathione-*S*-transferase (MPH-GST), was purified, characterized, and demonstrated to have cross reactivity with other OP substances i.e. paraoxon, parathion, fenitrothion, coumaphos, chlorpyrifos, and malathion [25].

Various types of enzyme-based biosensors have been developed which have different advantages and limitations. Inhibition-based AChE biosensors have been fabricated by certain researchers [33–36]; however, these biosensors have several limitations as the AChE is easily degraded. They have irreversible response, take a long time for responding during detection, are non-reusable in field analysis, and have low selectivity since they can be inhibited by either OPs or carbamates [37–39]. For catalytic biosensors, OPs can be directly detected using OP hydrolytic enzymes such as OPH and MPH. The principle for OP detection of these enzymes involves the hydrolytic activity as demonstrated in the degradation reaction of MP by MPH (Fig. 1). OP concentration can be determined by measuring PNP generated from the reaction, either by measuring the two protons generated from this redox hydrolytic reaction which happens during the cleavage of the P–X bonds, or by detecting the yellow color appeared [1]. OPH-based biosensors were fabricated using various methods such as electrochemical biosensors [40,41], amperometric biosensors [11,42–44], voltammetric biosensors [45], UV–vis based biosensors [46], and fluorescence-based biosensors [47–53]. MPH-based biosensors were also developed by electrochemical method [54–57], or by assessing for the changes in absorbance or fluorescence during OP hydrolysis through the appearance of the yellow color of PNP via optical method [6,50,58,59]. One of our previous works involved the development of an amperometric biosensor using the recombinant MPH-GST immobilized onto multi-walled carbon nanotubes (MWNTs) via electrostatic self-assembly [60–62]. This system was highly sensitive and efficient, having the linear range of 10–90 nM and the detection limit of 10 nM for MP detection. However, electrochemical biosensors in general have several disadvantages including interference by electrolytes in the sample solution causing false positive results, poor long-term stability, complicated assembly steps, and high development cost [6,63,64]. Optical biosensors, on the other hand, have advantages of having no such interference; lower development cost; as well as rapid, easy, and accurate determination [1,39]. Since our MPH-GST gave an efficient OP detection with amperometric biosensor, it is worth developing an optical biosensor with this enzyme.

There were several reports on absorbance-based OP optical biosensors [59,65,66]. Nevertheless, their detection limits were not low enough, being in the range of 4–80 μM. Hence, many researchers have nowadays gained more interests in developing fiber-optic [67–69] and fluorescence-based [48,70–74] optical biosensors. Many fiber-optic and fluorescence biosensors that have very low limit of detection were expensive and complicated when compared to absorbance-based biosensors, involving many fabrication steps and many substances, and, in some cases, the use of sophisticated technologies such as nanotechnology. They may be beneficial in some instances but may not be suitable for other situations. For large scale screening of OP contamination as a fast and easy means to monitor and control the environment in some countries, it is preferably performed without costing large amounts of funds. In so doing, a simple and cost-effective but reliable device would be a method of choice. Such device could also be utilized by individuals to easily examine whether their consumption products contain OP contamination. Our research is an attempt to develop an OP optical biosensor with simple and inexpensive method which has high detection efficiency and lowest possible detection limit, using our recombinant MPH-GST isolated from the clone BpGP as an enzyme of choice. Absorbance-based method for OP detection using immobilized MPH is a sensible option for this attempt, provided that a good immobilization method is exploited.

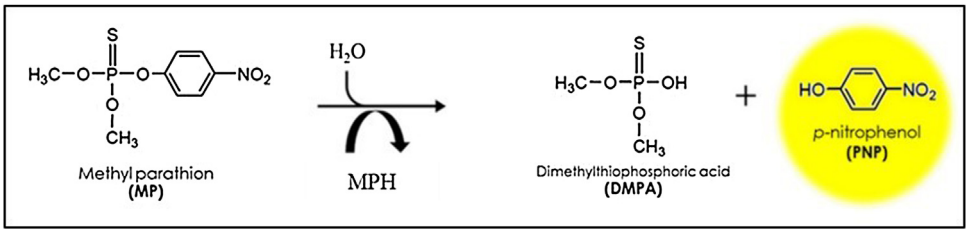

Fig. 1. MP degradation reaction by MPH.

The selection of the most suitable enzyme immobilization methods used for attaching biomolecules to the matrix surface is extremely important. Several methods like adsorption, entrapment, and cross-linking can easily lead to enzyme activity loss, discharge, and instability. Covalent immobilization is a technique capable of retaining enzyme activity in adverse conditions by enabling strong attachment between the enzyme and the matrix. Moreover, it can be reused more than the three methods mentioned and can increase the temperature stability when immobilizing with chitosan [39,75].

Here, an absorbance-based biosensor using the recombinant MPH-GST covalently immobilized onto a chitosan-coated microplate as an optical OP sensing device is proposed. It was connected with an appropriate transducer for the detection of MP, a representative of OP insecticides. The proposed optical biosensor is simple in design and usability, efficient, convenient, and capable of simultaneous multiple sample detection at site. Albeit its simplicity, this biosensor had a low limit of detection and demonstrated precise detection of MP comparable to that of conventional detection methods. Since MPH was shown to have cross reactivity with other OP compounds [25], the use of this absorbance-based MPH-GST biosensor is, therefore, a cost-effective means of OP detection practically suitable for fast and easy screening for toxic OP substances, especially in developing countries where such contamination is widely spread.

2. Materials and methods

2.1. Chemicals and materials

Methyl parathion (*O*,*O*-Dimethyl *O*-*p*-nitrophenyl phosphorothioate) (purity 98.3 %, analytical grade), medium molecular weight chitosan, glutaraldehyde (50 % in H_2O), bovine serum albumin (BSA), sodium borohydride ($NaBH_4$), and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (Singapore). A standard *p*-nitrophenol was purchased from Merck (Germany). Polystyrene microplates were ordered from SPL Life Sciences (Korea). LED light source with a wavelength value of 410 nm, TSL2561 luminosity sensor, and Arduino Uno R3 board were obtained from My arduino shop. Laser machine kit with internal working area of 15 × 10 cm was purchased from TomElectronics1 shop.

2.2. Construction of recombinant clone BpGP

The construction of recombinant clone BpGP was performed according to the procedure previously reported [25]. Briefly, the DNA fragment containing *mpdB* gene encoding for MPH, originally isolated from MP-degrading *Burkholderia cepacia* [25,31], was extracted and cut by BamHI and XhoI from the previous clone. The purified BamHI-XhoI DNA fragment was further subcloned into the pGEX 6P-1 expression vector (GE Healthcare Life Sciences, USA), designed for the production of a fusion protein with glutathione-*S*-transferase (GST) at the N-terminus. The recombinant plasmid was then transformed into *E. coli* BL21, yielding the recombinant clone BpGP, capable of expressing MPH in the form of MPH-GST fusion protein.

2.3. Production of the recombinant MPH-GST fusion protein

The recombinant clone BpGP was first tested for MPH activity using microtiter plate MPH assay [76], by adding it into the reaction mixture containing 50 μg mL^{-1} MP, 0.1 % TritonX-100, and 25 mM Tris–HCl (pH 8.5). The appearance of the yellow color of the MP hydrolytic product, PNP, was used as an indicator for MPH activity of the clone. The production of the recombinant MPH-GST fusion protein was done according to the previous report [25]. In brief, the recombinant bacteria were grown in Luria-Bertani (LB) broth containing 100 μg mL^{-1} ampicillin at 37 °C for 16–18 h. When the optical density at 600 nm of 0.5 was reached, it was subcultured into fresh LB broth and allowed to grow at 37 °C for 3–4 h. $CoCl_2$ at the concentration of 0.5 mM was added and the culture was induced with 1 mM ITPG. It was then grown further at 18 °C for 6 more hours. The culture was harvested and the pellet resuspended in 50 mM phosphate buffer saline (pH 8.0). Cell suspension was then disrupted by ultrasonication, and the cell debris was pelleted by centrifugation at 17,000×*g*, 4 °C for 20 min and discarded. This partially purified MPH-GST was then used in all the experiments.

2.4. Covalent immobilization of MPH-GST on polystyrene microplate

In order to immobilize MPH-GST onto the polystyrene microplate, the well surface of the plate was first coated with chitosan solution. The conditions for making the chitosan film were optimized. The chitosan concentration was varied within the range of 1–3 % (w/v) in 1 % (v/v) acetic acid, using the fixed volume of 50 μl. The coated microplate was dried at room temperature for about 6 h for chitosan film formation. The chitosan-coated microplate was treated with 2 % (w/v) BSA at 4 °C for 2 h. After washing with 50 mM PBS (pH 8.0), it was treated with 1 % glutaraldehyde in 50 mM PBS (pH 8.0) at 25 °C for 2 h. The MPH-GST solution was added to each well and was covalently immobilized at 4 °C overnight. The MPH-GST microplate wells were washed with 50 mM PBS (pH 8.0) and incubated with 0.1 M $NaBH_4$ in 50 mM PBS (pH 8.0) at 25 °C for 1 h. After that the wells were gently washed with 50 mM PBS (pH 8.0) containing 0.1 M NaCl and 0.5 % (v/v) Tween 20, and then with 50 mM PBS (pH 8.0) [77]. This MPH-GST immobilized, chitosan-coated polystyrene microplate was further used as a biosensing component of the MPH-GST optical biosensor fabricated.

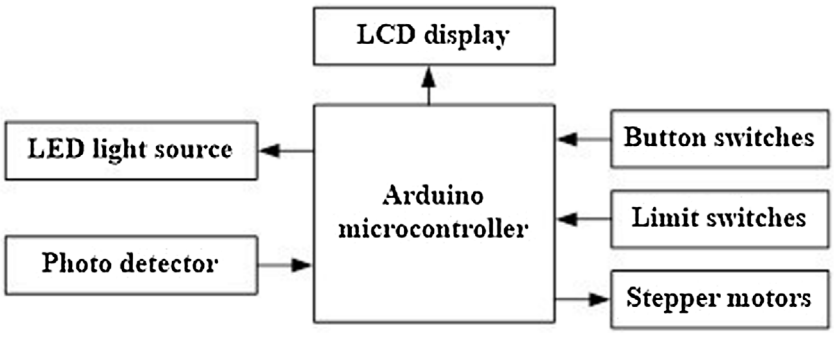

Fig. 2. Schematic operation of the optical transducer system.

2.5. MPH activity assay

The MP-degrading reaction was performed in the substrate mixtures consisting of assay buffer (pH 8.5) and 100 ppm MP at 37 °C for 5 min [25]. The appearance of the yellow color of PNP in each well was observed. The OD value at 410 nm, the maximum absorption wavelength of PNP, was determined using the fabricated MPH-GST optical transducer. A blank well, the chitosan-coated microplate well treated with 1 % glutaraldehyde containing assay mixture without immobilized MPH-GST, was used as a control.

2.6. Schematic operation of optical transducer system

The proposed system mainly consisted of a sensing unit, a microplate-well positioning control unit and an LCD display. The sensing unit comprised a blue light LED, which emitted at a wavelength of 410 nm corresponding to the maximum absorption of PNP, and a light-to-digital converter device, TSL2561. The LED was adopted as a light source, and the TSL2561 was adopted as a light sensor (photo detector). The microplate-well positioning control unit, consisting of limit switches and stepper motors, were cooperated with direction button switches in vertical and horizontal directions as a planar movement of the MPH-GST biosensing device. The result of MP detection was displayed on the LCD screen. The schematic operation of the designed optical transducer system was shown in Fig. 2.

2.7. Circuit design of optical transducer system

To fabricate the MPH-GST biosensor transducer, its circuit was designed with an embedded system using C language for the operational and control function. An Arduino microcontroller board was adopted as the main controller in our fabricated optical transducer system over the OP detection and calculation process. For the positioning system, 3 stepper motors were employed to move the microplate well in vertical and horizontal directions under the control of the motors and their drive circuits. The planar movement machine had an internal working area of $15 \times 10\ cm^2$ which was assembled with the 96-well support part to fix each microplate well position. For the display unit, 16 characters with 2 line liquid crystal display (LCD) communicated with the main controller board through the I^2C module. The power supply of the light sensor and motors with drive circuits was 3.3 V_{DC} and 12 V_{DC}, respectively. The rest of the circuit system were operated at 5 V_{DC}. The current mirror circuit was used to control the light intensity of the blue LED (light source) through an optimal resistor (R4). During MP-degrading reaction measurement, the emitted light passed through sample wells to the light sensor. The residue light intensity was converted to digital data for data processing via the microcontroller. The consequence of the MP detection was displayed on the LCD screen in terms of average absorbance value (A) and MP concentration (μM). The design of the detection circuit diagram was presented in Fig. 3.

To determine the effect of resistance (R) on the LED light intensity, resistors with different values of resistance (0, 150, 220,

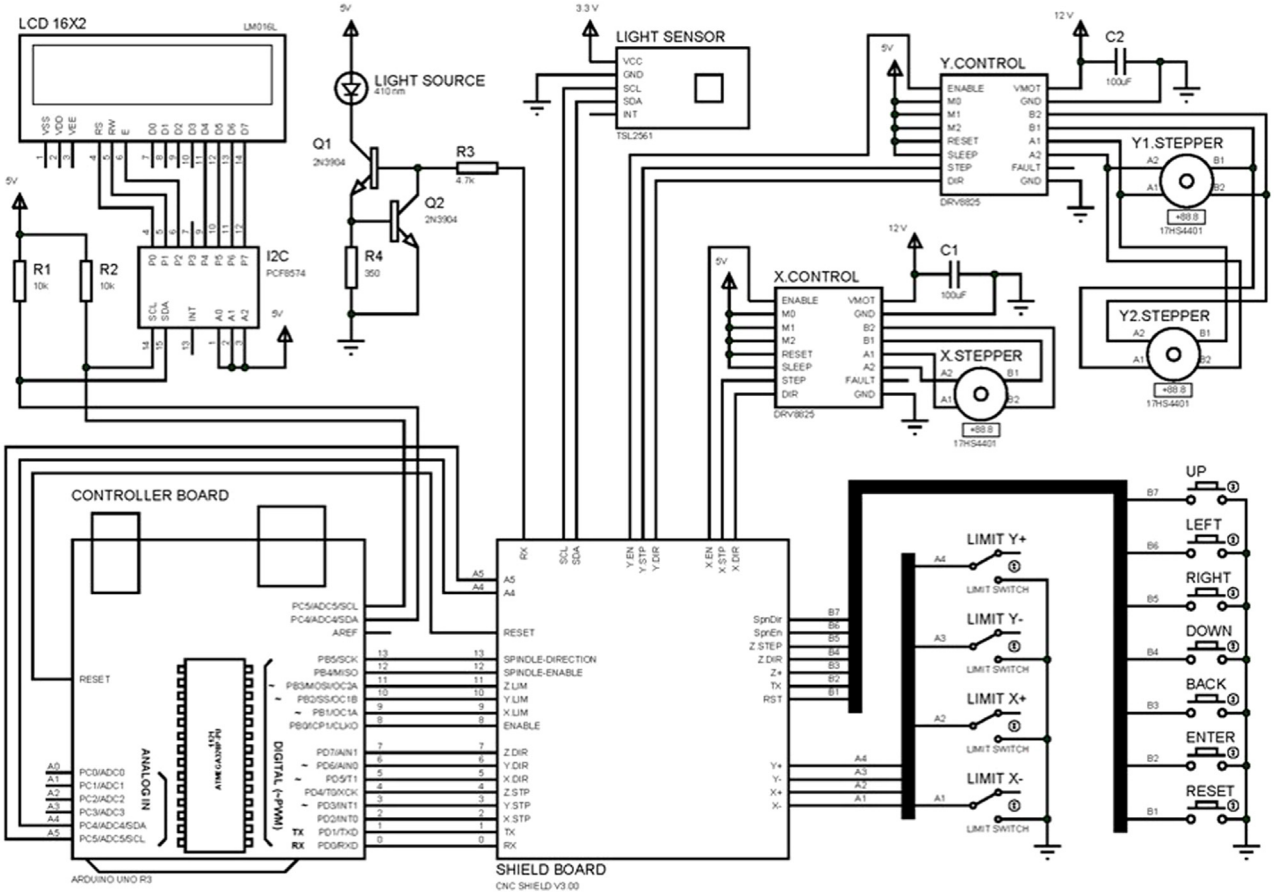

Fig. 3. Circuit diagram of optical transducer system.

300, 350, 370, and 400 Ω) were placed into the current mirror circuit at R4 position. Various concentrations of standard PNP in the range of 1–100 μM were prepared in appropriate assay buffers (0.05 M Tris–HCl, 0.1 M NaCl, and 0.1 % Tween 80), pH 8.0. The standard solution of PNP was added into individual wells. After 5 min incubation at room temperature, the OD_{410} value of each well was measured by the fabricated optical biosensor and the spectrophotometric microplate reader. A blank microplate well containing only the assay buffer without MPH was used as a control. The experiments were performed in triplicate.

2.8. Optimization of the MPH-GST biosensor

To optimize the MP detection conditions of the MPH-GST optical biosensor, optimum temperature and pH were determined. MP substrate at the concentration of 100 μg mL^{-1} in the assay buffer (pH 8.0) was added to each well of the MPH-GST sensing microplate, and the MP hydrolysis reaction was allowed to occur at different temperatures (4, 25, 37, 60, 70, and 80 °C). The OD_{410} value was measured after 5 min incubation and MPH activity was investigated. For optimum pH determination, the MPH activity was determined after 5 min incubation at optimal temperature and at different pH values ranging from 5 to 12 using 50 mM citrate buffer (pH 5.0), 50 mM phosphate buffer (pH 6.0, 7.0 and 7.5), 50 mM Tris-HCl (pH 8.0, 8.5, 9.0 and 9.5), 50 mM carbonate-bicarbonate buffer (pH 10.0), and 50 mM Na_2HPO_4-NaOH (pH 11.0–12.0). All optimization experiments were done in triplicate.

2.9. Determination of performance characteristics of MPH-GST biosensor

To determine the effect of MP concentration on OD_{410} value, various concentrations of MP (0.1–1 μM) were added to individual wells. After 5 min incubation at 25 °C, OD_{410} from MP hydrolytic reaction in each well was measured by the MPH-GST biosensor. The reactions were performed in triplicate.

To determine the effect of temperature on the stability of the MPH-GST sensing device for MP detection, 100 μg mL^{-1} MP in the assay buffer (pH 8.5) was added to the wells and the reactions were allowed to occur at different temperatures (4, 25, 37, and 60 °C). The OD_{410} values were measured, after 5 min incubation, every day for 30 days by the MPH-GST biosensor.

To determine the effect of pH on the stability of the MPH-GST sensing device for MP detection, the sensing device was stored at 4 °C in different buffers with varying pH values (pH 5, 6, 7, 7.5, 8, 8.5, 9, and 10). One hundred μg mL^{-1} MP in the assay buffer (pH 8.5) was added to individual wells, and the MP-hydrolytic reactions were allowed to occur at optimum temperature. The OD_{410} values were measured after 5 min incubation, for 30 days using the MPH-GST biosensor.

To test the reusability, 100 μg mL^{-1} MP in the assay buffer (pH 8.5) was added to the wells at each round of detection. After 5 min incubation at 25 °C, the OD_{410} values were measured. At each round of reaction, the wells were washed with 50 mM PBS (pH 8.0) before starting the new round until the total of 100 rounds were completed. All reactions were done in triplicate.

2.10. Gas chromatography-mass spectrometer (GC–MS) analysis of MP spiked in water sample

A tap water sample was collected and kept in an amber bottle using the method recommended by the Water Quality Management Division of Thailand [78]. Standard MP solution was spiked into the water sample to the final concentration of 38.02 μM. An MP extraction was performed according to the previous report [61,62]. The spiked water sample was transferred into a separating funnel, after which 10 mL of ethyl acetate was added. The funnel was vigorously shaken for 2 min. After the phases were clearly separated, an upper organic phase was decanted and 20 g of anhydrous sodium sulfate was added to eliminate water residue. The extract was filtered, and the MP filtrate was sealed in 1.5 mL vial and kept at room temperature until use. For calibration curve by GC, standard MP with varying concentrations (7.6, 15.2, 22.8, 30.4, and 38.02 μM) was prepared by dissolving in ethyl acetate. For GC–MS analysis, 7890A Agilent GC with a split/splitless injector system equipped with flame photometric detector (FPD) and 5975C mass spectrophotometer (Agilent Technologies, USA) were used according to the method previously reported [79]. Briefly, 1 μL of sample was injected through a 10 μL-microsyringe, using split mode with split ratio of 10:1 at 260 °C of injection port, into DB-5MS capillary column (5 % phenyl methylpolysiloxane, 30 m x 250 μm id x 0.25 μm film thickness, Agilent Technologies, USA). The temperature program used for MP separation was as follows. The temperature of 100 °C was used initially for 1 min, and then increased at 20 °C min^{-1} to 200 °C, kept for 8 min, and finally increased at 10 °C min^{-1} to 250 °C, kept for 7 min. Helium was used as carrier gas at the flow rate of 1 mL min^{-1}. The concentration of MP detected in the spiked water sample was derived from GC calibration curve. The percentage of MP recovery was determined. An MS was performed in electron ionization (EI) mode with the source temperature of 230 °C. The mass spectrums were obtained at a mass ratio scan from 50 to 550 *m/z*. The quality match analysis of MP peak was confirmed by the data from the National Institute of Standards and Technology (NIST) Mass Spectral Library.

2.11. Detection of MP spiked in water sample

To detect the concentration of MP spiked into the water sample using MPH-GST biosensor, standard MP at a concentration of 380.20 μM dissolved in absolute ethanol was spiked into the collected tap water sample. The spiked water sample was diluted with the assay buffer (pH 8.5) to the final concentration of 38.02 μM and was tested with fabricated optical biosensor under the optimal conditions. The spiked MP concentration was then measured from MP calibration curve and the percentage of MP recovery was determined. The experiment was done in triplicate.

2.12. Detection of MP in spiked and real (non-spiked) samples

Five grams of each agricultural product (i.e., tomatoes, grapes, Chinese kale, basil leaves, corianders, morning glories, yard long beans, red peppers, Chinese cabbages, asparagus, agasta, watermelons) obtained from various agricultural farmlands were washed with distilled water and chopped into small pieces. They were then stirred in 10 mL of 0.05 mM PBS (pH 8.5) and filtered with filter cloth. The filtrates were centrifuged and then the supernatants were collected for analysis. The spiked samples were added with MP at the concentration of 190 μM, and tested with the fabricated optical biosensor under optimal conditions. The spiked MP concentrations were then measured from MP calibration curve and the percentage of MP recoveries were determined. Real (non-spiked) samples were analyzed for MP residues using the same procedure. All tests were performed in triplicate.

3. Results and discussion

3.1. Covalent immobilization of MPH-GST

The recombinant MPH was covalently immobilized onto the chitosan-coated 96-well microplate. This would subsequently be used as the biocomponent part of the absorbance-based MPH-GST biosensor to detect the yellow color of the MP-hydrolytic product,

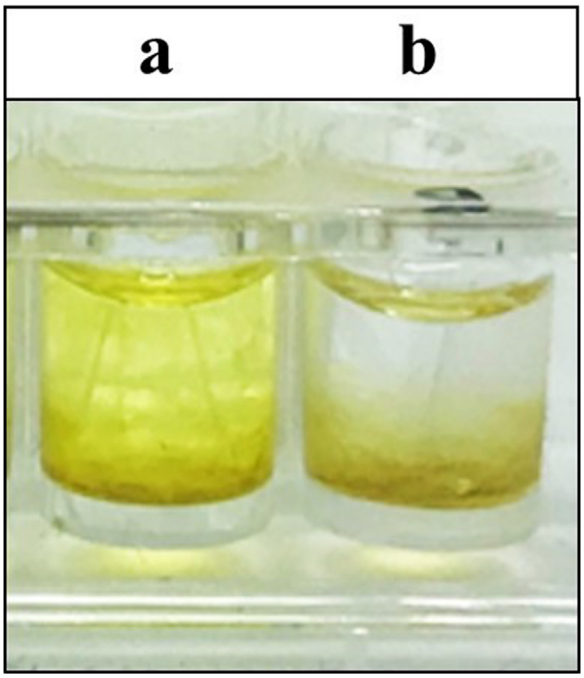

Fig. 4. MPH activity assay of the MPH-GST sensing device. (a) immobilized MPH-GST well; (b) blank well.

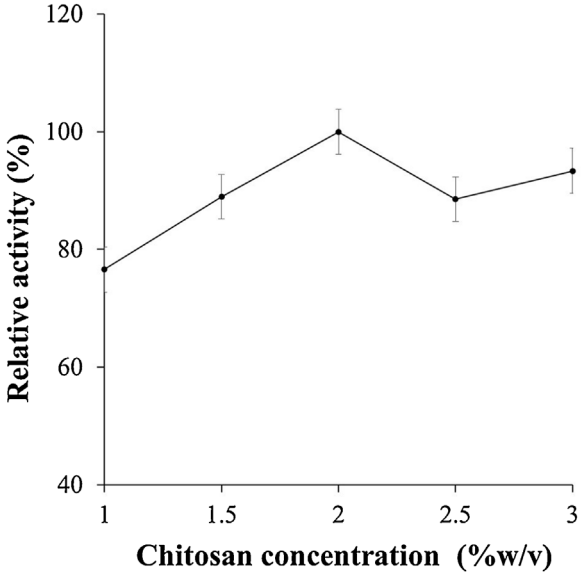

Fig. 5. Effect of chitosan concentration on the activity of the MPH-GST biosensing component.

PNP, at the wavelength of 410 nm. After the immobilization of MPH-GST was succeessfully done, the MPH-GST sensing microplate was then tested for MPH activity. Fig. 4 showed the appearance of the yellow color of PNP, indicating positive MPH activity of the sensing device. In order to optimize the effect of chitosan concentration on the activity, the concentration of chitosan was varied for MPH-GST immobilization and the MPH activity was determined. As shown in Fig. 5, the relative MPH activity was highest at 2 % chitosan. Subsequently, 2 % chitosan was used to investigate the appropriate chitosan volume within the range of 35–80 μl. As seen in Fig. 6, the relative MPH activity maximized at 40 μl chitosan, and slowly decreased thereafter.

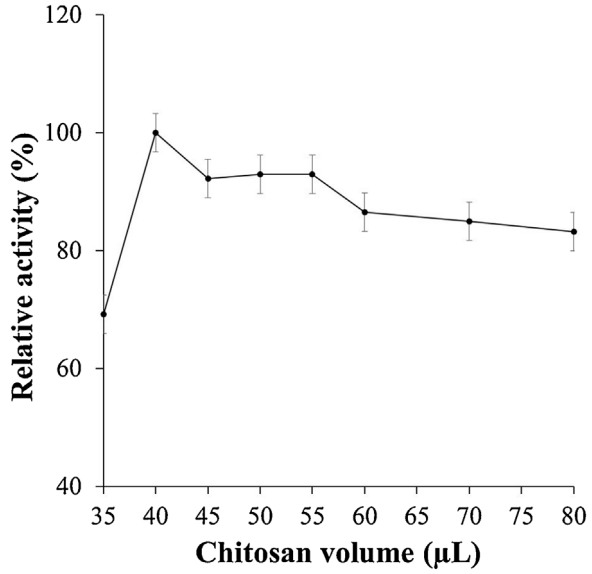

Fig. 6. Effect of chitosan volume on the activity of the MPH-GST biosensing component.

Consequently, the chitosan volume of 40 μl with the concentration of 2 % (w/v) in 1 % (v/v) acetic acid was used for the covalent immobilization of MPH-GST onto the polystyrene microplate which was further utilized as a biosensing device.

3.2. Effect of resistance on LED light intensity

The effect of resistance on the LED light intensity was determined. When the resistance of R4 was varied in the circuit system, the OD_{410} values of PNP arising from MP-hydrolytic reaction were measured, and each PNP calibration curve was made for each resistance tested. The sensitivity of the system was determined from its slope value corresponding to the appropriateness of resistance for practical use in optical transducer [80]. As seen in Table 1, the highest sensitivity (7.3×10^{-3}) was achieved with the 350 Ω resistor. This result was in agreement with that of the spectrophotometric microplate reader. Hence, with this resistance, the experimental results should be most reliable. The 350 Ω resistor was, thus, selected for controlling appropriate light intensity of the LED in the current mirror circuit.

3.3. Optimization of the MPH-GST biosensor

After the MPH-GST biosensing component was incorporated with the optical transducer, the resulting MPH-GST optical biosensor was subjected to optimization. Since temperature and pH are considered important factors affecting enzyme activity [81], the optimum temperature and pH were examined. It can be clearly seen in Fig. 7a that the relative MPH activity of MPH-GST optical sensing system was highest at 37 °C and rapidly decreased with higher temperatures. The MPH activity of the system was considerably high at the pH range of 8–9, with the optimum pH at 8.5 (Fig. 7b).

3.4. Performance characteristics of the MPH-GST biosensor for the detection of MP

The effect of MP concentration on OD_{410} for the detection of MP was examined under the optimum temperature and pH. The OD_{410} values were measured at the varying concentrations of MP (0.1–10 μM), and the calibration curve derived was shown in

Table 1
Effect of R4 resistance value on the sensitivity of MP detection.

Method	Resistance of R4 (Ω)	Sensitivity ($\times 10^{-3}$)	Regression equation	R^2
MPH-GST biosensor	0	4.5	y = 0.0045x + 0.0373	0.9429
	150	1.4	y = 0.0014x + 0.0076	0.8772
	220	2.8	y = 0.0028x + 0.0172	0.9249
	300	6.2	y = 0.0062x + 0.0284	0.9838
	350	7.3	y = 0.0073x - 0.0045	0.9972
	370	6.4	y = 0.0064x + 0.0458	0.9655
	400	2.3	y = 0.0023x + 0.0076	0.9390
Microplate reader		7.4	y = 0.0074x + 0.0056	0.9994

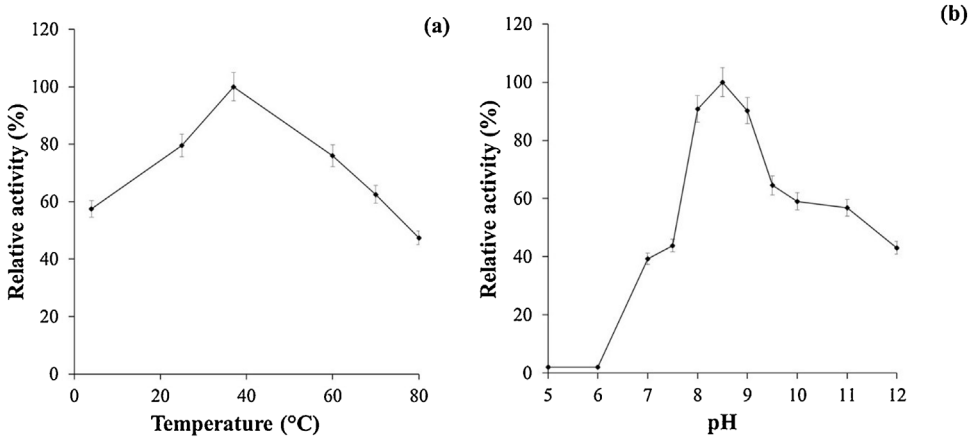

Fig. 7. Effect of temperature (a) and pH (b) on the activity of MPH-GST biosensor.

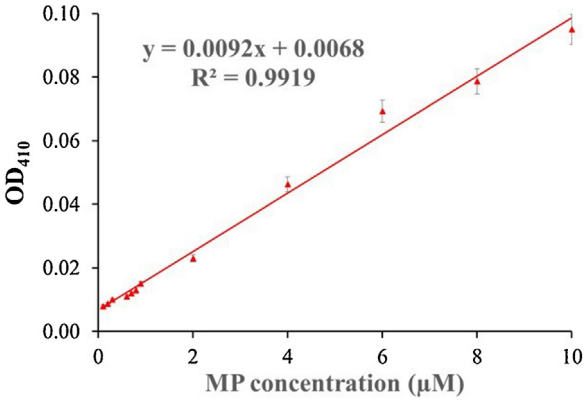

Fig. 8. Calibration curve of the MPH-GST biosensor.

Fig. 8. The straight line of the calibration curve clearly showed a linear regression, with the corresponding equation of y = 0.0092x +0.0068 (R^2 = 0.9919). The detection limit was found to be 0.1 μM and the linear range of the system for MP detection was 0.1–10 μM.

One of the most important characteristics for OP detection was the limit of detection [59]. Certain performance characteristics of the fabricated biosensor were compared with those of other optical enzymatic/microbial biosensors with different methods for the immobilization of bio-recognition elements (Table 2). Of all the biosensors in comparison, the optical biosensor fabricated from our work had the lowest limit of detection, except for the fluorescence-based biosensor of Wang et al. 2011 [82]. The works done by Mulchandani et al. [12,83] and Kuswandi et al. [84] involved the use of fiber optic biosensors for OP detection. There were certain drawbacks involving the limitation on stability of the biomolecules immobilized onto the biosensing surface and long response time [85,86]. For *E. coli* expressing enzymes, OPH and MPH were immobilized onto appropriate matrices [6,83]. Even though both enzymes were expressed on the cell surface and could readily react with OPs, there were limitations of this type of biosensors such as low selectivity and long response time. Moreover, the immobilized cells might be washed out from the matrix during continuous use [86]. The MPH-GST absorbance-

Table 2
Comparison of enzyme-based/microbial optical biosensor performance for OP detection.

Biosensor	Pesticide	Biomolecule	Immobilization method	LOD	Linear range	Ref.
Fiber-optic microbial biosensor	PT	OPH	*E. coli* expressing OPH on Biodyne A membrane	3 μM	0-0.6 mM	[83]
	PX				0-0.03 mM	
Optical microbial biosensor	MP	MPH	*E. coli* expressing MPH on polystyrene surface	7.6 μM	7.6-760 μM	[6]
Fluorescence-based biosensor	PX	AChE	–	3.5 pM	5.5 pM- 180 nM	[82]
Fiber optic biosensor	PX	OPH	Covalent binding	2 μM	0.02-0.5 mM	[12]
Fiber optic biosensor	CP	AChE	Entrapment	0.11 μM	1.4-4.3 μM	[84]
Absorbance-based biosensor	MP	MPH	Metal-chelation affinity	4 μM	0-0.1 mM	[59]
Absorbance-based biosensor	MP	MPH	Covalent binding	0.5 μM	0.1 – 10 μM	This work

based biosensor from our work yielded lower limit of detection than that of the work with similar principle of detection [59]. Fluorescence-based biosensors could generally detect analytes with higher sensitivity and specificity than absorbance-based biosensors [38,39]. However, although the results of Wang et al. 2011 [82] gave lower limit of detection, our absorbance-based optical biosensor had advantages in its simplicity, low cost, and ease for development.

This work has proved a success in developing a very simple MPH absorbance-based optical biosensor such that its limit of detection for MP could be improved to be lowest than those of any other biosensors of the same type ever reported. It is crucial to choose the most suitable enzyme immobilization method. The techniques used for the immobilization of biomolecules could significantly affect the biosensor characteristics [77]. El-Boubbou et al., 2012 [87] reported an enzyme immobilization method using silica matrix covalently modified with an ammonium organosilane for OPH. The immobilized OPH was done through the weak binding forces, Van der Waals and electrostatic interactions, in the pore of silica particle; hence, the size of the matrix pore has to be carefully optimized for the confinement of the enzyme. This immobilization approach could enhance OPH activity as compared to free enzyme. In our work, the immobilization of the MPH-GST was performed directly onto the chitosan-coated polystyrene microplate surface, using stable covalent bond. The increase in MP detection efficiency as determined by the limit of detection was demonstrated to be approximately 76 folds higher when compared to immobilized whole cells [6]. Covalent immobilization used in our fabricated biosensor had several advantages such as strong binding force [39] and long half-life and stability of the enzyme, even under adverse conditions [75]. Thus, this proposed biosensor compromised well between these advantages and its satisfactory limit of detection for OP in field work.

Temperature stability and pH stability of the MPH biosensor were investigated. As seen in Fig. 9, the MPH-GST sensing system was most stable at 4 °C. At 4 °C, the relative activity at day 5 increased to about 148 % and gradually decreased to the final activity of about 80 % at day 30. While at 25 °C, a high relative activity peak of about 155 % was observed at day 9. After that, it dropped to about 60 %–70 % and remained constant throughout the experiment. Thus, this is an advantage of this MPH-GST biosensor that allows its practical use at around room temperature. At 37 °C and 50 °C, the relative activities rapidly dropped to about 40 % at day 6 and to the final percentages of about 11 and 2, respectively, at day 30.

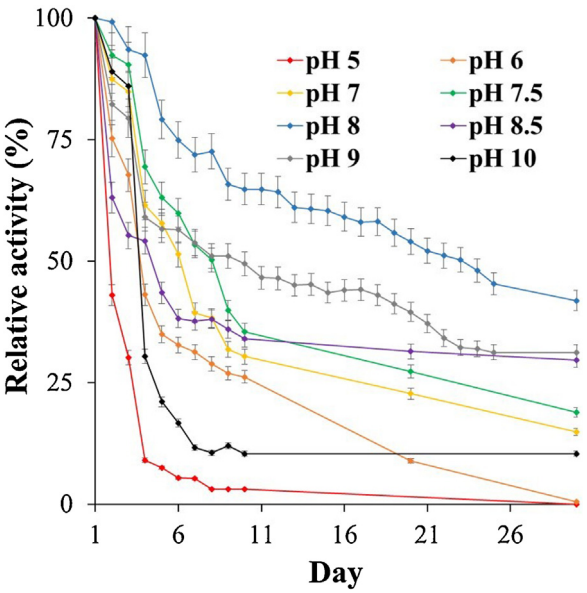

Fig. 10. pH stability of the MPH-GST biosensor.

It can be seen from Fig. 10 that this sensing system was most stable at pH 8, with more than 50 % of relative MPH activity remaining for 23 days, after which it gradually decreased to the final activity of about 42 % at day 30. At pH 9 the relative activity was lower than that at pH 8. Nevertheless, approximately more than 50 % of the activity was observed at day 10. Afterwards, it gradually dropped to about 30 % at day 30. At other lower and higher pH, the MPH activity was not as stable.

The reusability is another important performance characteristics of the application of biosensors. The experiment on reusability of the MPH-GST biosensor was performed under the optimum conditions, and the MPH activity determined for 100 rounds. As illustrated in Fig. 11, the relative activity remained at approximately 70 %–100 % for the first 86 rounds and then decreased to about 50 %–60 % until round 100. The decrease in

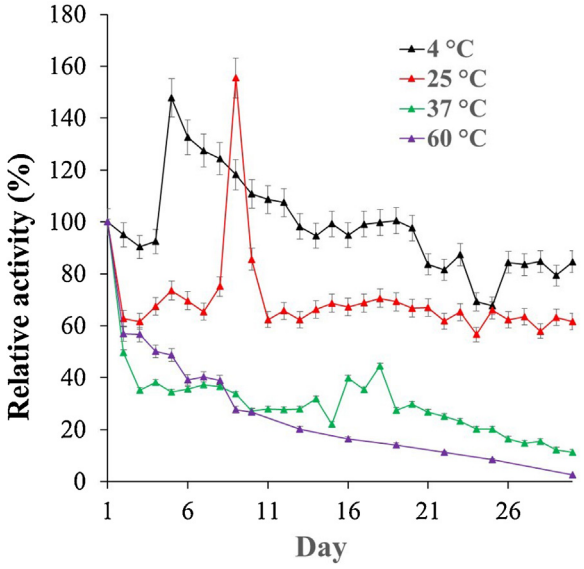

Fig. 9. Temperature stability of the MPH-GST biosensor.

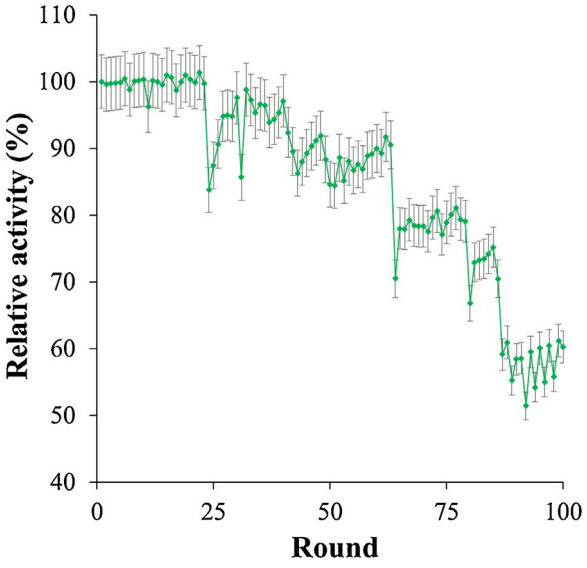

Fig. 11. Reusability of the MPH-GST biosensor for MP detection.

Table 3
Percentages of MP recovery determined by MPH-GST biosensor and GC–MS analysis.

Detection method	MP spiked (μM)	MP detected (μM)	Recovery (%)
MPH-GST biosensor	38.02	31.32	82.40
GC-MS analysis	38.02	32.32	85

Table 4
Percentages of MP recovery by MPH-GST biosensor on 50 ppm MP-spiked samples.

MP-spiked samples	Recovery (%)
Tomatoes	72
Grapes	76
Chinese kale	75
Basil leaves	70
Morning glories	75
Yard long beans	75
Red peppers	74
Chinese cabbages	71
Asparagus	77
Watermelons	75

MPH activity was possibly due to the fact that the MPH was inactivated and denatured [88]. Hence, this simply designed absorbance-based biosensor could be used repetitively up to 86 rounds with high activity and up to 100 rounds with approximately half the activity.

3.5. Comparison of MPH-GST biosensor and GC–MS analysis on MP recovery

The fabricated MPH-GST biosensor was used to test for the detection of MP spiked into the water sample in comparison with GC–MS analysis. GC–MS analysis is regarded as one of the conventional standard methods for pesticide detection with high precision though it is laborious, expensive, and time-consuming. From Table 3, it is evident that the percentage of MP recovery obtained from MPH-GST biosensor was very close to that derived from GC–MS analysis. The results indicated that our proposed MPH-GST absorbance-based biosensor could be efficiently utilized for such detection with high accuracy.

3.6. Determination of MP in spiked and real (non-spiked) samples

The MPH-GST biosensor was examined for the determination of MP that had been spiked into the samples. The agricultural samples tested were selected vegetables and fruits. The percentages of recovery were achieved in the range of 70–77 % as shown in Table 4, which were comparable to that determined in the MP-spiked water sample. Even for real samples without spiking, the MPH-GST biosensor was also capable of detecting MP as shown in Table 5. Hence, the system was proven to have high reliability and efficiency, with a great potential for applications in field works. Even though this work was performed using MP as a representative, the biosensor can be utilized to detect other types of phenyl-substituted OP insecticides due to the cross-reactivity of MPH. While it is not primarily developed to selectively differentiate MP or individual insecticide from the others within the OP group as a result of the common principle of detection mentioned above, this proposed MPH-GST biosensor can certainly give the overall image of OP contamination in the agricultural products and the environment which is beneficial for OP monitoring and control.

Table 5
Concentrations of MP in real (non-spiked) samples detected by MPH-GST biosensor.

Real (non-spiked) samples	MP concentration (ppm)
Tap water	1
Grapes	5
Chinese kale	2
Basil leaves	3
Coriander s	4
Chinese cabbages	4
Asparagus	2
Agasta	15

4. Conclusions

A simple, easy-to-use, and efficient absorbance-based MPH-GST optical biosensor for OP detection was successfully fabricated, with MP as a representative. For the optical sensing element, the covalent immobilization of MPH-GST onto chitosan-coated polystyrene microplate was achieved with good MPH activity. The easily designed operation circuit of the optical transducer demonstrated sensitivity similar to that measured by the spectrophotometric microplate reader. Our proposed biosensor yielded good performance with copious advantages. Uncomplicated as it was, the biosensor showed precision, stability, reusability, and low detection limit. By utilizing 96-well microplate as a part of sensing device, this sensor can detect multiple samples simultaneously. The MPH-GST biosensor was easily and successfully used to determine MP both in real, non-spiked and spiked samples, with high reliability. Its cost-effectiveness and multiple sample detectability added to its dominance. While sophisticated and expensive OP biosensors are demanded for particular circumstances, there are some other situations in which those with low cost and ease of use are needed. This MPH-GST biosensor developed here was exceptional in that it was a very simple OP biosensor, with the use of uncomplicated absorbance-based detection method, yet it could yield lowest possible limit of detection. Hence, the proposed biosensor has great potential to be an alternative device for easy screening of OP contamination in agricultural products and the environment without high expense, which is the desirable sceneario for many countries.

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CRediT authorship contribution statement

Witsanu Senbua: Conceptualization, Methodology, Investigation, Writing - original draft. **Jhirat Mearnchu:** Methodology, Software. **Jesdawan Wichitwechkarn:** Conceptualization, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

[1] M. Jain, P. Yadav, A. Joshi, P. Kodgire, Advances in detection of hazardous organophosphorus compounds using organophosphorus hydrolase based biosensors, Crit. Rev. Toxicol. 49 (2019) 387–410, doi:http://dx.doi.org/10.1080/10408444.2019.1626800.

[2] S.A. Ahmadizad Firozjaei, A.M. Latifi, S. Khodi, S. Abolmaali, A. Choopani, A review on biodegradation of toxic organophosphate compounds, J. Appl. Biotechnol. Rep. 2 (2015) 215–224.

[3] J. Kumar, A. Mishra, J. Melo, Biodegradation of methyl parathion and its application in biosensors, Austin J. Env. Toxicol. 4 (2018) 1–4.

[4] J.Y. Simon, The Toxicology and Biochemistry of Insecticides, CRC press, 2011.

[5] J. Sharma, A review on in situ biodegradation of methyl parathion through soil microbes, Int. J. Curr. Microbiol. Appl. Sci. 4 (2015) 632–649.

[6] C. Pootawee, W. Senbua, J. Wichitwechkarn, Immobilized whole cell system with methyl parathion-degrading activity for organophosphate insecticide detection, Online J. Biol. Sci. 18 (2018) 332–340, doi:http://dx.doi.org/10.3844/ojbsci.2018.332.340.

[7] W.J. Driskell, M. Shih, L.L. Needham, D.B. Barr, Quantitation of organophosphorus nerve agent metabolites in human urine using isotope dilution gas chromatography-tandem mass spectrometry, J. Anal. Toxicol. 26 (2002) 6–10, doi:http://dx.doi.org/10.1093/jat/26.1.6.

[8] D.B. Mawhinney, E.I. Hamelin, R. Fraser, S.S. Silva, A.J. Pavlopoulos, R.J. Kobelski, The determination of organophosphonate nerve agent metabolites in human urine by hydrophilic interaction liquid chromatography tandem mass spectrometry, J. Chromatogr. B 852 (2007) 235–243, doi:http://dx.doi.org/10.1016/j.jchromb.2007.01.023.

[9] N. Jaffrezic-Renault, New trends in biosensors for organophosphorus pesticides, Sensor 1 (2001) 60–74, doi:http://dx.doi.org/10.3390/s10100060.

[10] J.S. Melo, Overview on biosensors for detection of organophosphate pesticides, Curr. Trends Biomed. Eng. Biosci. 5 (2017) 1–11, doi:http://dx.doi.org/10.19080/ctbeb.2017.05.555663.

[11] J.H. Lee, J.Y. Park, K. Min, H.J. Cha, S.S. Choi, Y.J. Yoo, A novel organophosphorus hydrolase-based biosensor using mesoporous carbons and carbon black for the detection of organophosphate nerve agents, Biosens. Bioelectron. 25 (2010) 1566–1570, doi:http://dx.doi.org/10.1016/j.bios.2009.10.013.

[12] A. Mulchandani, S. Pan, W. Chen, Fiber-optic enzyme biosensor for direct determination of organophosphate nerve agents, Biotechnol. Prog. 15 (1999) 130–134, doi:http://dx.doi.org/10.1021/bp980111q.

[13] G. Schenk, I. Mateen, T.-K. Ng, M.M. Pedroso, N. Mitić, M. Jafelicci, R.F.C. Marques, L.R. Gahan, D.L. Ollis, Organophosphate-degrading metallohydrolases: structure and function of potent catalysts for applications in bioremediation, Coord. Chem. Rev. 317 (2016) 122–131, doi:http://dx.doi.org/10.1016/j.ccr.2016.03.006.

[14] N. Sethunathan, T. Yoshida, A *Flavobacterium* sp. that degrades diazinon and parathion, Can. J. Microbiol. 19 (1973) 873–875, doi:http://dx.doi.org/10.1139/m73-138.

[15] D.M. Munnecke, Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method, Appl. Environ. Microbiol. 32 (1976) 7–13, doi:http://dx.doi.org/10.1128/aem.32.1.7-13.1976.

[16] I. Horne, T.D. Sutherland, R.L. Harcourt, R.J. Russell, J.G. Oakeshott, Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate, Appl. Environ. Microbiol. 68 (2002) 3371–3376, doi:http://dx.doi.org/10.1128/AEM.68.7.3371-3376.2002.

[17] M.M. Pedroso, F. Ely, N. Mitić, M.C. Carpenter, L.R. Gahan, D.E. Wilcox, J.L. Larrabee, D.L. Ollis, G. Schenk, Comparative investigation of the reaction mechanisms of the organophosphate-degrading phosphotriesterases from *Agrobacterium radiobacter* (OpdA) and *Pseudomonas diminuta* (OPH), J. Biol. Inorg. Chem. 19 (2014) 1263–1275, doi:http://dx.doi.org/10.1007/s00775-014-1183-9.

[18] C. Jackson, H.-K. Kim, P.D. Carr, J.-W. Liu, D.L. Ollis, The structure of an enzyme–product complex reveals the critical role of a terminal hydroxide nucleophile in the bacterial phosphotriesterase mechanism, Biochim. Biophys. Acta, Proteins Proteom. 1752 (2005) 56–64, doi:http://dx.doi.org/10.1016/j.bbapap.2005.06.008.

[19] M. Thakur, I.L. Medintz, S.A. Walper, Enzymatic bioremediation of organophosphate compounds—progress and remaining challenges, Front. Bioeng. Biotechnol. 7 (2019) 289, doi:http://dx.doi.org/10.3389/fbioe.2019.00289.

[20] R.M. Dawson, S. Pantelidis, H.R. Rose, S.E. Kotsonis, Degradation of nerve agents by an organophosphate-degrading agent (OpdA), J. Hazard. Mater. 157 (2008) 308–314, doi:http://dx.doi.org/10.1016/j.jhazmat.2007.12.099.

[21] R. Hawwa, S.D. Larsen, K. Ratia, A.D. Mesecar, Structure-based and random mutagenesis approaches increase the organophosphate-degrading activity of a phosphotriesterase homologue from *Deinococcus radiodurans*, J. Mol. Biol. 393 (2009) 36–57, doi:http://dx.doi.org/10.1016/j.jmb.2009.06.083.

[22] D.A. Schofield, A.A. Dinovo, Generation of a mutagenized organophosphorus hydrolase for the biodegradation of the organophosphate pesticides malathion and demeton-S, J. Appl. Microbiol. 109 (2010) 548–557, doi:http://dx.doi.org/10.1111/j.1365-2672.2010.04672.x.

[23] Z. Cui, S. Li, G. Fu, Isolation of methyl parathion-degrading strain M6 and cloning of the methyl parathion hydrolase gene, Appl. Environ. Microbiol. 67 (2001) 4922–4925, doi:http://dx.doi.org/10.1128/AEM.67.10.4922-4925.2001.

[24] H. Liu, J.J. Zhang, S.J. Wang, X.E. Zhang, N.Y. Zhou, Plasmid-borne catabolism of methyl parathion and *p*-nitrophenol in *Pseudomonas* sp. strain WBC-3, Biochem. Biophys. Res. Commun. 334 (2005) 1107–1114, doi:http://dx.doi.org/10.1016/j.bbrc.2005.07.006.

[25] A. Ekkhunnatham, B. Jongsareejit, W. Yamkunthong, J. Wichitwechkarn, Purification and characterization of methyl parathion hydrolase from *Burkholderia cepacia* capable of degrading organophosphate insecticides, World J. Microbiol. Biotechnol. 28 (2012) 1739–1746, doi:http://dx.doi.org/10.1007/s11274-011-0985-y.

[26] R. Zhang, Z. Cui, X. Zhang, J. Jiang, J.D. Gu, S. Li, Cloning of the organophosphorus pesticide hydrolase gene clusters of seven degradative bacteria isolated from a methyl parathion contaminated site and evidence of their horizontal gene transfer, Biodegradation. 17 (2006) 465–472, doi:http://dx.doi.org/10.1007/s10532-005-9018-6.

[27] Y.J. Dong, M. Bartlam, L. Sun, Y.F. Zhou, Z.P. Zhang, C.G. Zhang, Z. Rao, X.E. Zhang, Crystal structure of methyl parathion hydrolase from *Pseudomonas* sp. WBC-3, J. Mol. Biol. 353 (2005) 655–663, doi:http://dx.doi.org/10.1016/j.jmb.2005.08.057.

[28] A.N. Bigley, F.M. Raushel, Catalytic mechanisms for phosphotriesterases, Biochim. Biophys. Acta, Proteins Proteom. 1834 (2013) 443–453, doi:http://dx.doi.org/10.1016/j.bbapap.2012.04.004.

[29] T.-K. Ng, L.R. Gahan, G. Schenk, D.L. Ollis, Altering the substrate specificity of methyl parathion hydrolase with directed evolution, Arch. Biochem. Biophys. 573 (2015) 59–68, doi:http://dx.doi.org/10.1016/j.abb.2015.03.012.

[30] J. Xie, Y. Zhao, H. Zhang, Z. Liu, Z. Lu, Improving methyl parathion hydrolase to enhance its chlorpyrifos-hydrolysing efficiency, Lett. Appl. Microbiol. 58 (2014) 53–59, doi:http://dx.doi.org/10.1111/lam.12155.

[31] C. Keprasertsup, S. Upatham, N. Sukhapanth, P. Prempree, Degradation of methyl parathion in an aqueous medium by soil bacteria, Sci. Asia 27 (2001) 261–270.

[32] S. Wongthianlai, S. Chimthong, K. Srirattana, J. Wichitwechkarn, Identification, cloning, and expression of *mpd* gene coding for methyl parathion hydrolase from *Burkholderia cepacia*, Proceedings of the 33th Congress on Science and Technology of Thailand, Nakhon Si Thammarat, Thailand, 2007.

[33] M. Trojanowicz, Determination of pesticides using electrochemical enzymatic biosensors, Electroanalysis 14 (2002) 1311–1328, doi:http://dx.doi.org/10.1002/1521-4109(200211)14:19/20<1311::AID-ELAN1311>3.0.CO;2-7.

[34] P. Raghu, B.E. Kumara Swamy, T. Madhusudana Reddy, B.N. Chandrashekar, K. Reddaiah, Sol–gel immobilized biosensor for the detection of organophosphorous pesticides: a voltammetric method, Bioelectrochemistry 83 (2012) 19–24, doi:http://dx.doi.org/10.1016/j.bioelechem.2011.08.002.

[35] P. Raghu, T. Madhusudana Reddy, K. Reddaiah, B.E. Kumara Swamy, M. Sreedhar, Acetylcholinesterase based biosensor for monitoring of malathion and acephate in food samples: a voltammetric study, Food Chem. 142 (2014) 188–196, doi:http://dx.doi.org/10.1016/j.foodchem.2013.07.047.

[36] J. Jiang, H. Zhang, C. Wang, Y. Xu, Electrochemical detection of methyl parathion in *Fritillaria thunbergii* based on acetylcholinesterase immobilized gold nanoshpere, Int. J. Electrochem. Sci. 11 (2016) 5481–5489, doi:http://dx.doi.org/10.20964/2016.07.31.

[37] A. Mulchandani, W. Chen, P. Mulchandani, J. Wang, K.R. Rogers, Biosensors for direct determination of organophosphate pesticides, Biosens. Bioelectron. 16 (2001) 225–230, doi:http://dx.doi.org/10.1016/S0956-5663(01)00126-9.

[38] S.O. Obare, C. De, W. Guo, T.L. Haywood, T.A. Samuels, C.P. Adams, N.O. Masika, D.H. Murray, G.A. Anderson, K. Campbell, K. Fletcher, Fluorescent chemosensors for toxic organophosphorus pesticides: a review, Sensor 10 (2010) 7018–7043, doi:http://dx.doi.org/10.3390/s100707018.

[39] N. Verma, A. Bhardwaj, Biosensor technology for pesticides-a review, Appl. Biochem. Biotechnol. 175 (2015) 3093–3119, doi:http://dx.doi.org/10.1007/s12010-015-1489-2.

[40] A. Gothwal, P. Beniwal, V. Dhull, V. Hooda, Preparation of electrochemical biosensor for detection of organophosphorus pesticides, Int. J. Anal. Chem. 2014 (2014)303641, doi:http://dx.doi.org/10.1155/2014/303641.

[41] A. Mulyasuryani, S. Prasetyawan, Organophosphate hydrolase in conductometric biosensor for the detection of organophosphate pesticides, Anal. Chem. Insights 10 (2015) 23–27, doi:http://dx.doi.org/10.4137/ACI.S30656.

[42] S.H. Chough, A. Mulchandani, P. Mulchandani, W. Chen, J. Wang, K.R. Rogers, Organophosphorus hydrolase-based amperometric sensor: modulation of sensitivity and substrate selectivity, Electroanalysis 14 (2002) 273–276, doi:http://dx.doi.org/10.1002/1521-4109(200202)14:4<273::AID-ELAN273>3.0.CO;2-5.

[43] R.P. Deo, J. Wang, I. Block, A. Mulchandani, K.A. Joshi, M. Trojanowicz, F. Scholz, W. Chen, Y. Lin, Determination of organophosphate pesticides at a carbon nanotube/organophosphorus hydrolase electrochemical biosensor, Anal. Chim. Acta 530 (2005) 185–189, doi:http://dx.doi.org/10.1016/j.aca.2004.09.072.

[44] V.A. Pedrosa, S. Paliwal, S. Balasubramanian, D. Nepal, V. Davis, J. Wild, E. Ramanculov, A. Simonian, Enhanced stability of enzyme organophosphate hydrolase interfaced on the carbon nanotubes, Colloids Surf. B Biointerfaces 77 (2010) 69–74, doi:http://dx.doi.org/10.1016/j.colsurfb.2010.01.009.

[45] J. Kumar, S.F. D'Souza, Microbial biosensor for detection of methyl parathion using screen printed carbon electrode and cyclic voltammetry, Biosens. Bioelectron. 26 (2011) 4289–4293, doi:http://dx.doi.org/10.1016/j.bios.2011.04.027.

[46] X. Tang, B. Liang, T. Yi, G. Manco, I. Palchetti, A. Liu, Cell surface display of organophosphorus hydrolase for sensitive spectrophotometric detection of *p*-

nitrophenol substituted organophosphates, Enzyme Microb. Technol. 55 (2014) 107–112, doi:http://dx.doi.org/10.1016/j.enzmictec.2013.10.006.
[47] A.L. Simonian, T.A. Good, S.S. Wang, J.R. Wild, Nanoparticle-based optical biosensors for the direct detection of organophosphate chemical warfare agents and pesticides, Anal. Chim. Acta 534 (2005) 69–77, doi:http://dx.doi.org/10.1016/j.aca.2004.06.056.
[48] L. Viveros, S. Paliwal, D. McCrae, J. Wild, A. Simonian, A fluorescence-based biosensor for the detection of organophosphate pesticides and chemical warfare agents, Sens. Actuators B Chem. 115 (2006) 150–157, doi:http://dx.doi.org/10.1016/j.snb.2005.08.032.
[49] S. Thakur, P. Kumar, M.V. Reddy, D. Siddavattam, A.K. Paul, Enhancement in sensitivity of fluorescence based assay for organophosphates detection by silica coated silver nanoparticles using organophosphate hydrolase, Sens. Actuators B Chem. 178 (2013) 458–464, doi:http://dx.doi.org/10.1016/j.snb.2013.01.010.
[50] R. Liu, C. Yang, Y. Xu, P. Xu, H. Jiang, C. Qiao, Development of a whole-cell biocatalyst/biosensor by display of multiple heterologous proteins on the *Escherichia coli* cell surface for the detoxification and detection of organophosphates, J. Agric. Food Chem. 61 (2013) 7810–7816, doi:http://dx.doi.org/10.1021/jf402999b.
[51] N. Kamelipour, A. Mohsenifar, M. Tabatabaei, T. Rahmani-Cherati, K. Khoshnevisan, A. Allameh, M.M. Milani, S. Najavand, B. Etemadikia, Fluorometric determination of paraoxon in human serum using a gold nanoparticle-immobilized organophosphorus hydrolase and coumarin 1 as a competitive inhibitor, Microchim. Acta. 181 (2014) 239–248, doi:http://dx.doi.org/10.1007/s00604-013-1103-7.
[52] R. Khaksarinejad, A. Mohsenifar, T. Rahmani-Cherati, R. Karami, M. Tabatabaei, An organophosphorus hydrolase-based biosensor for direct detection of paraoxon using silica-coated magnetic nanoparticles, Appl. Biochem. Biotechnol. 176 (2015) 359–371, doi:http://dx.doi.org/10.1007/s12010-015-1579-1.
[53] R. Karami, A. Mohsenifar, S.M. Mesbah Namini, N. Kamelipour, T. Rahmani-Cherati, T. Roodbar Shojaei, M. Tabatabaei, A novel nanobiosensor for the detection of paraoxon using chitosan-embedded organophosphorus hydrolase immobilized on Au nanoparticles, Prep. Biochem. Biotechnol. 46 (2016) 559–566, doi:http://dx.doi.org/10.1080/10826068.2015.1084930.
[54] S. Chen, J. Huang, D. Du, J. Li, H. Tu, D. Liu, A. Zhang, Methyl parathion hydrolase based nanocomposite biosensors for highly sensitive and selective determination of methyl parathion, Biosens. Bioelectron. 26 (2011) 4320–4325, doi:http://dx.doi.org/10.1016/j.bios.2011.04.025.
[55] Y. Zhao, W. Zhang, Y. Lin, D. Du, The vital function of Fe_3O_4@Au nanocomposites for hydrolase biosensor design and its application in detection of methyl parathion, Nanoscale 5 (2013) 1121–1126, doi:http://dx.doi.org/10.1039/c2nr33107a.
[56] G. Liu, W. Guo, Z. Yin, Covalent fabrication of methyl parathion hydrolase on gold nanoparticles modified carbon substrates for designing a methyl parathion biosensor, Biosens. Bioelectron. 53 (2014) 440–446, doi:http://dx.doi.org/10.1016/j.bios.2013.10.025.
[57] H. Ye, Z. Guo, M. Peng, C. Cai, Y. Chen, Y. Cao, W. Zhang, Methyl parathion degrading enzyme-based nano-hybrid biosensor for enhanced methyl parathion recognition, Electroanalysis 28 (2016) 1591–1596, doi:http://dx.doi.org/10.1002/elan.201501102.
[58] X. Sun, L. Qiao, X. Sun, X. Wang, Recent advance of biosensor instruments in pesticide residues rapid detection, Sens. Transducers 155 (2013) 136–149.
[59] W. Lan, G. Chen, F. Cui, F. Tan, R. Liu, M. Yushupujiang, Development of a novel optical biosensor for detection of organophoshorus pesticides based on methyl parathion hydrolase immobilized by metal-chelate affinity, Sensor 12 (2012) 8477–8490, doi:http://dx.doi.org/10.3390/s120708477.
[60] J. Wichitwechkarn, W. Surareungchai, A. Ekkhunnatham, Electrode for organophosphate compound detection and its fabrication protocol (in Thai), 2018, patent no. 62070. http://patentsearch.ipthailand.go.th/DIP2013/view_public_data.php?appno=11102000043.
[61] A. Ekkhunnatham, Development of Biosensor for Pesticide Detection Using Methyl Parathion Hydrolase From Mpd Clone, Master Degree Thesis, Silpakorn University, Nakhon Pathom, 2009.
[62] A. Ekkhunnatham, J. Wichitwechkarn, W. Surareungchai, Development of biosensor for pesticide detection using methyl parathion hydrolase from recombinant MPD clone, Pure and Applied Chemistry International Conference, Ubonratchathani, Thailand, 2010.
[63] M. Mehrvar, M. Abdi, Recent developments, characteristics, and potential applications of electrochemical biosensors, Anal. Sci. 20 (2004) 1113–1126, doi:http://dx.doi.org/10.2116/analsci.20.1113.
[64] M. Pohanka, P. Skládal, Electrochemical biosensors-principles and applications, J. Appl. Biomed. 6 (2008) 57–64, doi:http://dx.doi.org/10.32725/jab.2008.008.
[65] J. Kumar, S.F. D'Souza, An optical microbial biosensor for detection of methyl parathion using *Sphingomonas* sp. immobilized on microplate as a reusable biocomponent, Biosens. Bioelectron. 26 (2010) 1292–1296, doi:http://dx.doi.org/10.1016/j.bios.2010.07.016.
[66] I. Kim, H.G. Kim, S.C. Kim, J.H. Cha, G. Lim, Optical detection of paraoxon using single-walled carbon nanotube films with attached organophosphorus hydrolase-expressed *Escherichia coli*, Sensor 15 (2015) 12513–12525, doi:http://dx.doi.org/10.3390/s150612513.
[67] V.G. Andreou, Y.D. Clonis, A portable fiber-optic pesticide biosensor based on immobilized cholinesterase and sol–gel entrapped bromcresol purple for in-field use, Biosens. Bioelectron. 17 (2002) 61–69, doi:http://dx.doi.org/10.1016/S0956-5663(01)00261-5.
[68] J. Kumar, S.K. Jha, S.F. D'Souza, Optical microbial biosensor for detection of methyl parathion pesticide using *Flavobacterium* sp. whole cells adsorbed on glass fiber filters as disposable biocomponent, Biosens. Bioelectron. 21 (2006) 2100–2105, doi:http://dx.doi.org/10.1016/j.bios.2005.10.012.
[69] M. Arjmand, H. Saghafifar, M. Alijanianzadeh, M. Soltanolkotabi, A sensitive tapered-fiber optic biosensor for the label-free detection of organophosphate pesticides, Sens. Actuators B Chem. 249 (2017) 523–532, doi:http://dx.doi.org/10.1016/j.snb.2017.04.121.
[70] L. Yang, G. Wang, Y. Liu, M. Wang, Development of a biosensor based on immobilization of acetylcholinesterase on NiO nanoparticles–carboxylic graphene–nafion modified electrode for detection of pesticides, Talanta. 113 (2013) 135–141, doi:http://dx.doi.org/10.1016/j.talanta.2013.03.025.
[71] S. Thakur, M. Venkateswar Reddy, D. Siddavattam, A.K. Paul, A fluorescence based assay with pyranine labeled hexa-histidine tagged organophosphorus hydrolase (OPH) for determination of organophosphates, Sens. Actuators B Chem. 163 (2012) 153–158, doi:http://dx.doi.org/10.1016/j.snb.2012.01.024.
[72] J. Hou, J. Dong, H. Zhu, X. Teng, S. Ai, M. Mang, A simple and sensitive fluorescent sensor for methyl parathion based on L-tyrosine methyl ester functionalized carbon dots, Biosens. Bioelectron. 68 (2015) 20–26, doi:http://dx.doi.org/10.1016/j.bios.2014.12.037.
[73] Q. Long, H. Li, Y. Zhang, S. Yao, Upconversion nanoparticle-based fluorescence resonance energy transfer assay for organophosphorus pesticides, Biosens. Bioelectron. 68 (2015) 168–174, doi:http://dx.doi.org/10.1016/j.bios.2014.12.046.
[74] E. Luan, Z. Zheng, X. Li, H. Gu, S. Liu, Inkjet-assisted layer-by-layer printing of quantum dot/enzyme microarrays for highly sensitive detection of organophosphorous pesticides, Anal. Chim. Acta 916 (2016) 77–83, doi:http://dx.doi.org/10.1016/j.aca.2016.02.019.
[75] N.R. Mohamad, N.H.C. Marzuki, N.A. Buang, F. Huyop, R.A. Wahab, An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes, Biotechnol. Biotechnol. Equip. 29 (2015) 205–220, doi:http://dx.doi.org/10.1080/13102818.2015.1008192.
[76] G. Fu, Z. Cui, T. Huang, S. Li, Expression, purification, and characterization of a novel methyl parathion hydrolase, Protein Expr. Purif. 36 (2004) 170–176, doi:http://dx.doi.org/10.1016/j.pep.2004.04.019.
[77] Y. Zhang, L. Li, C. Yu, T. Hei, Chitosan-coated polystyrene microplate for covalent immobilization of enzyme, Anal. Bioanal. Chem. 401 (2011) 2311–2317, doi:http://dx.doi.org/10.1007/s00216-011-5306-7.
[78] Water Quality Management Division, Practical Method for Water Collection, Pollution Control Department, Ministry of Natural Resources and Environment, Bangkok, 2010.
[79] H.A. Sapahin, A. Makahleh, B. Saad, Determination of organophosphorus pesticide residues in vegetables using solid phase micro-extraction coupled with gas chromatography–flame photometric detector, Arab. J. Chem. 12 (2014) 1934–1944, doi:http://dx.doi.org/10.1016/j.arabjc.2014.12.001.
[80] M.C. Tran, General principles, in: Biosensors, Springer US, Boston, MA, 1993, pp. 7–19, doi:http://dx.doi.org/10.1007/978-0-585-37623-3_2.
[81] H. Bisswanger, General aspects of enzyme analysis, Pract. Enzymol., 2nd ed., Wiley, Honoken, 2012, pp. 5–91.
[82] K. Wang, L. Wang, W. Jiang, J. Hu, A sensitive enzymatic method for paraoxon detection based on enzyme inhibition and fluorescence quenching, Talanta 84 (2011) 400–405, doi:http://dx.doi.org/10.1016/j.talanta.2011.01.056.
[83] A. Mulchandani, I. Kaneva, W. Chen, Biosensor for direct determination of organophosphate nerve agents using recombinant *Escherichia coli* with surface-expressed organophosphorus hydrolase. 2. Fiber-optic microbial biosensor, Anal. Chem. 70 (1998) 5042–5046, doi:http://dx.doi.org/10.1021/ac980643l.
[84] B. Kuswandi, C.I. Fikriyah, A.A. Gani, An optical fiber biosensor for chlorpyrifos using a single sol–gel film containing acetylcholinesterase and bromothymol blue, Talanta 74 (2008) 613–618, doi:http://dx.doi.org/10.1016/j.talanta.2007.06.042.
[85] M. Mehrvar, C. Bis, J.M. Scharer, M. Moo-Young, J.H.T. Luong, Fiber-optic biosensors. Trends and advances, Anal. Sci. 16 (2000) 677–692, doi:http://dx.doi.org/10.2116/analsci.16.677.
[86] M.D. Marazuela-Lamata, M. Moreno-Bondi, Fiber-optic biosensors-an overview, Anal. Bioanal. Chem. 372 (2002) 664–682, doi:http://dx.doi.org/10.1007/s00216-002-1235-9.
[87] K. El-Boubbou, D.A. Schofield, C.C. Landry, Enhanced enzymatic activity of OPH in ammonium-functionalized mesoporous silica: surface modification and pore effects, J. Phys. Chem. C 116 (2012) 17501–17506, doi:http://dx.doi.org/10.1021/jp3023309.
[88] J. Kaushal, Seema, G. Singh, S.K. Arya, Immobilization of catalase onto chitosan and chitosan-bentonite complex: a comparative study, Biotechnol. Rep. 18 (2018)e00258, doi:http://dx.doi.org/10.1016/j.btre.2018.e00258.